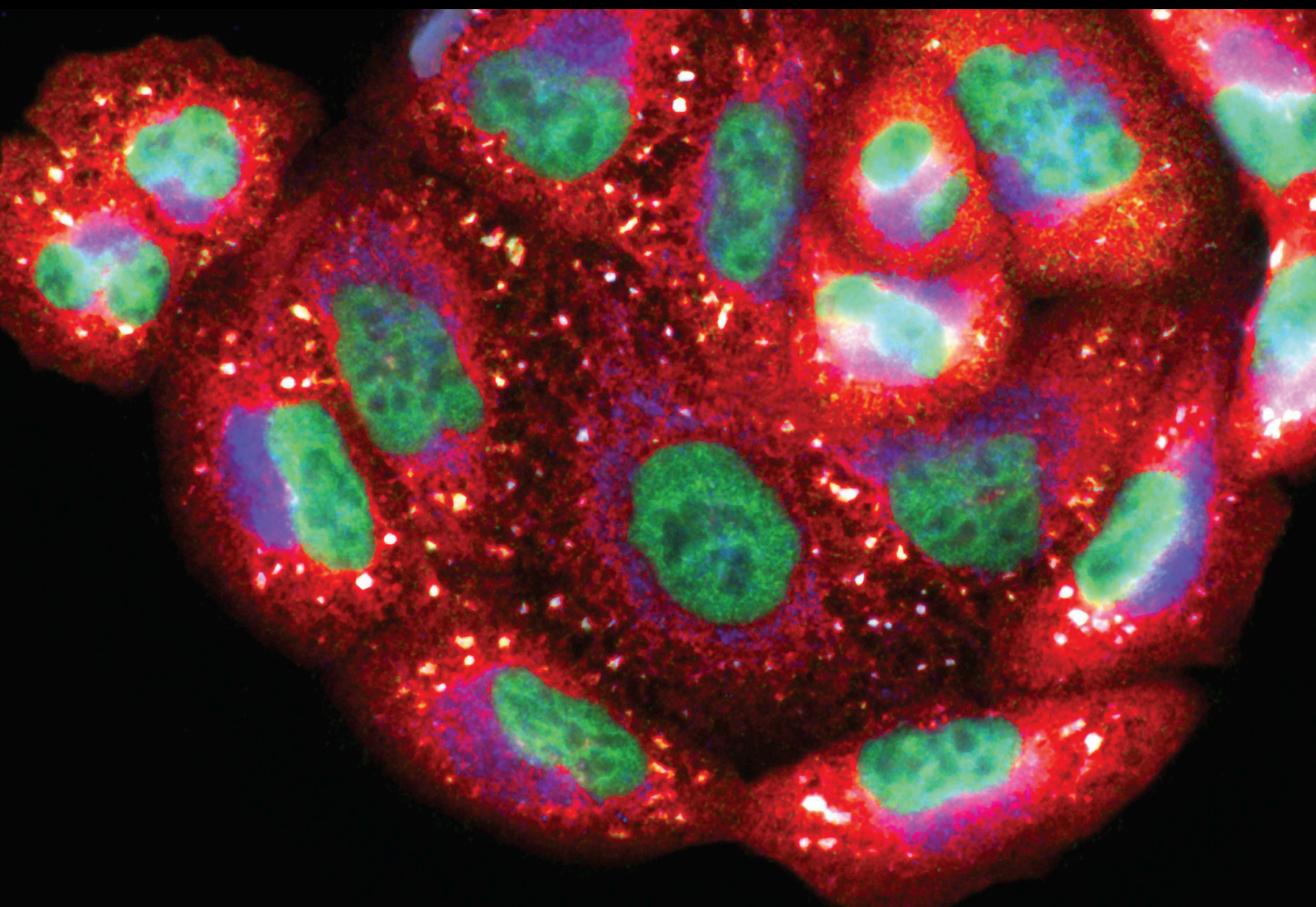
Oxidative Medicine and Cellular Longevity

Oxidative Stress and Reprogramming of Mitochondrial Function and Dynamics as Targets to Modulate Cancer Cell Behavior and Chemoresistance

Lead Guest Editor: Stefano Falone
Guest Editors: Michael P. Lisanti and Cinzia Domenicotti



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Editorial

Oxidative Stress and Reprogramming of Mitochondrial Function and Dynamics as Targets to Modulate Cancer Cell Behavior and Chemoresistance

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Among all the leading causes of mortality worldwide, cancer is responsible for approx. one in six deaths, and global cancer data show that the cancer burden has recently risen to 18.1 million people [1]. This represents a demanding challenge for healthcare systems and governments, whose actions are increasingly aimed at developing commitments for cancer treatment, on one hand, as well as at strengthening research activities on the mechanisms of carcinogenesis and cancer progression, on the other hand.

Fortunately, over the last decades, therapeutic strategies against cancer have taken significant steps forward, as demonstrated by the fact that age-standardized cancer death rates are falling globally [2]. However, medical treatments for oncological patients still encounter significant obstacles due to the development of radio- and chemoresistance, which, along with metastatic behavior, is thought to require extensive reprogramming of mitochondrial activity [3]. The balance of fission and fusion, along with the regulation of trafficking and autophagic removal, dictates mitochondrial morphology and function [4], and some researchers have suggested that mitochondrial dynamics could have a deep impact on redox homeostasis and antioxidant defense of cancer cells, as well as on their apoptotic response to oxidative stress-generating and DNA-damaging anticancer drugs [5].

This special issue comprises 4 review articles and 6 research articles that either investigated the role of mitochondria in mediating the proapoptotic response of malignant cells to anticancer drugs or examined ROS-dependent effects on redox-sensitive pathways controlling proliferation or viability of cancer cells.

Mitochondrial dynamics is in part regulated by the Liver Kinase B1- (LKB1-) AMP-activated protein kinase (AMPK) pathway [6]. LKB1 was identified as the critical upstream kinase required for AMPK activation thus providing a direct link between a known tumor suppressor and the regulation of metabolism. In fact, AMPK has a central role in the regulation of energy metabolism and coordinates glucose and lipid metabolism in response to alterations in nutrients and intracellular energy levels, contributing to maintain steady-state levels of intracellular ATP [7].

In their review article, F. Ciccarese et al. reported that loss of LKB1-AMPK signalling is able to confer sensitivity to energy depletion and to redox homeostasis impairment. Moreover, the authors have found an association between such a pathway and improved outcome in patients affected by advanced non-small-cell lung cancer (NSCLC) and treated with chemotherapy.

In this context, the review article of B. Poljsak et al. focused on the importance of understanding the origins of cancer in order to find successful strategies for effective cancer prevention and management.

In fact, it remains to be elucidated what exactly triggers the reprogrammed metabolism in cancer cells, and additional studies are needed to extend the knowledge about the relationships between metabolic abnormalities and the occurrence of genetic mutations in cancer.

As reviewed by B. Marengo et al., the metabolic reprogramming is the result of a complex network of mechanisms that, through the activation of oncogenes (i.e., MYC, HIF1, and PI3K) or the downregulation of tumor suppressors (i.e., TP53), induce an increased expression of glucose and/or glutamine transporters, along with an overexpression of glycolytic enzymes. The authors reported that among oncogenes, MYC is strongly involved in regulating cell metabolism since it facilitates glycolysis by inducing the activation of genes encoding for glycolytic enzymes and it is also able to promote mitochondrial biogenesis and function, thus increasing both oxygen consumption and ATP production.

In addition, it has been postulated that mitochondrial dysfunction in cancer cells would affect the cellular ATPase activities, ATP production, and subsequent apoptosis and migration processes [8].

In their research article, X. Zhang et al. demonstrated that the small molecule b-AP15 is an inhibitor of proteasome-associated deubiquitinase activity, which induced an increase in the generation of reactive oxygen species (ROS) in cancer cells. Oxidative stress (OS) induced by b-AP15 was found to be associated with a mitochondrial impairment and contributed to overcome resistance to bortezomib, which is an inhibitor of the 20S proteasome, in the clinical management of multiple myeloma.

Moreover, X. Li et al. have shown that KillerRed targeting mitochondria (mtKR) aggravated the mitochondrial dysfunction induced by radiation, thus suggesting a new strategy for ROS sensitization in future clinical cancer therapy. In this study, the N-terminal mitochondrial-targeting sequence (MTS) of PTEN-induced putative kinase 1 (Pink1) was used to mediate downstream mCherry and KillerRed to express in mitochondria, and the colocalization of mCherry (red) and mitochondrial tracker COX IV (green) was observed by fluorescence microscope analysis in COS-7 cells and human cervical cancer HeLa cells. In addition, the authors demonstrated in HeLa cells transfected with mtKR plasmids that mtKR induced mitochondrial ROS production, thus contributing to enhance apoptosis *via* the Cyt c/caspase-3 pathway in tumors treated with radiation.

Interestingly, evidence shows that natural molecules, such as curcumin and sulforaphane, are able to modulate the response of cancer cells to anticancer therapies. However, limited reports support the role of mitochondrial reprogramming in such a phenomenon, even though several natural chemosensitizers may act as regulators of mitochondrial dynamics and function. Further investigations on this may pave the way to diet-based approaches aimed at repressing the adaptive responses involving mitochondria following chemotherapy, thus contributing to an increase in the efficacy of anticancer strategies.

In their research article, B. George and H. Abrahamse, from University of Johannesburg, demonstrated that two phytochemicals isolated from roots of *Rubus fairholmianus* (1-(2-hydroxyphenyl)-4-methylpentan-1-one and 2-[(3-methylbutoxy) carbonyl] benzoic acid) were able to induce in human breast cancer MCF-7 cells an increase in ROS formation, cytochrome c release, and changes in mitochondrial membrane potential (MMP), thus activating the intrinsic apoptotic pathway. With specific focus on mitochondrion-dependent processes, the authors have quantitatively detected cytochrome c release by ELISA, as well as MMP by flow cytometry with a JC-1-based fluorescent kit.

The involvement of mitochondria in phytochemical-induced death response in cancer cells was even more evident in the original study of C. Antognelli et al., who identified in non-small-cell lung cancer (NSCLC) cells an interesting apoptogenic action of oleuropein (OP), a bioactive polyphenol found in olives. The authors found that OP was able to cause apoptotic death in A549 cells through depletion of mitochondrial superoxide anion, which in turn inhibited Akt signalling and activated the intrinsic apoptotic pathway *via* mitochondrial glyoxalase 2- (mGlo2-) mediated interaction with the proapoptotic protein Bax. This latter aspect is one of the most interesting findings of the work. In fact, the data provided by C. Antognelli et al. support the intriguing hypothesis that glyoxalase 2, an enzyme that is conventionally considered an enzyme committed to downregulate the formation of advanced glycation end products (AGEs) [9], is also able to form protein adducts with apoptosis-related factors. The critical role of mitochondrial redox reprogramming in the processes summarized above was demonstrated by silencing the mitochondrial superoxide dismutase (SOD2). This restored the normal $O_2^{\cdot-}$ levels and mGlo2 expression, and in such conditions, OP failed to induce apoptosis in cancer cells. The interest for OP in terms of clinical application is increased since the authors demonstrated that OP did not affect the viability of cells derived from human normal bronchial epithelium.

The demand for anticancer drugs with low systemic adverse effects and low impact on healthy cells is highly appreciated. In this context, the work from Y. Zheng et al. (Guangzhou University, China) presented novel molecular targets of betulinic acid (BA), a pentacyclic triterpene derived from birch bark extracts. BA is attracting increasing attention due to its high selectivity for cancer cells, with no apparent systemic toxicity in mice [10, 11]. BA proapoptotic effects in malignant cells have been traditionally linked to mitochondrial ROS generation and induction of DNA damage [12, 13]; however, Y. Zheng et al. revealed that BA attenuated migration and invasion of highly aggressive breast cancer cells *via* aerobic glycolysis inhibition, and glucose-regulated protein (GRP78), a major chaperone in the endoplasmic reticulum, was found to be critical for inhibitory effects of BA on glycolytic proteins. Moreover, Y. Zheng et al.'s findings indicated that the oxygen consumption rate (OCR) of breast cancer cell lines MDA-MB-231 and BT-549 decreased following BA treatment, thus suggesting that BA switched the cells from an energetic metabolic state to a relatively quiescent state. In their experiments, the authors obtained accurate profiles of cancer cell energy phenotypes by using a live cell metabolic assay platform for extracellular flux analyses.

The crucial roles of ROS scavenging systems and mitochondria in triggering the cancer cell death induced by dietary polyphenols have been extensively reviewed and summarized by S. NavaneethaKrishnan et al. In their review,

the authors focused their attention on some of the best known vegetable- and fruit-derived polyphenols with recognized pro-death properties against cancer cells. In particular, in their paper, S. NavaneethaKrishnan et al. provided interesting information about the cytotoxic effects of quercetin, curcumin, and resveratrol, with particular attention to the activation of ROS- and mitochondrion-dependent molecular pathways as possible mediators of such effects. In some cases, the redox-dependent cancer cell death is promoted through the activation of ROS-induced apoptosis, MMP reduction, cytochrome C release, and subsequent activation of caspase-3. In other cases, these polyphenols enhance TNF-related apoptosis-inducing ligand- (TRAIL-) induced apoptosis *via* the inhibition of ERK signalling pathway or by oxidatively modifying proteins that belong to the mitochondrial permeability transition pore (mPTP), thus causing mitochondrial depolarization, inhibition of ATP synthesis, and cell death. Furthermore, it was reported that some common plant-derived polyphenols exhibit a marked ROS-inducing capacity that leads to mitochondrial DNA damage and impairment of mitochondrial oxidative phosphorylation (OXPHOS). In addition, beyond exerting clear proapoptotic actions, some dietary polyphenols have also been proven to act as cell cycle arresting factors. Finally, the authors provided some interesting information about redox- and mitochondrion-targeting anticancer properties of less famous dietary polyphenols, such as capsaicin, coumaric acid, and phenethyl isothiocyanate, which leads to mitochondrial dysfunction in cancer cells but not in normal cells. Lastly, since poor absorption and fast metabolism of dietary polyphenols are concerning limiting factors in the administration to humans, promising strategies that include the use of novel formulations, prodrugs, and innovating delivery systems are proposed.

Hence, any strategy aimed at increasing ROS production or diminishing antioxidant capacity should be seen as a potential means by which the abnormal proliferation and growth of malignant cancer cells could be prevented or delayed. This topic was further investigated in the paper from K. Chen et al., who demonstrated that by deleting bloom syndrome protein (BLM), a DNA helicase belonging to the RecQ family, the proliferation of prostate cancer (PC) cells was repressed *via* downregulation of AKT signalling, and this was accompanied by enhanced ROS production. Of note, in their research, the authors used state-of-the-art techniques, such as isobaric tags for relative and absolute quantification (iTRAQ) proteomics, CRISPR/-Cas9-mediated gene editing, and automated western blot quantitative analysis.

We sincerely hope that the articles offered by this special issue may provide interesting mechanistic insights of the role of mitochondria and redox-related signalling pathways in determining the cancer metabolic reprogramming, the proliferative activity of cancer cells, or their apoptotic response to exogenous stressors (e.g., natural anticancer molecules). We also strongly hope that further efforts will be spent for expanding the scientific knowledge on such topics, with the aim of future development of diet-based co-therapies for cancer.

Finally, we wish to thank all the authors for sharing their novel findings or reviews, and all reviewers for their priceless support in processing all the manuscripts.

Conflicts of Interest

The editors declare they have no conflicts of interest regarding the publication of this special issue.

Authors' Contributions

Stefano Falone and Cinzia Domenicotti wrote the editorial. Michael P. Lisanti reviewed the editorial. All editors approved the content of the editorial.

Stefano Falone
Michael P. Lisanti
Cinzia Domenicotti

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Review Article

LKB1/AMPK Pathway and Drug Response in Cancer: A Therapeutic Perspective

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Inactivating mutations of the tumor suppressor gene Liver Kinase B1 (*LKB1*) are frequently detected in non-small-cell lung cancer (NSCLC) and cervical carcinoma. Moreover, LKB1 expression is epigenetically regulated in several tumor types. LKB1 has an established function in the control of cell metabolism and oxidative stress. Clinical and preclinical studies support a role of LKB1 as a central modifier of cellular response to different stress-inducing drugs, suggesting LKB1 pathway as a highly promising therapeutic target. Loss of LKB1-AMPK signaling confers sensitivity to energy depletion and to redox homeostasis impairment and has been associated with an improved outcome in advanced NSCLC patients treated with chemotherapy. In this review, we provide an overview of the interplay between LKB1 and its downstream targets in cancer and focus on potential therapeutic strategies whose outcome could depend from LKB1.

1. Introduction

The Liver Kinase B1 (*LKB1*, also known as *STK11*) is a tumor suppressor gene encoding a ubiquitously expressed and evolutionarily conserved serine threonine kinase, originally associated with the inherited cancer disorder Peutz-Jeghers Syndrome [1, 2]. Inactivating somatic mutations of *LKB1* are frequently reported in non-small-cell lung cancer (NSCLC) [3], malignant melanoma [4], and cervical carcinoma [5]. LKB1 positively regulates the AMP-activated protein kinase (AMPK) [6] and at least 12 additional AMPK-related downstream kinases, involved in the control of cell growth and metabolism and in the regulation of cellular response to energy stress and establishment of cell polarity [7]. Deregulation of LKB1 signaling has been implicated in oncogenesis across many cancer types [8–10], although the energy-sensing function of LKB1-AMPK may also confer a survival advantage under unfavourable conditions [11].

Several preclinical studies identified LKB1 signaling axis as a potential modifier of response of cancer cells to different drugs. Thus, understanding the different mechanisms that account for anti- or prooncogenic effect of LKB1 is essential to identify therapeutic strategies targeting this pathway.

In this review, we address the potential vulnerabilities of LKB1-deficient tumors and focus on recent scientific findings that support a role of this pathway in the modulation of drug response in cancer.

2. LKB1 Alterations in Human Cancers

Germline loss of LKB1 kinase activity accounts for the Peutz-Jeghers Syndrome, an autosomal dominant inherited disorder characterized by hamartomatous polyps in the gastrointestinal tract and mucocutaneous pigmentation [2]. Peutz-Jeghers Syndrome is associated with age-related increased risk of cancer development, principally involving the gastrointestinal tract but affecting also the breast, gynecologic tract, lung, and other sites [12], corroborating a *bona fide* tumor suppressor role for LKB1.

In the great majority of human cancers, somatic mutations of the *LKB1* gene are rare. However, *LKB1* is the most frequently mutated gene in cervical carcinoma (20% of cases

[5]) and the third most mutated gene in NSCLC (30% of cases in the Caucasian population [13]). Frequent somatic *LKB1* loss in lung adenocarcinoma is puzzling, as lung cancer is uncommon in Peutz-Jeghers patients. In contrast, *LKB1* somatic mutations are rare in colorectal cancer [14], the most frequent neoplasia associated with inherited *LKB1* loss. Several factors could account for these differences. First, *LKB1* loss in NSCLC is frequently homozygous [15], indicating that probably monoallelic LKB1 in Peutz-Jeghers patients is sufficient to limit lung tumorigenesis. Second, *LKB1* mutations coexist with several other genetic alterations in sporadic cancers. *TP53* and *KRAS* are, respectively, the first and the second most mutated genes in lung adenocarcinoma. About 12% of NSCLC cases have *LKB1* and *KRAS* comutations [16]. Moreover, *LKB1* mutations cooccur with gain-of-function *TP53* mutations in 8.2% lung adenocarcinomas [17]. Third, *LKB1* mutations are associated with smoking history of NSCLC patients [18]. Fourth, by interacting with breast cancer susceptibility 1 (BRCA1), LKB1 is involved in the DNA damage response, promoting homologous recombination (Figure 1) and fostering genomic stability [19]. In light of these considerations, *LKB*1 loss could be induced by and, afterwards, facilitate the mutagenic properties of carcinogens contained in tobacco smoke, being selected to promote lung tumorigenesis, while other malignancies—such as colon cancer—have evolved different protumorigenic alterations.

An interesting feature of NSCLC is its intratumor heterogeneity. Remarkably, somatic *LKB1* loss is an intermediate event during lung carcinogenesis, which arises clonally in lung cells with preexisting mutations in initiating drivers, such as *TP53* and *KRAS* [20]. The subclonal nature of *LKB1* highlights how the complexity of cancer genetics might impact on tumor progression and resistance to therapy.

Considering all the genetic and epigenetic events that can affect the *LKB1* gene, the estimated real frequency of *LKB1* alterations in NSCLC is as high as 90% [15], hinting at its fundamental role in lung cancer biology. Moreover, it should be emphasized that the frequency of LKB1 loss in other cancer types could be underestimated, due to rarely investigated epigenetic alterations. A paradigmatic example is breast cancer, whose aggressiveness and metastasis are promoted by LKB1 loss [9], even if *LKB1* mutations are detected with low frequency. The combination of sequencing and analysis of protein expression might overcome intrinsic limitations of sequencing and provide a comprehensive evaluation of LKB1 status in tumors.

3. Role of LKB1-AMPK Pathway in Cell Metabolism

LKB1 was identified as the critical upstream kinase required for AMPK activation [6, 21, 22] (Figure 1), thus providing a direct link between a known tumor suppressor and regulation of metabolism [23]. AMPK has a central role in the regulation of energy metabolism in eukaryotes and coordinates glucose and lipid metabolism in response to alterations in nutrients and intracellular energy levels, contributing to maintain steady-state levels of intracellular ATP [24].

Upon changes in energy availability, causing perturbations in the ATP-to-ADP or ATP-to-AMP ratio, AMPK is activated by an allosteric mechanism and by LKB1 via phosphorylation [7]. AMPK is also activated by increases in intracellular Ca^{2+} [25–27] and by DNA damage [28–30]. Moreover, a novel AMP-independent mechanism of AMPK activation under glucose starvation has recently been described by Zhang and colleagues who observed that, upon glucose starvation and the consequent decrease of fructose-1,6-bisphosphate (FBP) levels, aldolases promote the formation of a lysosomal complex containing v-ATPase, Ragulator, AXIN/LKB1, and AMPK [31], leading to LKB1-mediated AMPK activation before energy levels fall. This aldolase-dependent mechanism of AMPK activation could be at play under conditions where low glucose does not cause an increase of intracellular AMP-to-ATP or ADP-to-ATP ratios.

Once activated, AMPK redirects metabolism towards decreased anabolism and increased catabolism by phosphorylation of key proteins involved in several metabolic pathways [24], including lipid homeostasis, glycolysis, protein synthesis, and mitochondrial homeostasis.

AMPK was originally defined as the critical inhibitory upstream kinase for the metabolic enzymes acetyl-CoA carboxylase (ACC1 and ACC2) [32] (Figure 1) and HMG-CoA reductase [33], which serve as rate-limiting steps for fatty acid and sterol synthesis, respectively, in a wide variety of eukaryotes. Moreover, inactivation of ACC2 switches on fatty acid (FA) β-oxidation in mitochondria [34]. Through activation of FA oxidation and inhibition of FA synthesis, LKB1-AMPK pathway plays a pivotal role in the maintenance of intracellular NADPH levels, which is required to prevent oxidative stress and to promote cancer cell survival under energy stress conditions [35].

Moreover, when nutrient levels are low, AMPK acts as a metabolic checkpoint inhibitor of cell growth, by modulation of the master regulator of growth, the mammalian target of rapamycin (mTOR) pathway [36] (Figure 1). AMPK activation leads to inhibition of mTOR complex 1 (mTORC1), by activation of the negative mTORC1 regulator TSC2 and by inhibition of the mTORC1 subunit RAPTOR [36]. Importantly, activated mTORC1 is localized on the surface of lysosomes, where it is negatively regulated by AXIN through inhibition of the GEF (guanine nucleotide exchange factor) activity of Ragulator. Thus, AXIN/LKB1 complex inhibits mTORC1 through the glucose-sensing mechanism involving aldolase and FBP [31]. Moreover, AMPK activation caused G1 cell cycle arrest associated with activation of p53, followed by induction of the cell cycle inhibition protein p21 and by stabilization via phosphorylation of the cyclin-dependent kinase inhibitor $p27^{kip1}$ [37, 38]. Through mTOR inhibition, AMPK downregulates hypoxia-inducible factor 1α (HIF-1α), thus counteracting the Warburg effect [39].

In addition to its central role in the regulation of cell growth, mTORC1 controls autophagy, a lysosome-dependent catabolic program that maintains cellular homeostasis. Upon nutrient starvation, mTORC1 is inactivated through the energy-sensing mechanism of AMPK activation. Moreover, mTORC1 is also inhibited by direct dissociation from lysosomes through the glucose-sensing mechanism

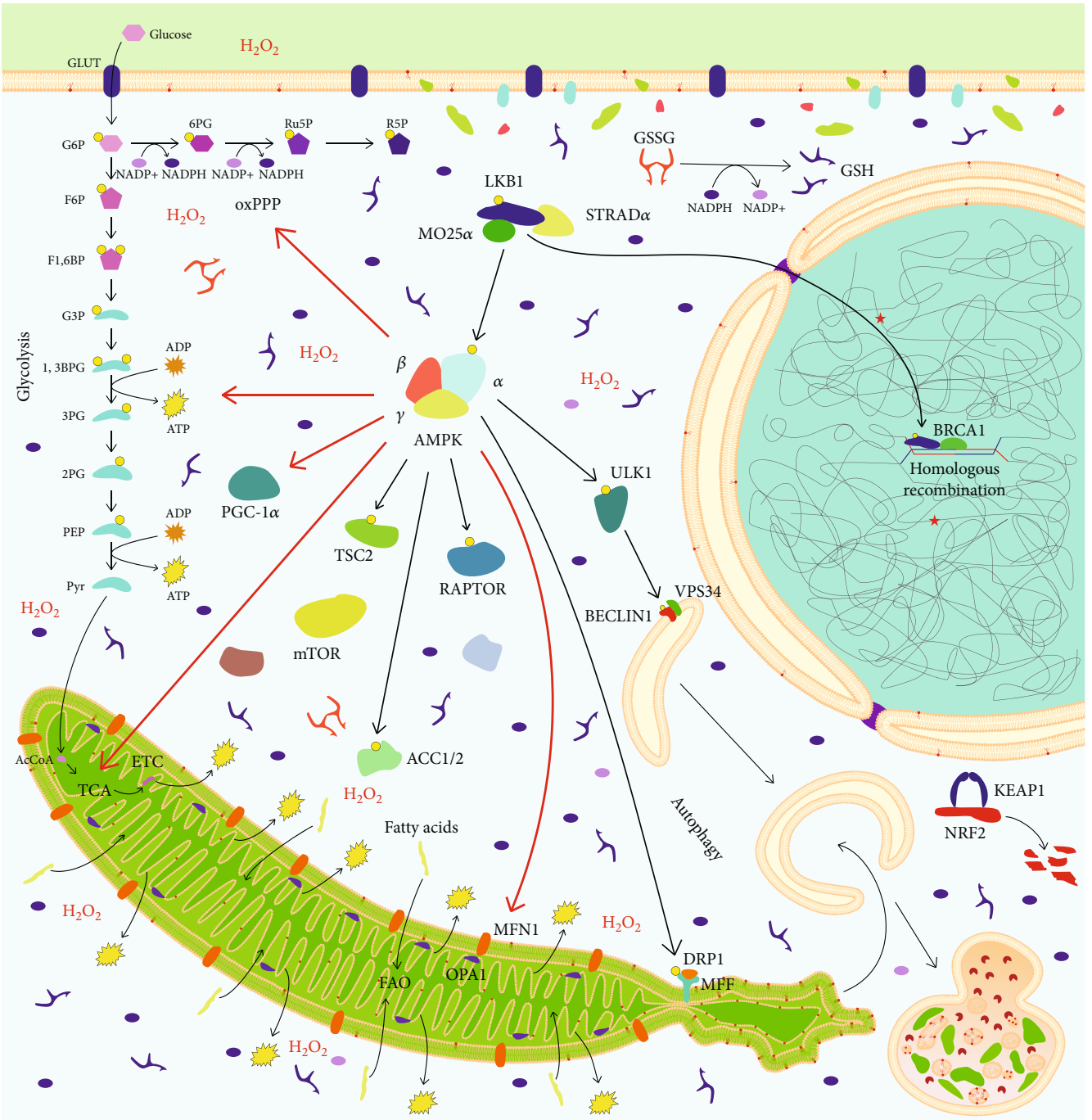

FIGURE 1: LKB1-proficient tumors display coordinated control of metabolism, DNA repair, and mitochondrial dynamics. LKB1 interacts with the pseudokinase STE20-Related Kinase Adaptor Alpha (STRADα) and with the armadillo-repeat containing protein MO25α. Once activated, LKB1 phosphorylates AMPK, which coordinates activation of catabolic processes—such as glycolysis, Krebs cycle, pentose phosphate pathway, fatty acid oxidation, and autophagy—and inhibition of anabolic processes—such as fatty acid synthesis and mTOR pathway. This maximizes ATP production and NADPH regeneration, thus controlling energy and redox homeostasis. Moreover, AMPK promotes mitochondrial fusion and mitophagy of damaged mitochondrial portions. In the nucleus, LKB1 fosters genomic integrity through sustaining homologous recombination. Black arrows from AMPK: direct phosphorylation. Red arrows: activation/upregulation. Yellow circles: phosphate groups. Red phospholipids in membranes: peroxidised phospholipids. Red stars in the nucleus: DNA damage sites. G6P: glucose 6-phosphate; F6P: fructose 6-phosphate; F1,6BP: fructose 1,6-biphosphate; G3P: glyceraldehyde 3-phosphate; 1,3BPG: 1,3-biphosphoglycerate; 3PG: 3-phosphoglycerate; 2PG: 2-phosphoglycerate; PEP: phosphoenolpyruvate; Pyr: pyruvate; AcCoA: acetyl-coA; 6PG: 6-phosphogluconate; Ru5P: ribulose 5-phosphate; R5P: ribose 5-phosphate; GLUT: glucose transporter; GSH: reduced glutathione; GSSG: oxidized glutathione; H_2O_2: hydrogen peroxide; oxPPP: oxidative pentose phosphate pathway; TCA: tricarboxylic acid cycle; ETC: electron transport chain; FAO: fatty acid oxidation. The names of proteins deriving from disassembly of mTORC1 and NADPH oxidase complexes are omitted. See the text for details.

[31]. This mTORC1 suppression relieves the inhibitory phosphorylation on Unc-51-Like Autophagy Activating Kinase 1 (ULK1), a kinase essential for autophagy induction [40, 41]. AMPK has also an important role in the regulation of autophagy through direct phosphorylation of ULK1 and of a second autophagy-initiating regulator, the lipid kinase complex PI32KC3/VPS34 [42]. Interestingly, AMPK triggers acute destruction of dysfunctional mitochondria through ULK1-dependent stimulation of mitophagy (Figure 1), and it stimulates *de novo* mitochondrial biogenesis through peroxisome proliferator-activated receptor gamma coactivator 1 α- (PGC-1α-) dependent transcription [38]. Interestingly, genetic deletion of *Lkb1* in the haematopoietic stem cell resulted in mitochondrial dysfunction and deregulation of bioenergetic processes through AMPK-dependent and independent mechanisms [43–45]. The interplay between AMPK and mitochondria is further discussed in a distinct section.

Besides AMPK, other 12 kinases, collectively termed AMPK-related kinases, are LKB1 substrates. However, little is known about what stimuli direct LKB1 towards any of these AMPK-related kinases. These enzymes include two family members, SNARK/Nuak2 and SIK2, both activated under low energy conditions, although only AMPK is activated under low ATP levels [36]. Moreover, other members, such as isoforms of PAR1/MARK, as well as SAD/BRSK, unlike AMPK, are not activated by energy stress but have been implicated in controlling cell polarity [46].

3.1. LKB1: An Unexpected Oncogenic Role for a Tumor Suppressor. Recently, the role of LKB1-AMPK to sense different types of stress has pointed at a conditional oncogenic role of this pathway. In fact, its ability to modulate cell metabolism in order to restore homeostasis may confer a survival advantage under selective pressure, by favoring adaptation to hostile conditions [47]. In this context, Lee and colleagues demonstrated that polyubiquitination of LKB1 by S-Phase Kinase-Associated Protein 2 (Skp2) ubiquitin ligase promotes its persistent activation, leading to cell survival and poor outcome in hepatocellular carcinoma patients [48]. A recent study showed that, although it negatively regulates the epithelial-to-mesenchymal transition (EMT)-inducing gene *ZEB1*, LKB1 expression is increased in spheroids obtained from breast cancer cell lines and its ablation induces anoikis, suggesting that LKB1 promotes survival of circulating tumor cells [49]. LKB1 activation can result in an oncogenic program based on the contextual oncogenic role of its targets. For instance, LKB1 upregulates the expression of miR-34a [50], which was found to promote survival in the context of adult T-cell leukemia/lymphoma (ATLL) [51].

Downstream of LKB1, also AMPK has been indicated as a contextual oncogene. In fact, AMPK activation promotes glioblastoma growth by inducing lipid internalization [52] and sustains bioenergetics of glioblastoma through HIF-1α signaling [53]. Moreover, AMPK activation results in increased AKT oncogenic signaling through Skp2 phosphorylation under stress [54] and promotes aberrant expression of PGC-1β and estrogen-related receptor α (ERRα) in colon cancer, supporting its survival [55]. Finally, AMPK activation promotes resistance of cancer cells to chemotherapy by induction of autophagy [56–59].

How can the contrasting role of LKB1 as a tumor suppressor or promoter of cancer survival be reconciled? It must be considered that this pathway has evolved to allow cell survival under energy stress. During the initial phases of tumorigenesis, stress is a critical event that alters cell physiology and induces genetic aberrations, genomic instability, and transformation. In this context, LKB1 and AMPK play a tumor suppressor role by dealing with metabolic stress. The maintenance of genomic integrity, activation of autophagy, which scavenges damaged organelles and proteins, and activation of TP53 [14] to eliminate aberrant cells blunt cancer initiation. However, stress is a double-edged sword in cancer, and if not solved, it would lead to tumor eradication. In this scenario, a functional LKB1-AMPK pathway is advantageous for growing cancer cells, as it promotes adaption to a hostile microenvironment and cell survival. The activation of catabolic pathways and increased recycling of cellular components through autophagy ensure maintenance of energy homeostasis [60]. Autophagy, which has both prosurvival and prodeath effects, is probably the main responsible for contextual tumor suppressor and oncogenic activities of LKB1-AMPK. It should be pointed out, however, that autophagic cell death is a concept that should be cautiously evaluated. Cell death occurs, likely, despite autophagy, rather than because of autophagy [61]. In fact, increased autophagy in dying cells could be a rescue mechanism that failed or a mechanism sustaining apoptosis through ATP production. Physiologic "tumor suppressor" autophagy, which degrades damaged organelles and suppresses tumor initiation, should be distinguished by aberrant "prosurvival" autophagy, which is coopted by cancer to sustain its growth. As degradation of cellular components that have been damaged by anticancer therapies is a widely adopted mechanism of resistance, activation of autophagy by LKB1-AMPK in advanced stage cancers could represent a rescue mechanism.

4. Mitochondrial Dynamics Is Affected by LKB1-AMPK Pathway

As master regulators of metabolism, LKB1 and AMPK are tightly intertwined with mitochondrial function and dynamics (Figure 1). Mitochondria are essential dynamic organelles that continuously shift from fusion to fission and vice versa. Mitochondrial dynamics is in part regulated by the LKB1-AMPK pathway (Table 1). Following stress, AMPK activates mitochondrial fusion to restore the function of damaged mitochondria. If the damage is too extensive, AMPK activates mitochondrial fission and mitophagy to separate and degrade damaged mitochondrial portions and promotes synthesis of new mitochondria, in order to preserve mitochondrial network function and maximize ATP production (Table 1). In contrast, in LKB1 defective tumors, hypoxic stress elicits activation of HIF-1α [62], which reduces the expression of Mitofusin-1 (MFN1) and Optic Atrophy 1 (OPA1) and increases activity of Dynamin-Related Protein 1 (DRP1), thus unbalancing mitochondrial dynamics towards fission (Figure 2). In endothelial cells, this promotes

Table 1: Mitochondrial dynamics control by LKB1-AMPK.

Target	Role of LKB1	Biological effects
(a) Role of LKB1/AMPK in mitochondrial fission		
MFF (mitochondrial fission factor)	AMPK-mediated phosphorylation	MFF phosphorylation relocalizes the cytosolic GTPase Dynamin-Related Protein 1 (DRP1) to mitochondria, leading to mitochondrial fragmentation [138]
ULK1 (Unc-51-Like Autophagy Activating Kinase 1)	AMPK-mediated phosphorylation	ULK1 phosphorylation initiates mitophagy of damaged mitochondria, providing cancer cells with an important loophole from therapy-induced cytotoxicity [139]
PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1 alpha)	AMPK-mediated activation	Activation of PGC-1α, the master regulator of mitochondrial biogenesis, promotes the biogenesis of new mitochondria, in order to preserve mitochondrial network functionality [139]
(b) Role of LKB1/AMPK in mitochondrial fusion		
MFN1 (Mitofusin-1)	AMPK-mediated upregulation	MFN1 mediates outer mitochondrial membrane fusion, protecting cells from mitochondrial dysfunction following a cytotoxic injury [140]
OPA1 (Optic Atrophy 1)	AMPK-mediated upregulation	OPA1 mediates inner mitochondrial membrane fusion, protecting cells from mitochondrial dysfunction following a cytotoxic injury [140]

migration, invasion, and tube formation, implying that hypoxia-induced mitochondrial fission activates angiogenesis [63].

Mitochondria fusion and fission are both involved in the response of cancer cells to therapies. Several studies observed that mitochondrial fission sensitizes cancer cells to chemotherapy. Inhibition of autophagy has been shown to enhance doxorubicin cytotoxicity in breast cancer cells through mitochondrial translocation of DRP1 and consequent mitochondrial fission [64]. Similarly, LKB1-deficient NSCLC cell line A549 resulted resistant to doxorubicin-induced apoptotic cell death due to dysfunctional DRP1 that impedes mitochondrial fission [65]. Notably, AMPK promotes the maintenance of mitochondrial membrane potential following stress [66], thus preventing the proteolytic cleavage of OPA1, which is involved in cell death induction [67].

In cancer cells, mitochondrial fission has also been described to trigger cell migration, leading to cell escape from stressful conditions, such as chemotherapy, metastasis, and chemoresistance. By decreasing reactive oxygen species (ROS) levels—as described later—AMPK inhibits the release of high mobility group box 1 (HMGB1), which is involved in mitochondrial fission [68], thus blunting these escape mechanisms.

5. Targeting the LKB1-AMPK Pathway

5.1. Activation of LKB1-AMPK Pathway by Biguanides. The biguanide metformin attracted considerable attention as a potential anticancer drug once the connection between LKB1 and AMPK was discovered [42]. Metformin is one of the most widely used type 2 diabetes drug worldwide, and epidemiological studies revealed that diabetic patients taking metformin show a statistically significant reduced tumor incidence [69].

Metformin and the related drug phenformin have been shown to inhibit complex I of the mitochondria [70], resulting in increased intracellular AMP and ADP levels, which trigger LKB1-dependent phosphorylation of AMPK [42]. Diabetic patients taking biguanides might have a lower incidence of cancer because of the role of the LKB1-AMPK pathway as a checkpoint inhibitor of cell growth and suppression of mTORC1 and other growth pathways. In addition, antitumor effects of metformin might be linked to its ability to lower circulating blood glucose and insulin levels, which also contribute to cancer risk and incidence in some contexts [69].

Tumor cells lacking functional LKB1 are acutely sensitive to metabolic stress, resulting in rapid apoptosis, likely a consequence of their inability to sense energy stress and activate mechanisms to restore energy homeostasis [6]. Taking advantage of these observations, Shackelford and colleagues tested the therapeutic potential of phenformin in *LKB1*-deficient NSCLC experimental tumors. Phenformin as a single agent reduced tumor burden in *KRAS*/*LKB1* comutated murine NSCLC. In particular, LKB1 inactivation renders NSCLC cells unable to modulate anabolic processes in conditions of metabolic stress caused by phenformin. The constitutive activation of KRAS pathway forced cells to duplicate their DNA and other intracellular structures, thus accelerating energy depletion and damage to intracellular components and triggering apoptosis [71].

In a recent study, it has been speculated that the metabolic frailty of *KRAS*/*LKB1* comutated NSCLC cells could be exploited pharmacologically by the combination of metformin with compounds that increase intracellular stress by interfering with DNA replication and repair, such as platinum compounds [72]. Metformin has been demonstrated to induce apoptosis in *KRAS*/*LKB1* comutated experimental tumors. On the contrary, in $KRAS^{wt}/LKB1^{wt}$ cells or in the

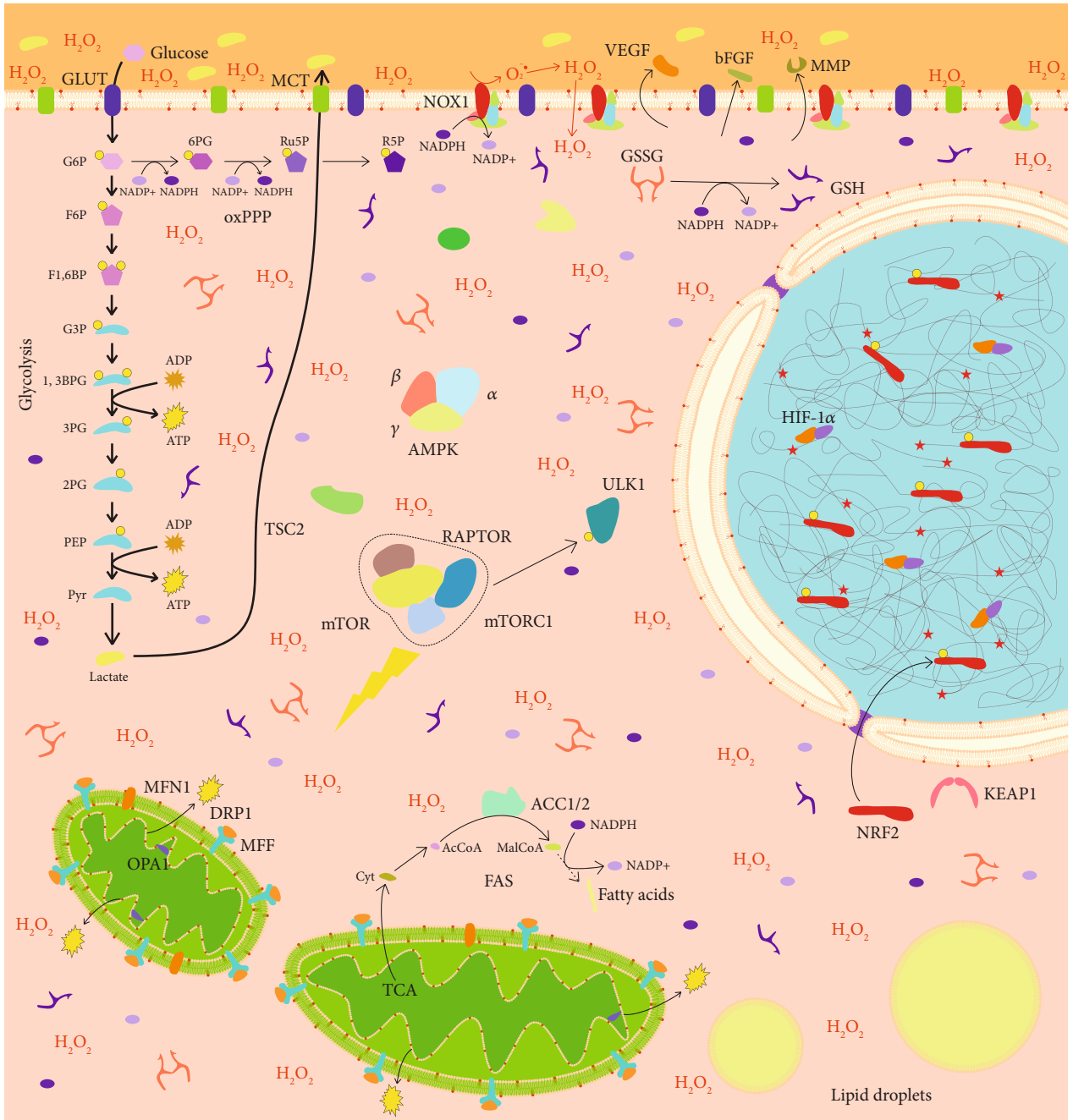

Figure 2: LKB1 loss alters cancer cell biology. LBK1 loss and consequent lack of AMPK activation lead to mTORC1 assembly, resulting in autophagy inhibition. High metabolic requirements imposed by sustained proliferation are met through aerobic glycolysis (i.e., Warburg effect), driven by HIF-1α stabilization, which provides cancer cells with ATP and intermediates for anabolic reactions (not shown). Pyruvate is preferentially converted to lactate, which is excreted in the tumor microenvironment. Activation of ACC1 and ACC2 promotes fatty acid synthesis in the cytosol, by using citrate coming from mitochondria. NOX1 expression drives the assembly of NADPH oxidase complex, which produces ROS in the microenvironment. NOX-produced ROS enter the cell, thus inducing oxidative stress and activating NRF2 through the oxidation of KEAP1. Reduced expression of MFN1 and OPA1 and increased activity of DRP1, induced by HIF-1α activation, lead to mitochondrial fragmentation. Increased ROS levels and mitochondrial fission promote the secretion of proangiogenic factors in the microenvironment. Yellow circles: phosphate groups. Red phospholipids in membranes: peroxidised phospholipids. Red stars in the nucleus: DNA damage sites. G6P: glucose 6-phosphate; F6P: fructose 6-phosphate; F1,6BP: fructose 1,6-biphosphate; G3P: glyceraldehyde 3-phosphate; 1,3BPG: 1,3-biphosphoglycerate; 3PG: 3-phosphoglycerate; 2PG: 2-phosphoglycerate; PEP: phosphoenolpyruvate; Pyr: pyruvate; 6PG: 6-phosphogluconate; Ru5P: ribulose 5-phosphate; R5P: ribose 5-phosphate; AcCoA: acetyl-coA; MalCoA: malonyl-coA; Cit: citrate; GLUT: glucose transporter; MCT: monocarboxylate transporter; GSH: reduced glutathione; GSSG: oxidized glutathione; H_2O_2: hydrogen peroxide; oxPPP: oxidative pentose phosphate pathway; TCA: tricarboxylic acid cycle; FAS: fatty acid synthesis. mTORC1 targets are omitted. See the text for details.

KRAS^{mut}/*LKB1*^{wt} experimental tumors, metformin determined activation of the LKB1/AMPK signaling pathway, thus reducing cell proliferation and metabolic requirements and preventing metabolic crisis in cancer cells. Treatment with metformin was also associated with enhanced cisplatin-induced *in vitro* proapoptotic and *in vivo* antitumor effects specifically in *KRAS*/*LKB1* comutated tumors [72].

The opportunity to target dysregulated metabolic features in *LKB1* mutated tumors could represent a strategy to improve therapeutic efficacy of other compounds affecting cell metabolism. In this regard, stable upregulation of glycolysis in tumor cells has been observed following antiangiogenic treatment [73], and as a master regulator of tumor cell metabolism and tumor microenvironment, LKB1/AMPK has a role in tumor response to VEGF neutralization [74]. Thus, sequential or simultaneous combination of antiangiogenic drugs and metformin might represent a new treatment opportunity for LKB1-deficient tumors. Although clinical and preclinical data are fragmentary, a case of a terminally ill patient with advanced endometrial cancer, showing radiological response to simultaneous administration of metformin and bevacizumab, was described by our group [75]. Interestingly, the high expression of MCT4—a marker of enhanced glycolysis—and loss of LKB1 expression were detected in the patient's liver metastasis sample. These findings suggest that metformin could modulate bevacizumab activity in tumors lacking LKB1 expression and deserves further validation in preclinical studies and clinical trials.

As previously described, autophagy represents a cellular process directed to preserve cellular homeostasis. Complementary with aforementioned findings, the ability to sense and counteract different types of stresses of LKB1 proficient tumor cells might be targeted by the combination of AMPK activators, such as metformin, and autophagy inhibitors, such as chloroquine, which has been recently repurposed as an anticancer agent [76]. Speculatively, this combination, currently evaluated in clinical trials [77], should potentiate the tumor suppressor activity of LKB1-AMPK by inhibiting its oncogenic prosurvival activity.

5.2. Targeting the Downstream Effectors of LKB1 Pathway

5.2.1. Inhibition of mTOR. Since LKB1 inactivation promotes mTORC1 signaling [46, 78] (Figure 2), mTOR inhibitors have been extensively tested as a therapeutic approach to target *LKB1* mutated tumors. However, preclinical studies produced controversial results. LKB1 inactivation in endometrial cancers resulted in high responsiveness to mTOR inhibitors [79], and rapamycin monotherapy (mTORC1 inhibitor) decreased polyp burden and size in LKB1$^{+/-}$ mice with polyposis [62]. In contrast, *LKB1* gene inactivation in NSCLC cells did not increase sensitivity to mTORC1 inhibitors, through negative feedback activation of AKT [80]. The same mechanism of escape to rapamycin could be at play in *Lkb1*-inactivated lung adenocarcinoma mouse model [81]. On the other hand, simultaneous inhibition of mTOR and glycolysis was significantly effective at reducing tumor volume and burden in a mouse model of spontaneous breast cancer promoted by loss of LKB1 in an ErbB2 activated model [82]. Given the master regulatory role of mTOR signaling in cell growth, additional preclinical and clinical studies are required in order to establish the appropriate genetic and molecular setting that could influence response to inhibition of mTOR pathway in the context of LKB1 status.

5.2.2. Inhibition of ACC Activity. *De novo* FA synthesis is essential to sustain rapid tumor growth, and reprogramming of lipid metabolism is a newly recognized hallmark of malignancy. Targeting altered lipid metabolic pathways has become a promising anticancer strategy [83]. Lipid-lowering drugs are being considered for clinical trials, showing their advantages in comparison with other anticancer drugs with high toxicity [83]. Since AMPK inhibits activity of ACC [32], the rate-limiting enzyme required for *de novo* FA synthesis, the latter might represent a potential metabolic target in tumors lacking LKB1. Inactivation of LKB1 in the adenocarcinoma mouse model determined accumulation of lipids and low levels of FA oxidation signature genes [81]. In preclinical models, ACC was required to maintain *de novo* FA synthesis needed for growth and viability of NSCLC cells, and its pharmacological inhibition results in robust inhibition of tumor growth [84]. Administration of ND-646—an allosteric inhibitor of the ACC enzymes ACC1 and ACC2 that prevents ACC subunit dimerization—as a single agent or in combination with the standard-of-care drug carboplatin markedly suppressed lung tumor growth in NSCLC xenograft from LKB1-deficient cells [84]. Effects of ACC inhibition on tumor growth fit its critical role in maintaining *de novo* FA synthesis and prompt further investigation to define new strategies to target LKB1-defective tumors.

5.3. Role of LKB1 in response to Therapy-Induced Oxidative Stress. ROS are signaling molecules that regulate several biological processes—such as autophagy, immunity, and differentiation—through reversible thiol oxidation [85]. On the other hand, excessive ROS levels induce irreversible modification of proteins, alongside with oxidation of lipids and nucleic acids, thus leading to oxidative stress and cell death [86]. Cell fate (i.e., growth arrest, proliferation, or death) is hypothetically decided by a ROS rheostat [87], which, in cancer cells, is set to intermediate levels to sustain tumor growth. A further increase in ROS levels induces extensive damage to cell structures and selective elimination of cancer cells, implying modulation of redox homeostasis as a promising anticancer strategy [88]. Several chemotherapeutic agents and radiotherapy, indeed, kill cancer cells by increasing ROS levels beyond the toxic threshold. Cisplatin [89], paclitaxel and other taxanes [90], doxorubicin [91], cytarabine [92], and arsenic trioxide [93] are some examples of traditional drugs that induce lethal oxidative stress in cancer cells. Moreover, several mitochondria-targeting compounds, such as capsaicin [94], betulinic acid [95], and curcumin [96], induce cancer cell death by increasing ROS levels.

Several studies reported that LKB1-AMPK pathway is involved in the maintenance of redox homeostasis by

contrasting ROS production and promoting ROS scavenging (Figure 1). Following metabolic stress, AMPK inhibits NADPH-consuming FA synthesis and increases NADPH-producing FA oxidation, thus maintaining elevated levels of NADPH, the universal electron donor used to regenerate ROS scavenging systems, leading to cancer cell survival [35]. ROS are able to activate AMPK, which, in turn, lowers ROS levels by inducing PGC-1α-mediated antioxidant response [97]. In response to ROS, AMPK activation also promotes glycolysis and pentose phosphate pathway (PPP), thus increasing NADPH levels [98]. Recently, it has been found that the mitochondrial NADPH pool is maintained by pathways other than the PPP [99]. AMPK activates Sirtuin-3 (SIRT3), which deacetylases isocitrate dehydrogenase 2 (IDH2), one of the principal contributors to NADPH production in mitochondria, thus increasing its activity [100]. Moreover, by increasing the activity of the tricarboxylic acid cycle and FA oxidation [7], AMPK could contribute to NADPH production in mitochondria through IDH2 and malic enzymes (ME) 2 and 3. LKB1 regulates oxidative stress response through p38-mediated upregulation of mitochondrial superoxide dismutase 2 (SOD2) and catalase, which scavenge ROS [101].

Given the established role of LKB1 and AMPK in maintaining redox homeostasis and the ability of ROS to kill cancer cells, one can speculate that functional LKB1-AMPK pathway could be a negative predictor of response to ROS-inducing therapies. Several evidences suggest that this is, in fact, the case.

In our recent work, we observed that LKB1 loss in NSCLC cells is associated with the increased expression of NADPH oxidase 1 (NOX1), leading to elevation of ROS levels (Figure 2) and exacerbated sensitivity to exogenous oxidative stress [102]. Preliminary results by our group indicate that LKB1 deficiency is associated with increased response to several ROS-inducing drugs commonly used in the clinic, such as arsenic trioxide, paclitaxel, and doxorubicin (Figure 3), thus suggesting that LKB1 status could predict tumor response to several chemotherapeutic regimens. Moreover, we found that LKB1-defective cancer cells undergo a decrease in reduced glutathione levels following exogenous oxidative stress and are more sensitive to cisplatin and γ-irradiation, compared with LKB1-proficient cancer cells. LKB1-defective NSCLC cells exposed to exogenous oxidative stress lose their mitochondrial membrane potential and undergo mitochondrial fragmentation, while LKB1-proficient cancer cells maintained polarized and fused mitochondria [103]. These results imply that LKB1-AMPK pathway exerts a protective effect towards oxidative stress, blunting the efficacy of ROS-inducing therapies. Remarkably, low-null LKB1 expression by IHC was retrospectively associated with the improved outcome in advanced NSCLC patients treated with first-line platinum-based chemotherapy [104]. This finding may be explained by considering the well-established role of LKB1 as a genomic sensor participating in the DNA damage response triggered by oxygen radicals. Consistently, LKB1-defective cells exposed to exogenous oxidative stress showed extensive macromolecular damage, measured as membrane lipid peroxidation, accumulation of nucleic acid oxidation marker 8-oxoguanine in mitochondrial DNA, and accumulation of DNA damage marker phosphorylated histone 2AX (γH2AX). Strikingly, LKB1-defective cells demonstrated oxidation of mitochondrial DNA even under basal culture conditions, alongside with more fragmented mitochondria compared to LKB1-proficient cells. These findings support that LKB1 and AMPK protect cells from excessive oxidation of lipids and nucleic acids both by decreasing NOX-mediated ROS production and by increasing ROS scavenging, thus blunting the efficacy of anticancer therapies aimed at impairing redox homeostasis. In line with our findings, Li and colleagues observed that LKB1 loss in lung adenocarcinoma is associated with increased ROS levels, which drive cancer plasticity and drug resistance through transdifferentiation to squamous cell carcinoma in the *KRAS-LKB1-* (KL-) mutant lung cancer mouse model [81]. Squamous cell carcinoma, compared to adenocarcinoma, upregulated the expression of genes involved in the metabolism of glutathione and of NRF2 target genes, thus reducing DNA oxidation. Interestingly, Li and colleagues observed an inverse correlation between LKB1 expression and 8-oxoguanine levels in human NSCLC, where a proportion of cells with LKB1 loss and high 8-oxoguanine staining expressed squamous cell carcinoma markers. Reexpression of AMPK in the KL adenocarcinoma model decreased ROS levels and DNA oxidation by increasing FA oxidation-derived NADPH production, indicating the involvement of AMPK in LKB1-mediated ROS decrease, according to our findings [103]. Interestingly, Li and colleagues observed that treatment with phenformin in KL model resulted in the selective survival of squamous cell carcinoma clones and in transdifferentiation of adenocarcinoma to squamous cell carcinoma. Findings from Li and colleagues imply that LKB1 loss in adenocarcinoma could select for clones resistant to oxidative stress through increased activity of the transcription factor NRF2. Interestingly, KEAP1 is frequently inactivated in NSCLC (about 20% of cases [105]), and LKB1-defective tumors have more than sixfold increased odds of bearing KEAP1 loss compared to LKB1-proficient cancers [106]. Consequently, LKB1 loss is frequently associated with aberrant activation of NRF2 pathway, which drives aggressiveness and resistance to therapy. Constitutive NRF2 activation in cancer is connected with transcriptional programs aimed at increasing NADPH and glutathione levels, such as the serine synthesis pathway [107], which fuels mitochondrial folate cycle, the principal contributor to NADPH production in cells [99]. Thus, constitutive NRF2 activation is frequently coselected with LKB1 loss in human cancers to compensate for increased oxidative stress induced by lack of AMPK activation.

5.4. Role of LKB1-AMPK in Therapy-Induced Senescence. Different types of stress, such as oxidative or oncogenic stresses, can induce an irreversible cell cycle arrest. Permanent blockade of cell proliferation, known as senescence, is a valuable anticancer strategy that could be achieved through sublethal chemotherapy and irradiation. High doses of chemotherapeutics or radiation cause massive

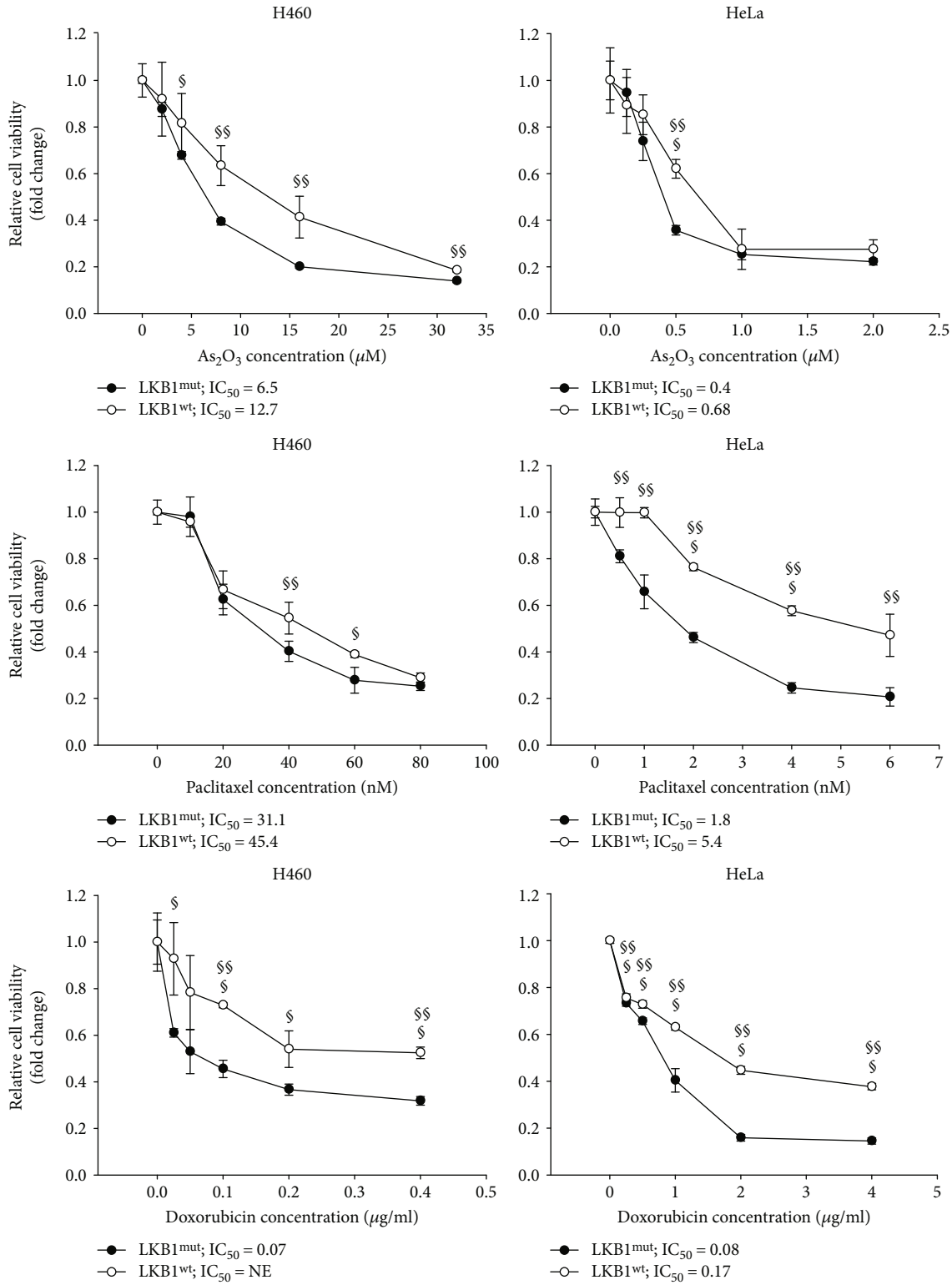

Figure 3: LKB1 expression regulates response to oxidative stress induced by prooxidant cytotoxic drugs. Isogenic pairs of H460 and HeLa cells (derived from NSCLC and cervical carcinoma, respectively) differing in LKB1 status and generated as described by Zulato et al. [103] were treated with arsenic trioxide, paclitaxel, or doxorubicin for 48 h. Viability was evaluated by the Sulphorhodamine B assay (for materials and methods, refer to [103]) in cells exposed to increasing concentrations of drugs. For each cell line tested, the IC_{50} values relative to $LKB1^{mut}$ and $LKB1^{wt}$ cells are reported. Results are representative of three independent experiments performed in triplicate ($^{\S}P < 0.05$, $^{\S\S}P < 0.01$, and $^{\S\S\S}P < 0.001$ $LKB1^{wt}$ versus $LKB1^{mut}$ cells). Results of SRB assay revealed that H460 and HeLa $LKB1^{wt}$ variants were more resistant than their $LKB1^{mut}$ counterparts to the drugs tested. NE: not evaluable.

damage to cell structures, leading to cell death not only in cancer cells but also in highly proliferating normal cells. On the contrary, low doses of anticancer drugs or radiation lead to therapy-induced senescence (TIS) only in cancer cells, thus decreasing side effects [108]. Noteworthily, several chemotherapeutics, including cisplatin, doxorubicin, etoposide, and resveratrol, induce senescence in cancer cells [109].

Contrasting data regarding the role of AMPK on senescence induction are reported in the literature. As oxidative stress is a senescence inducer and AMPK is involved in the maintenance of redox homeostasis, it is not surprising that LKB1-AMPK pathway could prevent senescence in cancer cells [110]. Han and colleagues observed that hydrogen peroxide-induced senescence is associated with inhibition of AMPK. Furthermore, pharmacological activation of AMPK prevented the induction of senescence by oxidative stress, through restoration of autophagy. Interestingly, the authors observed that inhibition of autophagy through chloroquine aggravated senescence induced by hydrogen peroxide and blunted the protective role of AMPK activation. Moreover, NAD^+ levels are decreased in senescent cells as a consequence of NAD^+ salvage pathway reduction and increased NAD^+ consumption by PARP-1. Pharmacological activation of AMPK promoted synthesis of NAD^+ through salvage pathway, thus increasing the activity of NAD^+-consumer SIRT1, which positively regulates autophagy. The results from Han and colleagues have important implications for cancer therapy. First, AMPK could have a protective role against TIS when the latter arises from a chemotherapeutic regimen that triggers oxidative stress. In this regard, metformin could increase the efficacy of chemotherapy, as described above, but could impair TIS, thus favouring the burden of surviving cells and tumor relapse. Second, autophagy emerges as an important escape mechanism from TIS, confirming its central role in the oncogenic properties of LKB1-AMPK pathway. The use of chloroquine or other inhibitors of lysosomal acidification in the clinic should enhance TIS, thus achieving remarkable anticancer activity.

On the other hand, the activation of SIRT1 and AMPK has been associated with the induction of senescence in colorectal carcinoma cells [111]. Jung and colleagues observed that aspirin induced senescence in two colorectal carcinoma cell lines, but not in normal colonic cells, through the increased expression and deacetylase activity of SIRT1 and the increased activation of AMPK. The enhanced activity of SIRT1 and AMPK was induced by a decrease of ATP levels in aspirin-treated cancer cells, as observed with irradiation. Interestingly, the authors demonstrated that knockdown of SIRT1 or inhibition of its deacetylase activity decreased aspirin-induced and irradiation-induced senescence. The same results were obtained through knockdown or inhibition of AMPK. On the contrary, activation of SIRT1 through resveratrol or of AMPK through AICAR promoted the induction of senescence. The data from Jung and colleagues are consistent with the known senescence-inducing activity of resveratrol. Thus, it is reasonable that in certain cellular contexts SIRT1 and AMPK induce senescence rather than inhibit it, as observed by Han and colleagues. The decreased levels of ATP observed in aspirin-treated cells, however, suggest that in this context autophagy could not play a central role. Although aspirin induces autophagy [112], it is possible that the latter was a rescue mechanism only in the context described by Han et al., thus profoundly altering the outcome of AMPK activation. The positive role of LKB1-AMPK pathway on senescence is supported by different studies. Yi and colleagues observed that low doses of metformin induced senescence of hepatoma cells through activation of AMPK [113]. Metformin also induced the acetylation of p53 as a consequence of AMPK-mediated inhibition of SIRT1 deacetylase activity on p53. Similarly, Liao and colleagues demonstrated that AMPK activation is involved in the metabolic alterations associated with radiation-induced senescence [114].

In conclusion, AMPK positively regulates TIS, implying that LKB1-proficient tumors could be more susceptible to a radiochemotherapeutic regimen that induces senescence. It should be considered, however, that AMPK-induced autophagy could be an escape mechanism that impairs TIS, thus curbing the efficacy of anticancer treatments. In this regard, a recent study provides evidence for a role of AMPK as a predictive factor of response to senescence-inducing therapies. In fact, Wang and colleagues observed that trametinib radiosensitized LKB1-defective NSCLC cells, while LKB1-proficient cells were protected by senescence through AMPK-mediated autophagy [115]. The central role of autophagy as a rescue mechanism—as recently confirmed by the observation of autophagy-mediated protumorigenic effects in the context of mitotic slippage-induced senescence [116]—suggests that the use of chloroquine in association with senescence inducers should be considered in the clinic.

Interestingly, as cancer cells could recover from senescence and senescent cells secrete soluble factors that promote tumor growth [117], the use of drugs that selectively kill senescent cells (known as senolytics), such as the BCL-xL inhibitor navitoclax, in combination with senescence inducers and chloroquine should be a highly effective anticancer strategy against both LKB1-proficient and defective cancers.

6. Exploiting Selective Vulnerabilities in LKB1-Defective and LKB1-Proficient Tumors

A great effort focused on the identification of novel potential therapeutic targeting in highly aggressive *LKB1*/*KRAS* comutated NSCLC. Kim and colleagues tested 230,000 synthetic small molecules in a panel of 91 lung cancer-derived cell lines, identifying coatomer complex I (COPI) as necessary for the survival of *LKB1*/*KRAS* double mutant NSCLC. COPI is involved in the acidification and maturation of lysosomes, essential organelles in the maintenance of proper mitochondrial function. In fact, LKB1 inactivation and KRAS activation drive dependency on autophagy to fuel the Krebs cycle with carbon sources [118]. These interesting findings imply that autophagy inhibition through chloroquine, which blocks lysosome acidification, could be highly effective in killing *LKB1*/*KRAS* comutated NSCLC cells through the induction of mitochondrial dysfunction. Notably, although chloroquine has been tested in some studies aimed at targeting NSCLC [119–122], no reports in the literature refer to

LKB1/*KRAS* mutations as a patient stratification criterion for the treatment of NSCLC.

Deoxythymidilate kinase (DTYMK) silencing has been identified as synthetically lethal with LKB1 loss in *LKB1*/*KRAS* double mutant NSCLC [123]. DTYMK catalyses the conversion of deoxythymidine monophosphate (dTMP) to deoxythymidine diphosphate (dTDP) and plays a fundamental role in nucleotide synthesis. Liu and colleagues demonstrated that LKB1 loss is associated with deficits in nucleotide metabolism. DTYMK inhibition in *LKB1*-mutated NSCLC cells leads to dUTP misincorporation in DNA, thus blocking replication. As dTMP derives from folate cycle-mediated conversion of deoxyuridine monophosphate (dUMP), hypersensitivity of *LKB1*-mutant tumors to antifolates, such as pemetrexed, raltitrexed, or pralatrexate, can be speculated. To the best of our knowledge, therapeutic efficacy of antifolates in *LKB1*-mutant lung cancer has not been evaluated in patients so far.

Another selective vulnerability in *LKB1*-mutated cancer cells is related to endoplasmic reticulum (ER) stress. Pharmacological induction of ER stress in *LKB1*/*KRAS* double mutant cancer cells triggers proapoptotic unfolded protein response and ROS-induced cell death [124]. HSP90 inhibitors and the proteasome inhibitor bortezomib are ER stress inducers currently used in the clinic. Cron and colleagues observed that proteasome inhibitors radiosensitize *LKB1*/*KRAS* double mutated NSCLC cell lines [125]. However, radiosensitization by bortezomib is a consequence of the accumulation of damaged proteins, which likely occurs independently from LKB1 status. It was observed that inactivation of LKB1 is associated with increased sensitivity to the HSP90 inhibitor 17-AAG [126–128]. Unfortunately, HSP90 chaperone protects LKB1 from proteasomal degradation [129], raising safety concerns about the exposure of normal cells to HSP90 inhibitors. Consequently, only bortezomib is a safe ER stress inducer, and more efforts should be devoted to the investigation of its efficacy in *LKB1*-mutated cancers.

Given the role of LKB1 in the maintenance of genomic integrity through the regulation of homologous recombination, its inactivation sensitizes cancer cells to PARP inhibitors [19]. PARP-1 is involved in the repair of single-strand breaks through the base excision repair (BER) pathway [130]. Ablation of PARP leads to the conversion of single-strand breaks to double-strand breaks during DNA replication, inducing cell death in homologous recombination-defective *LKB1*-mutated cancer cells. PARP inhibitors are promising anticancer drugs, some of which have been approved by the Food and Drug Administration (FDA) for the treatment of *BRCA*-mutated cancers. The use of PARP inhibitors in *LKB1*-mutated human cancers holds promise of therapeutic efficacy.

Some evidence suggests that LKB1 loss is involved in the upregulation of antiapoptotic proteins of the B-cell lymphoma 2 (BCL-2) family [131, 132], implying mitochondrial priming in LKB1-defective cancer. In particular, the activation of mTORC1 in LKB1-defective tumors drives the overexpression of myeloid cell leukemia 1 (MCL1) [62, 133]. In the last decade, a novel class of drugs, BH3 mimetics, was developed. BH3 mimetics mimic the structure of BH3 domain in BCL-2 family proteins, thus displacing proapoptotic BH3-only proteins from antiapoptotic proteins and inducing apoptosis. The upregulation of antiapoptotic proteins of the BCL-2 family following LKB1 loss suggests that LKB1-defective cancers could be sensitive to BH3 mimetics, particularly to MCL1 inhibitors, some of which—such as AZD5991—are currently in clinical trials for the treatment of hematological malignancies. The combination of MCL1 inhibitors with the BCL-2 specific inhibitor venetoclax should be effective against *LKB1*-mutated cancers and should induce a pronounced sensitization to standard chemotherapy.

Additional vulnerabilities in LKB1-defective cancers are even more speculative. The increased activation of NF-κB and STAT3 pathways due to LKB1 loss could drive sensitivity to NF-κB and STAT3 inhibitors in clinical trials, such as TAS4464 and TTI-101, respectively. Inhibition of these pathways should increase mitochondrial fragmentation and sensitivity to conventional therapies.

In contrast, figuring out selective vulnerabilities in LKB1-proficient cancers is not obvious. However, autophagy inhibition seems to be the most promising strategy to target drug resistance following AMPK activation, as mentioned above. In fact, the central role of ULK1 phosphorylation in the induction of angiogenesis, in the clearance of damaged mitochondria, and in maintenance of mitochondrial metabolism provides the rationale of targeting VPS34 kinase, whose activity is promoted by ULK1-mediated phosphorylation of Beclin-1. SAR405, a recently identified specific inhibitor of VPS34 kinase activity, inhibits fusion of late endosomes with lysosomes and autophagosome formation, exerting synergistic anticancer activity with the mTOR inhibitor everolimus in renal cancer cell lines [134]. Inhibition of autophagosomes leads to the accumulation of damaged and dysfunctional mitochondria, increasing the accumulation of mitochondrial ROS and inducing cell death [135]. Autophagy inhibition in LKB1-proficient tumors can be achieved with chloroquine, with some anticancer effects. However, blockade of lysosomal acidification does not impede engulfment of mitochondria in autophagosomes, which results in isolation of damaged mitochondria from the mitochondrial network. Moreover, ROS produced by damaged mitochondria inside autophagosomes must overcome two lipid membranes to reach the cytosol; thus, engulfed mitochondria release less ROS than free mitochondria.

Activated AMPK phosphorylates NRF2, thus promoting its nuclear accumulation [136]. The resulting activation of an antioxidant program is responsible for the resistance to oxidative stress observed in LKB1-proficient cancers. In fact, NRF2 activates the transcription of genes involved in the production of NADPH and induces cytoprotective autophagy [137]. Speculatively, pharmacological NRF2 inhibition should revert the resistance of LKB1-proficient tumors to ROS-inducing therapies, increasing lipid peroxidation, DNA damage, loss of mitochondrial membrane potential, and mitochondrial fragmentation, ultimately leading to cell death.

In conclusion, amongst several vulnerabilities affected by LKB1 status, dependency on cytoprotective autophagy and

on NRF2-driven antioxidant response is shared by LKB1-proficient cancers and by LKB1-defective cancers driven by additional genetic alterations (i.e., activation of KRAS and loss of KEAP1).

7. Concluding Remarks

In the era of personalized medicine, the key role of LKB1 as a central sensor of stress opens new possibilities to target cancer cell metabolism, with important clinical implications.

The precise definition of LKB1 status represents a challenge for patient stratification. A comprehensive approach considering genetic, epigenetic, and LKB1 protein expression analysis should be taken into account.

Cancer cell metabolism is plastic and adaptable, and LKB1 plays a central role in its modulation (Figure 1). Several evidences pointed out its contextual oncogenic and tumor suppressor role. Moreover, a key function of LKB1 in modulation of tumor microenvironment is emerging. LKB1 loss is associated with a metabolic deregulation (Figure 2) that could be exploited from a therapeutic point of view.

Therefore, a better understanding of the pathways presided over by LKB1, through metabolomics and proteomics analyses, together with LKB1 status evaluation, is required to develop personalized treatment strategies. Such an approach could help to unravel the heterogeneity of cancer and to identify concurrent pathway alterations which could be targeted to overcome acquired resistance to molecular targeted therapies.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Ciccarese F. and Zulato E. contributed equally to this work.

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Review Article

Cancer Etiology: A Metabolic Disease Originating from Life's Major Evolutionary Transition?

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A clear understanding of the origins of cancer is the basis of successful strategies for effective cancer prevention and management. The origin of cancer at the molecular and cellular levels is not well understood. Is the primary cause of the origin of cancer the genomic instability or impaired energy metabolism? An attempt was made to present cancer etiology originating from life's major evolutionary transition. The first evolutionary transition went from simple to complex cells when eukaryotic cells with glycolytic energy production merged with the oxidative mitochondrion (The Endosymbiosis Theory first proposed by Lynn Margulis in the 1960s). The second transition went from single-celled to multicellular organisms once the cells obtained mitochondria, which enabled them to obtain a higher amount of energy. Evidence will be presented that these two transitions, as well as the decline of NAD+ and ATP levels, are the root of cancer diseases. Restoring redox homeostasis and reactivation of mitochondrial oxidative metabolism are important factors in cancer prevention.

1. Introduction

Could cancer causation be interpreted as an allegory not to the damaged hardware (damaged genetic material caused by chance mutation) but to an incorrect function of a software (a metabolic program)? Do we thence use wrong approaches to treat the cancer disease with chemotherapy and radiation therapy, which are aimed at destroying the hardware (killing cells), instead of a more sophisticated approach aimed at reprogramming the software inside the cells in order to restore the normal mitochondrial function and metabolism?

There are carcinogenic and tumorigenic cells with zero mutations [1], and there are many somatic mutations in cancer-driver genes in healthy tissue, which does not become a cancer [2], with so-called driver mutations [3]. Furthermore, experiments on the nucleus and mitochondrial transfer revealed that tumorigenic phenotype is upgraded when tumor mitochondria are transferred to a normal cell cytoplasm and vice versa. This can be illustrated by the transplantation of noncancerous mitochondria which can inhibit tumor properties of metastatic cells [4–9]. Additionally, tumorigenesis may be suppressed by normal mitochondrial function [10–12], and metabolic enzymes of the Krebs cycle have been recognized as oncosuppressors [13].

Both abnormalities in tumor suppressor genes (anticogene acting to inhibit cell proliferation and tumor development) and oncogenes can be caused by impaired mitochondrial function [14]. Aerobic glycolysis of tumors is in some measure displayed by activation of oncogenes or absence of tumor suppressors, which are then additionally intensified by stabilization of the hypoxia-inducible factor (HIF) [15], which encodes for all of the glycolytic enzymes. It seems that fully operating mitochondria regulate apoptosis by releasing cytochrome c [16] and suppressing genes of cancer-like metabolism, which have been conserved from 500,000 million years ago and persist in cells of multicellular organisms. Such a program, which enables the development of cancer, preexists in genes in the nucleus from the season of low O_2 atmosphere and single-celled life. Namely, cancer cells shift their metabolism toward glycolysis, a strategy that allows for their survival when oxygen is limited [17], and

consequently increase the availability of biosynthetic intermediates needed for cellular growth and proliferation [18]. Du [19] proposed a hypothesis that "the survival style of cancer cells was the reevolution from eukaryotic to prokaryotic cells by the alteration of energy metabolism." A human body is a sum of colonies of cells and their mitochondria. The cells composing the human body are similar to single-celled eukaryotes (existing 500,000 million years ago) although human cells can no longer survive on their own and generally do not use the primitive source of energy, e.g., substrate-level phosphorylation, to produce ATP. The first life emerged on Earth around 3.5 billion years ago, when the early biosphere was more reduced. The increased amounts of dioxygen (O_2) emerged approximately 2.4 billion years ago when cyanobacteria, as a product of oxygenic photosynthesis, triggered the "Great Oxidation Event" [20]. Due to the elevated O_2 in the atmosphere, methods of mitigating its toxicity inside cells had to evolve [21], and the existing metabolic pathways had to be reshaped in early aerobic organisms, which adapted to use O_2 as a high-potential redox couple. Multicellular life appeared more than a billion-and-a-half years ago, and the Cambrian explosion (somewhere around 542 million years ago) resulted in the divergence of major animal groups. Both metabolic transitions have allowed divergence of life forms on Earth, but evolution has not provided a way to prevent the onset of cancer. Since the entire history of humanity, with the exception in the last 100 years, the average lifespan was between 20 and 30 years; consequently, there might not be much evolutionary pressure to eradicate cancer as a disease of mostly elderly persons.

1.1. Somatic Mutation Theory vs. Metabolic Impairment Theory/Mitochondrial Theory of Cancer. At present, cancer is regarded a genetic disease arising from numerous mutations in oncogenes and tumor suppressor genes. Are gene mutations in the cell nucleus the causal event in the origin of cancer (as suggested by the somatic mutation theory) or is the damaged genetic material just the consequence and not the primary cause of cancer? Is cancer caused by damaged mitochondria (impaired mitochondrial function) and metabolic dysfunction, which activates the divergence of the glucose metabolism away from the energy production and stimulates cell growth (transition from oxidative phosphorylation to glycolysis/fermentation)? Is it genomic instability or debilitated energy metabolism that is essentially in charge of the cause of cancer? While tumor growth could be explained by the classical multistage model of carcinogenesis, the model does not provide rationale for the beginning of tumor development [22]. In the last 50 years, it has been accepted that initiation is the one event during which one or more mutations transform a normal somatic cell into a latent neoplastic cell, that is, a tumor cell still lacking multiplicative autonomy. This phase is then followed by promotion in which further mutations and proliferative stimuli induce the initiated cell to give rise to the progeny constituting the tumor. However, it remains to be elucidated what is the effect and what is the cause of normal-to-tumor cell transformation. Cancer was primarily considered as a type of somatic genetic disease in accordance with Boveri's cancer theory [23, 24] where harm to a cell's nuclear DNA underlies the change of a normal cell into a cancer cell [25–27]. Indeed, multiple and heterogeneous mutations are found in cancer cells [22]. The question however remains whether DNA mutations are the initiating event causing cancer or are they merely necessary contributors to the progression of tumor after its initiation? Are we battling cancer from the right front considering the hypothesis that DNA mutations as drivers are not that significant in initiation of tumors? Can tumors arise with regular division and mutation rates? Namely, spontaneous mutations are of the order 10^{-5} [28]. Estimated probability of mutating five genes, such as both alleles of a particular tumor suppressor gene and an oncogene, is 10^{-20} [29]. Thus, in terms of genetic hits in one cell, it is difficult to explain cancer formation as a result of the acquirement of random genetic mutations.

On the other hand, Seyfried et al. [27] explain cancer as essentially a metabolic disease related to disturbances in energy production through respiration and fermentation. According to the metabolic impairment theory/mitochondrial theory of cancer [4, 27, 30–34], cancer can best be explained as a class/kind of mitochondrial disease. As indicated by Warburg's hypothesis, cancer cells emerge from normal body cells through steady and irreversible harm to their respiratory capacity. Just those body cells which are able to increase glycolysis during intermittent respiratory damage are viewed as fit for forming cancers [31, 32]. The gene theory of cancer suggests that dysfunctional mitochondria could be the resultative and not the causative factor of cancers. On the other hand, the metabolic impairment theory indicates the contrary. Abnormal energy metabolism characterises most tumor cells in all types of tissues [14]. It was further observed that genes for glycolysis are excessively expressed in the major part of cancers explored into [35, 36]. What is more, the cancer cell metabolism is regulated also by metabolic oncogenes and tumor suppressor genes (e.g., K-ras, p53, PI3K, Akt, and MYC) which have evolved to regulate the Warburg effect [37]. Several studies indicate that the structure and function of tumor mitochondria are not normal and as such not capable to generate the adequate levels of energy [38–47]. The mitochondrial structure is intimately related to mitochondrial function. Abnormalities in both the content and composition of mitochondria have been observed in different tumor tissues *in vivo*. On the contrary, in different human and animal tumor cells, when they are grown in the *in vitro* conditions, in contrast to structural defects, reduced numbers or the absence of mitochondria is commonly not observed [27]. Moreover, some researchers observed that in different tumor types, mitochondria and OXPHOS are normal. However, such results were noticed mainly from the *in vitro* studies measuring oxygen consumption rates in tumor cells [48–53]. Already half a century ago, Warburg suggested that oxygen consumption could be comparable in normal and tumor cells although ATP formation is significantly lesser in tumor cells. The fact that the oxygen consumption rate can be similar or even greater in cultured tumor cells than in nontumorigenic cells was claimed also by different other authors [40, 54, 55]. However, it has been established that the oxygen consumption rate alone cannot

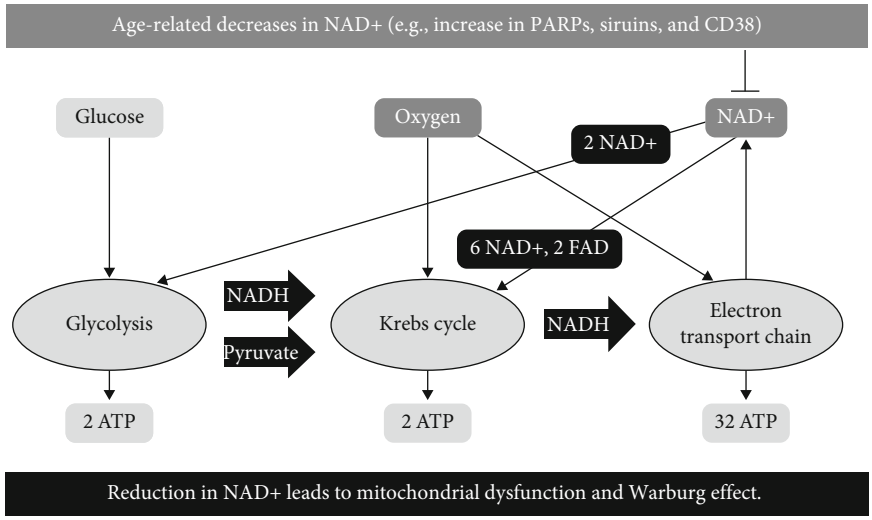

Figure 1: O_2 and NAD+ as limiting factors in driving oxidative phosphorylation. The figure presents a hypothesis that in situations with limited availability of NAD+, the cells will activate the program which switches off Krebs cycle and electron transport chain (process consumes 6 NAD+) and favors glycolysis (process consumes 2 NAD+) in order to obtain energy, preserve NAD+, and avoid cell death through reduced ATP production and activation of apoptosis. *Abbreviations: PARP: poly(adenosine diphosphate [ADP] ribose) polymerases; CD38: NAD+ glycohydrolases; sirtuins: NAD-dependent histone deacetylase ("HDAC") enzymes.

be considered as an indicator of coupled respiration. This can be explained by the fact that some tumor cells consume oxygen while the glycolytically derived ATP is imported and hydrolysed through the mitochondrial adenine nucleotide transporter 2 so as to preserve the proton motive gradient [56]. Moreover, the cultured cell lines are usually derived from only a single cell or a few cells of a heterogeneous tumor. It can be concluded that mitochondria might appear functionally normal in many types of cultured tumor cells but appear structurally abnormal when evaluated in the tumor cells of many primary malignant cancers.

1.2. Mitochondrial Substrate-Level Phosphorylation (mSLP) Provides Energy Source for Cancer Cells: The Missing Link in Warburg's Theory. Reduced ATP formation through impaired oxidative phosphorylation or hypoxia must be compensated by tumor cells with an alternative source of energy. Glucose and glutamine represent available fermentable fuels, since acetate and branched chain amino acids are not present in adequate quantities and other amino acids can be used only with the presence of high-energy phosphates for the metabolic conversion to succinyl-CoA, which is the substrate for mSLP [57]. mSLP produces high-energy phosphates through glutaminolysis and represents a compensatory energy mechanism for cancer cells with insufficient or defective OXPHOS [58]. According to Seyfried et al. [57, 58], the missing link in Warburg's theory is the succinic acid fermentation which uses glutamine as a major substrate through sequential conversion of glutamine → glutamate → alpha-ketoglutarate → succinyl-CoA → succinate.

1.3. Deficiency of Energy: From Respiration to Fermentation. In order to enable multicellular life, cells must adapt to strict control of cell division and differentiation. Such cooperation works until there is enough energy supply in the form of NAD+ and ATP. However, both NAD+ levels and energy production in the form of ATP decline with age [59–61], and the incidence of many types of cancer increases with aging [62, 63].

Age-related decline of NAD+ leads to mitochondrial dysfunction (Figure 1), which leads to the Warburg effect [64]. NAD+ or NAD+/NADH ratio can have an impact on the frequency of DNA mutation, epigenetic changes in DNA, and also metabolic programming [65]. The role of NAD+ is in accepting hydride equivalents, from glycolytic and TCA cycle metabolites, to form reduced NADH, which enables mitochondrial electron transport chain (ETC) to fuel oxidative phosphorylation [66]. In addition, high NAD+ levels regulate SIRT activity which influences metabolism, DNA repair, stress resistance, cell survival, inflammation, mitochondrial function, and lipid and glucose homeostasis, by targeting FOXO, PGC-1α, p53, NF-κB, HIF-1α, and many other cellular targets [65].

According to Warburg's theory of cancer, the energy through fermentation gradually compensates for insufficient respiration [31, 67] which allows a cell to stay alive. NAD+ content is a basic protective factor at the beginning of carcinogenesis, and decreased NAD+ intracellular concentration might play a significant role in the process of cancer development by limiting energy production which negatively affects genomic stability by altering responses to stress and efficiency of the DNA repair [65, 68].

1.4. Potential Protumorigenic Side Effects of Increased NAD+. NAD+ can act as both pro- and antitumorigenic due to its mediated reactions on the mechanism of apoptotic cell death and inflammation. Different inflammatory soluble molecules secreted by senescent cells that could promote tumor growth and progression as well as NAD+ metabolism might influence the senescence-associated secretory phenotype (SASP)

as discussed in the recent paper of Nacarelli et al. [69]. In their research, it was shown that increased NAD+ influences the inflammatory signaling of senescent cells in vivo in mouse models of pancreatic and ovarian cancers through the higher HMGAs and nicotinamide phosphoribosyltransferase (NAMPT) expression, which promotes the proinflammatory SASP through NAD+-mediated suppression of AMPK kinase, leading to suppression of the p53-mediated inhibition of p38 MAPK and enhanced NF-κB activity [69]. Moreover, FK866, a compound which inhibits nicotinamide-recycling enzyme NAMPT/PBEF, which is the bottleneck for NAD biosynthesis, resulted in anticancer effect [70] as a tumor apoptosis inducer due to NAD+ depletion [71].

It seems that NAD+ levels are a critical protective factor in early carcinogenesis and might become a detrimental factor later in the cancer progression and promotion phase. Namely, during cancer promotion, progression and treatment-increased NAD+ levels could have deleterious effects on the malignancy process due to increased cell survival, growth advantage, increased resistance to radio and chemotherapy, and promotion of inflammation (reviewed in [65]). The tumor promoting vs. inhibiting properties of NAD+ depend on the stages of cancer development and NAD+ concentration/time-dependent activation of PARPs and sirtuins, which interfere with the cell survival. Sirtuins and PARPs could have both procancer and anticancer effects, and their role in cancer prevention and promotion remains to be fully elucidated [72–77].

1.5. Cancer and Mitochondrial Damage. There are many environmental agents (e.g., radiation, pollutants, and hypoxia) that humans are exposed to through their lives which damage mitochondria and cellular respiration through increased generation of reactive oxygen species (ROS). Therefore, ROS-induced damage to the respiratory system promotes a hypoxic-like state [31], stabilizes the transcription factor HIF, and upregulates glucose transporters into the cell. Additionally, oncogenes have to turn on because they are the transcription factors that upregulate the transporters for glucose and glutamine. The efficiency of mitochondrial oxidative phosphorylation decreases with age, and pseudohypoxia increases which leads to increased apoptosis (every day, 50-70 billion cells of a human body activate apoptotic death). However, in rare cases, a "renegade cell" decides not to sacrifice itself and undergo apoptotic cell death for higher purposes—to preserve the organism. Contrarily, in order to preserve its own life, a "selfish renegade cell" activates a prehistoric program in order to obtain enough energy levels. The aforementioned program activates fermentation and consequently shuts down genomic stability, tumor-suppressive control mechanisms, and mitochondrial apoptotic response [78] allowing such a cell to enter its primitive state. Activation of such processes results in a higher entropy state level inside the cell. A typical cell is a highly ordered low entropy system and invests much energy to keep the entropy of the system low. So as to keep up stable entropy, which is far from thermodynamic balance, living systems use information and energy. Energy loss due to impaired mitochondria limits supply of energy invested for damage repair, and genomic stability increases entropy and impairs order of the cell organization. Namely, glycolysis generates only two moles of ATP per one mol of glucose whereas oxidative phosphorylation generates about 36 mols of ATP per mol of glucose [79] (Figure 1). Hence, carcinogenesis represents a reverse process with the progressive functional decline, disordered morphology, and accumulation of mutations. Energy restriction due to mitochondrial dysfunction might represent the metabolic initiator that "triggers the genetic mutations that drive the somatic evolution of the malignant phenotype" [80].

In cases of glucose deprivation, efficient glucose consumption and catabolism are critical for survival. It was observed that cells switch to glycolysis in combination with lactate dehydrogenase as an adaptation to limited glucose availability [81]. When NAD+ levels within the cell become critically limited, both the TCA cycle in the mitochondria and glycolysis in the cytoplasm can be halted. Despite having an excess of available glucose, this can lead to cell death [82–85]. A less severe reduction in NAD+ levels (e.g., from 30 to 85%) has been observed in the muscle tissue of aged mice with an associated deterioration in mitochondrial function but not glycolysis [6, 64, 86–88]. It seems that cytoplasmic NAD+ pool is less susceptible to scarcity since "cytoplasmic NAD/NADH ratios range between 60 and 700 in a typical eukaryotic cell, while mitochondrial NAD/NADH ratios are maintained at 7 to 8" [89, 90]. The availability of NAD+ is thus critical for mitochondrial function [91–93].

1.6. Is the "Default" Metabolic Program Incorporated in the Cells of Multicellular Organisms' Glycolysis or Oxidative Phosphorylation? It seems that cancer does not develop as a result of hypoxia due to damaged mitochondria or cell mass growth (hypoxic regions of tumors) that leads to impaired aerobic respiration as was first hypothesized by Warburg [31]. Some studies suggest that mitochondria are not damaged in some cancer cells [94–96], as discussed in the previous paragraph, and cancer cells seem to use glycolytic metabolism prior to the exposure to hypoxic conditions [97] as observed in leukemic cells [50, 98] and lung tumors which use aerobic glycolysis even though these tumor cells are exposed to high oxygen levels during tumorigenesis [99, 100]. Alteration in the metabolic switch to the aerobic glycolysis by cancer cells may thus result in the prehistoric (re)program that reverses premalignant cells to an embryonic program that supports cell growth by nutrient acquisition and metabolism. Before oxygen was formed in the atmosphere, proliferation and fermentation was the dominant phenotype and the default state of metazoan cells [101]. According to Szent-Györgyi [101], cancer is a condition of unrestricted cell development, which is typical of free-living cells 500,000 million years ago, before the existence of multicellular life. Cancer is a normal growth from before half a billion years ago, preceding the Cambrian time frame. That was before plants and before oxygen-rich atmosphere; life was just fermentation, with boundless telomerase. When nutrients are available, the unicellular organisms have evolutionary pressure to multiply as soon as possible by

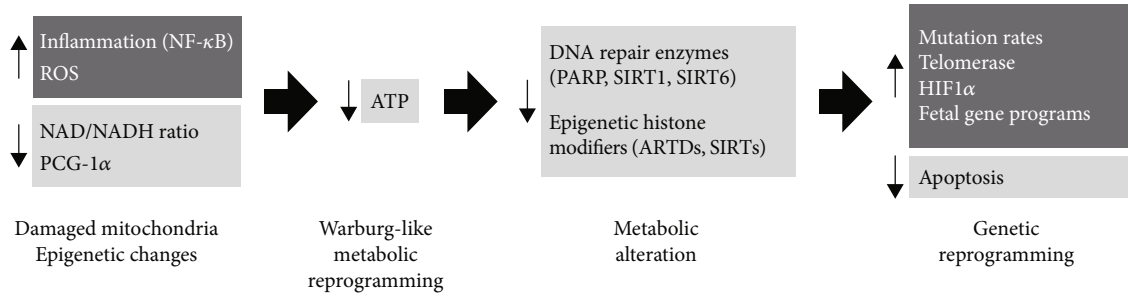

Figure 2: Metabolic alterations produce genetic alterations (activation of oncogenes and repression of tumor suppressor genes) which influence cancer development. What the causes are of metabolic alteration is still a matter of debate. Potential candidates involved in the metabolic switch from respiration to fermentation are increased inflammation, increased ROS formation, overstimulation of PARPs, decreased intracellular energy levels, and damaged respiration.

fermentation of glucose to generate biomass, which enables them to maintain the building blocks needed to produce a new cell [97]. In 1940, the French biologist Jacques Monod was the first to discover that genes can be regulated by metabolic readjustment in the experiment with *E. coli* fed on glucose or lactose sugar [102]. Although anaerobic glycolysis is less efficient, it is much more rapid than oxidative phosphorylation. Warburg observed that in the same amount of time a normal cell takes to consume one glucose molecule through OXPHOS, the cancer cell consumes 13 glucose molecules, only one of which through OXPHOS [103]. Such a switch can be explained from the evolutionary viewpoint as this may have helped a unicellular organism to speedily monopolize sugars when available and create an unfavorable environment for competing microorganisms [104].

1.7. Why Do Cancer Cells Prefer a Relatively Inefficient Way (in terms of ATP Production) of Extracting Energy from Glucose? Warburg effect enables cancer cells to convert nutrients into building blocks to form different macromolecules in order to divide fast. Cancer cells must be directed either to cell death or to adaptation to a glycolytic phenotype once their cells reach the oxygen diffusion limit and become hypoxic. If "renegade cells" do not shift to such a primitive form of energy, they will die from apoptosis or lack of ATP. Therefore, cells deficient in ATP often undergo apoptosis [105]. Contrary, by activating glycolysis, "a renegade cell" stimulates cell division and suppresses apoptosis and differentiation [14] as well as the "multiunit teamwork." Such a cell evolved to survive on its own. When cooperation is stopped, and fermentation is preferred, differentiation and specialization are reversed to a more primitive form, and transition to dedifferentiated cells is favored. Such a cell passes the energy needed for self-preservation/regeneration to increased reproduction; consequently, energy for the repair of cells and also the adaptive response to stress as cell cycle arrest regulation and apoptotic removal of damaged cells is depleted. Furthermore, glycolysis significantly diminishes cellular oxidative stress [106]. Both glucose and glutamine-derived glutamate are needed for synthesis of glutathione, which provides high antioxidant capacity and protect cancer cells from elevated ROS formation during chemo- and radiotherapies [107–109].

1.8. Why Did Evolution Preserve the Ability of Cells to Activate Aerobic Glycolysis? The antagonistic pleiotropy hypothesis would explain fermentation as a beneficial process to the organism's fitness at the first week of embryo life when fast-growing cells of an embryo resemble more a cancer mass than normal differentiated tissue. An embryo must survive the first days without blood supply and oxygen. When the ovum reaches the uterus, it develops into a blastocyst consisting of over 100 cells. Upon entering the uterus, the embryo attaches into the uterine lining. Only after the embryo reaches the womb does it obtain blood supply and oxygen, which enables its organized growth [110]. However, later in life, the ability to activate "cancer genes" to drive glycolysis could become detrimental to the organism's fitness as a cell might become cancerous.

What is more, anaerobic glycolysis is activated during short, intense exercise, providing energy to escape during fight-or-flight response. After only 10-30 seconds of short-duration high-intensity anaerobic exercise, the majority of cellular energy come from the anaerobic glycolytic system manifested in the elevation of the blood-lactate level. This system provides ATP for up to 2–3 minutes. Then, the generation of energy switches back to oxidative phosphorylation [111–113]. While the acute switch from oxidative phosphorylation to anaerobic glycolysis is triggered by high-intensity anaerobic training, the cause of the permanent switch to chronic glycolysis remains unknown (Figure 2). One trigger might be increased and permanent inflammation and oxidative stress, which stabilize HIF-1-alpha, which advances a hypoxic-like (Warburg effect) state in the cell resulting in metabolic reinventing toward glycolysis and thus encouraging tumor development [114–116]. Anaerobic glycolysis and imperfect respiratory chain produce a lot of ROS and frame an endless loop which creates significantly more damage to mtDNA and decreases energy formation from oxidative phosphorylation and further invigorates fermentation.

1.9. CSC Metabolic Reprogramming. The cancer stem cell (CSC) hypothesis states that malignant tumors are initiated and maintained by a population of tumor cells that share similar biologic properties to normal adult stem cells [117]. Transformation of a normal stem cell into a CSC may occur

through dysregulation of the proliferation and differentiation pathways or by inducing oncoprotein activity [118]. An alternative is the potential dedifferentiation of mutated cells so that these cells acquire stem cell-like characteristics [119], which is applicable to cells of all origins. It was observed that non-CSCs could be shifted to CSCs and vice versa in response to intrinsic and/or microenvironmental signals (e.g., oncogenes, tumor suppressor genes, hypoxia, oxidative stress, nutrient starvation, and epigenetics), which means that metabolic reprogramming might play a significant role during CSC transition [37]. Menendez et al. [37] argue that CSC bioenergetics might be another cancer and that metabolic reprogramming of CSCs has cancer-causing action. Increased glycolytic activity observed in early embryonic cells and high proliferation and diffusion are similar (or being reactivated) in cancer stem cells, which resume a more primitive metabolic pattern of energy production [13]. Cancer stem cells express the same metabolic defect as seen in all types of cancer cells. Mitochondrial function, redox status, and ROS formation play an important role in differentiation, maintenance, and self-renewal of CSC[13]. As in cancer cells, the stimulation of aerobic glycolysis supports, while the blockade of glycolytic enzymes blunts cancer-like metabolic reprogramming, phenomena observed in Induced Pluripotent Stem Cells (iPSCs) [120–123]. Even in the absence of genetic alterations, the Warburg effect and inhibition of OXPHOS are triggered in iPSCs by two primum movens: downregulation of the expression of the catalytic subunit of the AMP-activated protein kinase (AMPK) [124] and H+-ATPase synthase-geared metabolism switch [125–127]. Increased glycolysis in the presence of O_2 and impaired oxidative phosphorylation are observed in both embryonic cells and CSC and other tumor cells [128].

1.10. Reprogramming of the Glycolytic Metabolism and Oxidative Phosphorylation: Is the Trigger the Inflammatory Stresses? Numerous studies indicate a strong link between chronic inflammation and cancer (reviewed in [129–138]). Although mechanisms of chronic inflammation are very complex and the precise role of increased inflammation and cancer remain largely unknown, a nuclear factor-κB (NF-κB), considered as the master activator of inflammation [139], and p53, the major tumor suppressor, play a pivotal role. Activation of the NF-kappaB system increases the apoptotic resistance, activates the chronic inflammatory response, and reduces the autophagic cleansing [140]. Besides, macrophages that secrete cytokines and growth factors are attracted by the inflammatory response which promotes tumor cell growth and metastasis [141].

Chronic inflammation and accumulation of oxidative stress during aging also lead to NAD+ depletion [142], resulting in loss of sirtuin and PARP activity. Chronic inflammation will result in increased ROS formation leading to a decrease in intracellular NAD+ and cell death via energy restriction as a result of DNA strand breaks and PARP activation [143]. For example, in the brain cells, increased PARP activity, which leads to decreased NAD+, has been shown to decrease ATP as well as cause cell death [144, 145]. In particular, DNA repair enzyme PARPs utilize a lot of intracellular NAD+ (100 molecules of NAD+ when activated by one DNA break) and are in this manner in rivalry with sirtuins for the constrained supply of NAD+. Deacetylation by SIRT1 reprograms inflammation and cancer [146]. Constrained accessibility of NAD+ and reduced expression of SIRT1 may sustain aberrant chromatin structure and functions. Subsequently, reduced cellular NAD+ limits the efficacy of sirtuins (SIRT1), possibly deacetylating tumor suppressor proteins such as p53 [147]. p53 differentially controls a cluster of its target genes, encompassing the arrest of cell cycle, autophagy, apoptosis, and senescence, to apply its function in the damage of DNA and suppression of tumors. Consequently, a depletion of p53 gives a growth advantage to tumor cells; for example, it empowers cell survival under constraining nutrient conditions [148]. Moreover, NAD+-dependent tankyrases (PARP-5a and PARP-5b), which control telomerase activity and telomere maintenance, may likewise impact the cancer-causing process [149].

SIRT1 likewise impacts inflammation and cancer by straightly deacetylating targets like p65, p53, and NF-κB, which produce proinflammatory products. NAD+ levels steadily decline with age [129] due to loss of SIRT3 activity in mitochondria, loss of PARP activity, and increased levels of NADase CD38 during aging [86, 142]. Since NF-κB regulates the CD38 expression [150], the increase in low-grade inflammation with age might be the reason for NAD+ decline. Consequently, cells with high levels of CD38 use less oxygen, have increased lactate, and have dysfunctional mitochondria [142]. During chronic inflammation, NAD+ levels and SIRT transcription and/or protein levels are persistently reduced in different tissues [151]. Chronic inflammation and the release of proinflammatory mediators might thus reprogram cellular metabolism and energy production. For example, the induction of anabolic glycolysis is observed in cells of the immune system (e.g., monocytes and macrophages) exposed to inflammatory stress [152–155]. With increased age, the innate immune system does not efficiently clears the senescent cells as emitters of signals that drive inflammation and the vicious cycle initiates [156].

1.11. Prevention of Glycolysis and Reactivation of Mitochondrial Oxidative Metabolism: Approaches That Target Cell Energy Metabolism and Restore Mitochondrial Function

1.11.1. Targeting Aerobic Glycolysis Pathways and the Warburg Effect. Many compounds affect aerobic glycolysis and would be efficient in depleting ATP in cells with mitochondrial defects and triggering cell death. Different small molecules target aerobic glycolysis and could be used as novel tumor therapeutics, for example, 2-deoxyglucose [157], lonidamine, 3-bromopyruvate [158, 159], imatinib, oxythiamine, and 6-aminonicotinamide [160–162]. Another way of action is to inhibit glucose transport by phloretin [163] or stimulation of mitochondrial oxidative metabolism through overexpression of mitochondrial frataxin, which inhibits tumor growth [164, 165]. Already in clinical use are imatinib and trastuzumab (Herceptin), which target signaling pathways linked to glucose metabolism [98, 166], primarily in those

individuals with mutations in specific receptors linked to the insulin-like growth factor 1-Akt/protein kinase B (IGF-1/PI3K/Akt) pathway. Many studies are showing that the Warburg effect can be targeted with dichloroacetate (DCA) and increased mitochondrial activity of glutaminolysis with arsenic trioxide (ATO). It was observed that DCA induces apoptosis in cancer cells but does not induce apoptosis in normal cells [167–172].

There are many agents that can act as anti-Warburg agents. Their way of acting is to increase the NAD+ levels and promote the oxidative metabolism [173]. For example, SIRT3 can restrain the "Warburg effect" by controlling HIF-1α and change the cancer cell metabolism programming from highly glycolytic toward oxidative phosphorylation [116, 174, 175]. Besides, by inactivating HIF-1α, SIRT1 represses HIF-1 target genes and adversely effects tumor growth and angiogenesis [176]. By increasing levels of sirtuins, PARPs, and PGC-1α, oxidative metabolism, inflammation, epigenetic gene silencing, cell cycle control, genome stability, apoptosis, stress resistance, energy efficiency, DNA repair, cell death, genome integrity, cellular differentiation, gene expression, and antiaging could be promoted.

Finally, mitochondria could be used as a potential anticancer drug target. The apoptotic process could be regulated by reactivating or by transferring mitochondria [5].

1.11.2. Enhancing Mitochondrial Biogenesis and Efficacy and Boosting Oxidative Metabolism. By enhancing the bioavailability of NAD+, oxidative capacity of mitochondria could be restored. NAD+ levels could be raised with exercise, restriction of calories (CR), and ingestion of NAD+ precursors and intermediates. Alternatively, NAD+ bioavailability can be increased by using poly-ADP-ribose polymerase (PARP), CD 38, and SAM1 inhibitors [60, 65, 177–185]. Consequently, increased NAD+ levels could activate PARPs and sirtuins which control the genes that play a role in the process of DNA repair and maintenance [173]. Additionally, different NAD(+) precursors can be used through distinct metabolic pathways to form NAD(+), such as nicotinamide, nicotinamide mononucleotide, tryptophan, nicotinic acid, and nicotinamide riboside. Further, consumption of foods that contain molecules necessary for respiratory enzyme function (riboflavin, nicotinamide, iron salts, and pantothenic acid) could help to maintain health when it is combined with dietary energy restriction [186] since CR increases the efficiency of the electron transport in the mitochondrial respiratory chain [187]. Pyrroloquinoline quinone (PQQ) might increase the number and efficiency of mitochondria. PQQ interacts with cell signaling pathways and influences energy-related mitochondrial metabolism [188]. The mitochondrial biogenesis is stimulated through a pathway that activates the cAMP response element-binding protein (CREB) and peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1alpha) [189].

While raising NAD+ levels for cancer prevention might be beneficial, increasing NAD+ levels might be detrimental during the precancerous stage or once the cancer is formed [65] (discussed in the previous paragraph).

1.11.3. Increasing the Intracellular Oxygen Level with Hyperbaric Oxygen Therapy. Hyperbaric oxygen therapy raises oxygen levels in tumors and reverses the cancer-promoting effects of tumor hypoxia [190, 191]. By enhancing oxygen delivery to cells, more ATP can be obtained through oxidative phosphorylation since cells make use of oxygen acting as a final electron acceptor in the process of generating ATP in their mitochondria and mitochondrial integrity could be preserved [192]. Poff et al. [193] observed that a combination of the ketogenic diet with hyperbaric oxygen therapy resulted in a noticeable drop in blood sugar and the rate of tumor development and increased mean survival of mice with systemic metastatic cancer.

1.11.4. Increasing Regulation of Contact Inhibition (Density-Dependent Inhibition) and Proliferation. Due to the loss of growth control, the growth and division of cells are uncontrolled. Cells should be informed that they are a part of a multicellular organism and that they have to obey the control of proliferation or to activate apoptosis if being damaged. This could be achieved by increasing the response to the signals that cause healthy cells to cease proliferation and enter the G0 phase and by decreasing the production of growth factors that stimulate cancer cells to own proliferation [194–196].

1.11.5. Targeting Glucose and Elevating Blood Ketone Bodies through a Calorie-Restricted Ketogenic Diet (KD-R). The energy metabolism in glycolysis-dependent tumors can be targeted by a transition from carbohydrate to ketones. Healthy cells can be protected from such glycolytic inhibition, and the brain can be protected from hypoglycaemia by elevating blood ketones, which occurs when a low-carbohydrate and a high-fat ketogenic diet is carried out in limited amounts [34]. Exogenous ketone supplementation on its own inhibits cancer cell proliferation and viability *in vitro*, slows tumor growth, and prolongs survival *in vivo* [197]. Caloric restriction/KD-R reduces carbons needed in glycolytic and pentose phosphate pathways in order to provide ATP, precursors for lipid and nucleotide synthesis and formation of antioxidant glutathione. Due to impaired mitochondrial function, cancer cells are depending on substrate-level phosphorylation, and during ketone body metabolism, mSLP is bypassed. Ketone bodies may elicit their anticancer effects, most likely by glycolytic enzyme inhibition [198]. Numerous research studies documented that *in vitro* cancer cells were deficient in metabolizing of ketone bodies [199, 200]. Ketone bodies generate ATP energy only through oxidative respiration in the mitochondria and cannot be fermented. While dietary energy reduction lowers blood glucose levels and restricts the energy supply to cancer cells, some of the tumor cells might still obtain enough energy to survive due to the endogenous glucose and amino acid influx.

1.11.6. Targeting Glutamine. For cell growth and division, cells need a supply of carbon, nitrogen, free energy, and reducing equivalents, which can be obtained through glucose and glutamine metabolism [97]. Glutamine functions as a

significant energy metabolite for some cancers. For example, tumors with deregulated MYC expression may be less sensitive to inhibition of glycolysis than tumors with overactivation of the Akt pathway [178, 201]. Glutamine provides a source of carbon and nitrogen needed for nucleotide synthesis, and targeting glutamine metabolism with the glutamine antagonist 6-diazo-5-oxo-norleucine (DON) might be used in cancer treatment [202–204]. For example, it was shown in two glioblastoma mouse models that administration of DON and calorically restricted ketogenic (KD-R) diet killed tumor cells, reversing disease symptoms and increasing overall mouse survival. Simultaneous administration of DON and KD-R both targeted substrate-level phosphorylation reactions in mitochondria (glutaminolysis) and in the cytoplasm (glycolysis), respectively, thus enabling ATP formation and synthesis of proteins, nucleotides, and lipids [205]. It should be stressed that glutamine is needed for appropriate functioning of the immune system and the gut [206]; thus, glutamine targeting is more demanding than glucose targeting.

1.11.7. "Press-Pulse" Therapeutic Strategy. The team of Seyfried developed a so-called "press-pulse" therapeutic strategy [107, 207]. The general concept of press disturbances (chronic metabolic stress on tumor cell energy disturbance) and pulse disturbances (acute metabolic stressors that restrict glucose and glutamine availability) could be applied for the management of cancer. Press therapies reduce systemic glucose concentrations and elevate ketone bodies; pulse therapies use cocktails which interfere with glycolysis and glutaminolysis metabolic pathways [107, 207].

1.11.8. Targeting Insulin/Insulin-Like Growth Factor (IGF) Signaling, Mammalian Target of Rapamycin (mTOR), and AMP-Activated Protein Kinase (AMPK) Pathways. Metabolic therapies that lower circulating glucose levels were reported to significantly reduce growth and progression of numerous tumor types [14]. A number of epidemiological studies initially concluded that in patients with diabetes who controlled their blood sugar levels by taking metformin, the development of cancer was less likely. It was also observed that their survival rate was improved once cancer was initiated. Several retrospective studies indicated that people with diabetes had increased cancer mortality compared with nondiabetics and that people with diabetes on metformin had a substantially (~40%) reduced cancer burden compared with diabetics on other treatments [208]. For example, glucose reduction lowers insulin and IGF-1 levels, which is required for driving tumor cell metabolism and growth [209, 210]. Caloric restriction specifically influences the IGF-1/PI3K/Akt/HIF-1α signaling pathway, which regulates several cancer hallmarks like evasion of apoptosis, cell proliferation, and angiogenesis [14]. Diabetes drugs metformin and phenformin might have benefit in cancer prevention as activators of AMPK in cells. AMPK is activated by also salicylate in vitro and by "nutraceuticals" such as resveratrol, epigallocatechin gallate, and berberine, which activate AMPK by inhibiting mitochondrial ATP production [211]. AMPK is also activated in the resting muscle with 5-aminoimidazole-4-carboxamide-riboside (AICAR), which enters the muscle and is phosphorylated to ZMP (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl-5′-monophosphate, an AMP analog). ZMP is a nucleotide that mimics the effect of 5′-AMP [212–214]. 3,3′-Diindolylmethane (DIM) from cruciferous vegetables and epigallocatechin gallate (EGCG) from green tea have been reported to be effective AMPK activators in a prostate or breast cancer model system, both *in vitro* and *in vivo* [215]. Additionally, AMPK was demonstrated to suppress tumor growth in vivo as a negative regulator of the Warburg effect [216]. AMPK in muscles is activated, in response to both *in vivo* exercise and *ex vivo* contraction [217, 218]. The varied role of AMPK on cancer cell survival and tumor progression and suppression is explained in detail elsewhere [219]. The induction of AMPK activity inhibits the activity of rapamycin (mTOR) [220]. Mammalian target of rapamycin (mTOR) regulates a translational control over cell division, growth, and energy metabolism, while IGF-1/Akt regulates the transcriptional regulators of these processes. The inhibition of apoptosis and the promotion of growth and division are, therefore, the result of the activated IGF/Akt pathway [221]. A serine/threonine protein kinase mTOR controls the growth, proliferation, motility, and survival of cells; protein synthesis; and transcription [222, 223] in response to nutrients (e.g., glucose and amino acids), growth factors (e.g., increased levels of insulin, IGF-1, and platelet-derived growth factor (PDGF)), and cellular energy status (ATP). CR and p53 (a nuclear transcription factor with a proapoptotic function) may also inhibit mTOR activity [148].

1.11.9. Shifting from Anabolic to Catabolic Metabolism Suppresses High Rates of Proliferation. Anabolic pathways that advance growth are stimulated in cancer by means of tumorigenic mutations, especially PI3K-mTOR signaling [224]. PI3K-Akt-mTOR network signaling, where many oncogenes and tumor suppressors reside, is acquired with minimal reliance on external stimulation by growth factors [225]. Additionally, glucose metabolism generates glycolytic intermediates (hexosamine pathway, PPP, and one-carbon metabolism) which promote anabolic pathways that support cell growth [226]. On the other hand, only a couple of short periods of fasting activates AMPK, which triggers repair and catabolic processes. Alongside, AMPK-mediated inhibition of mTOR activity [226] and downstream anabolic pathways establishes separation of anabolic and catabolic processes [227]. Tumor cells have aberrant activation of mTORC1 that evokes an anabolism leading to nucleotide, protein, and lipid synthesis. A depletion of tumor suppressors, such as p53, or activation of oncogenes, e.g., MYC, to a greater extent enhances an anabolic growth program by metabolic gene transcriptional regulation.

Currently, there are many other strategies under investigation targeting mitochondrial energy metabolism to 2inhibit or delay tumor growth. Some of them deal with DNA methylation pattern, epigenetic reprogramming, and aberrant microRNA (miRNA) levels and/or investigate the role of intermediates of the Krebs cycle on "nonmetabolic" signaling which alters the immune system, the role of DJ-1 (Parkinsonism-associated deglycase) as a modulator

of mitochondrial metabolic efficiency and a switch between glycolysis and oxidative phosphorylation, and the role of bouchardatine in suppressing cancer by disrupting its metabolic pathways via activating the SIRT1-PGC-1α-UCP2 axis. Detailed descriptions of their principles are beyond the scope of this paper. More information can be found elsewhere [228–231].

1.12. Chemoresistance. Drug-resistant tumor cells arise in a large part from the damage to respiration in bystander precancerous cells. While cytotoxic drugs and radiation create tumor cells that become highly resistant to the classical treatment approaches, this is not probable when dietary energy reduction and approaches aimed at reversing abnormal energy metabolism and growth behavior in tumor cells are used [107, 232]. Chemoresistance is the result of the fermentation metabolism in the tumor cells. Glucose and glutamine contribute to the synthesis of glutathione, which protects tumor cells from oxidative stress [205]. Inhibition of glycolysis in cancer cells increases the sensitivity to common anticancer agents and overcomes the drug resistance [232]. Dietary restriction, periodic fasting, and fasting-mimicking diets are emerging as interventions used to prevent and treat cancer in combination with chemo- and radiotherapy [233–235].

2. Conclusion

A clear understanding of the origins of cancer is the basis of successful strategies for effective cancer prevention and management. Results are indicating that the carcinogenic process is not driven by the accumulation of random or stochastic genetic mutations, but instead, a mitochondrial metabolic disease [4] was presented. However, it remains to be elucidated what exactly triggers the reprogrammed metabolism in cancer cells. Additional studies are needed to investigate the causation-consequence relationship between metabolic abnormalities and the causation of the genetic mutations and, on the other hand, the mutation ability to trigger the metabolic abnormalities.

Both metabolic and standard cytotoxicity-based treatment approaches should be coupled. Strategies that restore mitochondrial metabolism/functions could have both tumor preventive (e.g., caloric restriction or intermittent fasting) and therapeutic implications in cancer (use of drugs, such as glutamine antagonist and 6-diazo-5-oxo-L-norleucine (DON), and others including KD-R). Evidence was presented that restoring redox homeostasis and reactivation of mitochondrial oxidative metabolism are important factors in cancer prevention. Preclinical studies are needed, followed by controlled-randomized clinical trials, investigating strategies to restore mitochondrial metabolism as well as synergistic effect of metabolic and standard cytotoxicity-based treatment approaches. Without findings of additional studies, no specific therapy can be currently favorited. The efficacy of the proposed treatment approaches should be further studied to determine their potential for clinical use in the future.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Betulinic Acid Suppresses Breast Cancer Metastasis by Targeting GRP78-Mediated Glycolysis and ER Stress Apoptotic Pathway

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Targeting aberrant metabolism is a promising strategy for inhibiting cancer growth and metastasis. Research is now geared towards investigating the inhibition of glycolysis for anticancer drug development. Betulinic acid (BA) has demonstrated potent anticancer activities in multiple malignancies. However, its regulatory effects on glycolysis and the underlying molecular mechanisms are still unclear. BA inhibited invasion and migration of highly aggressive breast cancer cells. Moreover, BA could suppress aerobic glycolysis of breast cancer cells presenting as a reduction of lactate production, quiescent energy phenotype transition, and downregulation of aerobic glycolysis-related proteins. In this study, glucose-regulated protein 78 (GRP78) was also identified as the molecular target of BA in inhibiting aerobic glycolysis. BA treatment led to GRP78 overexpression, and GRP78 knockdown abrogated the inhibitory effect of BA on glycolysis. Further studies demonstrated that overexpressed GRP78 activated the endoplasmic reticulum (ER) stress sensor PERK. Subsequent phosphorylation of eIF2α led to the inhibition of β-catenin expression, which resulted in the inhibition of c-Myc-mediated glycolysis. Coimmunoprecipitation assay revealed that BA interrupted the binding between GRP78 and PERK, thereby initiating the glycolysis inhibition cascade. Finally, the lung colonization model validated that BA inhibited breast cancer metastasis *in vivo*, as well as suppressed the expression of aerobic glycolysis-related proteins. In conclusion, our study not only provided a promising drug for aerobic glycolysis inhibition but also revealed that GRP78 is a novel molecular link between glycolytic metabolism and ER stress during tumor metastasis.

1. Introduction

Breast cancer is the most diagnosed malignancy among women worldwide. In 2018, it is estimated that 2.1 million new cases will be diagnosed. Breast cancer accounts for cancer occurrences in almost 1 in 4 females and for 11.6% of all sites of malignancies in both men and women [1]. Importantly, breast cancer is also the leading cause of cancer deaths among women in over 100 countries. There will be 62679 breast cancer deaths globally in 2018, which accounts for 6.6% of all site cancer deaths in both sexes [2, 3]. According to cancer death cause analysis, metastasis is always the leading reason and tremendous endeavor has been dedicated to its underlying mechanisms, such as cancer stem cells, immune depression, and metabolic alteration. However, candidate drugs approved for the inhibition of

metastasis are very limited, and natural phytochemicals have become an important resource to discover precursors of metastasis inhibitors.

Metabolic reprogramming is one of the hallmarks of cancer [4], especially for aerobic glycolysis. Since the first report of cancer glycolysis activity by Otto Warburg in 1920, several studies have demonstrated that cancers prefer glycolysis even in the presence of oxygen, a phenomenon known as "Warburg effect" [5, 6]. At present, the Warburg effect of tumor could be monitored by 18F-fluorodeoxyglucose positron-emission tomography (FDG-PET) to indicate metastasis information [7]. At the same time, a number of molecular targets have been identified in the glycolysis pathway and been paid with great interests for metastasis inhibition and anticancer drug development. For example, lactate dehydrogenase A (LDHA) was found to promote breast cancer metastasis and its inhibitor oxamate was effective in inhibiting cancer cell invasion in multiple malignancies [8]. Pyruvate dehydrogenase kinase 1 (PDK1) overexpression could enhance head and neck squamous carcinoma metastasis via the upregulation of fibronectin [9]. The hexokinase II inhibitor 2-DG or 3-BrPA had the inhibitory effect on tumorigenesis and metastasis in multiple malignancies such as lung cancer, liver cancer, and breast cancer [10, 11]. Consequently, glycolysis inhibition may be a promising new strategy for antimetastasis. However, the internal mechanisms of aerobic glycolysis have not been fully elucidated. Identification of prime carcinogenic signaling is critical for the development of glycolysis inhibition strategy. Recent studies suggested that glucose-regulated protein (GRP78) serves as a molecular hub in mediating metabolism regulation and cancer metastasis [12].

GRP78, a major chaperone in the endoplasmic reticulum, is a central sensor of cellular stress and is frequently highly expressed in most solid tumors [13]. High expression of GRP78 contributes to the acquisition of metastatic phenotypes including apoptosis resistance, immune escape, angiogenesis, and drug resistance [12]. It has been reported that GRP78 is involved in the development of metastatic breast cancer as a multifunctional receptor when it is expressed on the cancer cell surface [14]. In fact, GRP78 also participates in cancer cell metabolism regulation. Glucose deficiency usually leads to GRP78 overexpression, which enhances glutamine metabolism to support cell survival by modulating β-catenin signaling [15]. A recent report demonstrated that GRP78 regulated metabolic reprogramming by modulating acetyl-CoA production and histone acetylation in prostate cancer cells [16]. In addition, another study indicated that GRP78 induction could result in enhanced *HIF-1a* transcription and GLUT1 expression, which are the key factors contributing to glycolysis [17]. Given the membrane translocation of GRP78 under cellular stress and its biofunction in controlling glycolysis and metastasis, it is interesting and promising to develop candidate inhibitors targeting GRP78 from natural phytochemicals, which may overcome the limitations of existing glycolytic inhibitors. For example, although 2-deoxyglucose and 3-bromopyruvic acid showed excellent anticancer effects in preclinical studies, their clinical applications were significantly limited due to the serious systemic adverse effects [18]. Therefore, the demand for developing a glycolysis inhibitor with high safety is highly appreciated.

BA, a pentacyclic triterpene widely found in birch bark extracts, has been reported to act anticancer activities in multiple cancers, including breast cancer [19]. What is more important, it was found that BA did not display apparent systemic toxicity in tumor-bearing mice even at 500 mg/kg [20]. Subsequent studies also suggested that BA did not exhibit discernable impact on normal cells at doses which killed cancer cells *in vitro* [21]. Therefore, BA attracts increasing attention due to its high selectivity for cancer cells. With regard to pharmacological mechanisms, current findings include (i) the induction of cancer cell apoptosis via the mitochondrial pathway induced by the release of soluble factors or generation of reactive oxygen species (ROS) [22, 23]; (ii) the inhibition of angiogenesis [24]; (iii) the degradation of transcription factor specificity protein 1 (Sp1) [25, 26]; and (iv) the induction of DNA damage by suppressing topoisomerase I [27, 28]. Notably, a recent report suggested that BA could change cellular glucose metabolism with concomitant reduction of glucose oxidation [29]. Besides, we also noticed that BA exerted antimetastatic potential by reversing EMT in melanoma cells via repressing the expression of neutrophil gelatinase-associated lipocalin (NGAL) [30]. However, the underlying molecular mechanisms of BA are far away from full elucidation. It is interesting to identify the molecular target of BA and the association with glycolysis regulation.

In the present study, we found that BA could attenuate migration and invasion of highly aggressive breast cancer cells via aerobic glycolysis inhibition. GRP78 silencing blocked the inhibitory effects of BA on glycolytic proteins including LDHA, PDK1, and c-Myc. Exploration of the molecular mechanism indicated that BA interrupted the binding between GRP78 and PERK, which subsequently activated eIF2α phosphorylation, and suppressed downstream signaling by β-catenin/c-Myc. *In vivo* studies also demonstrated that BA inhibited lung colonization of breast tumor. Our results provide novel insights of BA as a promising molecular inhibitor of breast cancer metastasis *via* glycolysis inhibition and also reveal a novel regulatory pathway between GRP78 and glycolytic metabolism in cancer cells.

2. Materials and Methods

2.1. Cell Culture. Breast cancer cell lines MDA-MB-231 and BT-549 and mammary epithelial cell line HBL-100 were purchased from the American Type Culture Collection (ATCC). The cells were cultured in the basal medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a humidified incubator with 5% CO_2 at 37°C.

2.2. Cell Viability. Cell viability was detected by CCK-8 assay. MDA-MB-231, BT-549, and HBL-100 were plated at a density of 3×10^3 cells into the 96-well plate. After attachment, the cells were treated by serial concentration gradients of BA (Xi'an Natural Field Bio-Technique Co. Ltd., Xi'an, China) for 24 h, 48 h, and 72 h. The cell viability was measured by the CCK-8 reagent (Beyotime Biotechnology,

Shanghai, China) according to the absorbance value. Three independent repetitive experiments were conducted.

2.3. Colony Formation Assay. 1000 cells were plated into each well of a 6-well plate to disperse homogeneously.

Cells were firstly treated by 20 or 40 μM BA for 4 h and then cultured with fresh medium for 2 weeks. The ultimately formed colonies were fixed with 4% paraformaldehyde and stained with Coomassie blue.

2.4. Wound Healing and Transwell Invasion Assay. For the wound healing assay, cells were seeded into the 6-well plate at a density of 4×10^5. When the cells grew to 100% confluence, a "wound" in a cell monolayer was created and its distance was compared at 0, 12, 24, and 48 h to quantify the migration rate of the cells with or without BA treatment. To exclude the antiproliferative effects of BA on cell migration, the MDA-MB-231 and BT-549 cells were treated with BA for 12 h before scratching. After removing BA, the same amounts of cells were then cultured in serum-free medium to avoid the influence of proliferation. For Transwell invasion assays, the chambers were coated with a layer of Matrigel prior to the experiment. Similarly, MDA-MB-231 and BT-549 cells were pretreated by BA for 12 h, then quantified and seeded into the upper compartment with 200 μl serum-free media (50000 cells per well). In contrast, the lower compartment contained 10% FBS. After 24 h incubation, the cells that penetrated the filter were fixed with 4% paraformaldehyde, followed by 0.1% Coomassie blue staining for 20 min.

2.5. TUNEL Analysis. MDA-MB-231 and BT-549 were treated with BA 20 and 40 μM for 48 h. Then, the cell apoptosis was detected in situ by fluorescence using TUNEL analysis as described previously [31].

2.6. Western Blotting Analysis. Equal amounts of protein lysates (50 μg) were loaded for SDS-PAGE and transferred to a PVDF membrane (Millipore, Billerica, MA). The signals were probed with primary antibodies and amplified by the secondary antibodies. The primary antibodies included E-cadherin antibody (20874-1-AP, Proteintech, Rosemont, IL, USA), N-cadherin antibody (22018-1-AP, Proteintech, Rosemont, IL, USA), vimentin antibody (10366-1-AP, Proteintech, Rosemont, IL, USA), MMP-2 antibody (A6247, ABclonal Technology Cambridge, Boston, USA), MMP-9 antibody (sc-13520, Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin antibody (4970, Cell Signaling Technology, Danvers, MA, USA), β-catenin antibody (51067-2-AP, Proteintech, Rosemont, IL, USA), c-Myc antibody (A1309, ABclonal Technology Cambridge, Boston, USA), LDHA antibody (3582, Cell Signaling Technology, Danvers, MA, USA), LDHB antibody (sc-100775, Santa Cruz Biotechnology, Santa Cruz, CA, USA), PDK-1 antibody (sc-293160, Santa Cruz Biotechnology, Santa Cruz, CA, USA), p-PDK-1 antibody (3061, Cell Signaling Technology, Danvers, MA, USA), GRP78 antibody (11587-1-AP, Proteintech, Rosemont, IL, USA), caspase-12 antibody (55238-1-AP, Proteintech, Rosemont, IL, USA), CHOP antibody (15204-1-AP, Proteintech, Rosemont, IL, USA), PERK antibody (5683, Cell Signaling Technology, Danvers, MA, USA), p-PERK antibody (DF7576, Affinity Biosciences, Cincinnati, OH, USA), eIF2α (11233-1-AP, Proteintech, Rosemont, IL, USA), and p-eIF2α (AP0635, ABclonal Technology Cambridge, Boston, USA). Finally, the bands were imaged though the ECL Advance reagent (Tanon Science & Technology, Shanghai, China) and quantified by optical densities using the ImageLab software (Bio-Rad, Hercules, CA).

2.7. Gelatin Zymography. Cells were cultured in the 6-well plate in 10% fetal bovine serum (FBS) with or without BA treatment. At 70-80% confluence, the FBS was removed and continue to grow cells in FBS-free media. After 48 h, the conditioned media were centrifuged and collected. Adjust conditioned media in all samples to the same protein concentration at 10 μg/ml before SDS-PAGE. The 7.5% acrylamide gel contained 8 mg gelatin. The gel was washed 2×30 min with washing buffer (2.5% Triton X-100, 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$) after electrophoresis and then incubated in an incubation buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$) for 24 h at 37°C. Finally, the gel was stained with 0.5% Coomassie blue solution and in turn destained until bands can clearly be seen.

2.8. RT-qPCR Analysis. Total RNA was extracted with RNAiso Plus Reagent (Takara BIO, Japan) and transcribed to complementary DNA in reverse using the reverse transcription reagent kit with gDNA eraser (Takara BIO, Japan). The RT-PCR was performed by Applied Biosystems ViiA7 Real-Time PCR System (Thermo Fisher Scientific, Hudson, USA) using SYBR® Premix Ex TaqTM II kit (Takara BIO, Japan) in accordance with the manufacturer's instruction. The relative mRNA levels were compared using the $2^{-\Delta\Delta Ct}$ method.

2.9. Lactate Production Assays. Cells were treated with gradient concentration of BA for 48 h and then lysed in lactate assay buffer using VCX105 ultrasonic cell crusher (SONICS, USA). The lactate production in cell lysates was measured using the Lactate Assay Kit (Sigma-Aldrich, Shanghai, China) according to the manufacturer's instructions.

2.10. Cell Energy Phenotype Analysis. The cell energy phenotype profiles were analyzed though the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) values that were obtained by the Seahorse XF24 extracellular flux analyzer (Seahorse Bioscience). Briefly, 4×10^4 cells per well were seeded into XF24 cell culture microplates and cultured overnight. Meanwhile, the XF24 cartridge was equilibrated with the calibration solution overnight at 37°C. On the second day, cells were treated with 40 μM BA for 3 h prior to the measurement. XF assay medium (containing 10 mM glucose, 2 mM glutamine, and 1 mM pyruvate in XF base medium, pH = 7.4) was used to prepare the cellular stress-inducing reagents, including 1.0 μM oligomycin, 1.0 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), 0.5 μM antimycin A, and 0.5 μM rotenone (final concentration). All the reagents were loaded in the ports according to the manufacturer's instructions. After the

measurement, cell numbers in each well were counted and were used to normalize the OCR and ECAR values.

2.11. DARTS. The molecular target of BA was identified by the DARTS strategy according to the protocol provided by Lomenick et al. [32] and improved by ourselves [33]. Briefly, the breast cancer cell lines MDA-MB-231 or BT-549 were treated with gradient concentrations of BA (0.1-100 μM) or DMSO control for 3 h. The cells were then lysed with protease and phosphatase inhibitors and diluted to the same concentration of protein. Each sample was proteolyzed at 4°C for 30 min with 0.05 mg/ml pronase (Roche Diagnostics, Indianapolis, USA). To find the protected bands, SDS-PAGE was applied, and the gels were stained with Coomassie blue. Protected bands were ultimately cut out and digested by trypsin for mass spectrometry analysis.

2.12. Plasmid Construction and Transfection. shRNAs were purchased from GenePharma (Shanghai, China), and recombinant plasmids of GRP78 were obtained from Vigene Biosciences (Maryland, USA). The scrambled plasmids and empty vector were used as control. MDA-MB-231 and BT-549 cells were transfected with lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The protein expressions were verified by western blotting after transfection of 48 h.

2.13. Coimmunoprecipitation Analysis. Coimmunoprecipitation assay was carried out by the Pierce® Co-Immunoprecipitation Kit (Thermo Fisher Scientific, Hudson, NH, USA) according to the manufacturer's instructions. In brief, GRP78 antibody was immobilized with resin. Then, the immobilized resin was incubated with the MDA-MB-231 and BT-549 cell lysates for detection of target protein PERK by immunoblotting.

2.14. Mouse Procedures. Five-week-old female Balb/c nude mice were obtained from the Beijing Vital River Laboratory Animal Technology Co. Ltd. Experimental treatments of all mice were reviewed and approved by the supervision of the Institutional Animal Research Ethics Committee in Guangzhou University of Chinese Medicine (Approval No. 20180912013). The mice were fed in the specific pathogen-free ventilation chambers under an ambient temperature of 20-25°C and 45-50% relative humidity and given sterilized food and water. To establish the lung colonization model of breast cancer in mice, luciferase gene-tagged MDA-MB-231 cells were injected through the tail vein at the density of 2×10^5 once a week and continuing for 6 weeks. Starting from the third week, the mice were segregated into 3 groups randomly ($n = 6$), including vehicle (0.5% CMC-Na) and BA 125 and 250 mg/kg. The dosage of BA is rationalized according to previous studies [20]. BA was dissolved in DMSO and then dispersed in 0.5% CMC-Na. The final amount of DMSO was less than 5%. BA was administered by intraperitoneal injection every other day for 4 weeks. At the end of treatment, the mice were anesthetized by isoflurane inhalation and injected intraperitoneally with D-luciferin (PerkinElmer, Boston, USA) at 150 mg/kg for luminescent imaging. We imaged photonic emission with the IVIS-spectrum system (PerkinElmer, Boston, USA) and quantified bioluminescence of the lung colonization.

2.15. Immunohistochemistry and Hematoxylin-Eosin Staining. Tumor specimens were fixed in 4% paraformaldehyde for 24 h, followed by the protocol as we described previously [31]. Hematoxylin-eosin staining was conducted using the Hematoxylin and Eosin Staining Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions.

2.16. Immunofluorescence Analysis. The lung tissue specimens were processed same as the immunohistochemistry assays and then permeabilized with 0.25% Triton X-100, following being blocked with 5% bovine serum albumin (Sigma-Aldrich, Shanghai, China) for 30 min at room temperature. Afterwards, the specimens were incubated with primary antibodies overnight at 4°C and fluorescence-conjugated secondary antibody for 1 h at room temperature in the dark. The nucleus was stained by DAPI (Sigma-Aldrich, Shanghai, China) for 20 min at room temperature. In the end, the fluorescence was visualized by the LMS710 confocal microscope (ZEISS, Jena, Germany).

2.17. Statistical Analysis. All statistical analyses were performed using Statistical Product and Service Solutions (SPSS) 20.0 software. The one-way ANOVA and the Dunnett post hoc test were performed for comparison among multiple groups. ANOVA for repeated measurement was performed towards repeated measures data. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. BA Inhibits Metastasis of Highly Aggressive Breast Cancer Cells. Our previous study had shown that BA suppressed glycolysis metabolism of breast cancer cells [34]. Moreover, emerging evidence implied that targeting tumor cell glycolysis may be a promising strategy to inhibit metastasis. To investigate the activity of BA against breast cancer metastasis, two basal-like highly aggressive breast cancer cell lines MDA-MB-231 and BT-549 were treated by BA for 24, 48, and 72 h. The viability of both cell lines was inhibited in a time- and dose-dependent manner (Figure 1(a)). However, BA had a minimal influence on the proliferation of the nonmalignant mammary epithelial cell line HBL-100 from 24 to 72 h, confirming its highly selective inhibitory effect on malignant cells (Figure 1(b)). We next performed colony formation assays to evaluate the long-term inhibitory effects of BA. Obviously, colonies of MDA-MB-231 and BT-549 cells were significantly suppressed by BA treatment (Figure 1(c)). In contrast, BA only had a modest effect on the colony growth of HBL-100, further validating the high safety profile of BA over a long exposure period (Figure 1(d)). Based on these results, the influence of BA on cancer cell migration and invasion was analyzed by wound healing and Transwell migration assays. We found that the extent of wound healing was impaired (Figure 1(e)) and the number of invading cells passing through the membrane was significantly reduced following BA treatment (Figure 1(f)). These findings suggested that

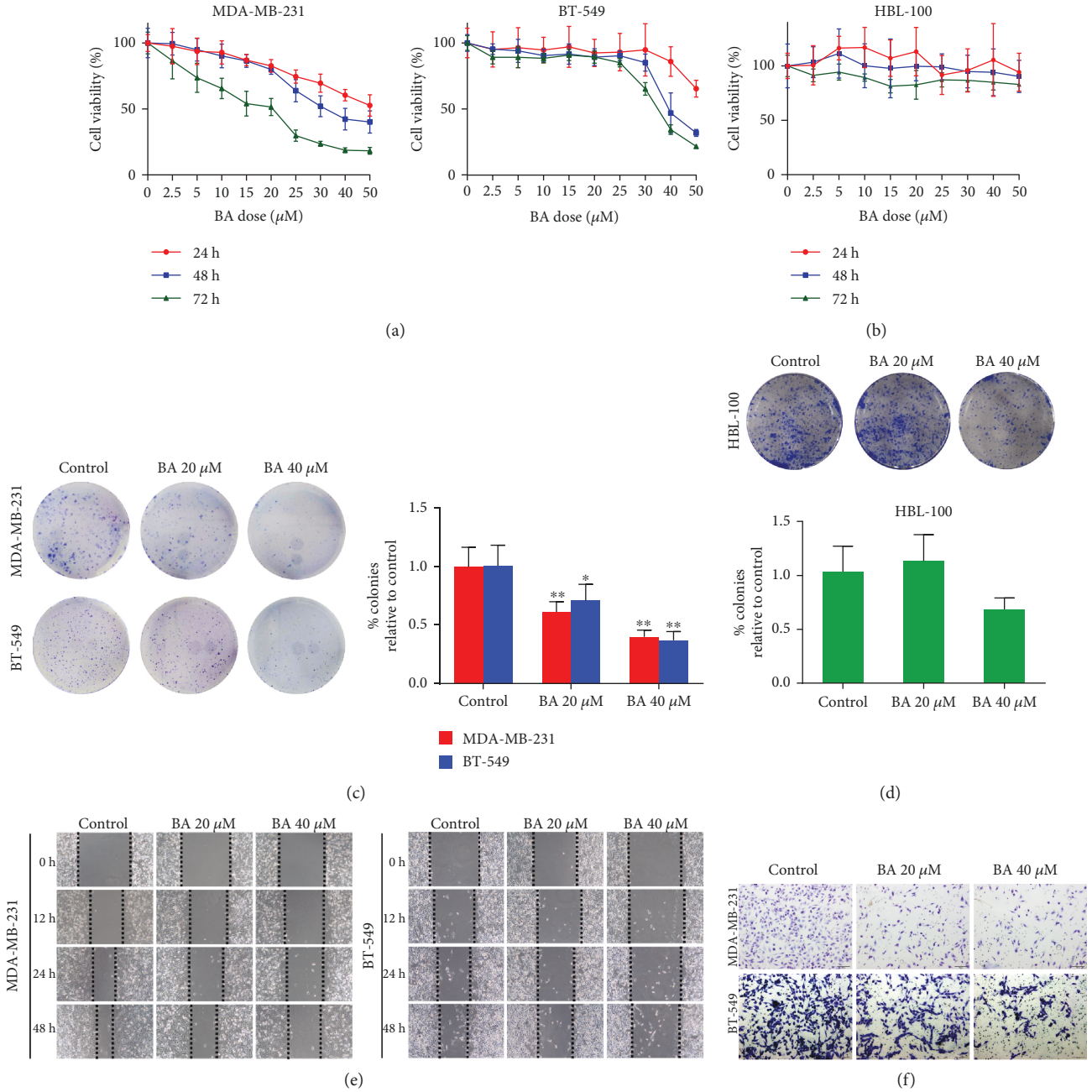

Figure 1: BA inhibits breast cancer cell proliferation and metastasis. (a) BA inhibited MDA-MB-231 and BT-549 cell viability in a dose- and time-dependent manner. (b) BA exerted minimal inhibitory effects on HBL-100. (c, d) 20 and 40 μM BA significantly suppressed colony growth of both MDA-MB-231 and BT-549, while it did not apparently affect the colony formation of HBL-100. (e) BA significantly slowed down the confluence of wound healing, revealing its ability of migration resistance. (f) Transwell assay indicated that the number of invasive cells was reduced by BA (the results were obtained from triplicate experiments and were represented as mean values ± SD; $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with control).

BA may also have the effect of inhibiting breast cancer metastasis, in addition to glycolysis suppression.

3.2. BA Blocks Breast Cancer EMT and MMP Secretion. Previous studies demonstrated that BA induced cancer cell apoptosis and DNA damage directly. Similarly, our study also identified the role of BA in inducing MDA-MB-231 and BT-549 cell apoptosis via the TUNEL assay (Figure 2(a)). Besides, H2AX was activated in response to BA treatment, reflected by the presence of double-strand DNA breaks in breast cancer cells (Figure 2(b)). On the other hand, western blotting analysis also indicated that BA downregulated the levels of N-cadherin and vimentin as the mesenchymal markers, while increased E-cadherin which is an epithelial marker (Figure 2(c)), validating the EMT inhibition effects of BA in breast cancer cells. Since matrix metalloproteinases

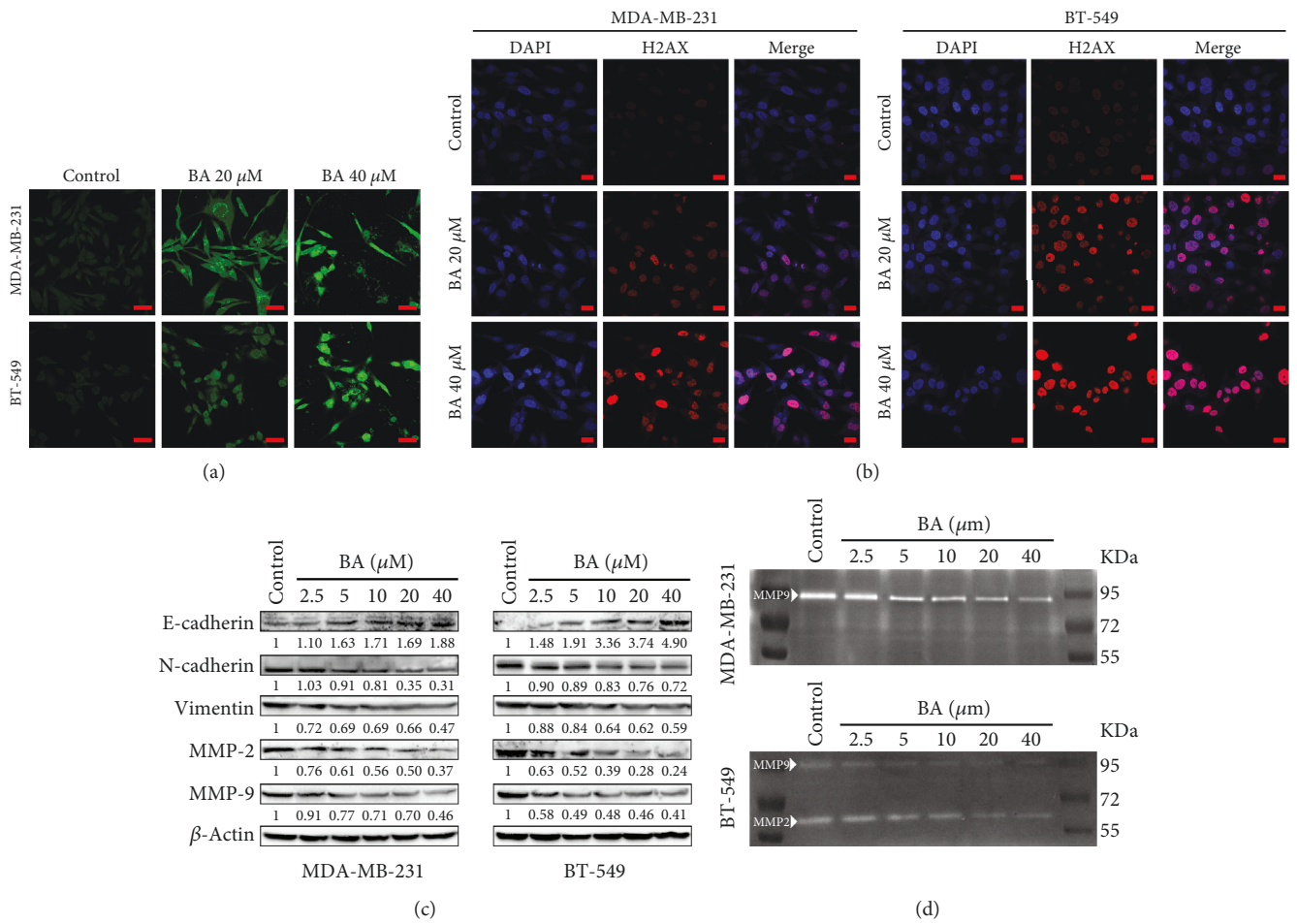

Figure 2: BA induces breast cancer cell DNA damage and inhibits EMT and MMPs. (a) TUNEL assay showed that BA induced MDA-MB-231 and BT-549 apoptosis (the scale bars indicate 50 μm). (b) BA broke double-strand DNA in MDA-MB-231 and BT-549 cells, as represented by H2AX activation (the scale bars indicate 20 μm). (c) BA reversed EMT in breast cancer cells, represented by a dose-dependent decrease in N-cadherin and vimentin and an increase in E-cadherin. MMP-2 and MMP-9 were also downregulated by BA treatment. (d) Gelatin zymography assay indicated that BA downregulated MMP-2 and MMP-9 secreted by breast cancer cells.

(MMPs) promote tumor metastasis by degrading the extracellular matrix (ECM), western blotting and gelatin zymography were used to measure the relative amounts of MMP-2 or MMP-9. The results indicated that BA significantly decreased the expression of MMP-2 and MMP-9 secreted by breast cancer cells (Figures 2(c) and 2(d)). All these findings further validated the activity of BA against breast cancer metastasis.

3.3. BA Suppresses Metastasis through β-Catenin-Mediated Aerobic Glycolysis. Based on the above findings, further studies were needed to clarify the intrinsic association of BA in glycolysis and metastasis inhibition. Therefore, a panel of metastasis-related genes was selected to identify the most responsive gene influenced by BA using qPCR. Although the relative expression of all metastasis-associated genes was downregulated, *β-catenin* ranked first among the top five responsive genes in both MDA-MB-231 and BT-549 (Figure 3(a)). Interestingly, aberrant β-catenin accumulation and the activated downstream target gene of *c-Myc* are critical to cancer metastasis and metabolic alteration. We therefore assessed changes in their protein expression level in response to BA treatment. Western blotting results showed that BA dose-dependently downregulated the expression of β-catenin and c-Myc (Figure 3(b)). Meanwhile, the levels of glycolytic enzymes, including LDHA and p-PDK1/PDK1, were all decreased in a dose-dependent manner by BA. In contrast, LDHB that catalyzes the conversion of lactate to pyruvate was increased (Figure 3(c)). Consistently, lactate production in both MDA-MB-231 and BT-549 cells was significantly reduced following BA administration (Figure 3(d)), indicating that the glycolysis pathway may be inhibited by BA. What is more important, the cell energy phenotype of MDA-MB-231 and BT-549 was profiled by the extracellular flux analyzer. The results demonstrated that the extracellular acidification rate (ECAR), which reflects the glycolysis activity, was retarded following BA administration. Additionally, the oxygen consumption rate (OCR), which is a marker of mitochondrial respiration, was also decreased simultaneously (Figure 3(e)). Overall, these results implied that BA switched the cells from an energetic metabolic state to a relatively quiescent state, which might be closely correlated with

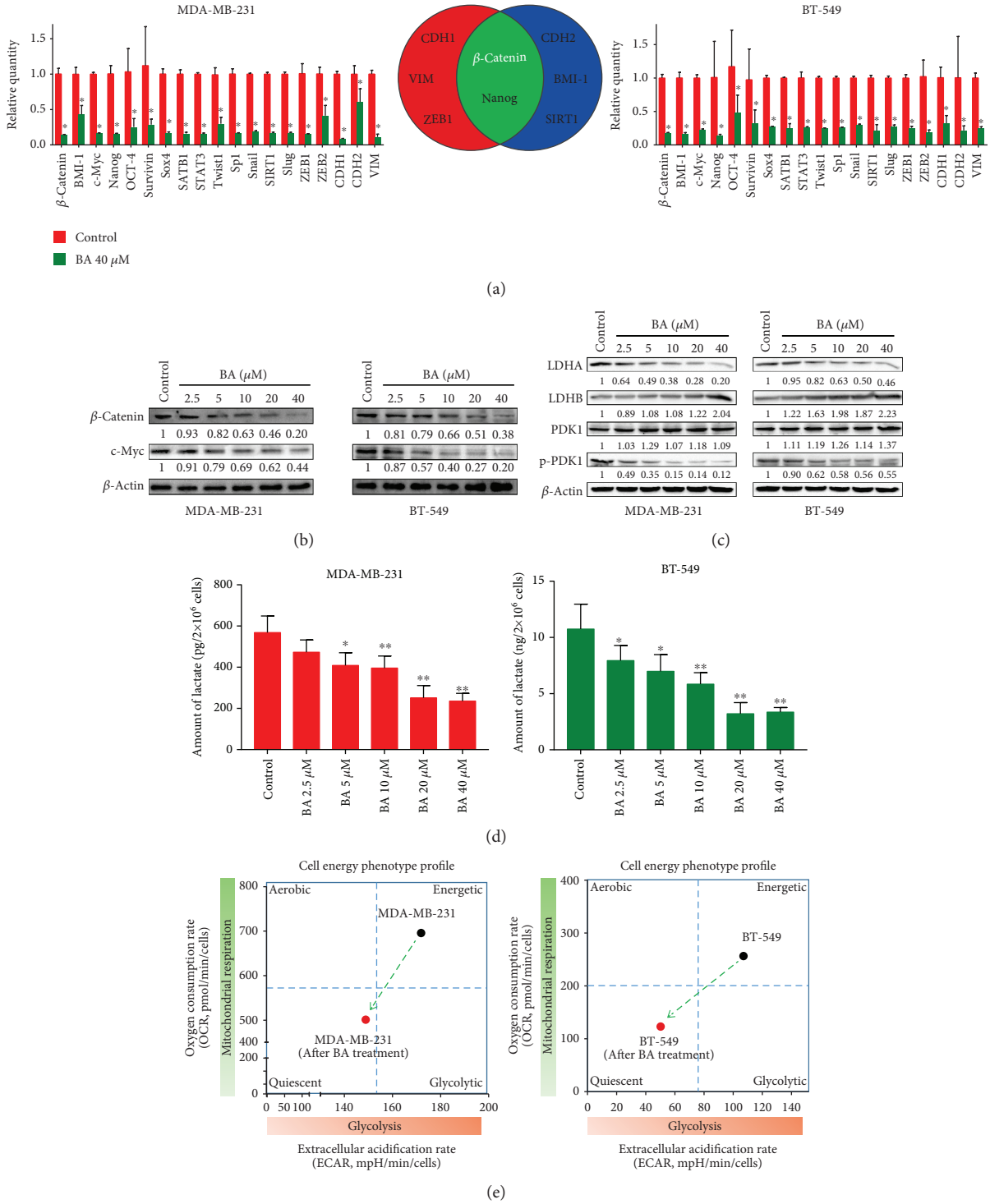

Figure 3: BA suppresses metastasis through β-catenin-mediated glycolysis. (a) The transcription levels of metastasis-related genes were screened by qPCR after BA treatment. Almost all genes were suppressed, among which β-catenin ranked as the most repressed gene in both cell lines (the results were obtained from triplicate experiments and were represented as mean values ± SD; $^{*}P < 0.05$ as compared with control). (b) Western blotting further confirmed that β-catenin and its downstream target c-Myc were downregulated by BA in a dose-dependent manner. (c) BA dramatically attenuated the levels of glycolysis-related proteins including LDHA and p-PDK1/PDK, whereas LDHB was elevated due to its function of converting lactate into pyruvate. (d) BA reduced the lactate production of MDA-MB-231 and BT-549 cells in a dose-dependent manner (values were represented as mean ± SD; $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared with control). (e) The cell energy phenotype was profiled by the extracellular flux analyzer. BA reduced ECAR and OCR values, keeping breast cancer cells in a relative quiescent energetic state.

the downregulation of the metabolic switch c-Myc. And β-catenin might be a panel point between the two hall markers of glycolysis and metastasis in tumor cells.

3.4. BA-Induced GRP78 Overexpression Restrains Aerobic Glycolysis of Breast Cancer Cells. Our previous study has uncovered that GRP78 is a molecular target for chemosensitizing effects of BA [31]. In the current study, it was also identified that GRP78 was the direct binding protein of BA in highly aggressive breast cancer cells by DARTS strategy. Following BA treatment at 0.1-100 μM, a protected band around 70 kDa was presented (Supplementary Figure 1A). The protected gel was subsequently identified as GRP78 by LC/MS analysis (Supplementary Figure 1B).

Western blotting further confirmed that BA treatment significantly enhanced GRP78 expression in MDA-MB-231 and BT-549 cells (Figure 4(a)). To determine the functional relevance of GRP78 to BA inhibition of aerobic glycolysis in breast cancer, MDA-MB-231 and BT-549 cells were transfected with a recombinant GRP78 plasmid. Consistent with the pharmacological action of BA, overexpressed GRP78 attenuated the levels of glycolytic-related proteins, including LDHA, p-PDK1/PDK1, and c-Myc (Figure 4(b)). In contrast, GRP78 knockdown promoted the expression of glycolysis-related proteins and abolished the inhibitory effects of BA (Figure 4(c)), indicating the critical role of GRP78 in mediating the pharmacological action of BA. We next studied the possible mechanism by which GRP78 induction inhibited the switch activation of β-catenin/c-Myc for glycolysis. It is known that GRP78 would activate PERK signaling under ER stress and the activated PERK would phosphorylate eIF2α, resulting in the inhibition of protein translation. Therefore, we first verified that BA activated PERK signaling and in turn promoted phosphorylation of eIF2α (Supplementary Figure 2A), which was also in line with our previous study that BA could activate ER stress apoptotic pathway. In addition, BA-induced downregulation of β-catenin was reversed by ISRIB, a specific inhibitor of PERK (Figure 4(d)). Conversely, salubrinal, a small molecule compound enhancing eIF2α phosphorylation, attenuated β-catenin expression (Figure 4(e)). Altogether, these results suggested that BA-induced downregulation of β-catenin was mediated by the GRP78/PERK/eIF2α pathway. Moreover, GRP78 and PERK could be coprecipitated in the highly aggressive breast cancer cells, and their interaction was interrupted following BA treatment in a dose-dependent manner (Figure 4(f), Supplementary Figure 2B). These findings suggested that BA inhibited the β-catenin/c-Myc pathway by interrupting the binding between GRP78 and PERK and ultimately suppressed the glycolysis of breast cancer cells.

3.5. BA Suppresses Breast Cancer Metastasis In Vivo. Given the above results and the inhibitory effects of BA on cancer cell invasion and migration *in vitro*, a lung colonization model of breast cancer was established by injecting luciferase-labeled MDA-MB-231 cells through the lateral tail vein. The doses of BA were chosen according to the literatures [20] and our preliminary experiment. In fact, BA at 250 mg/kg did not induce observable morphological variations in primary organs of mice. In addition, BA at 250 mg/kg did not lead to noticeable changes on hematological, hepatic, and renal functions in mice (data not shown). After 4 weeks of treatment by BA, bioluminescent imaging demonstrated that BA significantly inhibited breast cancer cell colony growth in the lungs, representing as reduced luminescent intensity compared with vehicle-treated controls (Figures 5(a)–5(c)). In addition, hematoxylin and eosin staining confirmed that the pulmonary metastasis lesions were remarkably suppressed after BA administration (Figure 5(d)).

3.6. BA Retards Breast Cancer Lung Colonization by GRP78/β-Catenin/c-Myc Signaling. Based on the *in vivo* results, immunohistochemistry analysis further revealed that MMP-2 and MMP-9 expressions were reduced in the lung colonization lesions after BA treatment, implying that BA might diminish the aggressiveness of breast cancer cells *in vivo* (Figure 6(a)). Immunofluorescence results further validated that BA suppressed the levels of vimentin and elevated E-cadherin expression (Figures 6(b) and 6(c)), confirming the blocking effect of BA in EMT process *in vivo*. The expressions of GRP78, β-catenin, and c-Myc were also detected in the lung colonization lesions after BA treatment. In line with previous *in vitro* findings, BA was found to enhance GRP78 expression and significantly inhibit β-catenin and c-Myc expression in the lung lesions (Figures 6(d)–6(f)), suggesting that the metastasis inhibition effects of BA were closely correlated with GRP78-mediated glycolysis inhibition. In conclusion, our results suggested that BA inhibits breast tumor metastasis *in vivo* and GRP78 might be the critical target of BA associating with its anticancer pharmacological action.

4. Discussion

Metabolic reprogramming is required for both malignant transformation and tumor development, including invasion and metastasis [35]. In this study, we found that BA restrained breast cancer metastasis by inhibiting aerobic glycolysis. Moreover, relatively high doses of BA applied *in vitro* and *in vivo* seemed to be acceptable due to its low toxicity. Also, numerous derivatives of BA have been validated with satisfactory anticancer efficacy. It is worth noting that a PEGylated derivative of BA possessed excellent water solubility of 160.2 mg/ml (about 750-fold higher than BA) and showed a high therapeutic index in a lung cancer xenograft model [36]. In addition, the structure-activity relationship analysis found that chemical modifications on the C-2 site enhanced the antitumor potency of BA [37]. However, few studies have examined why BA possesses selective cytotoxicity against cancer cells. Intriguingly, one study implied that BA might exert higher efficiency in low pH environments (around 6.8) [38]. Similar with this finding, our study also found that BA remarkably decreased cancer cell lactate production and the expression of glycolytic enzymes, which resulted in cell energy phenotype switching to a quiescent status. More importantly, our study identified GRP78, which is a glucose-regulated protein, as a direct interacting target of

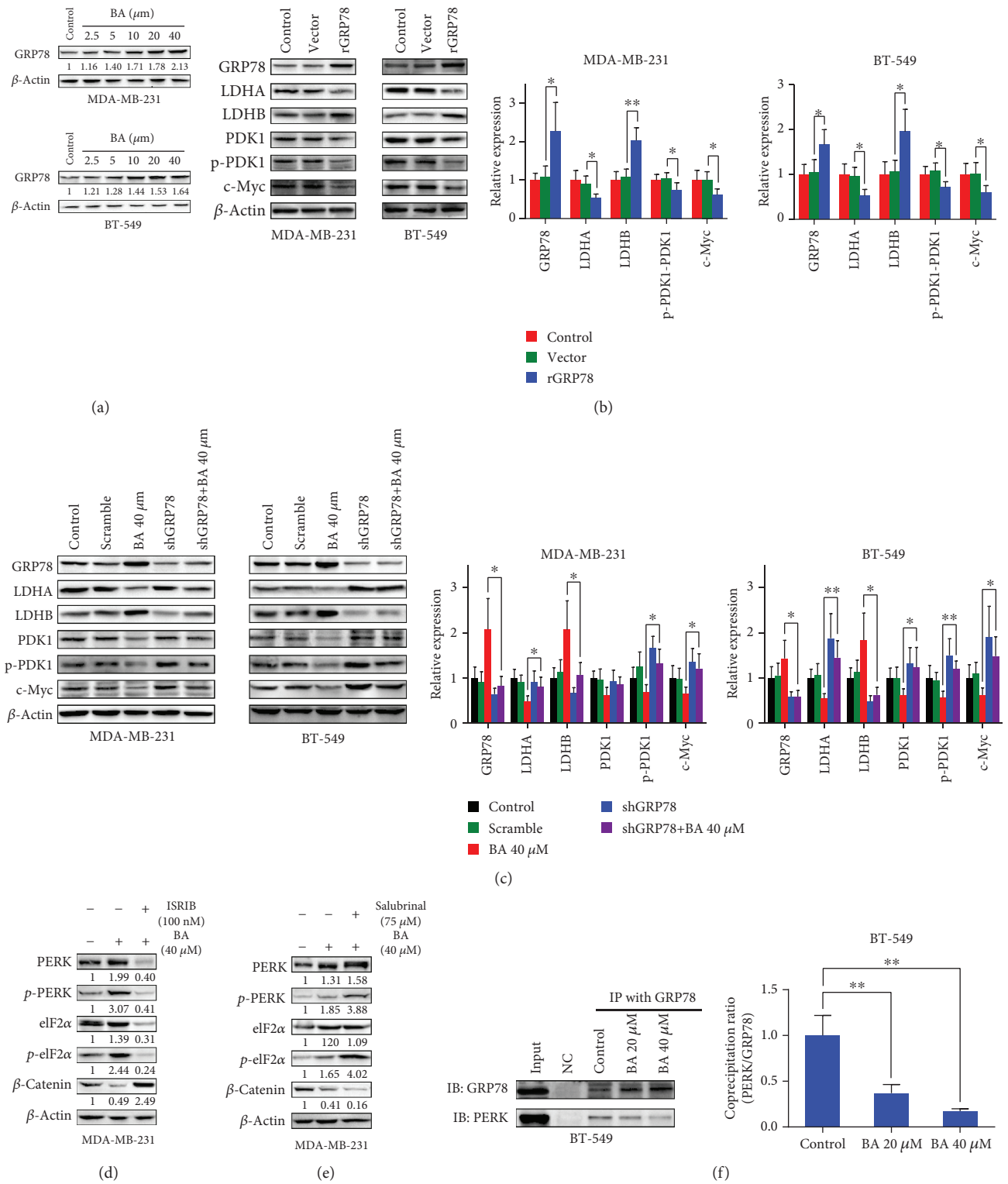

Figure 4: GRP78 overexpression suppresses aerobic glycolysis by activating PERK signaling to inhibit β-catenin. (a) Western blotting analysis verified that BA significantly enhanced GRP78 expression. (b) Overexpressed GRP78 led to the downregulation of c-Myc and subsequently decreased LDHA and p-PDK1/PDK1 but increased LDHB expression. (c) On the contrary, GRP78 knockdown reversed the inhibition of c-Myc, LDHA, and p-PDK1/PDK1 and the enhancement of LDHB induced by BA. (d) ISRIB (100 nM), the specific PERK inhibitor, inhibited eIF2α phosphorylation and reversed β-catenin inhibition induced by BA. (e) Like BA, salubrinal (75 μM) inhibited eIF2α dephosphorylation and therefore downregulated β-catenin expression. (f) Coimmunoprecipitation assay revealed the binding of GRP78 and PERK, which was disrupted by BA in a dose-dependent manner (the results were obtained from triplicate experiments and were represented as mean values ± SD; $^{*}P < 0.05$ and $^{**}P < 0.01$).

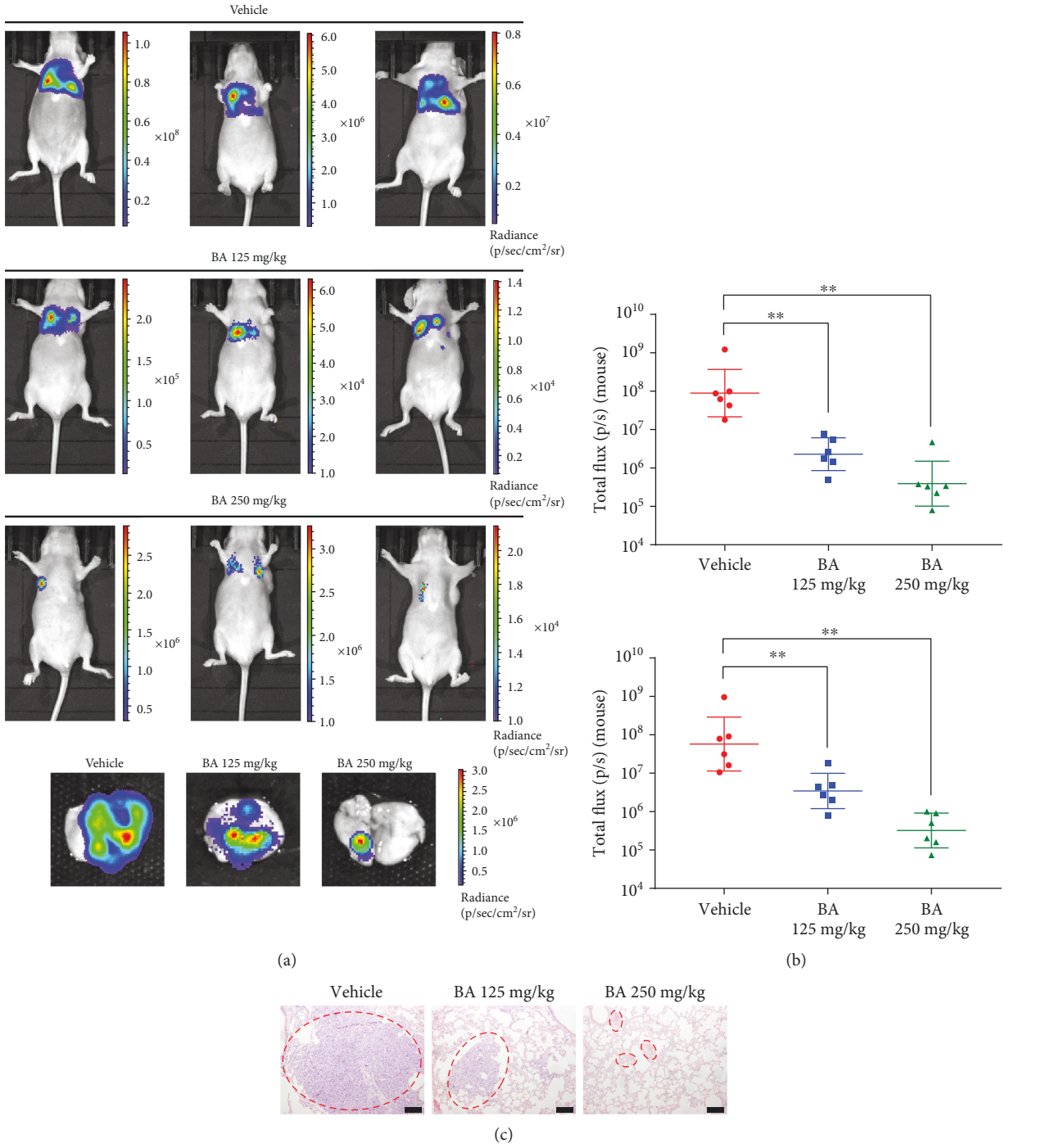

FIGURE 5: BA inhibits breast cancer lung colonization *in vivo*. (a) Bioluminescent imaging indicated that BA significantly reduced breast cancer cell lung colonization compared with vehicle-treated controls. (b) Logarithmic value of luminescent intensity after treatment with vehicle or BA (values represented as the mean ± SD, $n = 6$, $^{**}P < 0.01$). (c) Hematoxylin and eosin staining demonstrated the reduction of lung metastatic lesions in BA-treated mice (the scale bars indicate 100 μm).

BA, further manifesting the central role of glucose metabolism in mediating the selective killing effects of BA in cancer cells. Actually, other pharmacological studies have also demonstrated that BA could reduce prostate cancer angiogenesis *via* inhibiting the HIF-1α/stat3 pathway [39]. A proteomic study also implied that a ROS-mediated pathway was the main target responsible for mediating the anticancer activities of BA [40]. All these findings suggested that the anticancer pharmacological mechanism of BA might be associated with stress signaling.

Aberrant cellular stress is another hallmark of cancer [41]. The rapid proliferation of cancer cells creates a relatively nutrient-starved microenvironment, causing cancer cells to adapt to this "stressful" condition by activating ER

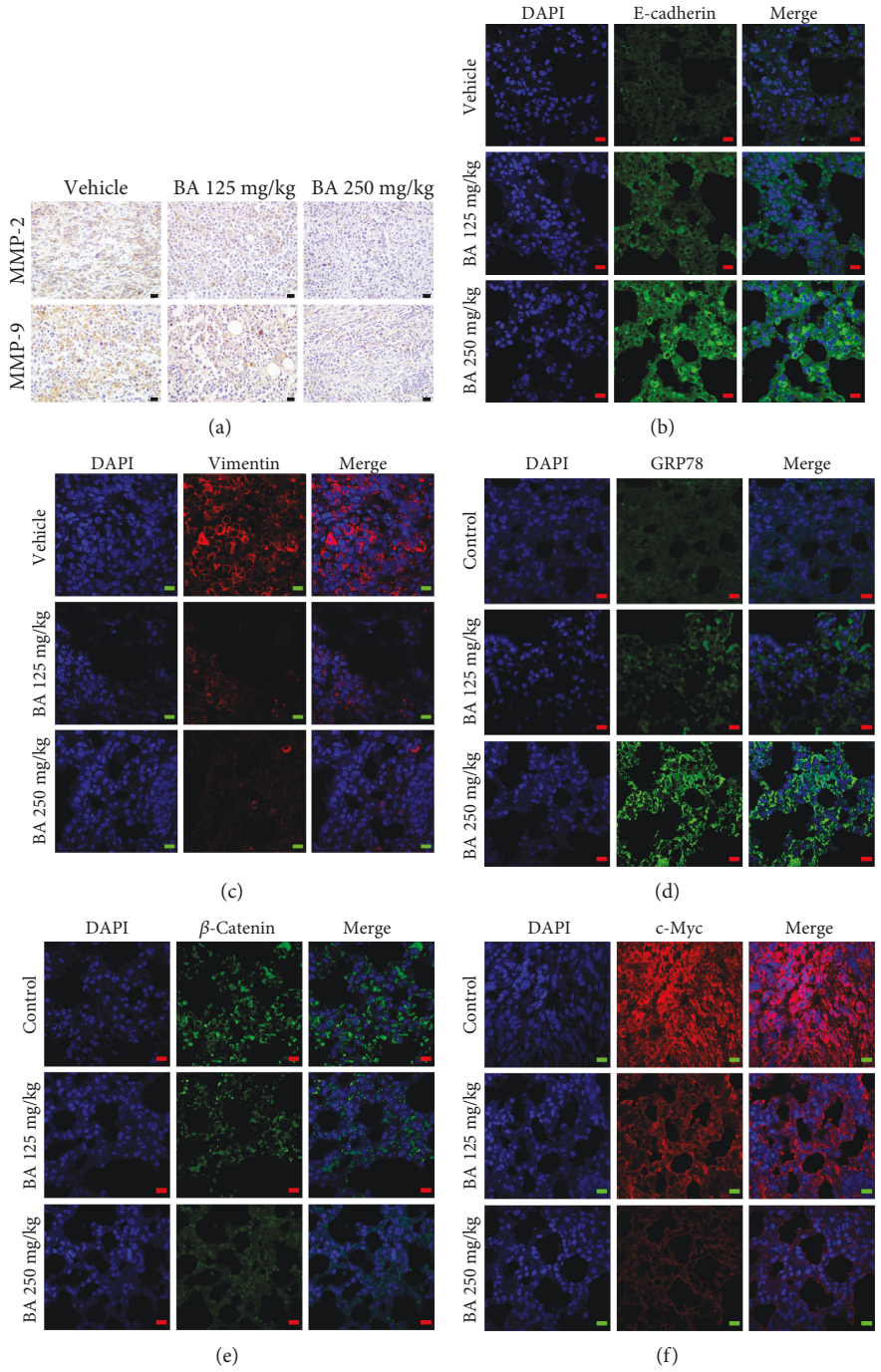

Figure 6: BA inhibits glycolysis signaling in metastatic lesions by targeting GRP78/β-catenin/c-Myc signaling. (a) MMP-2 and MMP-9 expressions were attenuated by BA administration in the lung colonization lesions (the scale bars indicate 20 μm). (b, c) Immunofluorescence showed that BA increased E-cadherin expression in the lung tissue but reduced the expression of vimentin, suggesting that EMT in breast cancer was blocked by BA *in vivo* (the scale bars indicate 10 μm). (d–f) Immunofluorescence demonstrated that BA significantly elevated GRP78 levels and decreased β-catenin/c-Myc signaling in lung metastatic lesions (the scale bars indicate 10 μm).

stress signaling [42]. Emerging evidence has demonstrated that ER stress influences cellular metabolism through various mechanisms. ER stress is known to stimulate lipogenesis through the unfolded protein response (UPR), thereby providing lipids for ER expansion. Mechanistic studies have revealed that ER stress can promote fatty acid and cholesterol biosynthesis through two major transcriptional regulators: SREBP1 and SREBP2 [43, 44]. In addition, PERK, as the sensor of ER stress, has also been reported to be significant for lipogenic tissue development, since PERK knockout impairs mouse mammary gland lipogenesis during pregnancy, which leads to a reduction of free fatty acid in milk

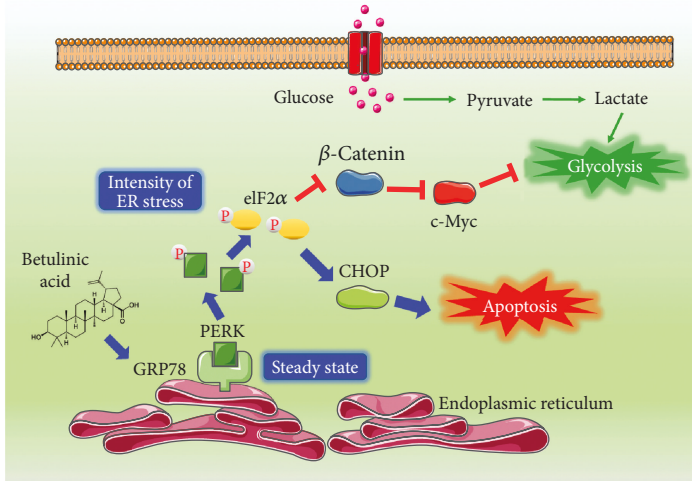

Figure 7: Diagram illustrating how BA inhibited glycolysis by targeting GRP78. BA interrupted the binding of GRP78 and PERK, which initiated ER stress, and subsequently activated eIF2α phosphorylation, resulting in β-catenin inhibition and c-Myc-mediated aerobic glycolysis. Meanwhile, the ER stress apoptotic pathway was triggered.

[45]. Furthermore, ER stress would facilitate physical coupling and calcium transfer from ER to mitochondria, resulting in augmented mitochondrial respiration and bioenergetics, thereby enhancing adaptive ATP production [46]. However, it is still unclear how ER stress affects cancer aerobic glycolysis. In the present study, we reported that the ER stress protein GRP78 inhibited aerobic glycolysis by promoting PERK activation, which impaired β-catenin production and consequently inhibited downstream c-Myc-mediated glycolysis (Figure 7). Our study proposes a regulatory relationship between ER stress and glycolysis metabolism in tumor cells and highlights that targeting ER stress to inhibit cancer aerobic glycolysis might be a novel strategy for cancer therapy.

GRP78, a biomarker of ER stress, is highly expressed in multiple malignancies. Additionally, GRP78 has also been reported to play an important role in cancer metastasis. For example, GRP78 was identified as a potential diagnostic biomarker for the early detection of melanoma metastasis [47]. High expression of GRP78 was also detected in the metastatic phenotype of prostate cancer [14], hepatocellular carcinoma [48], and esophageal squamous cell carcinoma [49]. In our study, the precise target of BA was identified as GRP78 by using DARTS technology. However, the antimetastasis effects of BA were found to be correlated with enhanced GRP78 expression. Meanwhile, GRP78 overexpression could suppress the expression of glycolytic proteins including LDHA, PDK1, and c-Myc. These results indicated that GRP78 overexpression could inhibit cancer glycolysis. Our findings kept consistency with the report by Li et al. [17], which found that GRP78 overexpression induced a decline in the PKM2 level in colorectal cancer cells. What is more important, the overexpression of GRP78 also downregulated the LDHA mRNA expression, accompanied by reduced lactic acid secretion. However, some studies also reported that GRP78 overexpression could enhance glycolysis activity. For example, Miharada et al. found that Cripto/GRP78 signaling could improve glycolysis activity in hematopoietic stem cells by regulating HIF-1α [50, 51]. As well known, GRP78, as the stress-responsive chaperone, plays dual roles in cancer initiation and development. To adapt to the hypoxic and glucose-deprivation microenvironment in tumor tissues, GRP78 was activated to degrade the unfolded protein to satisfy the nutrient requirements of cancer cells. Therefore, GRP78 upregulation was usually found in multiple cancer cells and associated with drug resistance and metastasis and angiogenesis. However, when GRP78 was overactivated, it will trigger the ER stress apoptotic pathway and induce cell death. Furthermore, the activation of the PERK/eIF2α pathway in the ER stress signaling would result in the inhibition of β-catenin translation, which led to c-Myc-mediated glycolysis suppression (Figure 7). Since the expression of GRP78 is significantly higher than normal cells, further exogenous overexpression of GRP78 would make cells to be in an excessive stressful situation and towards apoptosis finally. Therefore, selectively triggering ER stress by targeting GRP78 in cancer cells might be a promising approach for future drug discovery. Although BA demonstrated selective killing effects in cancer cells, its specificity towards GRP78 and targeting for drug delivery still needs to be investigated.

5. Conclusions

In conclusion, this study uncovered the mechanism of BA in inhibiting breast cancer metastasis by targeting GRP78 to trigger ER stress signaling, subsequently suppressing aerobic glycolysis. Our results shed new light on BA antimetastasis through suppressing aerobic glycolysis and also highlight GRP78 as a potential regulatory target for tumor glucose metabolism.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplementary Figure 1: identification of GRP78 as a target of BA in highly aggressive breast cancer cells. (A) After treatment with BA, a protected band around 70 kDa emerged by Coomassie blue staining. The black arrow indicates the protected band. (B) The most probable protein was identified as GRP78 through mass spectroscopy analysis. The representative peptide mass fingerprints were shown. Supplementary Figure 2: BA promotes the dissociation of GRP78 and PERK to activate the PERK signaling of ER stress. (A) PERK signaling and eIF2α phosphorylation were provoked by BA in a dose-dependent manner. (B) The binding between GRP78 and PERK was found in MDA-MB-231 cells by coimmunoprecipitation assay, which was attenuated after BA treatment (the results were obtained from triplicate experiments and were represented as mean values ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$). *(Supplementary Materials)*

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Research Article

Oleuropein-Induced Apoptosis Is Mediated by Mitochondrial Glyoxalase 2 in NSCLC A549 Cells: A Mechanistic Inside and a Possible Novel Nonenzymatic Role for an Ancient Enzyme

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Oleuropein (OP) is a bioactive compound derived from plants of the genus Oleaceae exhibiting antitumor properties in several human cancers, including non-small-cell lung cancer (NSCLC). Recent evidence suggests that OP has proapoptotic effects on NSCLC cells via the mitochondrial apoptotic pathway. However, the exact molecular mechanisms behind the apoptogenic action of OP in NSCLC are still largely unknown. Glyoxalase 2 (Glo2) is an ancient enzyme belonging to the glyoxalase system involved in the detoxification of glycolysis-derived methylglyoxal. However, emerging evidence suggests that Glo2 may have also nonenzymatic roles in some malignant cells. In the present study, we evaluated whether and how Glo2 participated in the proapoptotic effects of OP in NSCLC A549 cells. Our results indicate that OP is able to induce apoptosis in A549 cells through the upregulation of mitochondrial Glo2 (mGlo2), mediated by the superoxide anion and Akt signaling pathway. Moreover, our data shows that the proapoptotic role of mGlo2, observed following OP exposure, occurs via the interaction of mGlo2 with the proapoptotic Bax protein. Conversely, OP does not alter the behavior of nonmalignant human BEAS-2B cells or mGlo2 expression, thus suggesting a specific anticancer role for this bioactive compound in NSCLC. Our data identify a novel pathway through which OP exerts a proapoptotic effect in NSCLC and suggest, for the first time, a novel, nonenzymatic antiapoptotic role for this ancient enzyme in NSCLC.

1. Introduction

1.1. OP and Cancer. Oleuropein (OP) is an olive-derived polyphenol with an array of pharmacological properties, including anti-inflammatory and antioxidant effects [1], which have fostered intense interest in cancer research as a putative anticancer agent. Specifically, several lines of *in vitro* [2, 3] and *in vivo* [1] evidence demonstrate both antiproliferative and proapoptotic effects of this secoiridoid.

1.2. Lung Cancer and OP Effect on Non-Small-Cell Lung Cancer (NSCLC). Lung cancer is the leading worldwide cause of cancer mortality [4]. While recent years have seen notable advances in the treatment of many cancers, the prognosis for non-small-cell lung cancer (NSCLC), which accounts for 85% of all lung cancer cases, remains dire. The lack of therapies capable of curing or even prolonging survival (estimated survival rate of only 15% at 5 years) of NSCLC highlights the urgent need for the identification of proteins and pathways responsible for the development and progression of the disease as potential drug targets for novel, effective, and safe therapeutic options. Epidemiologic studies have shown an inverse correlation between olive oil consumption and the risk of lung cancer [5–7], and recent findings have shown that OP exerts cytotoxic effects, by inducing mitochondrial apoptosis, in NSCLC cells [8, 9]. However, the molecular mechanisms driving the apoptogenic action of OP in NSCLC remain unclear.

1.3. Glyoxalases and Cancer. Glyoxalase 2 (Glo2) and glyoxalase 1 (Glo1) are together responsible for the detoxification of methylglyoxal (MG), a metabolic by-product of glycolysis with strongly proapoptotic effects [10]. Specifically, using glutathione (GSH) as a cofactor, Glo1 converts MG to S-D-lactoylglutathione (LSG) which is then hydrolyzed to D-lactate, regenerating GSH [10]. Recent evidence suggests that cancer cells show bioenergetic versatility depending on the genetic heterogeneity of the tumor [11, 12]. If on the one hand, many types of tumors consume larger amounts of glucose, compared to normal tissues, as Warburg originally observed, on the other hand, high glycolytic rates in tumors and mitochondrial respiration often operate simultaneously [11]. Moreover, it has been reported that cancer cells can also use a range of fuels including glutamine, fatty acids, heme flux, and acetate to sustain their growth and progression [11]. Cancers with enhanced glycolysis, such as prostate cancers [13, 14], are characterized by an increase in expression and enzyme activity of glyoxalases that reduce the production of MG, favoring escape from apoptosis [10, 15]. We have recently demonstrated, in prostate cancer cells, that Glo2 is involved in the control of apoptosis, in a Glo1-independent and possibly nonenzymatic manner, through the modulation of intracellular levels of p53 [16]. Here, we examined whether and how Glo2 might be involved in the proapoptotic effect of OP in NSCLC A549 cells and nonmalignant BEAS-2B cells.

2. Materials and Methods

2.1. Cells and Reagents. Human NSCLC A549 cells and human noncancerous BEAS-2B cells were purchased from Merck Spa (Milan, Italy). Cells were grown in RPMI-1640 (Thermo Fisher Scientific, Monza, Italy) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, Monza, Italy) and 1% antibiotics (penicillin-streptomycin) (Thermo Fisher Scientific, Monza, Italy) at 37°C in a humidified incubator with 5% CO_2. Confluent cells were treated with OP (Vinci-Biochem Srl, Florence, Italy) at final concentrations of 50 and 150 μM (in dimethyl sulfoxide, DMSO) for 24 h [8, 9]. The most robust biological effects were obtained using 150 μM OP 24 h post exposure, so mechanistic studies were carried out using this concentration. Untreated cells, incubated for the same time period, were used as controls. Separately, the effects of joint treatment with the specific Akt inhibitor MK2206 (10 μM in DMSO for 48 hours) or SC79 Akt activator (4 μg/ml in DMSO for 30 minutes) followed by 150 μM OP for a further 24 hours were also examined. At the concentration, used MK2206 or SC79 exhibited no significant toxicity to cells. Unless otherwise stated, the biochemical evidence of inhibitor efficacy was established in preliminary experiments mainly by western blot analysis (data not shown). For agents in DMSO, final DMSO concentration in incubations was 0.01%. Controls contained an identical volume of DMSO vehicle. Laemmli buffer and the bicinchoninic acid (BCA) kit for protein quantification were from Thermo Fisher Scientific (Monza, Italy); Roti-Block was from Prodotti Gianni (Milan, Italy). The antibodies used in this study included the following: rabbit anti-Glo2 polyclonal antibody (pAb), mouse anti-Glo1 (D6) monoclonal antibody (mAb), mouse anti-rabbit anti-Bcl-XL pAb, rabbit anti-Bax (N20) pAb, rabbit anti caspase-3 pAb, and mouse anti-β-actin mAb from DBA Italia srl (Milan, Italy). Rabbit anti-p-Akt (Ser473) mAb was from Sigma-Aldrich (Milan, Italy); mouse anti-Bcl-2 mAb was from Dako (Milan, Italy); mouse anti-cytochrome c (Cyt c) mAb and mouse anti Apaf-1 mAb (clone 24) were from BD Pharmingen (Milan, Italy); mouse anti-Cyt c oxidase subunit IV (Cox IV) mAb was from Molecular Probes (Monza, Italy); mouse anti-SOD2 mAb was from Abcam (Milan, Italy).

2.2. Apoptosis Detection. Apoptosis was quantified by two methods, firstly by measuring the activation of caspase-3, using an enzyme-linked immunosorbent assay (ELISA) (Thermo Fisher Scientific, Monza, Italy), specific for activated human caspase-3 following the manufacturer's instructions, and secondly by DNA fragmentation using agarose gel electrophoresis as previously described [17, 18].

2.3. Enzymatic Activity Assays. Enzymatic activity assays were conducted on cell extracts prepared as previously described [19]. Briefly, cells treated (24 h) with OP were harvested and resuspended (10^7 cells/ml) in 10 mM phosphate buffer pH 7.0, containing 1 mM dithiothreitol (for the detection of Glo2 and Glo1 enzymatic activities) and 0.1 mM phenylmethanesulphonylfluoride (PMSF). Cells were then homogenized with a Potter-Elvehjem homogenizer, and cell debris removed by centrifugation (13,000 x g for 30 min) and the resulting cell supernatants were assayed for protein content and enzymatic activity. Mitochondrial extracts were prepared using a Mitochondria/Cytosol Fractionation Kit (BioVision, Florence, Italy), according to the manufacturer's instructions. Protein concentration was determined with a bicinchoninic acid (BCA) kit (Pierce), by reference to a standard curve prepared with bovine serum albumin. Glo1 activity was assayed according to Mannervik et al. [20]. The assay solution contained 0.1 M sodium-phosphate buffer pH 7.2, 2 mM MG, and 1 mM GSH. Activity was measured spectrophotometrically by monitoring the increase of absorbance at 240 nm at 25°C. One unit of activity was defined as 1 μmol of S-D-lactoylglutathione produced min^{-1}. Conversely, Glo2 activity was assayed spectrophotometrically, at 25°C by recording the decrease in absorbance at 240 nm due to S-D-lactoylglutathione (0.3 mM) hydrolysis [21, 22]. One unit activity was defined as 1 μmol S-D-lactoylglutathione hydrolyzed/min. Finally, SOD activity was measured using Calbiochem's Superoxide Dismutase Assay Kit II (EMD Chemicals, Gibbstown, NJ) according to the manufacturer's directions. One unit of SOD activity was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

2.4. Cell Lysate Preparation for Western Blot. Cells (10^6) were lysed in precooled radioimmunoprecipitation assay (RIPA) lysis buffer, proteins separated by SDS-PAGE, and subjected to Western blot analysis as previously described [23, 24]. Briefly, samples of equal protein concentration were mixed with Laemmli buffer and boiled for 5 minutes then resolved

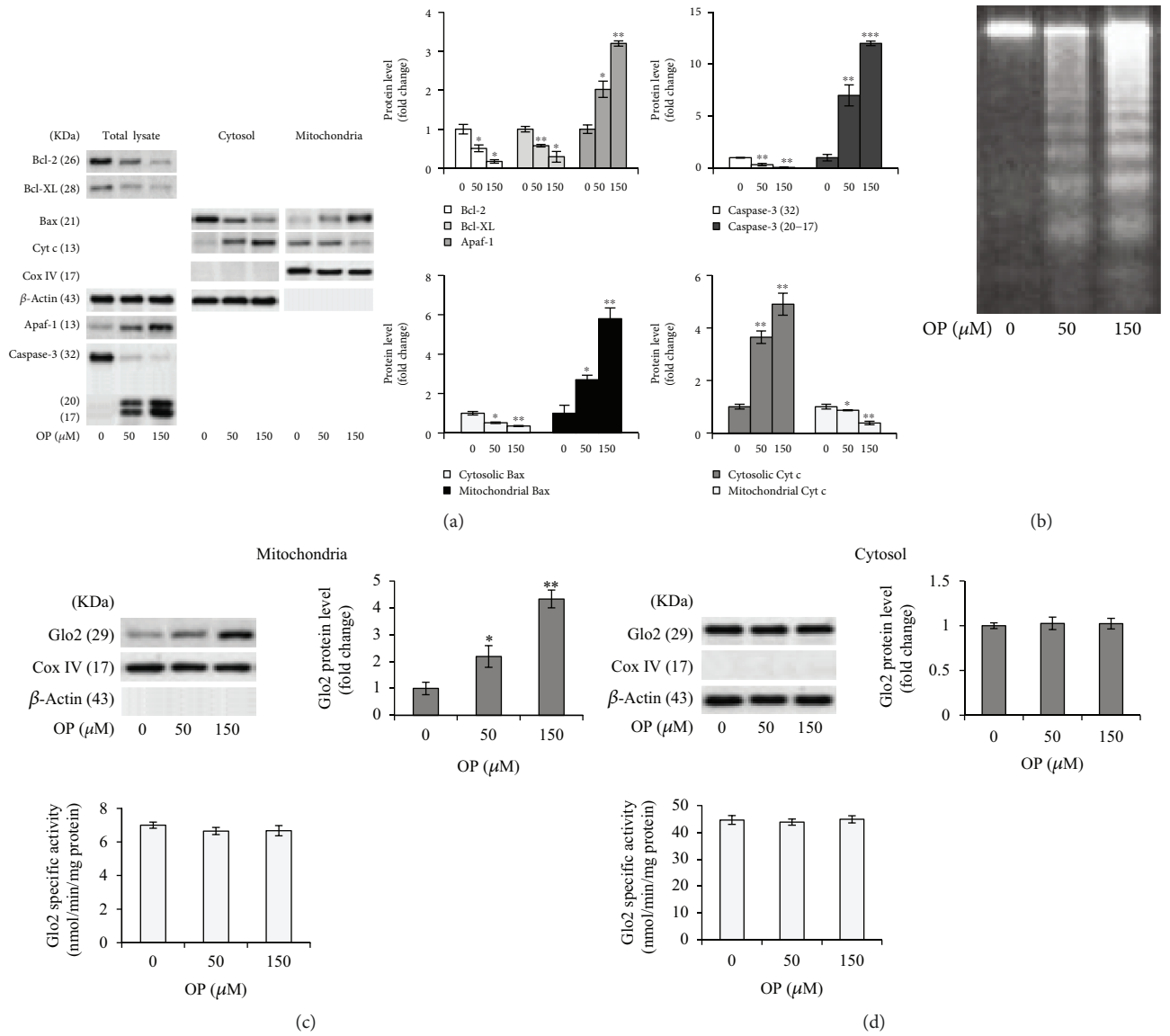

Figure 1: The proapoptotic effect of OP is associated with increased expression of mitochondrial Glo2 in NSCLC A549 cells. (a) Antiapoptotic Bcl-2 and Bcl-XL, proapoptotic Bax, cytochrome c (Cyt c), Apaf-1, and caspase-3 (intact protein, 32 kDa molecular weight; active fragments, 20 and 17 kDa molecular weight) protein expression in untreated (0 μM) and oleuropein- (OP-) treated (50 and 150 μM) A549 cells. (b) Apoptosis was confirmed at morphological level by DNA fragmentation, evaluated by gel electrophoresis. Electrophoresis is a representative of three independent experiments providing the same result. Evaluation of Glo2 expression, by western blot, and enzyme specific activity, by a spectrophotometric assay, in the (c) mitochondria and (d) cytosolic lysates of A549 cells. Histograms indicate the means ± SD of three different cultures, each of which was tested in quadruplicate and expressed as a percentage of control. Western blot analysis of β-actin or CoxIV expression is provided to show equal loading of the samples and to demonstrate successful enrichment of mitochondria in fractionated extracts. Blots are representative of three independent experiments, which gave the same results. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, significantly different from control untreated cells.

on 4-15% SDS-PAGE and blotted onto a nitrocellulose membrane (iBlot Dry Blotting System, Thermo Fisher Scientific, Monza, Italy). Membranes were blocked in Roti-Block for 1 h at room temperature, incubated overnight at 4°C with an appropriate dilution of the primary Abs. After washing with TBST, membranes were incubated (1 h, RT) with the appropriate HRP-conjugated secondary Ab and visualized using ECL (Amersham Pharmacia, Milan, Italy). The primary Ab was then stripped by incubating membranes in stripping buffer (100 mM 2-ME, 2% SDS, and 62.5 mM Tris-HCl, pH 6.8) and reprobed with an Ab against an appropriate housekeeping protein as an internal loading control.

2.5. Superoxide Anion Detection. Intracellular $O_2^{\cdot-}$ production after OP treatment was detected using dihydroethidium (DHE) (Sigma-Aldrich, Milan, Italy) [25, 26]. DHE enters cells and reacts with the superoxide anion to form ethidium, which exhibits red fluorescence. Briefly, confluent A549 cells were exposed to OP and then incubated with 5 μM DHE in HBSS (2 mM $CaCl_2$, 1 mM $MgSO_4$) at 37°C for 30 min. At

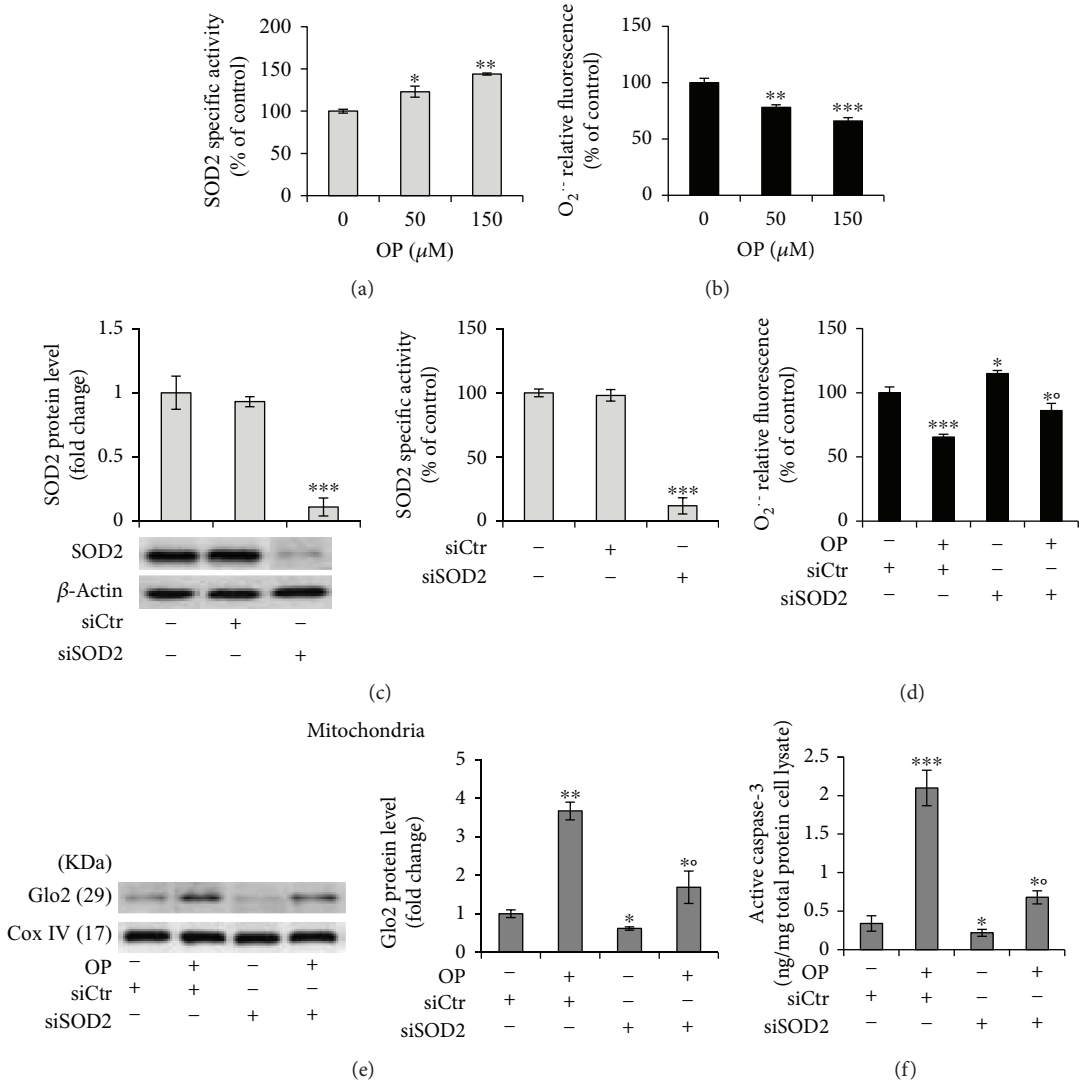

Figure 2: OP induces apoptosis through a mechanism involving SOD2-mediated superoxide anion- ($O_2^{\cdot-}$-) dependent mGlo2 upregulation in NSCLC A549 cells. (a) Superoxide dismutase 2 (SOD2) activity and (b) superoxide anion ($O_2^{\cdot-}$) level in untreated (0 μM) and oleuropein- (OP-) treated A549 cells. Under 150 μM OP exposure, SOD2 silencing by small interfering RNA (siSOD2) (c) significantly reversed (d) $O_2^{\cdot-}$ levels, (e) mitochondrial Glo2 expression, and (f) apoptosis, evaluated by active caspase-3 expression. Western blots were stripped of the bound Abs and reprobed with anti-β-actin or anti-Cox-IV, to confirm equal loading. The western blots shown are representative of three separate experiments. Histograms indicate the means ± SD of three different cultures each of which was tested in triplicate. siCtr: control (nonspecific siRNA), (-) untreated, and (+) treated cells; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus unexposed cells; $^{\circ}p < 0.05$ versus OP-treated cells.

the end of the incubation, cells were detached by scraping and disrupted by sonication. After clarification (13000 x g, 5 min, 4°C), supernatants were collected and fluorescence was measured using a fluorimeter (Kontrol Instrument, SFM 25, Eching, Germany) (excitation 488 nm, emission wavelength 512 nm). The results were confirmed using an HE-based HPLC assay (data not shown).

2.6. Gene Silencing. Pools of four small interfering RNA (siRNA) oligonucleotides targeting SOD2 (siSOD2) (ON-TARGET plus SMART pool siRNA) or non-targeting siRNA oligonucleotides (siCtr) (ONTARGET plus siCONTROL) as a negative control (all from Dharmacon RNA Technologies, Carlo Erba, Milan, Italy) were transiently transfected into NSCLC A549 cells using DharmaFECT 1 transfection reagent (Dharmacon RNA Technologies, Carlo Erba, Milan, Italy), according to the manufacturer's instructions. Potential effects due solely to the transfection reagent were controlled by performing mock transfections without any siRNA (data not shown). Since the biological readouts examined here were indistinguishable in nontransfected, mock-treated, or siCtr-treated cells, the observed changes resulting from siSOD2 treatment were reported relative to siCtr-exposed cells only.

2.7. Immunoprecipitation. Immunoprecipitation (IP) was performed using Dynabeads Protein G Immunoprecipitation Kit (Thermo Fisher Scientific, Monza, Italy) according to the

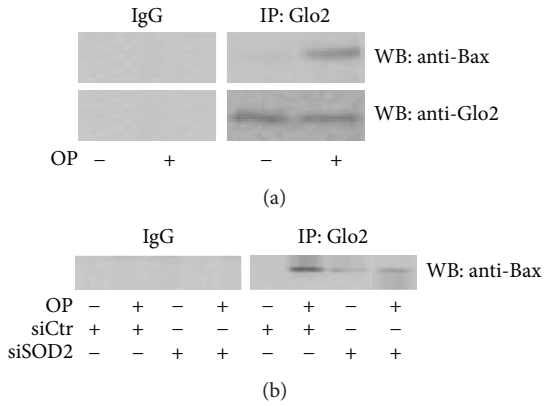

Figure 3: OP drives apoptosis in NSCLC A549 cells by promoting mGlo2 association to Bax. (a) Mitochondrial lysates from A549 cells treated with 150 μM oleuropein (OP) were immunoprecipitated with protein G agarose-coupled anti-Glo2 (IP : Glo2) and subjected to western blotting (WB) with the anti-Bax antibody (WB : anti-Bax). Blots were then stripped and reprobed with the anti-Glo2 antibody to ensure equal immunoprecipitation of Glo2 protein. Mouse IgG was used as a negative control for immunoprecipitation; (b) mitochondrial lysates from A549 cells treated with 150 μM OP under SOD2 silencing (siSOD2) or control (siCtr, nonspecific siRNA) were immunoprecipitated with protein G agarose-coupled anti-Glo2 (IP : Glo2) and subjected to western blotting (WB) with the anti-Bax antibody (WB : anti-Bax).

manufacturer's instructions. Briefly, supernatants of cells lysed in ice-cold RIPA lysis buffer containing a protease inhibitor cocktail were incubated (4 h, 4°C) with protein G Dynabeads to which a rabbit anti-Glo2 Ab had been pre-bound (O/N, 4°C). Immune complexes were recovered magnetically and analyzed by Western blot using either anti-Bax or anti-Glo2 antibodies as described above.

2.8. Statistical Analysis. All data were generated from three independent biological replicates and expressed as the means ± standard deviation (SD). One-way analysis of variance with Dunnett's correction was used to assess differences among groups when appropriate. The statistical significance, determined by Student's t-test, was set at $p < 0.05$.

3. Results and Discussion

3.1. The Proapoptotic Effect of OP Is Associated with Mitochondrial Glo2-Increased Expression in NSCLC A549 Cells. The proapoptotic effect of OP in NSCLC A549 cells and the related intrinsic apoptosis mechanism were evaluated by measuring the levels of major proteins typically activated in a mitochondrial apoptotic pathway, namely, the antiapoptotic Bcl-2 or Bcl-XL or the proapoptotic Bax proteins, Cyt c, Apaf-1, and the final executioner caspase-3 [18] by immunoblotting. As shown in Figure 1(a), we found a significant dose-dependent decrease in the levels of the antiapoptotic Bcl-2 or Bcl-XL proteins paralleled by a marked increase in the levels of the proapoptotic Bax protein as well as its translocation from the cytosol to the mitochondria, in OP-exposed cells compared to controls. Concomitantly, Cyt c release into the cytosol, as well as the activation of Apaf-1 and caspase-3, was observed (Figure 1(a)). DNA fragmentation into oligonucleosomes, a hallmark of apoptosis, confirmed the apoptotic responses at the morphological level, as evidenced by the typical DNA laddering response (Figure 1(b)). Hence, in line with the literature [8, 9], our results show that OP induces apoptosis in NSCLC A549 cells through a mitochondrial pathway. Glo2 is an ancient enzyme that together with Glo1 participates in the removal of cytotoxic MG [10]. Very little is known about Glo2, including its role in MG scavenging and its functional significance in health and disease [10, 15, 16]. We have recently demonstrated, in prostate cancer cells, that Glo2 is involved in the control of apoptosis, in a Glo1-independent and possibly nonenzymatic manner, through the modulation of intracellular levels of p53 [16]. In particular, Glo2 protected cancer cells from apoptosis [16]. In humans, two Glo2 isoforms have been identified, one in the cytosol (cGlo2) and one in the mitochondrion (mGlo2) [27]. These isoforms are encoded by a single Glo2 gene through alternate translational start sites [28]. In order to investigate whether Glo2 could be involved in OP-induced apoptosis, we studied the protein expression and specific activity of the Glo2 enzyme in the mitochondrial and cytosolic fractions of A549 cells exposed to OP. Unexpectedly, we found that OP induced a dose-dependent and statistically significant increase in mGlo2 protein levels without affecting the enzyme's specific activity (Figure 1(c)). Similarly, OP did not modify cGlo2 expression either at protein or at functional level (Figure 1(d)). Overall, these results indicated that the proapoptotic effect of OP is associated with an increase in mGlo2, suggesting a novel mechanism by which this natural bioactive compound exerts its apoptogenic function in NSCLC A549. Moreover, our findings suggest a proapoptotic role of Glo2, at least of the mitochondrial isoform and at least following OP exposure in NSCLC A549 cells. This role turns out to be opposite to that observed for Glo2 in prostate cancer cells, where conversely, an antiapoptotic nonenzymatic role of this protein was described [10]. Even though the antiapoptotic action of Glo2 in prostate cancer cells has been shown without discriminating between the mitochondrial or cytosolic isoform, our findings highlight a potential and intriguing complexity of Glo2 biology, providing powerful motivation for further research. Moreover, these data showed that mGlo2 involvement in the apoptosis driven by OP is independent from its traditional function as a metabolic enzyme, positing this isoform as a "moonlighting" protein (a protein with more than one function), as has been documented for other "ancient" metabolic enzymes [29, 30], and in agreement with the emerging role for Glo2 in other malignant cells [16].

3.2. OP Induces Apoptosis through a Mechanism Involving SOD2-Mediated Superoxide Anion-Dependent mGlo2 Upregulation in NSCLC A549 Cells. It has been shown that OP exerts antioxidant effects either directly by reducing the generation of reactive oxygen species (ROS) or indirectly through modulating endogenous antioxidant enzymes [31, 32]. In particular, it has been reported that OP has a potent superoxide anion scavenging activity [32]. In addition,

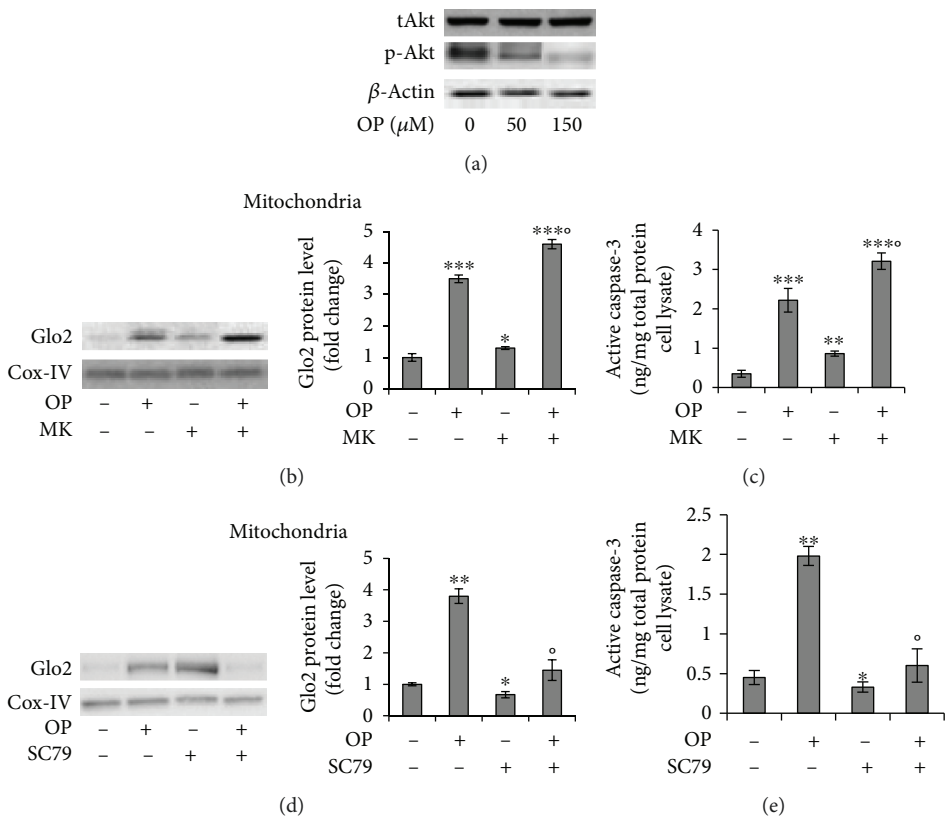

Figure 4: OP-induced mGlo2 upregulation is dependent on p38 MAPK and Akt signaling pathways. (a) Protein level of total Akt (tAkt) phospho-Akt (p-Akt) by western blot (WB) analysis after oleuropein (OP) treatment for 24 hours, (b, d) mitochondrial Glo2 expression, and (c, e) apoptosis, evaluated by WB and active caspase-3 expression, respectively, in A549 cells pretreated with the selective Akt inhibitor MK2206 (MK) (b, c) or the Akt activator SC79 (d, e) and following OP exposure (150 μM). Western blots were stripped of the bound Abs and reprobed with anti-β-actin or Cox-IV, to confirm equal loading. The western blots shown are the representative of three separate experiments. Histograms indicate the means ± SD of three different cultures each of which was tested in triplicate. (-) untreated and (+) treated cells; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus unexposed cells; $^{\circ}p < 0.05$ versus OP-treated cells.

because the mitochondria are the major sources of ROS production, mGlo2 may be somehow affected by the redox status of these organelles. Finally, it is known that Mn-superoxide dismutase (SOD2) plays a crucial role in the control of the mitochondrial apoptotic pathway [33]. Hence, we investigated whether OP-induced apoptosis occurred through the SOD2-mediated depletion of the superoxide anion ($O_2^{\cdot-}$). As shown in Figure 2, OP induced a significant increase in SOD2 activity compared with untreated cells (Figure 2(a)) and this was paralleled by a marked decrease in $O_2^{\cdot-}$ levels (Figure 2(b)). More importantly, striking SOD2 silencing, demonstrable by measurements of both protein expression and levels of enzyme activity (Figure 2(c)), was able, following 150 μM OP exposure, to restore normal $O_2^{\cdot-}$ levels (Figure 2(d)) and to decrease mGlo2 expression (Figure 2(e)) and apoptosis (Figure 2(f)).

Collectively, our results define a novel mechanism, based on the involvement of SOD2, $O_2^{\cdot-}$, and mGlo2 in the proapoptotic effect of OP, thus adding further insight into the molecules activated by this bioactive compound in inducing apoptosis. The issue of whether the action of OP on malignant cells is predominantly antioxidant or prooxidant remains to be established. While in many cases, the proapoptotic effects of OP on tumor cells are elicited via pathways involving ROS generation and oxidative stress [34, 35], in others, OP has been reported to mediate antioxidant effects. Our results are in agreement with the studies supporting this last assessment [36, 37]. Moreover, our data suggest a role for mGlo2 in the group of the proteins participating in ROS-mediated apoptosis, thus providing further insight to the mechanisms underlying ROS-dependent apoptosis, which are still far from being completely understood [38].

3.3. OP Drives Apoptosis in NSCLC A549 Cells by Promoting mGlo2 Association to Bax. To begin to address the mechanism by which mGlo2 could promote apoptosis after OP exposure, we decided to address whether mGlo2 could interact with proteins involved in the known mitochondrial apoptosis pathway. We found that OP promoted mGlo2 association with the proapoptotic Bax protein (Figure 3(a)) and this was partially reversed by SOD2 silencing (Figure 3(b)).

Thus, our data provide evidence of a proapoptotic role for mGlo2 after OP exposure as well as a physical association with a known mediator of apoptosis, Bax. It has been demonstrated that during apoptosis, Bax and Bak mediate the release of cytochrome c from the mitochondria by clustering on the outer mitochondrial membrane and thereby increasing its permeability [39]. However, it remains unclear how

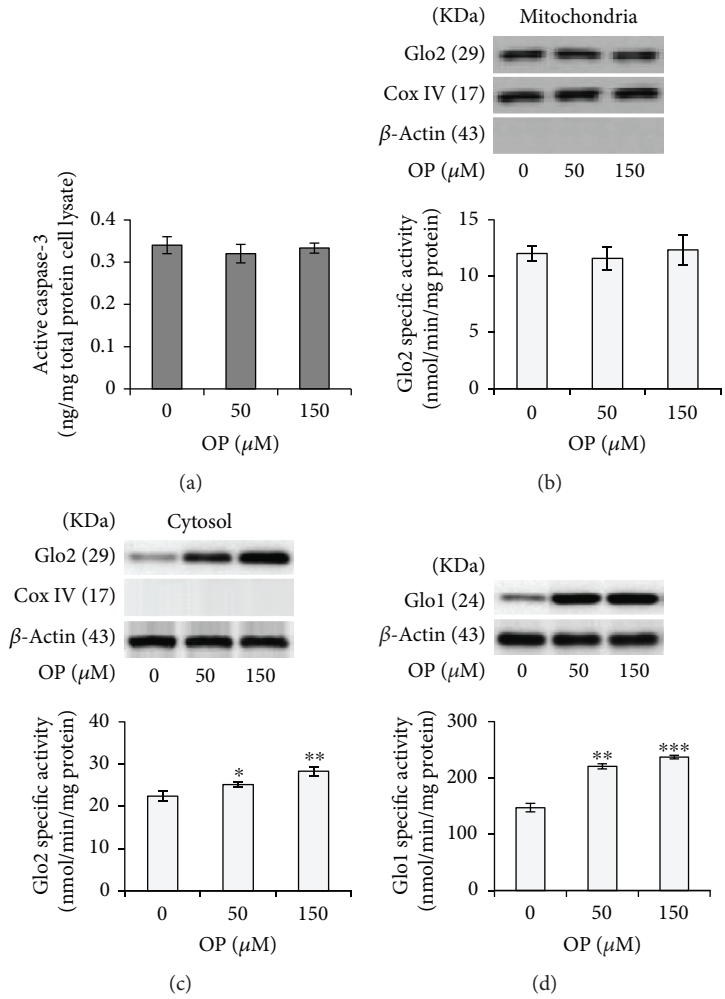

Figure 5: OP effect on the viability and Glyoxalase expression in nonmalignant BEAS-2B cells. (a) Apoptosis, evaluated by detecting active caspase-3 (b) mitochondrial and (c) cytosolic Glo2 expression and enzyme activity. (d) Glo1 protein levels and activity in A549 cells treated with OP. Protein expression and enzyme activities were evaluated by western blotting (WB) and specific spectrophotometric methods, respectively. Western blots were stripped of the bound Abs and reprobed with anti-β-actin or Cox-IV, to confirm equal loading. The western blots shown are the representative of three separate experiments. Histograms indicate the means ± SD of three different cultures each of one was tested in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus unexposed cells.

outer membrane openings form. Based on our data, we hypothesize that Glo2 might help in achieving this goal, a possibility which needs further investigation. Our data on the interaction between Glo2 and Bax, although novel, are in fact in agreement with the proposal that Glo2 might form specific protein-protein interactions with its enzyme substrates as reported by Ercolani et al. [40]. Glyoxalases are ubiquitous enzymes. In yeast, it has been reported that the mGlo2 complements the cytosolic form in the detoxification of MG [28]. The role of mGlo2 in humans has been less investigated, and in agreement with our results, it does not appear to have an MG-scavenging function [16]. Recently, Navarro et al. have described an antiglycative role of OP in the HepG2 cell line [41]. In particular, they found that OP was able to trap MG, the cytotoxic metabolite preferentially detoxified by cGlo2 in cooperation with Glo1. We speculate that cGlo2 might not participate in the apoptotic effects of OP since OP directly reduces levels of MG.

3.4. OP-Induced mGlo2 Upregulation Is Dependent on p38 MAPK and Akt Signaling Pathways. One of the most frequent events in carcinogenesis is the hyperactivation of the Akt signaling pathway [42, 43]. In NSCLC, activation of the Akt pathway promotes tumor progression by inducing evasion of apoptosis [44]. Here, we wanted to investigate whether the OP-induced increase in mGlo2 expression was paralleled by Akt desensitization and apoptosis induction. We first showed that Akt signaling is active in basal A549 cells and that OP reduced its activation (Figure 4(a)). Subsequently, by using the selective MK2206 (MK) Akt inhibitor, we demonstrated that OP-induced mGlo2 expression was upregulated by Akt deactivation and this was associated with a reversal of apoptosis. In fact, following OP administration, MK treatment potentiated mGlo2 protein expression (Figure 4(b)) and apoptosis (Figure 4(c)). Akt activation by SC79 [45] further confirmed that OP-induced mGlo2 upregulation is Akt-dependent (Figures 4(d) and 4(e)). The control

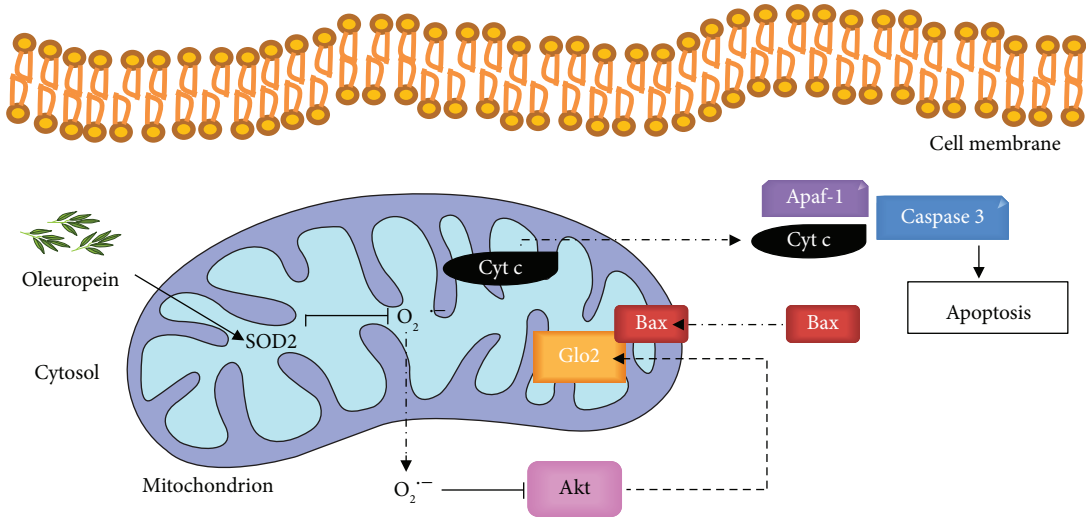

Figure 6: Oleuropein induces apoptosis in NSCLC A549 cells through mitochondrial glyoxalase 2 (Glo2). Oleuropein induces (→) SOD2 upregulation and consequent scavenging (⊣) of superoxide anion $O_2^{\cdot-}$. The depletion of $O_2^{\cdot-}$ inhibits (⊣) the Akt signaling pathway that, in turn, induces (→) the upregulation of mitochondrial Glo2 expression. Glo2 interacts with the proapoptotic protein Bax activating apoptosis through the intrinsic pathway [cytochrome c (Cyt c) release from the mitochondrion, activation of Apaf-1, and eventually caspase-3 activation].

of mGlo2 expression by Akt deactivation in our model is further supported by our observation that $O_2^{\cdot-}$ depletion mediated an additional increase in the mGlo2 protein level after OP exposure. In fact, it was previously reported that $O_2^{\cdot-}$ sustains A549 cell survival by supporting Akt activation [46]. Hence, it is plausible to assume that OP-induced depletion of $O_2^{\cdot-}$ in our model leads to Akt deactivation and, in turn, to the upregulation of mGlo2.

3.5. OP Effect on the Viability and Glyoxalase Expression in Nonmalignant BEAS-2B Cells. Most conventional anticancer therapies do not categorize between cancerous and normal cells, leading to unwanted side effects and toxicity. In agreement with the literature [8], we found here that OP did not affect the viability of normal BEAS-2B cells (data not shown). Moreover, no apoptosis was observed after OP exposure (Figure 5(a)), thus suggesting a selective toxicity against A549 cancer cells while sparing healthy, nonmalignant cells. Moreover, in BEAS-2B cells, OP did not affect mGlo2 expression or enzyme activity (Figure 5(b)), thus suggesting that the viability of BEAS-2B cells and malignant A549 cells is regulated by different proteins and mechanisms. Intriguingly, OP increased the protein expression and enzyme activity of both the cytosolic Glo2 isoform (Figure 5(c)) and Glo1 (Figure 5(d)), which needs further investigation.

4. Conclusions

The data reported here show that OP induces apoptosis in NSCLC A549 cells through a novel mechanism involving the SOD2/$O_2^{\cdot-}$/Akt/mGlo2 axis (Figure 6), thus identifying mGlo2 as a crucial protein in OP-driven apoptosis and extending the limited information available on the anticancer effect of OP in NSCLC models [8, 9]. In addition, our results further supporting the idea that the pro-apoptotic role of OP in NSCLC cells, together with the absence of toxic effects on healthy cells, make this bioactive natural compound an excellent candidate for treating this malignancy.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Review Article

MYC Expression and Metabolic Redox Changes in Cancer Cells: A Synergy Able to Induce Chemoresistance

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Chemoresistance is due to multiple factors including the induction of a metabolic adaptation of tumor cells. In fact, in these cells, stress conditions induced by therapies stimulate a metabolic reprogramming which involves the strengthening of various pathways such as glycolysis, glutaminolysis and the pentose phosphate pathway. This metabolic reprogramming is the result of a complex network of mechanisms that, through the activation of oncogenes (i.e., MYC, HIF1, and PI3K) or the downregulation of tumor suppressors (i.e., TP53), induces an increased expression of glucose and/or glutamine transporters and of glycolytic enzymes. Therefore, in order to overcome chemoresistance, it is necessary to develop combined therapies which are able to selectively and simultaneously act on the multiple molecular targets responsible for this adaptation. This review is focused on highlighting the role of MYC in modulating the epigenetic redox changes which are crucial in the acquisition of therapy resistance.

1. Cancer Metabolic Reprogramming

Metabolic reprogramming is an early event in the carcinogenic process, and it is involved in the development of malignancy and the acquisition of most cancer hallmarks [1]. The first metabolic phenotype observed in cancer cells was described by Otto Warburg, a German biochemist, as a shift from oxidative phosphorylation (OXPHOS) to aerobic glycolysis to generate lactate and ATP even in the presence of O_2 (i.e., Warburg effect) [2]. Since the Warburg effect is also found in tumor cells with intact and functional mitochondria, it is reasonable to assume that it could represent a strategy adopted by cancer cells, not only to cope with the greater energy demands but also to reduce oxidative stress, preserving cells from oxidative death [3]. In this regard, reactive oxygen species (ROS), maintained at "physiological" levels, have been demonstrated to activate redox signaling pathways involved in cell proliferation and survival [4, 5].

Over the past decade, numerous studies have supported the hypothesis that the Warburg effect can be explained by the alterations in multiple signaling pathways resulting from mutations of oncogenes and tumor suppressor genes [6, 7]. Indeed, tumor metabolic reprogramming involves the activation of key metabolic pathways such as glycolysis, the pentose phosphate pathway, and glutaminolysis [8].

In this regard, it has been demonstrated that the glycolytic metabolic switch is due to a marked slowing down of the conversion of phosphoenolpyruvate into pyruvate, a reaction catalyzed by pyruvate kinase (PKM) [9]. Furthermore, in cancer cells, it has been observed that the presence of the low-activity dimeric form of PKM2 promotes the conversion of pyruvate to lactate [10] and that the increased levels of lactic acid detected in cancer patients are related to rapid tumor growth and high levels of metastases [11]. Moreover, considering that most chemotherapeutic agents are weak bases, the presence of lactic acid, generating acidity, induces the ionization of the drugs which, in their modified

chemical structure, are not able to enter the tumor cells, thus facilitating the onset of chemoresistance [12, 13].

PKM2, which makes cells less susceptible to oxidative stress and enhances NADPH production [14, 15], has been found to have a role in chemoresistance. In fact, a recent study showed that this kinase promotes gemcitabine resistance on one hand by inhibiting the transcriptional activation of p53 and the p38-mediated signaling pathway and on the other by increasing the expression of the antiapoptotic protein bcl-xl [16]. In addition, it has been reported that many cancer cells in order to satisfy their bioenergetic and metabolic needs depend on glutamine which is the main source of tricarboxylic acid (TCA) cycle precursors (Figure 1). For example, at the mitochondrial level, glutamine is converted to glutamate by glutaminase (GLS). In turn, glutamate can be converted to α-ketoglutarate (KG) by glutamate dehydrogenase (GDH) or transaminase, resulting in sustaining the TCA cycle. In addition, glutamate can serve as a precursor not only of nonessential amino acids such as aspartate, alanine, proline, and arginine but also of the most important intracellular antioxidant, glutathione (GSH), which is a tripeptide consisting of glutamate, cysteine, and glycine. In addition, malate, which is derived from glutamine, can be converted into pyruvate, leading to NADPH formation [17]. Therefore, the production of NADPH and GSH, derived from glutamine, allows cancer cells to reduce oxidative stress levels associated with mitochondrial respiration and rapid cell proliferation (Figure 1).

In this regard, our recent studies on human neuroblastoma (NB) cells [18], as well as other studies carried out on brain tumor samples [19] and ovarian cancer cells [20], have all demonstrated that the acquisition of chemoresistance is associated with high levels of GSH that enable cancer cells to counteract the prooxidant action of many chemotherapeutic agents [4, 21, 22].

It is noteworthy that the dependency of tumors on specific metabolic substrates, such as glucose or glutamine, is determined by alterations in oncogenes and oncosuppressor genes which are responsible for the tumor metabolic phenotype, while also supporting tumorigenesis. Among oncogenes, MYC has been found to have a pivotal role in the metabolic reprogramming of tumor cells by enhancing glucose uptake and glycolysis, lactate production and export, glutamine uptake and glutaminolysis, mitochondrial biogenesis, and oxidative phosphorylation [1].

2. Role of MYC in Cancer Metabolic Reprogramming and Adaptation to Therapy

MYC is a family of protooncogenes (i.e., c-MYC, L-MYC and N-MYC) which encode transcription factors that have roles in both normal and cancer cell physiologies. MYC requires dimerization with the protein MAX for DNA binding and for the assembly of transcriptional machinery. MAX can also interact with Mxd members which are transcriptional repressors and act in antagonism with MYC/MAX complexes. In addition, Mxd members can also bind to Mlx proteins that can interact with transcription activators of the Mondo family [23]. The MondoA/Mlx complex, located in the cytosol, translocates to the nucleus where, in response to an increase in extracellular glucose levels, it stimulates the expression of the thioredoxin-interacting protein (TXNIP) which suppresses the glucose uptake by limiting the expression of glucose transporters (GLUT) in the membrane [24, 25].

MYC is strongly involved in regulating cell metabolism and facilitates glycolysis by inducing the activation of genes encoding for glycolytic enzymes and GLUT (Figure 1) [26]. It is also able to promote mitochondrial biogenesis and function, thus increasing both oxygen consumption and ATP production [27–29].

Furthermore, it has been found that MYC upregulates the expression of glutamine transporters, facilitating glutaminolysis [30, 31], which is also stimulated by repressing microRNA-23a/b transcription leading to GLS1 overexpression [32]. As reported above, GLS converts glutamine to glutamate [32] which either enters the TCA cycle for the production of ATP or serves as a substrate for GSH synthesis [30]. In this regard, it has been reported that S6K1, a downstream effector of mTORC1, facilitates the translation of MYC, further contributing to the increase of GLS and GDH [33, 34]. In addition, it has been shown that mTORC1 expression, in response to stress conditions, is inhibited by FOXO transcription factors [35] and an increased expression of FOXO3a is able to antagonize the MYC binding to promoters, reducing the mitochondrial mass, oxygen consumption, and ROS production [36].

Regarding the enhancement of the mitochondrial function, it has been found that MYC can activate the PPARγ coactivator-1α (PGC-1α) and the mitochondrial transcription factor A (TFAM), mediators of mitochondrial biogenesis and mitochondrial gene expression, respectively [28, 37]. Interestingly, although the role played by MYC/PGC-1α axis is controversial [38], several reports have demonstrated that PGC1-α is involved in chemoresistance [39] and the inhibition of the PGC-1α pathway has been found to activate glycolysis [40] and to sensitize melanoma to oxidative damage [41].

Therefore, as reported above, the MYC-overexpressing tumors depend on glutamine [30, 31], and it has been demonstrated that glutamine depletion leads to the reduction of GSH levels and consequently triggers apoptosis. In fact, buthionine sulfoximine- (BSO-) induced depletion of GSH was able to induce apoptosis of N-MYC-amplified NB cells through a ROS-mediated activation of PKCδ-dependent pathways (Figure 2) [5, 42, 43]. Accordingly, PKCδ overexpression sensitized NB cells to the proapoptotic effects of BSO and of etoposide [18, 44–46].

Clinical studies carried out on NB patients have demonstrated that N-MYC amplification correlates to a reduction in the survival rate of those patients undergoing a multidrug therapy protocol consisting of etoposide, vincristine, carboplatin, adriamycin, and cyclophosphamide [47].

3. Molecular Mechanisms of MYC-Dependent Metabolic Changes

In N-MYC-amplified NB tumors, Akt has been found to be hyperactivated [48] and Akt activation has been demonstrated

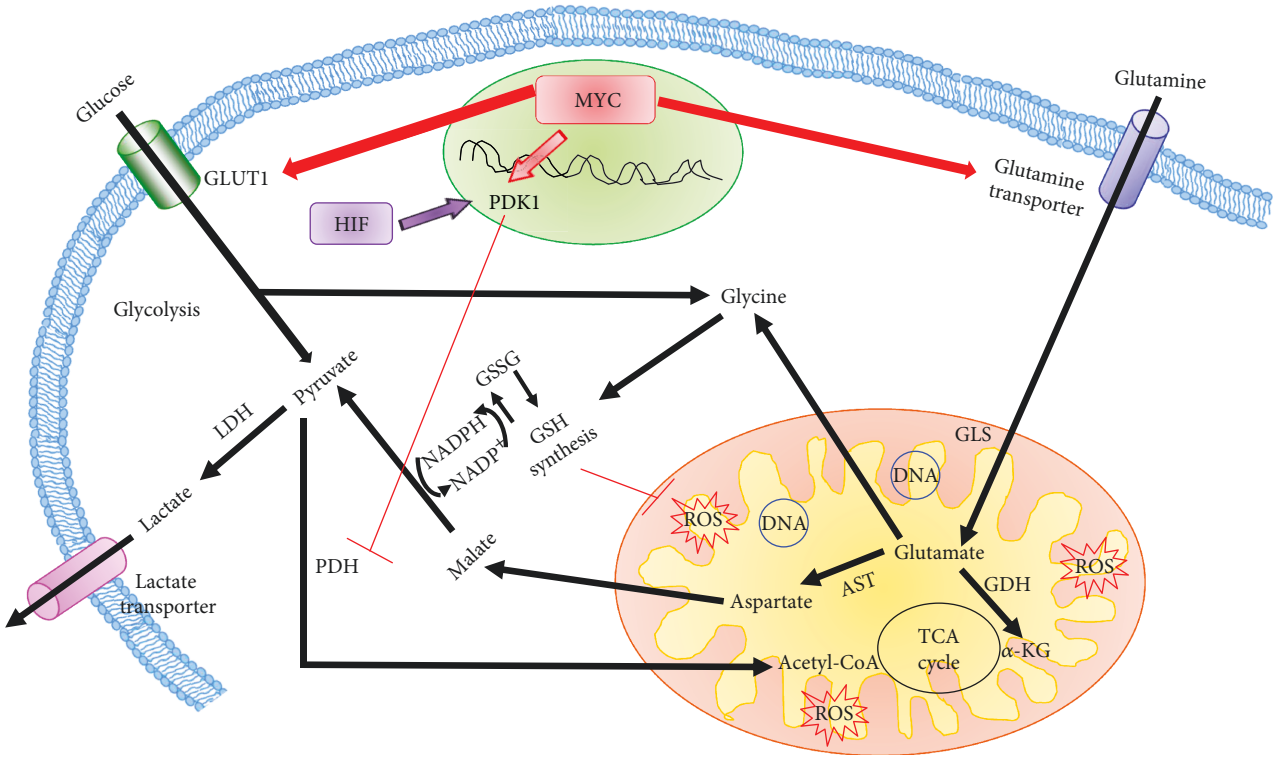

Figure 1: Role of MYC in the modulation of cancer metabolic reprogramming. MYC is involved in the modulation of both glycolysis and glutaminolysis. MYC, in order to carry out this double role, upregulates membrane transporters and enzymes involved in these metabolic processes (indicated in red). AST: glutamic-oxaloacetic transaminase; GDH: glutamate dehydrogenase; GLS: glutaminase; GLUT1: glucose transporter 1; GSH: reduced glutathione; GSSG: oxidized glutathione; α-KG: α-ketoglutarate; LDH: lactic dehydrogenase; PDH: pyruvate dehydrogenase; PDK1: pyruvate dehydrogenase kinase 1; ROS: reactive oxygen species; TCA: tricarboxylic acid.

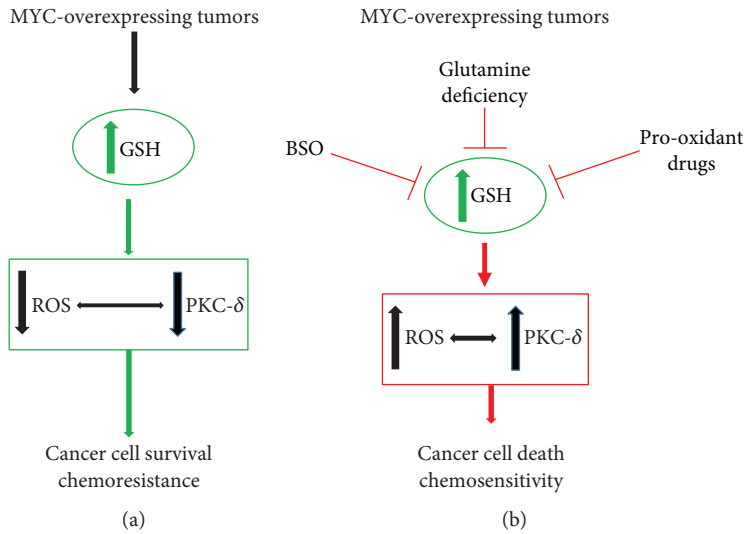

Figure 2: MYC overexpression and increase of glutathione levels in the acquisition of chemoresistance. (a) Chemoresistance of MYC-overexpressing tumors is associated with an enhancement of intracellular glutathione (GSH) levels. (b) In order to promote cell death, it is helpful to deplete GSH by using depleting agents such as buthionine sulfoximine (BSO) or prooxidant drugs. These strategies stimulate reactive oxygen species (ROS) production which modulate, and are modulated by, the proapoptotic protein kinase C-delta (PKC-δ).

to be strongly involved in etoposide resistance [46, 49–51], as well as being related to the expression of CD133, a marker of staminality associated with the most aggressive cancer phenotype [52]. Accordingly, it has been shown that Akt inhibition sensitizes NB cells to the cytotoxic action of etoposide [53], doxorubicin, vincristine, and cisplatin [52]. In addition, under conditions of nutrient deficiency, the reduced activity of Akt decreases the amount of MDM2,

the p53 endogenous inhibitor, resulting in an increase in p53 levels [54]. In fact, it has been found that the activation of p53 limits glycolysis and promotes OXPHOS in cancer cells while the loss of function of mutated p53 contributes to the development of the Warburg effect [55, 56]. Therefore, p53, in repressing PGC-1α, which is involved in mitochondrial biogenesis, and modulating other genes implicated in autophagy, in glucose metabolism, and also in the pentose-phosphate pathway [57–66], can play a role as a regulator of tumor cell metabolism and chemoresistance.

Interestingly, our recent studies have shown that chronic treatment of N-MYC-amplified NB cells with etoposide does not modify the homozygous p53 mutation (A161T), previously found in etoposide-sensitive NB cells, and therefore, in this context, p53 is responsible neither for OXPHOS activation nor for the metabolic adaptation of etoposide-resistant NB cells [67].

Moreover, several studies have demonstrated that the metabolic reprogramming might be the result of the "molecular interplay" between N-MYC and hypoxia-inducible factors (HIFs) [68]. HIF1 and HIF2 provide transcriptional homeostatic responses to limited oxygen levels in both physiological and pathological conditions. Although physiological HIF1 can inhibit the activity of normal MYC, the altered expression of the oncogenic MYC collaborates with HIF to confer the propensity to cancer cells to convert glucose to lactate, even in the presence of adequate O_2 levels [69–72]. In fact, at normal MYC levels, it has been observed that HIF1α can compete for MAX, displacing MYC, while, at higher MYC levels, the formation of MYC-MAX heterodimers is maintained through mass action. Similar to MYC, HIF1 activates all genes involved in glycolysis, but unlike MYC, HIF1 actively inhibits mitochondrial respiration by promoting mitochondrial autophagy [73, 74] and preventing mitochondrial biogenesis [29]. In this context, it has been reported that HIF1 induces the expression of pyruvate dehydrogenase kinase (PDK1) which phosphorylates and inactivates pyruvate dehydrogenase, a mitochondrial enzyme catalyzing the conversion of pyruvate to acetyl CoA [75, 76]. Moreover, it has been found that MYC, when overexpressed in human tumors, cooperates with HIF1 to induce PDK1 and hexokinase 2 (HK2) expression, altering cellular metabolism in favor of glycolysis with an increased production of lactate [70, 75]. HIF1 and MYC independently activate GLUT1 and lactate dehydrogenase A (LDHA), resulting in an increased glucose influx and higher glycolytic rates [75].

Interestingly, HK2, which plays a key role for the Warburg effect in cancer, binds competitively to the voltage-dependent anion channel (VDAC), in the outer mitochondrial membrane, preventing its union with proapoptotic Bax and thereby avoiding apoptosis [77].

Apoptosis and senescence represent two tumor-suppressive mechanisms which can be modulated by MYC and RAS oncogenes. In fact, RAS inhibits MYC-induced apoptosis *via* PI3K activity and MYC suppresses RAS-induced senescence *via* CdK2, a cyclin-dependent kinase which phosphorylates MYC at Ser62 residue [78]. Accordingly, CdK2 inhibition has been shown to slow down the growth of MYCN-amplified neuroblastoma cells [79] and of other MYC-driven tumors [80].

Many chemotherapeutic drugs exert their cytotoxic effects on cancer cells by reactivating apoptosis and/or senescence [81]. In this context, it has been hypothesized that therapy-induced senescence (TIS) could be useful in the treatment of tumors with an impairment of the apoptotic pathways.

Interestingly, it is relevant to know that the presence of TIS cells can stimulate immunosurveillance and also induce chemoresistance [82, 83]. In fact, TIS cells have features of stemness that is regulated by the Wnt-dependent pathways [84–86] and undergo a metabolic reprogramming characterized by an increase in the glycolytic activity [2, 3] and an impairment of proteasome activity and autophagy [87]. In this context, the treatment of oncologic patients with anthracyclines and alkylating agents has been shown to induce cellular senescence and the secretion of cytokines, chemokines, growth factors, and proteases that can contribute to the side effects of chemotherapy [82, 88].

Recently, it has been reported that downregulation of p21, a cell cycle inhibitor, leads to MYC upregulation which represses the expression of CD47 receptor generating a subpopulation of cells that escape senescence [89]. However, further studies are necessary to determine if senescence is a general adaptive pathway to chemotherapy and if this response concerns only a specific subpopulation of cancer cells.

4. Inhibition of MYC Effectors as a Potential Strategy to Block Cancer Metabolic Reprogramming

Although MYC is considered the "most-wanted" target for anticancer therapy [90], the targeting of this oncogene has not yet obtained any positive outcomes. In fact, the inhibition of MYC can interfere with its physiological functions and therefore an alternative approach inhibiting MYC effectors could be more useful. More specifically, given that MYC drives the glucose and glutamine metabolism of cancer cells, the use of small molecules, able to inhibit enzymes involved in glycolysis and glutaminolysis, might be effective in slowing down tumor cell proliferation. Among them, several drugs targeting the MYC effectors are currently being tested in clinical practice [91–97] (Table 1).

Interestingly, a promising approach could be to indirectly modulate MYC through the "synthetic lethality" [90], and in this regard, the development of MK-3475 (pembrolizumab or keytruda) might offer new therapeutic opportunities. In fact, this latter compound is an inhibitor of the programmed death-1 (PD-1) protein and MYC modulates the expression of its ligand (PD-L1) [98], which, when overexpressed, stimulates glucose metabolism [99] by increasing GLUT1 expression [100]. MK-3475 has been, and is currently, the subject of over 900 clinical trials, and two of these have even reached Phase 4 (NCT03715205; NCT03134456). In addition, in Phase 3 studies, it should be noted that this compound per se is efficacious in treating recurrent or metastatic head-

Table 1: Drugs targeting glucose or glutamine metabolism currently used in clinical trials.

Drug	Target	Effect on MYC	Cancer type	Phase trials	NCT
Silibyn	GLUT	Reduction [91]	Prostate cancer	II	00487721
Gossypol	Lactate dehydrogenase (LDH)	Reduction [92]	Small-cell lung carcinoma	II	00773955
			Prostate cancer	II	00666666
			Esophageal/gastroesophageal cancer	I/II	00561197
			Glioblastoma	I	00390403
				II	00540722
Dichloroacetate	Pyruvate dehydrogenase (PDH)	Reduction [93]	Breast cancer and non-small-cell lung carcinoma	II	01029925
			Head and neck cancer	I	01163487
			Brain cancer	II	00540176
Deoxyglucose	Hexokinase II	Reduction [94]	Prostate cancer	I/II	00633087
			Lung cancer and breast cancer	I	00096707
Apigenin	Pyruvate kinase M (PKM)	Reduction [95]	Breast cancer	—	03139227
Diclofenac	GLUT1 and LDH	Reduction [96]	Basal cell carcinoma	II	01358045
CB-839	Glutaminase1 (GLS1)	Reduction [97]	Leukemia	I	02071927
			Colorectal cancer	I/II	02861300
			Hematological tumors	I	02071888
			Melanoma	I/II	02771626
			Triple negative breast cancer and solid tumors	I	02071862

and-neck squamous cell carcinoma (NCT02252042) [101], advanced urothelial carcinoma (NCT02256436) [102], non-small-cell lung cancer (NCT01905657) [103], and melanoma (NCT02362594) [104].

5. Conclusions

Tumor metabolic reprogramming is a direct result of the reengineering of intracellular signaling pathways that are altered by activated oncogenes or downregulated oncosuppressors and by epigenetic changes, conferring a proliferative advantage to cancer cells.

Indeed, tumors may prefer either a glycolytic or an oxidative metabolism, depending on the activation of oncogenes or repression of oncosuppressors but also on the tumor microenvironment. Therefore, it is conceivable that in the tumor niche there is a strong "metabolic competition" due to high nutritional requirements and also an intense "molecular interplay" able to maintain an efficient metabolism. The balance between these factors could paradoxically guarantee the development and the survival of cancer even under therapy-induced stress conditions. Consequently, therapies that block glucose metabolism might be more effective towards tumors with high glycolytic rates, while they might develop therapy resistance in tumors whose metabolism depends on oxidative phosphorylation [105].

Therefore, anticancer therapy must take into account that most chemotherapeutic drugs are prooxidant agents and are able to induce a metabolic reprogramming that alters the redox homeostasis of cancer cells activating signaling pathways responsible for cell survival.

Considering the crucial role of MYC in driving the metabolic reprogramming of cancer which has been shown to be strictly related to drug resistance, several studies have been carried out in order to focus MYC-dependent metabolic pathways. Even though the efforts are multiple, to date the applicability of MYC inhibitors is still a utopia. However, the use of small molecules, able to inhibit MYC-related enzymes involved in glycolysis and glutaminolysis, might result effective in slowing down tumor cell proliferation and counteracting chemoresistance.

However, the characterization of the metabolic reprogramming of tumors and its connection with oncogenic signaling is a promising strategy to identify novel molecular approaches in anticancer treatment.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

Oxidative Stress Induced by the Deubiquitinase Inhibitor b-AP15 Is Associated with Mitochondrial Impairment

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Inhibitors of the 20S proteasome such as bortezomib are cytotoxic to tumor cells and have been proven to be valuable for the clinical management of multiple myeloma. The therapeutic efficacy of bortezomib is, however, hampered by the emergence of acquired resistance. Available data suggest that blocking proteasome activity at the level of proteasome-associated deubiquitinases (DUBs) provides a mechanism to overcome resistance to bortezomib and also to other cancer therapies. The small molecule b-AP15 is an inhibitor of proteasome-associated DUB activity that induces both proteotoxic stress and increases in the levels of reactive oxygen species (ROS) in tumor cells. Antioxidants have been shown to decrease apoptosis induction by b-AP15 and we here addressed the question of the mechanism of redox perturbation by this compound. We show that oxidative stress induction by b-AP15 is abrogated in cells deprived of mitochondrial DNA (ρ^0 cells). We also show associations between the level of proteotoxic stress, the degree of mitochondrial dysfunction, and the extent of induction of hemeoxygenase-1 (HO-1), a target of the redox-regulated Nrf-2 transcription factor. Decreased expression of COX5b (cytochrome c oxidase subunit 5b) and TOMM34 (translocase of outer mitochondrial membrane 34) was observed in b-AP15-treated cells. These findings suggest a mitochondrial origin of the increased levels of ROS observed in cells exposed to the DUB inhibitor b-AP15.

1. Introduction

Aberrant accumulation of misfolded or damaged proteins is associated with reduced cell survival [1]. Protein quality control is primarily mediated by the ubiquitin-proteasome system (UPS), the major eukaryotic proteolytic pathway, and is essential for cell viability [2, 3]. The proteasome degrades the bulk of cellular protein and is instrumental to the regulation of essential cellular processes such as cell cycle progression [4–7]. Misfolded, damaged, or temporally regulated proteins are marked for removal by the destruction tag ubiquitin that signals traffic to the proteasome for degradation. Once at the proteasome, ubiquitin is removed from polyubiquitinated proteins by deubiquitinases (DUBs) localized in the 19S regulatory particle, to facilitate translocation into the 20S core particle where degradation takes place [8, 9]. Cancer cells, characterized by rapid protein synthesis and unlimited proliferation, face an extreme load of misfolded proteins and therefore have an increased requirement for UPS-mediated protein turnover [10, 11]. Under conditions of proteasome inhibition, misfolded proteins accumulate in tumor cells, resulting in pleiotropic effects such as induction of cytosolic chaperones, endoplasmic reticulum (ER) stress, and oxidative stress [12–14].

Inhibitors of the 20S proteasome such as bortezomib and carfilzomib are used for treatment of multiple myeloma and have changed the clinical course of this disease [15, 16]. However, both intrinsic and acquired resistance to bortezomib limit its therapeutic efficacy [17]. A number of different mechanisms have been described to result in bortezomib

resistance, including mutations in the PSMB5 subunit and overexpression of this subunit [17].

The redox state is important for cell survival, proliferation, and apoptosis [18]. Reactive oxygen species (ROS) may be harmful to cells leading to oxidative damage such as lipid peroxidation but may also be second messengers controlling signaling pathways [19]. The three major sources of ROS in the cell are mitochondria, peroxisomes, and the endoplasmic reticulum (ER) [20, 21]. Oxidative stress has been described to be induced by proteasome inhibitors [13, 14, 22], and antioxidants have been shown to decrease the apoptotic effects of these drugs [12, 13]. Oxidative stress resulting from proteasome inhibition has been attributed to ER stress [23] and to mitochondrial dysfunction [13] by various investigators.

We and the others have shown that the dienone compounds b-AP15, VLX1570, and RA-9 inhibit the activities of proteasome-associated DUBs [24–27], in particular USP14 [27]. This class of compounds induce apoptosis in tumor cells defective in TP53 [28] and overexpressing BCL2 [24, 29]. The ability of these compounds to selectively kill tumor cells while being largely insensitive to TP53 mutational status and defects in apoptotic machinery is interesting both from a mechanistic and therapeutic point of view, in particular considering their anticancer activities *in vitro* and in animal models [24, 25, 27, 29–41]. Interestingly, b-AP15 shows antiproliferative activity on myeloma cells resistant to bortezomib [33] and melanoma cells resistant to MAPK-targeting therapies [27]. We recently showed that the strong proteotoxicity induced by b-AP15 resulted in mitochondrial toxicity [42]. We and the others have shown that b-AP15 induces reactive oxygen species in tumor cells and that antioxidants decrease the apoptotic response [22, 27, 43]. These findings prompted us to examine whether oxidative stress induction by b-AP15 is mechanistically linked to mitochondrial dysfunction. We here provide experimental evidence in support of this notion.

2. Material and Methods

2.1. Chemicals and Antibodies. b-AP15 was obtained from OnTarget Chemistry (Uppsala, Sweden), Velcade (bortezomib, Selleck Chem) and CpdA [44] from Novartis. Antibodies used were anti-actin (Sigma-Aldrich catalogue number A5441), anti-Ub-K48 (Merck Millipore catalogue number 05-1307), anti-HMOX (BD Biosciences catalogue number 610713), anti-Hsp60 (Cell Signaling catalogue number 12165), anti-HSP40 (Cell Signaling catalogue number 4868), anti-Nrf-2 (Cell Signaling catalogue number 12721), anti-CHOP (Cell Signaling, catalogue number 5554), anti-HSP70B′ (Abcam catalogue number ab69408), and anti-MTCOXII2 (Abcam catalogue number ab110258).

2.2. Cell Culture and Drug Treatment. HCT116 colon carcinoma cells were maintained in McCoy's 5A modified medium with 10% FBS and 1% penicillin. HeLa cells were cultured in DMEM medium with supplemented 10% FBS and 1% penicillin. Cell lines were used at low passage numbers and checked for absence of mycoplasma. Drugs were dissolved in DMSO for final concentrations of DMSO 0.5%. CpdA was used at a concentration of 10 μM as described previously [45].

2.3. Western Blot Analysis. Cell extract proteins were resolved by 3-8% Tris-Acetate protein gels (Invitrogen, Carlsbad, CA) to detect polyubiquitinated proteins and 4-12% Bis-Tris protein gels to detect other proteins mentioned in the text, then transferred onto a PVDF membrane for western blotting [46]. Blots were developed by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

2.4. Electron Microscopy. Cells were treated with b-AP15 for different times and fixed with 2.5% glutaraldehyde. Cells were postfixed in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin. Ultrathin sections were prepared for analysis in a transmission electron microscope. Electron microscopy was performed by Kjell Hultenby at the Department of Laboratory Medicine, Clinical Research Center, Karolinska Universitetssjukhuset Huddinge, Sweden.

2.5. Glutathione Assays. For measurement of glutathione, cells were treated with 1.0 μM b-AP15 for 6 h. Cells were collected and concentrations of GSSG and total glutathione (GSH + GSSG) were analyzed using the quantification kit for oxidized and reduced glutathione (#38185, Sigma) as described. The final concentration of GSH was determined by equation of $\mathrm{GSH} = \text{total glutathione}\,(\mathrm{GSH} + \mathrm{GSSG}) - \mathrm{GSSG} \times 2$. The data was analyzed using GraphPad Prism 7.

2.6. Measurements of G6PD, Glutathione Peroxidase, and Malondialdehyde. Drug-treated cells were washed with PBS and frozen at -80°C as cell pellets. Pellets were shipped to Biochemikon SAS, 94000 Créteil, France (study director Marc Conti). Cell pellets were sonicated, and enzymatic activities and substrate concentration measurements were performed. Glutathione peroxidase activity was measured according to Paglia and Valentine [47]. G6PD activity was adapted/optimized from Beutler [48]. MDA measurements were determined according to Conti et al. [49].

2.7. Measurements of Oxygen Consumption. OCR (oxygen consumption rates) were measured using a Seahorse XF24 extracellular flux analyzer in real time as recommended by the manufacturer (Seahorse Bioscience, North Billerica, MA, USA). Cells (60,000 cells/well) were plated in 100 μL culture medium in XF24-well cell plates with blank control wells. Prior to the measurements, the medium was replaced with 500 μL Seahorse assay media (1 mM pyruvate, 25 mM glucose, and 2 mM glutamine) at 37°C without CO_2 for 1 h.

2.8. Generation of HeLa Rho^0 (ρ^0) Cells. Hela cells were grown in DMEM medium supplemented with 100 ng/mL EtBr and 50 μg/mL uridine [50]. DNA was isolated using PureLink® Genomic DNA Mini Kit (Thermo Fisher Scientific), and mtDNA and nDNA were amplified by Human Mitochondrial DNA (mtDNA) Monitoring Primer Set (Takara). Copy number was measured using a 7500/7500 Fast Real-Time PCR System (Applied Biosystems), and

mitochondria DNA to nuclear DNA ratios were calculated by the program supported by Takara. The absence of mtDNA-encoded protein MTCOXII2 in Rho^0 cells was confirmed by immune blotting.

2.9. Isolation of Mitochondria. Mitochondrial isolation process was performed as [42].

2.10. Proteomics. Proteomic analysis was performed as described [42]. The raw data from LC-MS were analyzed by MaxQuant, version 1.5.6.5 [51]. The Andromeda search engine [52] searched MS/MS data against the International Protein Index (human, version UP000005640_9606, 92957 entries). Protein abundances were normalized by the total protein abundance in each sample. Mitochondrial proteins were further selected from total detected protein pool using MitoCarta (http://www.broad.mit.edu/publications/MitoCarta) supplied by [53].

2.11. Statistical Analysis. Statistical significance was evaluated by Student's two-tailed paired t-test (parametric) or Mann-Whitney U test (nonparametric). Protein expression data were compared using Spearman correlation coefficients.

3. Results

3.1. The Deubiquitinase Inhibitor b-AP15 Affects Mitochondrial Structure and Function. We have previously reported that b-AP15, an inhibitor of proteasome-associated deubiquitinases, generates both proteotoxic stress and oxidative stress [22, 24, 26, 27] and also induces mitochondrial dysfunction [42]. As shown in Figure 1(a), treatment of HCT116 cells with 1 μM b-AP15 resulted in increased levels of K48-linked polyubiquitin conjugates and induction of the chaperones HSP70B′ and HSP40 as well as the ER marker CCAAT-enhancer-binding protein homologous protein (CHOP) [54, 55]. Consistent with previous results, mitochondria became increasingly deformed during exposure to b-AP15 (Figure 1(b)). Mitochondrial function was examined by monitoring oxygen consumption rates using a Seahorse XF24 analyzer. Confirming previous results [42], the stimulation of oxygen consumption by carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP) was reduced in b-AP15-exposed cells (Figure 1(c)), showing a decrease in maximal respiration capacity.

3.2. b-AP15 Induces Oxidative Stress but Not Lipid Peroxidation. Previous studies demonstrated increased levels of intracellular ROS in b-AP15-treated HCT116 cells [22] and in melanoma cells [27]. Exposure of HCT116 cells to b-AP15 resulted in increased levels of the redox-regulated transcription factor Nrf-2 (nuclear factor erythroid 2-related factor 2) and its downstream target HO-1. This increases occurred at doses that induced the accumulation of high molecular weight K48-linked polyubiquitin conjugates (Figure 2(a)). Furthermore, a significant increase in the GSSG/GSH ratio was observed in b-AP15-exposed HCT116 cells, whereas no significant increases in total GSH levels were observed (Figures 2(b) and 2(c)). Increased glucose 6-phosphate dehydrogenase (G6PD) enzyme activity, the rate-limiting enzyme of the pentose phosphate pathway, was observed in b-AP15-treated HCT116 cells ($p = 0.015$) (Figure 2(d)). In contrast, glutathione peroxidase (GPx) activity levels were not significantly altered by b-AP15 treatment (Figure 2(e)). Malondialdehyde is a product of lipid peroxidation and a marker of oxidative damage [56]. Increased levels of malondialdehyde were not observed in HCT116 cells exposed to b-AP15 for 6 h (Figure 2(f)).

3.3. Induction of Oxidative Stress Is Dependent on Functional Mitochondria. Attempts to generate HCT116 cells deficient in mitochondrial DNA (ρ^0 cells) by exposure to ethidium bromide were unsuccessful (not shown), possibly due to HCT116 cells being dependent on oxidative phosphorylation [57]. We therefore used HeLa cells, for which ρ^0 derivatives have been described [58]. Similar to the response in HCT116 cells, increases in polyubiquitinated proteins, chaperones, and CHOP were observed in HeLa cells exposed to b-AP15 (Figure 3(a)) and b-AP15 induces an apoptotic response in HeLa cells (Supplementary Fig. 1). Furthermore, Nrf-2 and HO-1 induction was observed also in b-AP15-treated HeLa cells (Figure 3(b)). Continuous exposure of HeLa cells to low doses of ethidium bromide resulted in cells with a reduced copy number of mitochondrial DNA (Figure 3(c)), aberrant mitochondrial morphology (Figure 3(d)), low oxygen consumption rates (Figure 3(e)), and no detectable expression of the mitochondria genome-encoded protein MTCOXII (Figure 3(f)). Exposure of HeLa ρ^0 cells to b-AP15 resulted in a dramatic abrogation of Nrf-2 and HO-1 induction (Figure 3(f)). Furthermore, and in contrast to the response observed in HeLa parental cells, exposure of HeLa ρ^0 cells to b-AP15 did not result in an increased GSSG/GSH ratio (Figure 3(g)). These findings are consistent with the notion that oxidative stress induction by b-AP15 is dependent on functional mitochondria. We considered the possibility of decreased levels of protein synthesis in ρ^0 cells, resulting in reduced proteotoxic stress and, as a consequence, lower oxidative stress. However, the levels of polyubiquitinated proteins induced by b-AP15 or bortezomib were comparable in HeLa parental and ρ^0 cells (Figure 3(f)).

Our observations suggest an association between oxidative stress and mitochondria perturbation as a result of proteotoxic stress generated by b-AP15. One alternative mechanism of oxidative stress induction is inhibition of thioredoxin reductase (TrxR) activity, previously shown for b-AP15 [43]. To examine this possibility, we used a number of recently identified inhibitors of proteasome-associated DUBs that do not inhibit TrxR (Supplementary Fig. 2). We found that three different and chemically unrelated molecules that do not inhibit TrxR all induced the expression of the Nrf-2 target HO-1 (Figure 3(h)). Auranofin, a well-documented inhibitor of TrxR [59], induced HO-1 expression but did not induce accumulation of polyubiquitinated proteins (Figure 3(h)). These findings show that induction of the Nrf-2 target protein HO-1 by inhibitors of proteasome-associated DUBs does not require inhibition of TrxR.

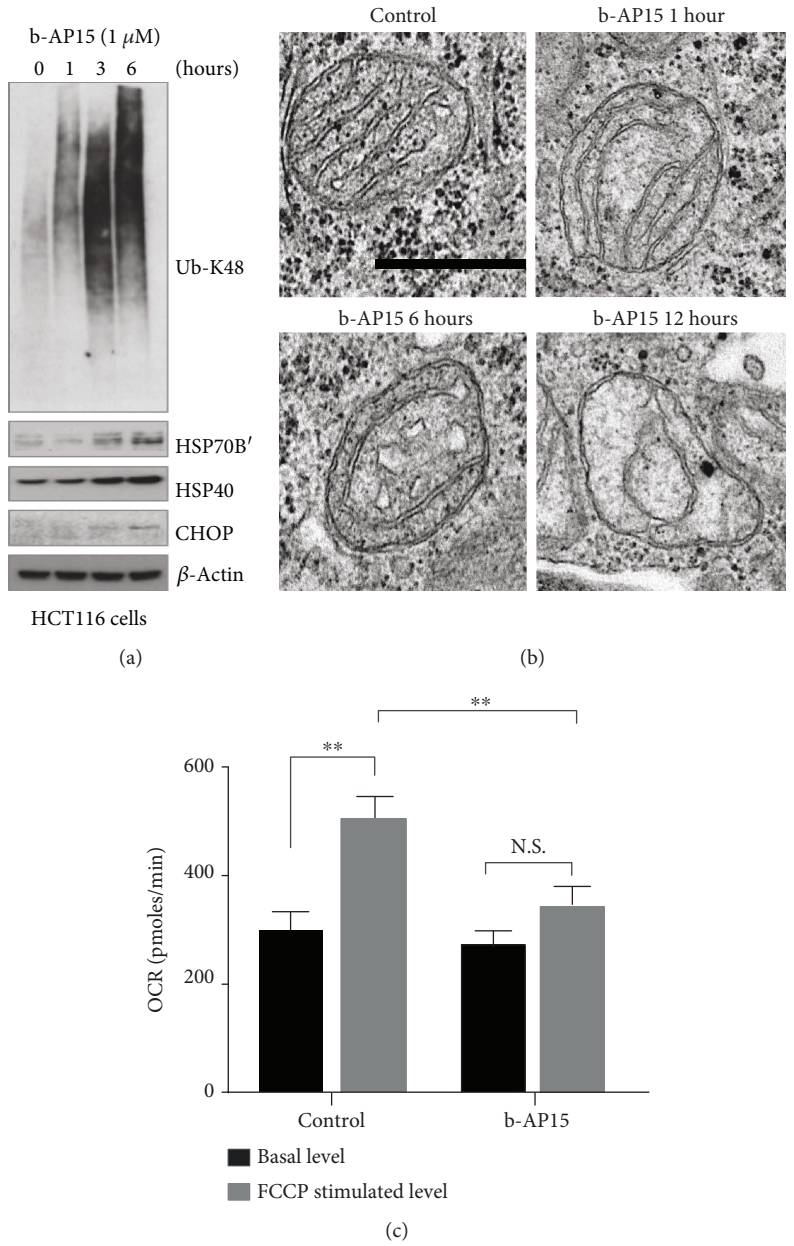


Figure 1: Induction of mitochondrial dysfunction in HCT116 cells by the deubiquitinase inhibitor b-AP15. (a) HCT116 cells were exposed to 0.5% DMSO or 1 μM b-AP15 for 1, 3, and 6 hours, and extracts were prepared and subjected to immunoblotting using the indicated antibodies. (b) Electron micrographs of HCT116 cells treated with b-AP15 for 1, 6, and 12 h. Scale bar = 0.5 μm. (c) Basal and maximal oxygen consumption rates (OCR) were measured after a 5-hour exposure of HCT116 cells to 1 μM b-AP15 using a Seahorse XF analyzer. Uncoupled respiration was measured after exposure to carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) (mean ± S.D.; $**p < 0.01$).

3.4. Increased Levels of Proteotoxic Stress Result in Decreased Oxygen Consumption and Increased Expression of HO-1. Further increases in the levels of proteotoxic stress are expected to result in increased mitochondrial damage and elevated oxidative stress. We used the CpdA, an inhibitor of Sec61-mediated anterograde protein translocation over the ER membrane [44], to test this prediction. Consistent with previous results [45], cotreatment of b-AP15 with CpdA induced strong accumulation of polyubiquitinated proteins and overexpression of HSP70 and HSP40 chaperones in HCT116 cells (Figure 4(a)). Cotreatment resulted in stronger reductions in oxygen consumption rates compared to treatments with b-AP15 or CpdA alone (Figure 4(b)). Cotreatment with b-AP15 and CpdA also resulted in stronger increases in HO-1 (Figure 4(a)), consistent with the notion of an association between proteotoxic stress and oxidative stress.

3.5. Alterations of the Mitochondrial Proteome Reveal Decreased Expression of COX5b. Damaged mitochondria in b-AP15-treated cells are not cleared by mitophagy and can

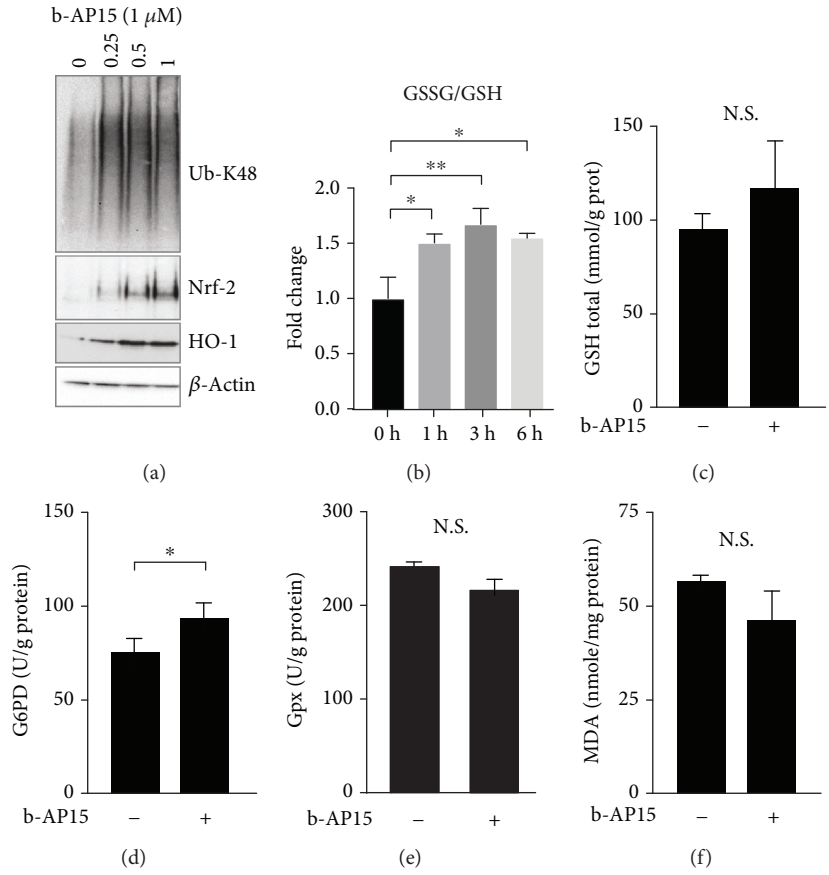


Figure 2: Evidence of oxidative stress in b-AP15-exposed HCT116 cells. (a) HCT116 cells were exposed to 0.5% DMSO or b-AP15 (0.25, 0.5, and 1.0 μM in 0.5% DMSO) for 6 h, and extracts were prepared and subjected to immunoblotting using the indicated antibodies. (b) Increases in the ratio of GSSG/GSH in HCT116 cells exposed to 1 μM b-AP15 for the indicated times (mean ± S.D.; $*p < 0.05$, $**p < 0.01$; $n = 3$). (c) Total levels of GSH were determined in vehicle-treated cells and in cells exposed to 1 μM b-AP15 for 6 h (mean ± S.D.; $n = 3$; N.S.: not significant at $p < 0.05$). (d) Glucose-6-phosphate dehydrogenase activity in HCT116 cells exposed to 1 μM b-AP15 for 6 h compared to vehicle-treated cells (mean ± S.D.; $*p < 0.05$; $n = 3$). (e) Glutathione peroxidase activity in HCT116 cells exposed to 1 μM b-AP15 for 6 h compared to vehicle-treated cells (mean ± S.D.; $n = 3$). (f) Malondialdehyde levels in HCT116 cells exposed to 1 μM b-AP15 or vehicle for 6 h. Statistical significance was calculated using Student's t-test in (b)–(f).

be purified and analyzed by proteomics and other methods [42]. Three mitochondrial proteins were found to be significantly downregulated in mitochondrial preparations from b-AP15-treated cells: TOMM34 (translocase of outer mitochondrial membrane 34), CHDH (choline dehydrogenase), and COX5b (cytochrome c oxidase subunit 5B) (Figures 5(a) and 5(b)). Cotreatment with b-AP15 and CpdA resulted in a similar or larger decrease in the levels of these proteins and significant downregulation of some additional proteins (Figures 5(c) and 5(d)). COX5b is a component of the electron transport chain and the decrease of this protein may explain the decreases in mitochondrial oxidative phosphorylation observed in b-AP15-treated cells.

Mitochondria possess a protein folding machinery (HSP60, HSP10, TRAP1, and mtHSP70) to respond to the misfolding stress inside of mitochondria (UPR^{mt}) [60]. We addressed the question of whether b-AP15 affects mitochondrial protein homeostasis, leading to induction of HSP60. However, HSP60 expression was not affected by treatment with b-AP15 in the absence or presence of CpdA (Figures 5(e) and 5(f)).

4. Discussion

b-AP15 and similar compounds have been shown to induce apoptotic responses in tumor cells overexpressing BCL2 family proteins and cells defective in TP53 [24, 29]. Previous reports have demonstrated induction of both strong oxidative stress and proteotoxicity by b-AP15 [22, 27, 61] and also showed evidence of mitochondrial toxicity [42]. These findings led to the hypothesis that oxidative stress induction by this class of compounds is mechanistically linked to mitochondrial dysfunction. We here found weaker induction of the Nrf-2 target HO-1 and decreased elevation of GSSG/GSH ratios in ρ^0 cells exposed to b-AP15, consistent with a mitochondrial involvement in b-AP15-induced oxidative stress. We also found that increasing the level of proteotoxic stress by inhibiting anterograde ER translocation resulted in increased induction of expression of HO-1. These findings are consistent with the hypothesis of a mitochondrial origin of the increased levels of ROS observed in cells exposed to the DUB inhibitor b-AP15.

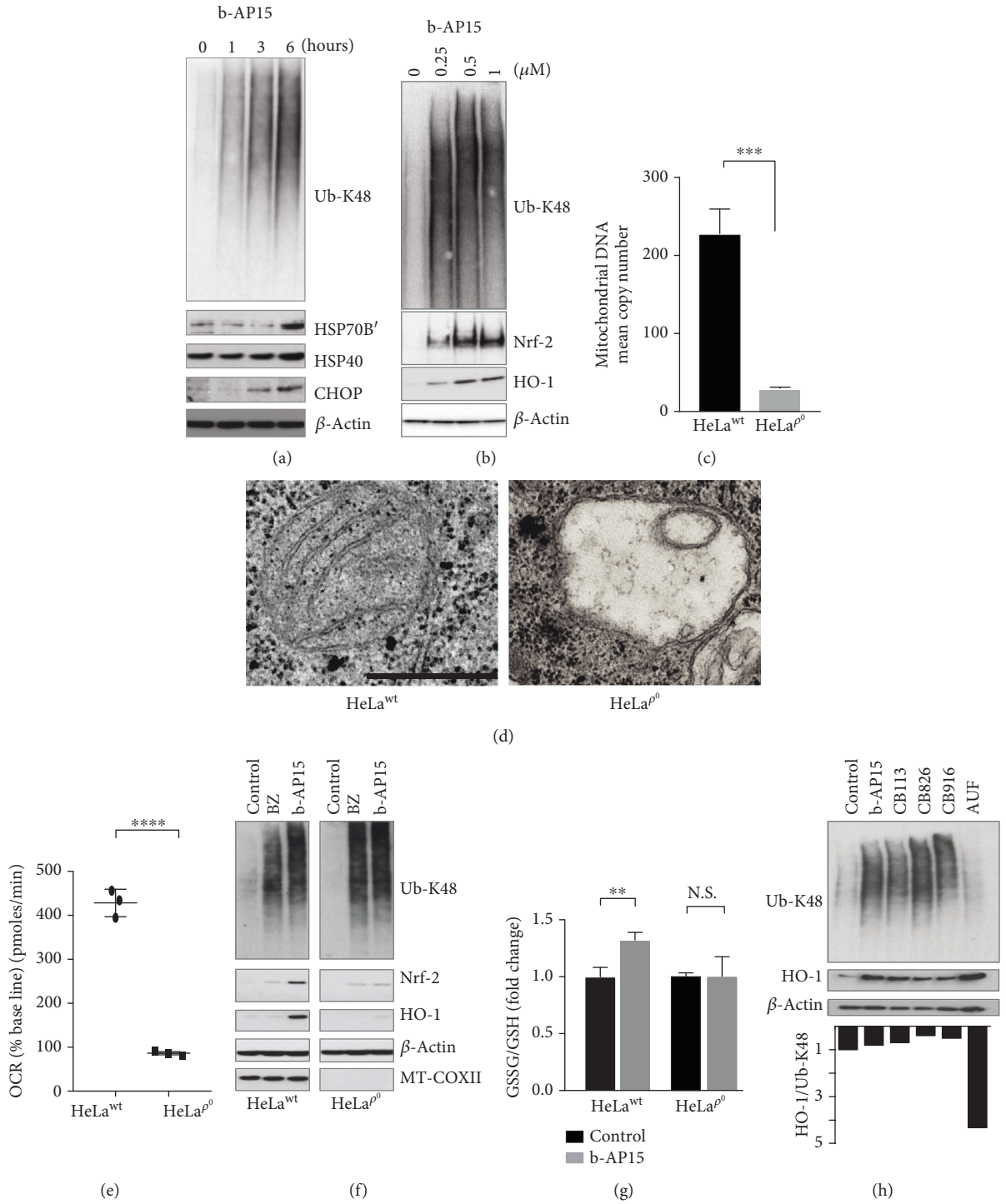


Figure 3: HeLa Rho^0 (ρ^0) cells show a decreased oxidative stress response to b-AP15. (a) HeLa cells were exposed to 0.5% DMSO or 1 μM b-AP15 for 1, 3, and 6 hours, and extracts were prepared and subjected to immunoblotting using the indicated antibodies. All cultures received 0.5% DMSO. (b) HeLa cells were exposed to 0.5% DMSO or b-AP15 (0.25, 0.5, and 1.0 μM in 0.5% DMSO) for 6 h, and extracts were prepared and subjected to immunoblotting using the indicated antibodies. (c) HeLa cells were exposed to EtBr and uridine to generate mitochondrial DNA depleted cells (HeLa ρ^0). The ratio of mtDNA to nDNA was compared in HeLa parental and ρ^0 cells using RT-PCR ($***p < 0.001$). (d) Electron micrographs of mitochondria in HeLa parental and ρ^0 cells. Scale bar = 0.5 μm. (e) Basal oxygen consumption rates (OCR) of HeLa parental and ρ^0 cells ($n = 3$; mean ± S.D.; $****p < 0.0001$). (f) HeLa ρ^0 cells were treated with 100 nM bortezomib (BZ) or 1μM b-AP15 for 5 h followed by western blot analysis for K48-linked polyubiquitin chains, Nrf-2, HO-1, MT-COXII, and β-actin. Note the impaired induction of Nrf-2 and HO-1 by UPS inhibitors in ρ^0 cells. (g) The ratio of GSSG/GSH was determined in parental HeLa and ρ^0 cells exposed to 1 μM b-AP15 or vehicle for 6 h ($n = 3$; mean ± S.D.; $**p < 0.01$). (h) HCT116 cells were exposed to 0.5% DMSO, 1 μM b-AP15, 5 μM CB113, 5 μM CB826, 5 μM CB916, and 1.5 μM auranofin (AUF) for 6 h, and extracts were prepared and subjected to immunoblotting using the indicated antibodies.

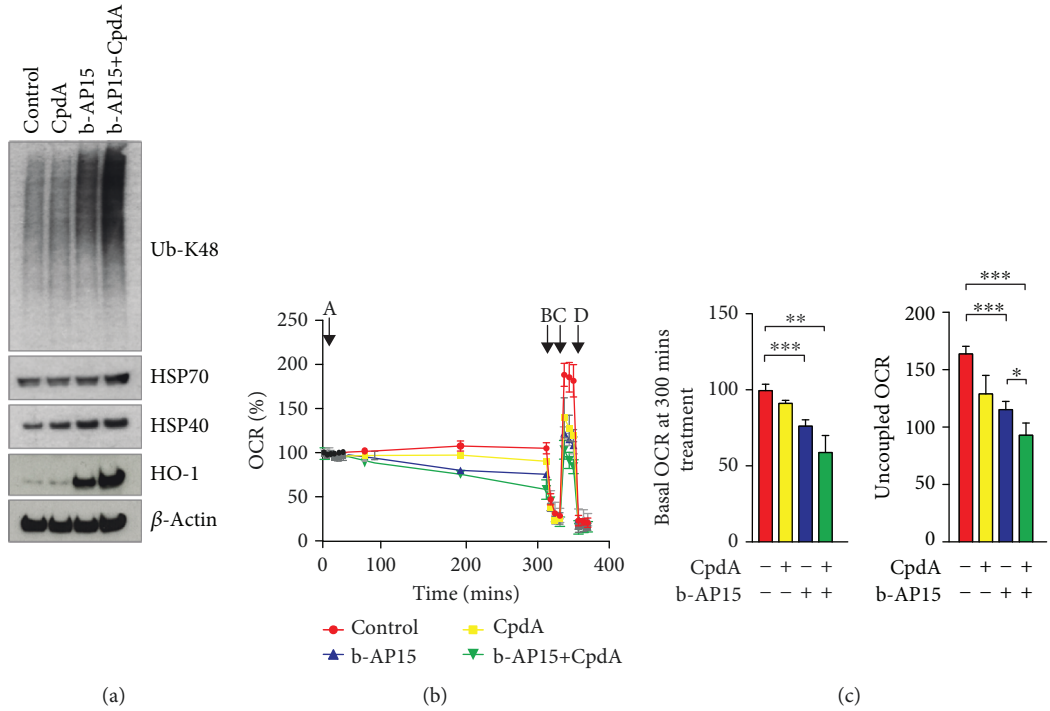
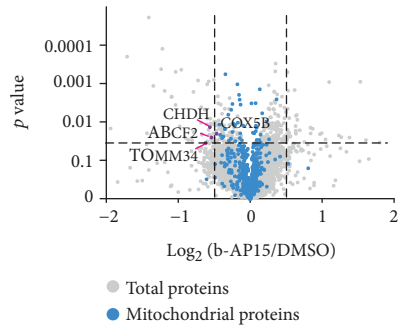


FIGURE 4: Increased levels of proteotoxic stress are associated with decreased mitochondrial function and increased induction of HO-1. (a) HCT116 cells were exposed to 0.5% DMSO, 1 μM b-AP15, and 10 μM CpdA for 6 h, as indicated. Extracts were prepared and subjected to immunoblotting using the indicated antibodies. Note the increased levels of polyubiquitinated proteins, Hsp70, and HO-1 in cells exposed to b-AP15 and the ER translocation inhibitor CpdA. (b, c) HCT116 cells were treated with b-AP15 (1 μM) and/or CpdA (10 μM) for 5 hours and oxygen consumption rates were measured using a Seahorse XF analyzer ($n = 3$ in each group). A: DMSO or compounds; B: oligomycin; C: FCCP; D: antimycin and rotenone. (b) Measurement of OCR in real time after exposure to different compounds; (c) *left*: basal OCR after 300 min of treatment with compounds (mean ± S.D.; $***p < 0.0001$; $n = 3$); *right*: uncoupled OCR after addition of FCCP (mean ± S.D.; $***p < 0.0001$; $*p < 0.05$; $n = 3$).

We and the others have shown that b-AP15 induces phosphorylation of JNK and that inhibition of JNK signaling decreases the apoptotic response [22, 27]. Both JNK signaling and apoptosis are decreased by antioxidant treatment [22]. These findings point to a perturbation of the intracellular redox state being involved in induction of apoptosis. The levels of malondialdehyde, a product of lipid peroxidation of polyunsaturated fatty acids [56], did not increase during b-AP15 treatment and available data suggest that activation of antioxidant systems prevents direct oxidative damage. We here found significant increases in glucose 6-phosphate dehydrogenase (G6PD) activity, leading to a larger potential of NADPH generation [62]. The lack of detectable lipid peroxidation can be argued to mean that b-AP15 does not induce "oxidative stress" by a more stringent definition but merely induces redox imbalances. Although these imbalances are sufficient to induce Nrf-2 and phosphorylation of JNK, they appear to be contained by antioxidant defenses. It should be pointed out, however, that the lack of detectable increases in lipid peroxidation does not necessarily mean that oxidative damage to macromolecules does not occur in specific cellular compartments. For a discussion of redox perturbations, oxidative stress, and oxidative damage, see [63].

We previously presented evidence favoring that the mitochondrial damage that occurs during exposure to b-AP15 is due to the accumulation of misfolded proteins on the outer mitochondrial membrane [42]. This observation did not explain the decrease in oxidative phosphorylation that occurs during drug treatment. We here show downregulation of the COX5b protein, an essential component of cytochrome c oxidase [64]. Cytochrome c oxidase is a key enzyme in the overall regulation of cellular energy production in eukaryotes [65]. Decreases in COX5b have been associated with mitochondrial dysfunction in various conditions [66], and upregulation of COX5b has been observed in energy-demanding cell types and healthy tissues. It has also been demonstrated that downregulation of COX5b by siRNA increases mitochondrial ROS generation [67]. The levels of the yeast homologue of COX5b, COX IV-1, have been shown to be posttranscriptionally regulated by the cardiolipin content of the mitochondria [68], and COX5b has also been reported to be regulated by carbon source and oxygen [64, 69].

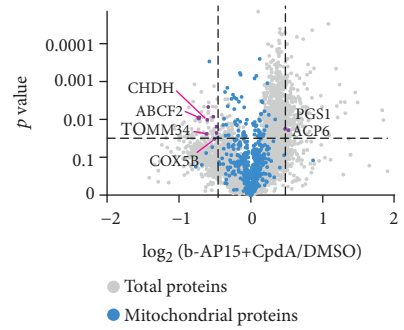
TOMM34 (34 kDa translocase of the outer mitochondrial membrane) was originally identified as a component of the mitochondrial import machinery for nucleus-encoded mitochondrial proteins and has been reported to form a complex with both Hsp70 and Hsp90 as a cytosolic scaffolding cochaperone [70–72]. We observed decreased levels of TOMM34 in mitochondrial preparations in parallel with elevated levels of HSP70B′. It is possible that an elevated demand of TOMM34 in assisting Hsp70/Hsp90 in different



(a)

Downregulated proteins			
Name	Log_2(fold change)	p value	Location in mitochondria
TOMM34	−0.62	0.05	Outer membrane
CHDH	−0.56	0.01	Inner membrane
COX5B	−0.41	0.02	Inner membrane

(b)

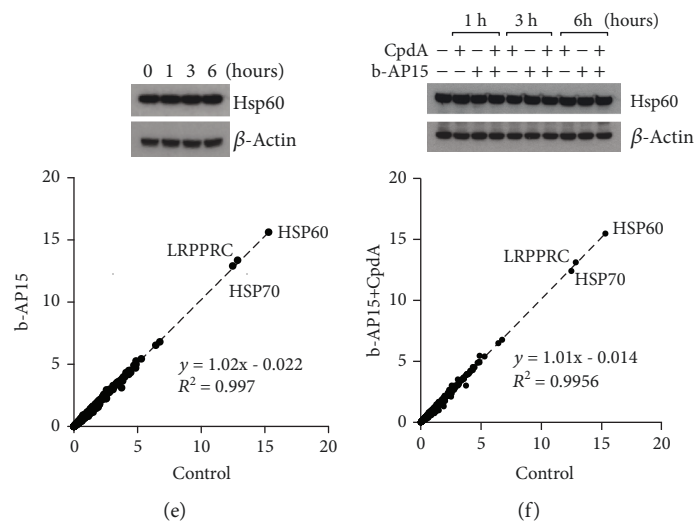


(c)

Downregulated proteins			
Name	Log_2(fold change)	p value	Location in mitochondria
TOMM34	−0.61	0.04	Outer membrane
CHDH	−0.59	0.01	Inner membrane
SLC25A15	−0.52	0.01	Inner membrane
COX5B	−0.51	0.04	Inner membrane
GLRX5	−0.48	0.02	Matrix
CHCHD2	−0.48	0.02	Intermembrane space
COQ4	−0.46	0.03	Inner membrane

Upregulated proteins			
Name	Log_2(fold change)	p value	Location in mitochondria
PGS1	0.48	0.02	Inner membrane
ACP6	0.52	0.02	Intermembrane space

(d)



(e) (f)

FIGURE 5: Proteomic analysis of mitochondrial proteins. (a) Volcano plot showing log_2(fold change) versus p values for proteins from isolated mitochondria prepared from HCT116 cells treated with DMSO or 1 μM b-AP15 for 6 h. (b) Top candidates with significant changes from (a) ($p \leq 0.05$, $log_2 \geq 0.4$ or $log_2 < -0.4$). (c) Volcano plot showing log_2(fold change) versus p values for proteins from isolated mitochondria prepared from HCT116 cells treated with DMSO or 1 μM b-AP15 and 10 μM CpdA for 6 h. (d) Top candidates with significant changes from (c) ($p \leq 0.05$, $log_2 \geq 0.4$ or $log_2 < -0.4$). (e, f) Upper part: HCT116 cells were exposed to 0.5% DMSO, 1 μM b-AP15 in the presence or absence of 10 μM CpdA for 1, 3, and 6 h, as indicated. Extracts were prepared and subjected to immunoblotting using antibodies to Hsp60 and β-actin. (e, f) Lower part: mitochondria were purified from cells treated with 1 μM b-AP15 in the presence or absence of 10 μM CpdA and analyzed by shotgun proteomics. Data was normalized to control samples (treated with 0.5% DMSO).

folding processes in the cytosol limits the availability of TOMM34 proteins on the outer mitochondrial membrane.

Despite its strong cytotoxicity to tumor cells, b-AP15 and similar compounds show limited activity against normal cells [3, 6] and its cytotoxicity is likely to be dependent on the elevated rate of protein turnover in tumor cells. The results presented here suggest that oxidative stress is coupled to proteotoxic stress, leading to an enhancement of the effects on proteasome inhibition. b-AP15 has shown activity in a number of tumor models, including multiple

myeloma [12, 13], Ewing's carcinoma [14], Waldenström's macroglobulinaemia [15], melanoma [9], and colon cancer [3]. The *in vivo* efficacy of b-AP15 is limited by the poor solubility of the compound, and efforts are ongoing to improve the pharmacological properties of this class of molecules. If these efforts are crowned by success, inhibitors of proteasome-associated DUBs could be important drugs in an increasing arsenal of therapeutic options for cancer.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

SL is a consultant of Vivolux AB. No other potential conflicts of interest were disclosed.

Authors' Contributions

X.Z. and S.L. were responsible for the conception and design. X.Z., B.E., and A.S. were responsible for the acquisition of data. X.Z., B.E., and A.S. were responsible for the analysis and interpretation of data. The manuscript was written by X.Z. and S.L. and reviewed/revised by X.Z., B.E., A.S., P.D., R.Z., and S.L.

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Supplementary Materials

Supplementary Fig. 1: induction of caspase cleavage activity by b-AP15 in HeLa cells. HeLa cells were exposed to 1 μM b-AP15 for 24 hours in the presence of absence of BIK siRNA or scrambled control siRNA as indicated. Exponentially growing HeLa cells were seeded in 100 mm dishes at 106 cells per plate and grown for 24 h and then transfected with BIK siRNA (Qiagen) at a final concentration of 10 nM using Lipofectamine RNAi MAX (Invitrogen). Cells were incubated for another 72 h and then treated with DMSO or b-AP15 for another 18 h. Apoptosis was determined by the measurement of the accumulation of caspase-cleaved apoptosis product in cells and culture medium using the M30 Apoptosense ELISA assay (VLVbio, Stockholm). The assay measures caspase-cleaved keratin 18 (K18-Asp396) (Bivén et al., Apoptosis 8 (2003) 263). BIK is a BH3-only protein that has been described to be associated with oxidative stress-induced apoptosis (Bodet et al., Br J Cancer 12 (2010) 1808). Means + S. D. are shown ($n = 3$). Statistical significance was determined using Student's t-test. Supplementary Fig. 2: oxidative stress induced by b-AP15 is not due to the inhibition of thioredoxin reductase (TrxR) activity. CB113 (ChemBridge ID 6943113), CB826 (ChemBridge ID 6556826), or CB916 (ChemBridge ID 6237916) was added to thioredoxin reductase enzyme assay mixes at 20 μM in Tris-EDTA and incubated for 5 hours. Reactions contained 50 nM rTrxR (21 U/mg), 200 μM NADPH, and 1 mg/mL BSA. Reactions were started by the addition of 2.5 mM of DTNB and absorbance was read at 412 nms. The TrxR inhibitor auranofin and Juglone were used as positive controls. Note, none of these three proteasome inhibitors targeted the TrxR activity at the concentration of 20 μM. *(Supplementary Materials)*

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Research Article

Increased Oxidative Stress Induced by *Rubus* Bioactive Compounds Induce Apoptotic Cell Death in Human Breast Cancer Cells

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Bioactive compounds from plants represent good candidate drugs for the prevention and treatment of various forms of cancer. Berries are rich sources of bioactive compounds, and there has been an increasing interest in the study of therapeutic action of wild berries. Oxidants are generated continuously in biological system as a result of physiological process. When there is an imbalance between oxidants and antioxidants, it leads to a condition called oxidative stress. Natural compounds as inducers of oxidative stress are able to modulate the physiological functions of cancer cells leading to cell death or survival. The aim of this study was to evaluate the induction of apoptosis by isolated bioactive compounds (1-(2-hydroxyphenyl)-4-methylpentan-1-one (C1) and 2-[(3-methylbutoxy) carbonyl] benzoic acid (C2)) from *Rubus fairholmianus* against MCF-7 breast cancer cells. The exposure of C1 and C2 reduced viability (IC_{50} of C1: 4.69; C2: 8.36 μg/mL) and proliferation. Cytochrome c release from mitochondria and changes in mitochondrial membrane potential of treated cells supported the intrinsic apoptotic cell death. Reactive oxygen species (ROS) production after treatment with C1 and C2 was found to be higher and induced nuclear damage. Expression of apoptotic proteins after the treatments was significantly upregulated as indicated using immunofluorescence (caspase 9, p53, and Bax), western blotting (p53, cleaved PARP, cytochrome c, and Bax), and ELISA (caspase 9) analysis. Overall, C1 was more cytotoxic, increased the ROS production in dichlorodihydrofluorescein diacetate assay, and induced apoptosis in breast cancer cells. These results illustrate that berry bioactive compounds have strong chemopreventive potential. In this article, we provide information on prooxidant and anticancer activities of *Rubus* bioactive compounds. Natural products have always demonstrated a significant contribution to the development of several cancer chemotherapeutic drugs. Most of these compounds are known to affect the redox state of the cell; and studies on these compounds have focused on their antioxidant property instead of prooxidant properties.

1. Introduction

Cancer is the leading cause of death in both developing and developed countries. Globally, cancers of the lung, breast, colon/rectum, and prostate are the most common types. Breast cancer is the most predominant, hormone-associated malignancy in women. The prevalence of breast cancer is growing in developing countries. Upregulation of growth hormone receptors such as estrogen in breast cells is the key reason and the stimulating factor for the development of breast cancer [1].

Historically, plants have been used for many health benefits. About 80-85% of worldwide population rely on traditional plant-based medicines for their health care needs. A number of plant extracts, isolated compounds, and their analogues have been used as effective anticancer drugs, and there has been an increasing interest in the study of therapeutic properties of plant-derived compounds [2]. The characterization and analysis of therapeutic values of plant extracts and the isolated bioactive compounds are a growing area of research. Epidemiological studies show that diets rich in plant-based foods protect against many diseases including

cancer. Among the bioactive compounds of plants, phenolics and flavonoid compounds are known to have cytotoxic properties against various tumor cells with low toxicity towards normal cells. Oxidative stress is a normal phenomenon. Normally, the intracellular levels of reactive oxygen species (ROS) are maintained low. Therefore, oxidative stress can be observed as an imbalance between prooxidants and antioxidants [3]. Some of the antioxidants act as prooxidants by inducing nuclear damage and lipid peroxidation if transition metal is available. The number of free OH substitutions initiates the prooxidant activity of a flavonoid. The OH exchange is essential for antioxidant properties, but the more OH substitutions, the stronger prooxidant activities [4].

Raspberries are excellent sources of vitamins such as ascorbic acid. They have been used in traditional and alternative medicine for various illnesses. Some antioxidants like ascorbic acid have both prooxidant and antioxidant effects depending upon the dose. Raspberry extracts, individual polyphenols or in conjunction with other compounds, are able to inhibit the proliferation of cancer cells. They have shown antiproliferative effects on human colon, prostate, breast, and oral cancers [5]. The prooxidant/antioxidant activity of carotenes and lycopene has also been found to depend on their interaction with biological membranes and other coantioxidant molecules. At higher oxygen tension, carotenoids tend to lose their effectiveness as antioxidants, whereas the prooxidant action of tocopherol is evident at low oxygen tension [6].

Apoptosis is the most common cell death mechanism that plays a vital role in normal metabolic function. Tumor cells are characterized by uncontrolled multiplication rates and loss of apoptosis. The activation of apoptotic pathways is one of the cell death pathways by which chemotherapeutic agents kill cancer cells. Agents that block or destroy tumor cell proliferation by inducing apoptosis are considered as promising antitumor mediators [7]. Apoptosis is also a gene-regulated cell death mechanism with well-described biological changes [8]. Initiation of apoptosis is the mode of action of numerous compounds employed in cancer chemotherapy. Hence, the compounds that induce apoptosis are likely to be perfect anticancer drugs. Many bioactive compounds from plants inhibit cancer cell growth through induction of apoptosis; therefore, elucidating the mechanism of apoptosis has a significant implication in cancer chemoprevention. In the present study, we examined the apoptosis induction of two bioactive compounds isolated from a *Rubus* species in human breast carcinoma cells. Most phytochemicals induce apoptosis via the initiation of the intrinsic apoptotic pathways, which includes a range of intracellular stimuli. The major players of this pathway include pro- and antiapoptotic proteins. A difference in the ratio of these proteins leads to mitochondrial membrane damage and results in cytochrome c release, which mediates the activation of caspase 9 [9]. Therefore, alleviating or preventing oxidative damage by ROS should be a potential therapeutic target. Many plant-derived natural compounds induce the controlled production of ROS, which initiates cell death in various cancer conditions through apoptotic pathways.

Previous studies have showed that increased Bax expression may induce apoptosis and increased Bcl-2 expression may inhibit apoptosis [10]. Caspases are a major class of proteases which play a crucial role in cell death. Cytochrome c release by the mitochondria is one of the key features of apoptosis. Bcl-2 proteins prevent apoptosis by decreasing the cytochrome c release to inhibit caspase 3 activation. Bax protein, a vital part of the mitochondrial membrane, helps in the transfer of cytochrome c across the membranes thereby forming apoptotic bodies and activates caspase 9 and caspase 3 that eventually leads to apoptosis [11]. Poly (ADP-ribose) polymerase (PARP) is a family of related enzymes, which induces posttranslational modification proteins, that exist in the nucleus and cytoplasm and is involved in DNA damage repair, gene transcription regulation, telomerase activity regulation, and protein degradation. PARP inhibition may be useful in cancer chemotherapy to selectively sensitize cancer cells to DNA damage agents and promote cell death. PARP is cleaved into two fragments, which causes the loss of its function and leads to apoptosis [12, 13].

Berries are rich in bioactive compounds including phenolics, flavonoids, and anthocyanins. Many bioactive compounds of berries possess antioxidant properties and are important in developing drugs. *Rubus fairholmianus*, the Himalayan raspberry, has been examined for its several pharmacological actions both *in vitro* and *in vivo*. The *R. fairholmianus* root extract induced cell death in colorectal, breast, and lung cancer and melanoma cells via caspase-dependent apoptotic pathways [14–16]. However, evidence regarding the anticancer effect of isolated bioactive compounds from *R. fairholmianus* is inadequate.

Therefore, the objective of this study was to determine the anticancer effects of the bioactive compounds of *Rubus* such as 1-(2-hydroxyphenyl)-4-methylpentan-1-one (C1) ($C_{12}H_{16}O_2$, 192.25 MW) and 2-[(3-methylbutoxy) carbonyl] benzoic acid (C2) ($C_{13}H_{16}O_4$, 472.53 MW) (Figure 1) on MCF-7 human breast cancer cells *in vitro*.

2. Materials and Methods

2.1. Extraction and Bioactive Compound Isolation. *Rubus fairholmianus* Gard. wild plants were collected, and a voucher specimen (BSI/SRC/5/23/2010-11/Tech.1657) was deposited in the Botanical Survey of India. Based on the higher radical scavenging activity, root acetone extract was selected for the isolation of bioactive compounds. The roots were extracted successively using polar and nonpolar solvents in a Soxhlet apparatus, and the extract was concentrated to dryness under reduced pressure in a rotary evaporator. *R. fairholmianus* root acetone extract showed higher antioxidant activity, and it was selected for the isolation of bioactive phytochemicals. The preliminary screening was done using thin layer chromatography (TLC) with various solvent combinations; the fractions with similar banding patterns were clubbed and the active fractions obtained were loaded on column chromatography for the compound isolation. The isolated compounds were purified by semipreparative TLC and preparative HPLC. Further ultraviolet, Fourier-transform infrared, nuclear magnetic resonance, and mass spectrometry methods characterized the compounds. Activity-guided column chromatographic isolation

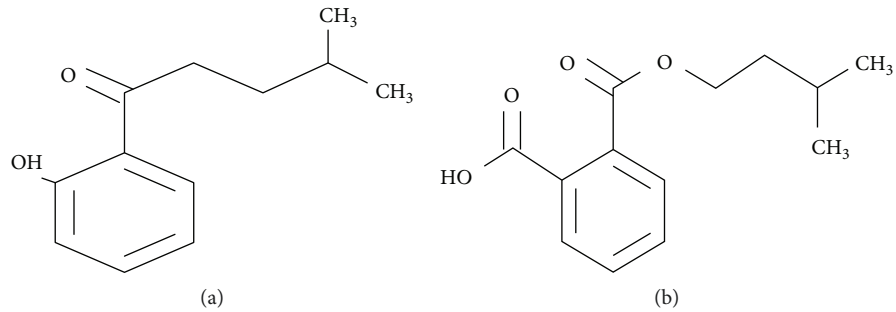

Figure 1: Chemical structure of isolated bioactive compounds from *Rubus fairholmianus*: (a) 1-(2-hydroxyphenyl)-4-methylpentan-1-one (C1) and (b) 2-[(3-methylbutoxy) carbonyl] benzoic acid (C2).

yielded 6 compounds [17]; among which, the most active 1-(2-hydroxyphenyl)-4-methylpentan-1-one (C1) and 2-[(3-methylbutoxy) carbonyl] benzoic acid (C2) were selected for the study of induction of cell death in MCF-7 breast cancer cells *in vitro*.

2.2. Cell Culture. Human breast cancer cells MCF-7 (ATCC HTB-22) were used for the *in vitro* studies. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (FBS; Gibco 306.00301), supplemented with 1% penicillin/streptomycin (PAA Laboratories GmbH, P11-010) and 1 μg/mL amphotericin B (PAA Laboratories GmbH, P11-001). Human skin fibroblast monolayer cultures (WS1-ATCC CRL1502) were grown in Eagle's minimal essential medium (Invitrogen 32360–026) supplemented with 2 mM L-glutamine (Gibco, 25030), 1 mM sodium pyruvate (Gibco, 11360), 0.1 mM nonessential amino acids (Gibco, 11140), 1% amphotericin B (Gibco, 104813), 1% penicillin-streptomycin (Gibco, 15140), and 10% *v*/*v* fetal bovine serum (FBS; Gibco, 306.00301). Cells were maintained in a CO_2 incubator (37°C, 5% CO_2, and 80% humidity) and used as control normal cells. Cells were washed with Hank's Balanced Salt Solution (HBSS, Invitrogen, 10-543F) when it became 80% confluent and detached with TrypLE Express (Gibco, 12604) and subcultured. Cells were seeded at a concentration of 5×10^5 cells/plate (MCF-7 cells) and 6.5×10^5 cells/plate (WS1 cells) in 3.5 cm^2-diameter culture plates for experimental purposes.

2.3. Morphological Analysis. After 24 h of incubation with different concentrations of C1 and C2 (2.5, 5, and 10 μg/mL), the cells were rinsed once with HBSS and replenished with fresh medium, then the morphological changes were carefully noticed using Wirsam Olympus CKX 41 inverted light microscope.

2.4. Assessment of Cell Viability, Proliferation, and Cytotoxicity. The effect of C1 and C2 on cell viability was measured by trypan blue assay (Sigma-Aldrich T8154). Ten microliters of cell suspension and 0.4% trypan blue were mixed and added to a haemocytometer and counted using automated cell counter (Countess™ Automated Cell Counter, Invitrogen). CellTiter-Glo[1] luminescent assay (Promega, G7571, Anatech Analytical Technology, South Africa) quantifies the ATP levels in metabolically active cells. The Cyto-Tox96 X assay (Anatech, Promega G 400) was used to evaluate the cytotoxic activity of C1 and C2 on MCF-7 cells. The membrane integrity and cytotoxicity were assessed by quantifying the lactate dehydrogenase (LDH) released to the culture media following the pretreatment with C1 and C2 bioactive compounds [18].

2.5. Annexin V/PI Staining. The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Becton Dickinson, 556570, Scientific Group, South Africa) was used to distinguish the population of apoptotic and nonapoptotic cells according to manufacturer's protocol. After the staining process, flow cytometry was performed using Fluorescence-Activated Cell Sorting (FACS) Aria flow cytometer (BD Biosciences) to quantify the population of various cells [18].

2.6. Cytochrome c Release and Mitochondrial Membrane Potential Analysis ($\Delta\Psi m$). An Enzyme-Linked Immunosorbent Assay (ELISA) (Human Cytochrome c Platinum ELISA Kit, Affymetrix eBioscience BMS263) was used to detect cytosolic cytochrome c levels [18]. The evaluation of changes in mitochondrial membrane potential is an indirect measure of intrinsic apoptosis pathway. The $\Delta\Psi m$ was analysed using BD™ Mito Screen flow cytometry mitochondrial membrane potential detection kit [18].

2.7. DCFH-DA ROS Assay and Hoechst Nuclear Staining. The cellular ROS produced was measured by dichlorodihydrofluorescein diacetate (DCFH-DA) (D-6883, Sigma-Aldrich) assay, and nuclear damage induced by C1 and C2 was observed by Hoechst staining [18].

2.8. Immunofluorescence. Cells were cultured in 3.4 cm^2-diameter culture dishes over sterile cover slips until 80% confluence and treated with C1 and C2 (IC_{50} of C1: 4.69; C2: 8.36 μg/mL) for 24 h. After the treatment, the cells were fixed in 4% formaldehyde for 15 min at room temperature and washed twice with 1x PBS, followed by incubating the cells with permeabilization buffer (0.01% *v*/*v* Triton X-100) for 15 min and then washed twice with 1x PBS. Cells were blocked in PBS containing 3% bovine serum albumin for 1 h, and primary antibody recognizing p53 (Santa Cruz Biotechnology, SC393031), Bax (Santa Cruz Biotechnology, SC493), and caspase 9 (Santa Cruz Biotechnology, SC56076) were added at 1 : 100 dilution for 2 h at room temperature. The cells were washed twice with 1x PBS and FITC

(Santa Cruz Biotechnology, SC2010; SC2359) conjugated secondary antibodies were added and incubated for 2 h. The nucleus was stained by DAPI and the slides were examined using the Carl Zeiss Axio Observer Z1 with the filter set 358Ex/461Em.

2.9. Caspase 9 Expression by Enzyme-Linked Immunosorbent Assay (ELISA). We used cell-based ELISA to analyse the expression of caspase 9 in cultured cells. Plates were incubated with 100 μL of 1 : 30 diluted primary antibody (caspase 9, Cat# SC-73548, Santa Cruz Biotechnology), and 100 μL of a 1 : 5000 secondary antibody was added (Goat anti-mouse IgG-HRP: Cat# SC-2005, Santa Cruz Biotechnology), and the colorimetric reaction was measured at 450 nm (Perkin-Elmer, Victor^3 plate reader) according to the manufacturer's protocol [18].

2.10. p53, PARP, Cytochrome c, and Bax Expression by Western Blot Analysis. The protein concentration in cell lysate was assessed using BCA Protein Assay Reagent (Thermo Fisher Scientific, Rockford, IL, USA). Authors used the respective primary antibodies (p53: mouse monoclonal antibody, Cat# SC-99, Santa Cruz Biotechnology; PARP: mouse monoclonal antibody, Cat# SCBSC-8007; Cytochrome c: mouse monoclonal antibody, Cat# SCBSC-13156; Bax: mouse monoclonal antibody, Cat# 336400, Life Technologies; and GAPDH: mouse monoclonal antibody, Cat# MA5-15738, Invitrogen) and the horseradish peroxidase-conjugated secondary antibody (Goat anti-mouse HRP, Cat# SC-2005, Santa Cruz Biotechnology) for the western blot experiments and followed the protocol explained by George et al. [18].

2.11. Statistical Analysis. Data were represented as mean ± standard error of the mean (SEM) of at least three tests done in duplicates. The statistical significance was analysed using SigmaPlot version 13.0. The treated groups were compared with the untreated groups by one-way ANOVA to find out the statistical significance. The p value less than 0.05 was considered as significant.

3. Results

3.1. Morphology. Morphological variations in MCF-7 cells followed by the treatment with C1 and C2 were compared with control cells (Figures 2(a)–2(g)) and WS1 fibroblast normal cells (Figures 3(a)–3(g)). The pretreated cells presented with an irregular shape, and the number of dead cells in the treated groups was found to be more compared to that in the control group. Total loss of membrane integrity and detachment from the culture plate were observed in the cells treated with C1 and C2. Compared to C2, the C1-treated cells indicated an increased impact with a greater number of dead cells. The most identifiable morphological features of apoptosis such as loss of membrane integrity and cellular shape were observed in the MCF-7 cells treated with bioactive compound C1. The WS1 cells did not show any significant change in morphology after 24 h treatment with C1 and C2.

3.2. Viability, Proliferation, and Cytotoxicity. The treatment with C1 and C2 compounds decreased cell viability in MCF-7 cells. We examined the effect of C1 and C2 on cell viability by trypan blue viability test. MCF-7 cells were treated with several concentrations of C1 and C2, and the viability was measured based on the uptake of blue dye and expressed as percentage (Figure 4(a)). Compounds induced a dose-dependent decrease in viability with an inhibitory concentration (IC_{50}) of 4.69 and 8.36 μg/mL for C1 and C2, respectively. A significant ($p < 0.001$) reduction in cell number was observed in cells treated with higher concentration of C1 ($21.75 \pm 0.85\%$) compared with higher dose of C2 ($48.25 \pm 2.32\%$). The decrease in percentage viability was significant with all tested concentrations ($p < 0.001$).

Uncontrolled cell division is a primary contributor to the progression of cancer. The energy levels in MCF-7 cells were high, which was notable from the increased ATP level in untreated cells. Exposure of MCF-7 cells to C1 and C2 leads to the decrease in the intracellular ATP, which shows decreased proliferation rates. We found that the proliferation of MCF-7 cells was inhibited in a concentration-dependent manner after exposure to C1 and C2 (2.5, 5, and 10 μg/mL) (Figure 4(b)). C1 showed relatively high antiproliferative activities on MCF-7 cells compared to C2. However, both compounds were significant in reducing the cellular proliferation compared to untreated cells.

Similar effects were also found when the LDH assay was performed to check the cytotoxic effects of C1 and C2 on MCF-7 cells. The LDH assay was used to measure the cellular membrane integrity following treatment with C1 and C2. The cell membrane damage was measured by LDH release into the culture medium. The WS1 cells (Figure 3(h)) and untreated control cells (Figure 4(c)) displayed reduced LDH release compared to the treated MCF-7 cells. A significant ($p < 0.001$) increase in toxicity was noticed at higher concentrations of C1 and C2 (Figure 4(c)). A 3.08-fold increase in cytotoxicity was observed in MCF-7 cells when treated with C1 at a higher concentration compared to 10 μg/mL of C2 (2.87). Thus, our results suggest that the bioactive compounds isolated from *Rubus* were able to decrease the viability and proliferation in breast cancer cells, while increasing the cytotoxicity of cells after 24 h treatment.

3.3. Annexin V/PI Staining. Apoptosis plays a vital role in the homeostasis, and several morphological and biochemical changes in cells characterize this process. The bioactive compounds C1 and C2 significantly ($p < 0.001$) induced cell death in a dose-dependent manner after 24 h treatment; the Annexin V/PI staining determined the population of apoptotic and nonapoptotic cells. The results of staining displayed a substantial increased uptake of Annexin V by an increased cell population percentage in the lower and upper right quadrants in the pretreated groups (Figures 5(a)–5(h)). C1 and C2 induced early cell apoptosis in MCF-7 cells (2.95 ± 68 and $1.97 \pm 0.76\%$) at the highest concentrations. Flow cytometric analysis of cells displayed that there is a substantial increase in the percentage of late apoptotic cells treated with C1 and C2 ($35.45 \pm 2.13\%$ and $18.08 \pm 1.4\%$) at 10 μg/mL concentrations, indicative of apoptotic cell death (Figure 5(i)). In

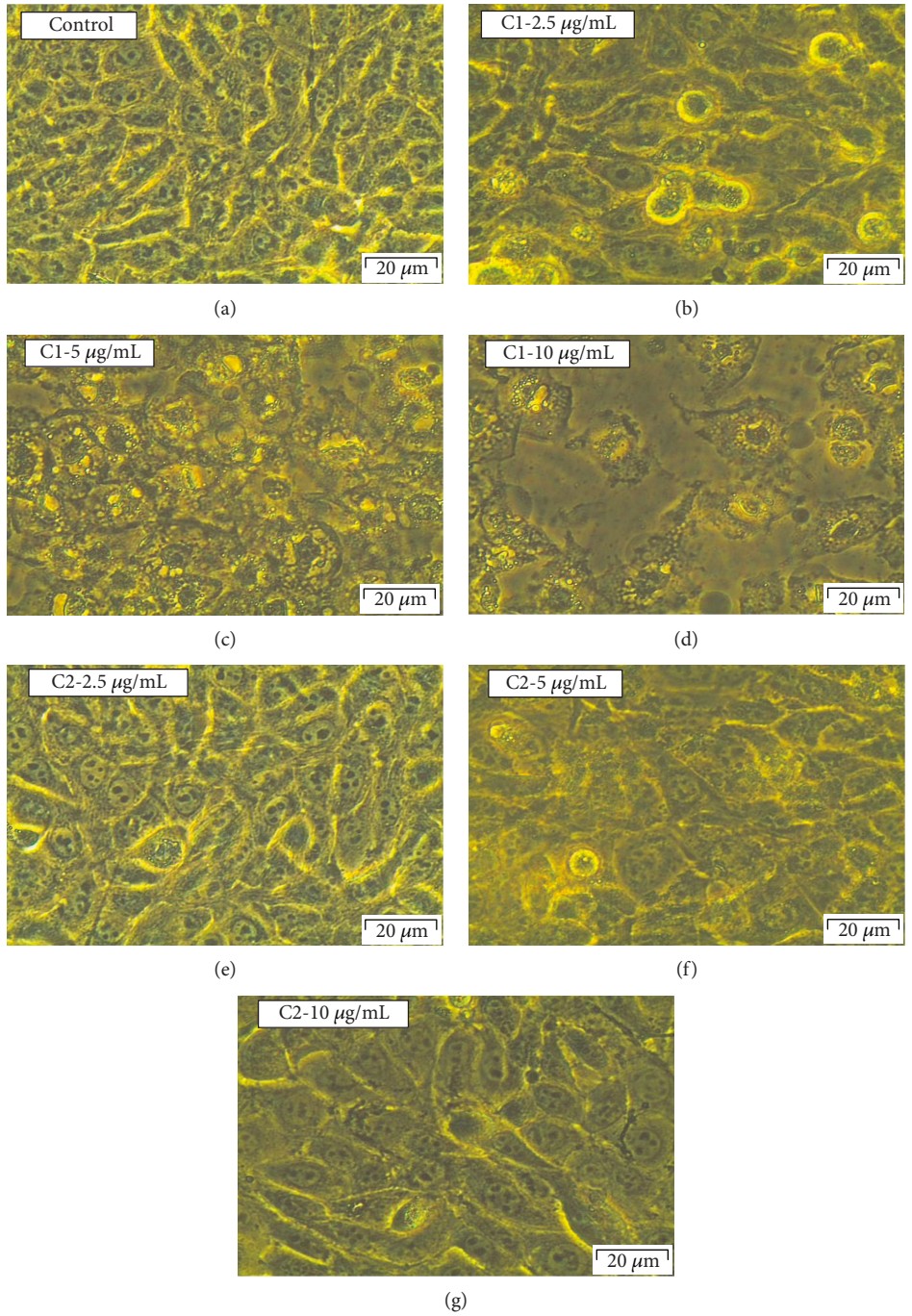

Figure 2: Morphological evaluation of C1- and C2-treated MCF-7 cells. There were no significant visible differences in control (a) and 2.5 μg/mL C2-treated groups (e); the cells did not show any cellular shrinkage and apoptotic bodies after the treatment. However, more dead cells were observed at all concentrations of C1 and higher concentration of C2 (5 and 10 μg/mL). The treated cells showed loss of intact membrane and loss of contact with neighbouring cells and were condensed; being detached from the culture plate showed the features of apoptotic cells.

contrast, the number of early and late apoptotic and necrotic cell concentrations in untreated cells was found to be very low compared to C1 and C2 treatments.

3.4. Cytochrome c Release. Cytochrome c release from mitochondria is a critical event in cell death via the intrinsic apoptotic pathway. A difference in various apoptotic and nonapoptotic proteins in cells leads to damage of the mitochondrial membrane resulting in discharge of cytochrome c and followed by caspase activation. The cytochrome c release was determined after 24 h treatment with C1 and C2. The ELISA results of cytochrome c release revealed that in the control, the cells were unable to initiate such a damaging event and low cytochrome c release was observed

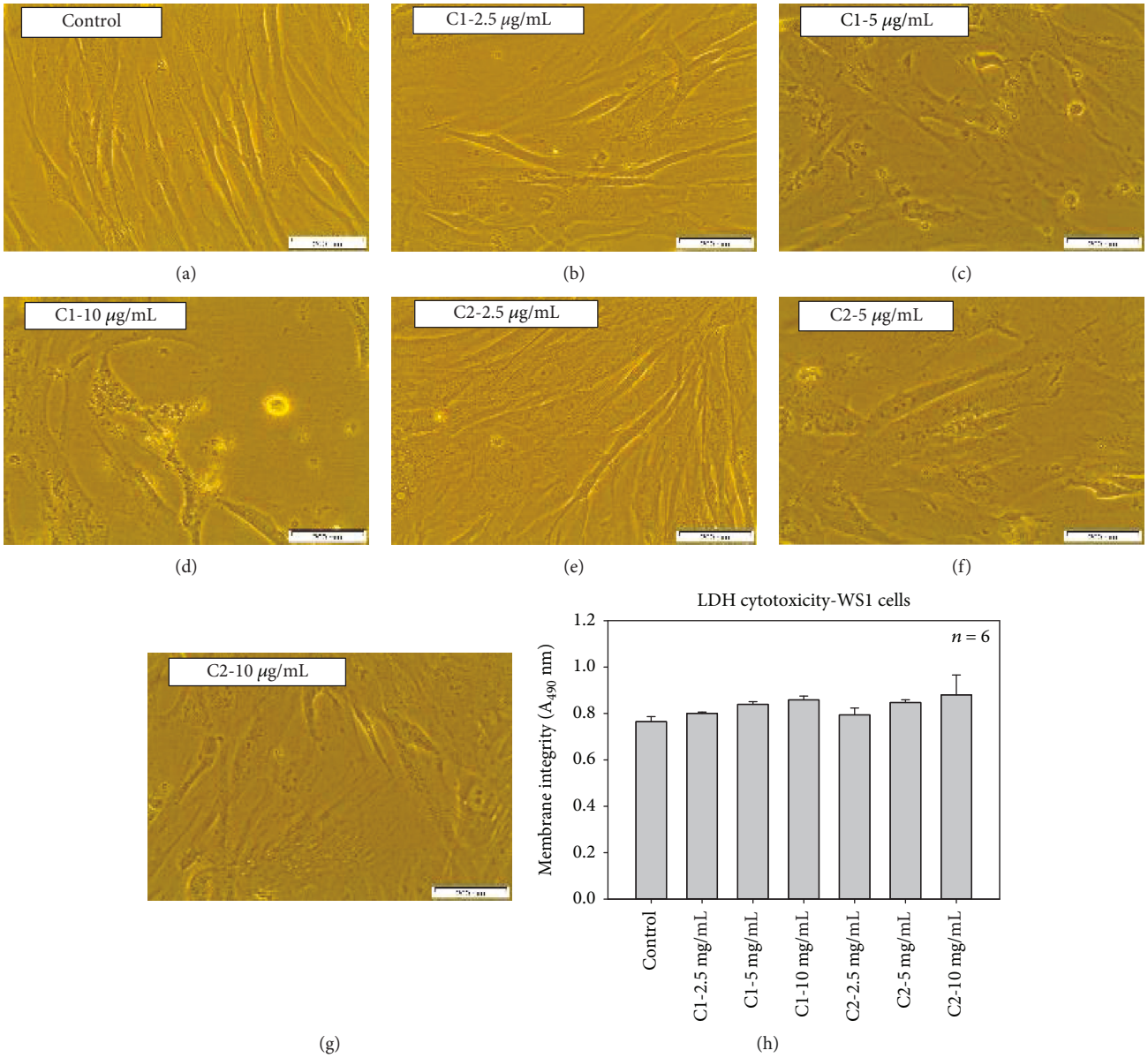

Figure 3: Morphological evaluation of C1- and C2-treated WS1 cells. There were no significant visible differences in control (a) and treated groups (b–g); the cells did not show any cellular shrinkage and apoptotic bodies after the treatment. The lactate dehydrogenase (LDH) cytotoxicity. The LDH cytotoxicity test showed that there is no significant increase in cytotoxicity in WS1 cells after 24 h treatment with C1 and C2 compared to control cells (h).

(Figure 6(a)). All doses of C1-treated cells showed significant ($p < 0.001$) release of cytochrome c compared to C2. However, the treatment with C1 and C2 initiated cell damage in MCF-7 cells and led to an increased cytochrome c release.

3.5. Mitochondrial Membrane Potential. To further confirm that C1 and C2 induced apoptosis, mitochondrial membrane potential or mitochondrial destabilization was measured in MCF-7 cells after treatment with C1 and C2. Figure 6(b) shows that both compounds induced significant damage to mitochondria after 24 h incubation. Compared to C2, C1 showed highly significant results. The percentages of polarized and depolarized membrane potential in each group were determined and compared to the respective percentage of the control cells. After 24 h of incubation with JC-1 stain, no change in membrane potential was detected in control cells. However, changes in both polarized (black) and depolarized (grey) cell populations were noticed in C1- and C2-treated cells. The treated cells increased the depolarized mitochondrial membrane and decreased the polarized membranes. The higher concentrations of C1 (5 and 10 μg/mL) significantly ($p < 0.001$) increased the depolarized mitochondrial membrane and decreased the polarized mitochondrial membrane compared to C2 ($p < 0.05$).

3.6. ROS Production and Nuclear Damage. The ROS generation was evaluated after 20 h exposure to C1 and C2. A significant increase in the level of intracellular ROS was

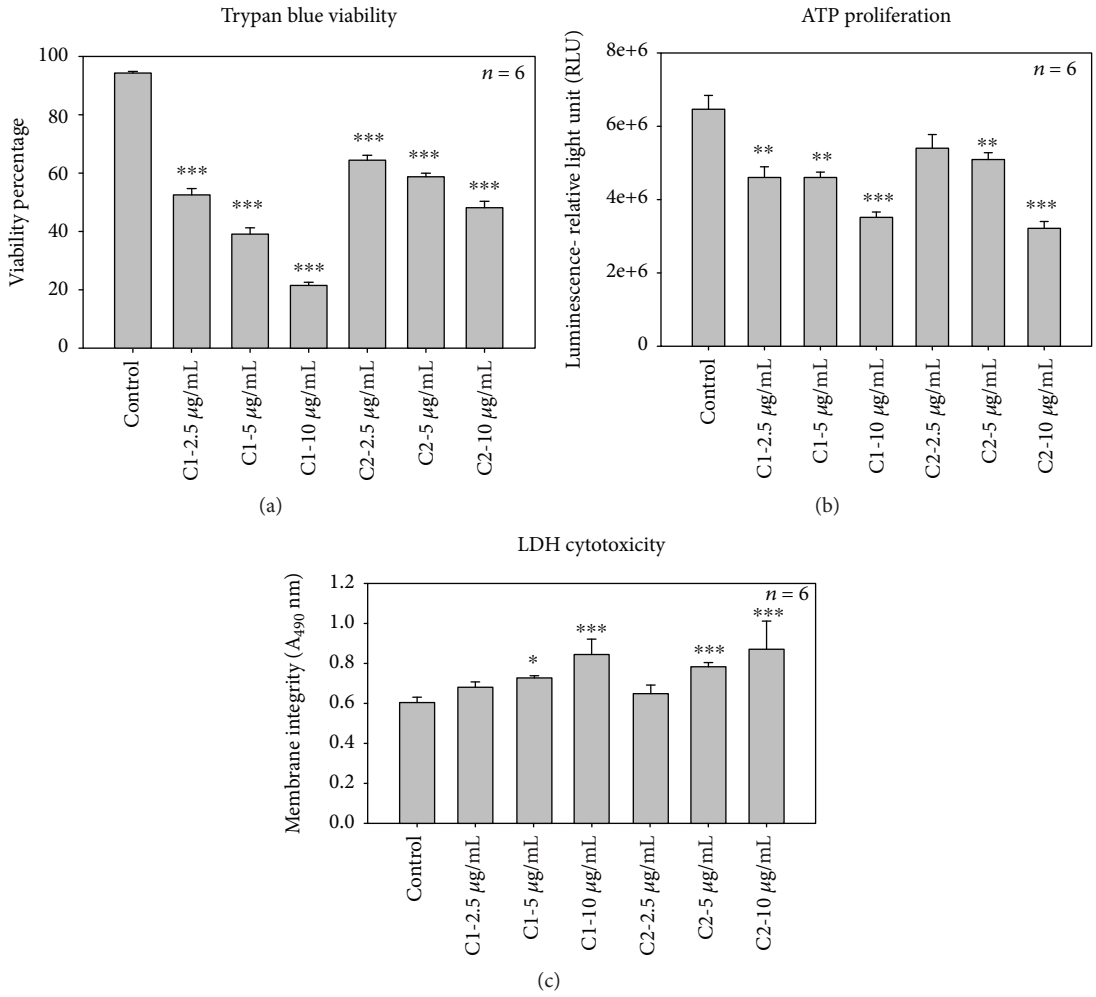

Figure 4: Effect of C1 and C2 on cellular viability, proliferation, and cytotoxicity. (a) Trypan blue viability. Trypan blue viability test showed a significant ($***p < 0.001$) decrease in the viability of the MCF-7 cells after C1 and C2 treatment compared to the control cells. (b) The ATP proliferation assay. ATP luminescent cell assay was used to determine MCF-7 cell proliferation after the treatment with C1 and C2. Control cells showed an increased ATP level, whereas a dose-dependent significant ($***p < 0.001$ and $**p < 0.01$) decrease in ATP level was observed in both experimental groups. (c) The lactate dehydrogenase (LDH) cytotoxicity. The LDH cytotoxicity test showed a significant increase in cytotoxicity of cells after the 24 h treatment with C1 and C2 compared to control cells. The significant differences between the treated and control groups are shown as $***p < 0.001$ and $*p < 0.05$.

observed in fluorescent and ELISA methods, as shown in Figures 7(a)–7(e). However, the H_2O_2-treated group showed the highest ROS production compared to other groups. This finding indicates that the ROS production is enhanced by the treatment with C1 and C2, which could be one of the reasons behind the cell death. In the quantitative ROS production analysis, using ELISA also showed the increased production when the cells were treated with C1 bioactive compound compared to C2, H_2O_2 used as the positive control in these experiments. Some of the important features of apoptosis such as nuclear condensation and DNA damage were examined by Hoechst nuclear staining.

The Hoechst nuclear stain was used to measure the DNA damage after the treatment with C1 and C2. Results from the control group showed dense spherical and homogenously blue-stained nuclei, while cells treated with C1 and C2 (2.5, 5, and 10 μg/mL) showed irregular-shaped nucleus, suggestive of nuclear condensation as shown in Figures 8(a)–8(g). The treated cells showed shrinkage of chromatin granules with smaller nucleus and scattered nuclear granules, with irregular shape, indicative of nuclear fragmentation. Hence, it suggests that the treatment with C1 and C2 induces apoptotic cell death in MCF-7 cells, as the Hoechst stain was able to show the nuclei changes, which were characteristics of apoptosis.

3.7. C1 and C2 Treatment Upregulates Apoptotic Proteins. To investigate the effect of C1 and C2 in inducing the intrinsic apoptotic pathway, we examined the expression of caspase 9, p53, and Bax proteins (Figures 9(a)–9(i)) by immunofluorescence. The apoptotic protein levels will be low in cancer cells compared with the normal dying cells. In our experiments, the apoptotic proteins such as caspase 9, p53, and Bax expressions were high in treated groups than control cells. Upon treatment, active p53 and caspase 9 were highly expressed in the nucleus whereas the activated Bax was

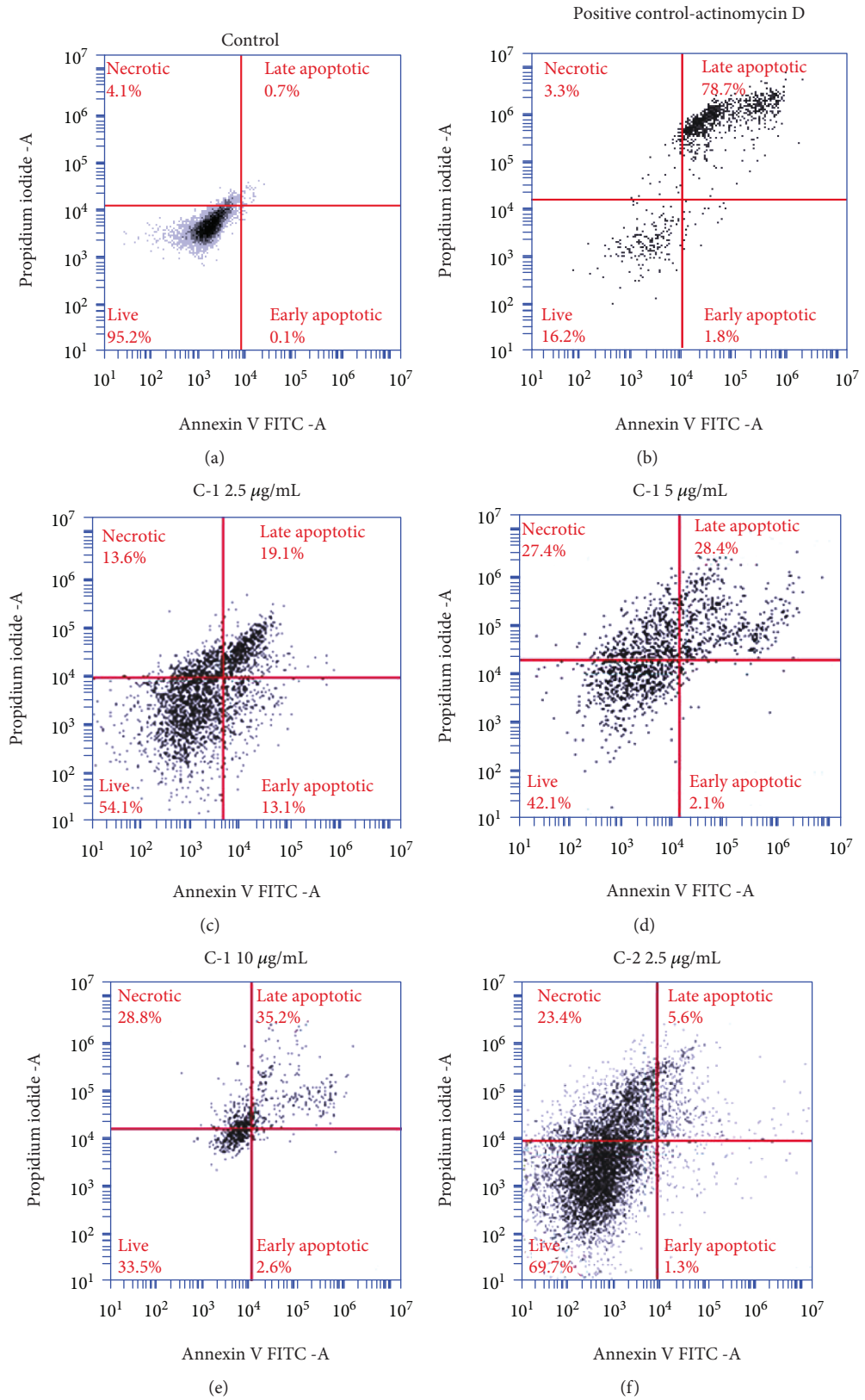

Figure 5: Continued.

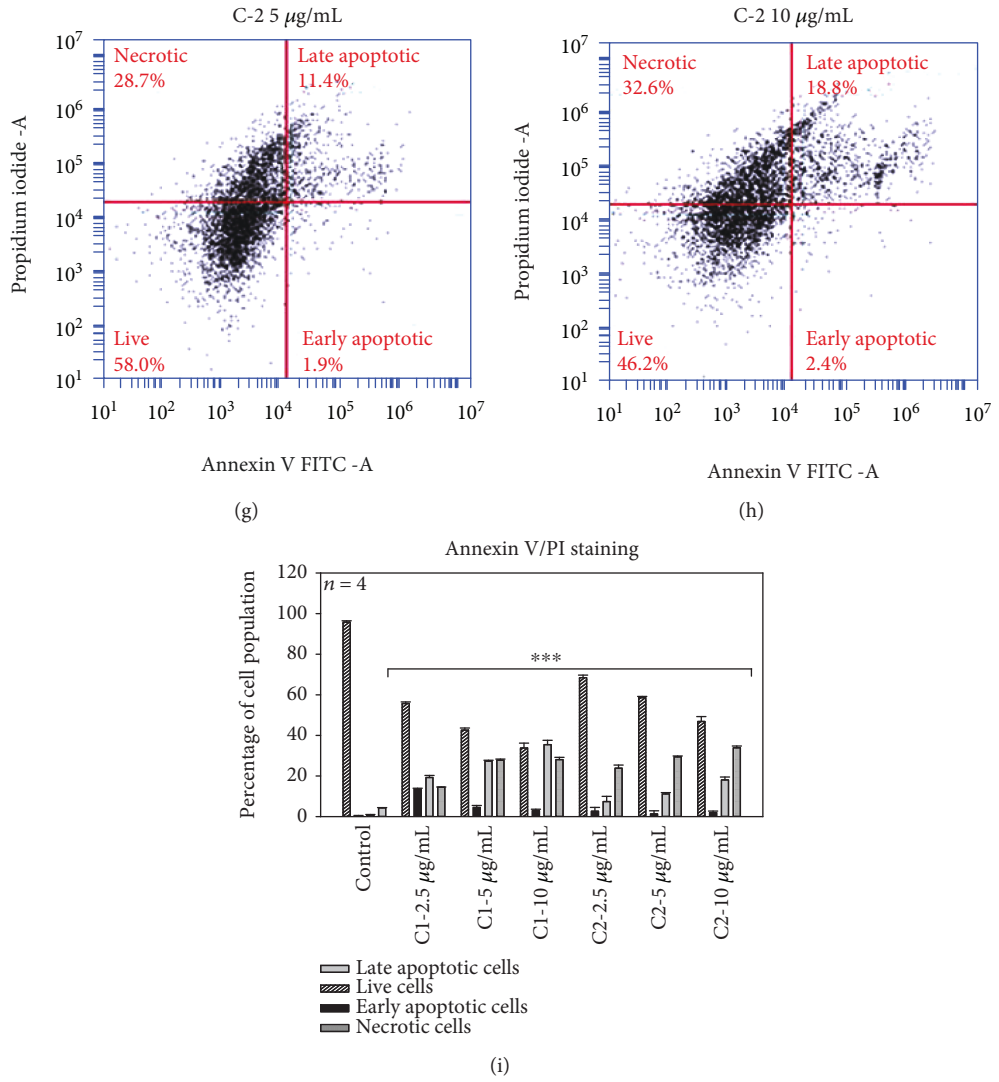

Figure 5: Annexin V FITC/PI staining. Annexin V-PI staining was used to assess the mode of cell death. C1- and C2-treated MCF-7 cells showed an increased percentage of apoptotic cell population after 24 h incubation. The population of early and late apoptotic cell percentage in the control group was found to be lower and the live cell percentage was higher in control cells compared with the experimental groups. A significant decrease (***$p < 0.001$) in the live cell and increase in the early and late apoptosis and dead cell percentage were observed in C1- and C2-treated MCF-7 cells. (a–h) Dot plots and (i) the graph with statistical significance.

highly expressed in the cytoplasm. Bax activation leads to mitochondrial permeability; this was supported by the mitochondrial membrane potential assay and cytochrome c release. Among the bioactive compounds, C1-treated groups showed higher expression of all the four proteins tested than the compound C2.

3.8. ELISA and Western Blotting Analysis of Apoptotic Proteins. To investigate the effect of C1 and C2 in inducing the intrinsic apoptotic pathway, we examined caspase 9 expression by ELISA (Figure 10(a)) and the expression of p53, Bax, PARP, and cytochrome c by western blotting (Figures 10(b)–10(d)). In cancer cells, the apoptotic protein level will be low compared with apoptotic cells. In our experiments, the expression of apoptotic proteins such as p53, Bax, cleaved PARP, and caspase 9 levels was high in treated groups than control cells, after the treatment with C1 and C2, which favours the apoptosis in MCF-7 cells. The C1-treated groups showed significant ($p < 0.05$ and $p < 0.001$) expression of all four proteins tested. Upon treatment, active p53, Bax, cleaved PARP, cytochrome c, and caspase 9 were highly expressed. The activation of these proteins leads to mitochondrial permeability; these results were supported by the mitochondrial membrane potential, cytochrome c release assay, and immunofluorescence.

4. Discussion

Dysregulation of normal apoptotic mechanisms favours cancer cell growth. The dysregulated apoptotic pathways such as downregulation of death receptors and p53 mutations lead to increased incidence of breast cancer. Breast cancer treatments such as chemo, radiation, and hormone therapy induce various apoptotic mechanisms to initiate cell

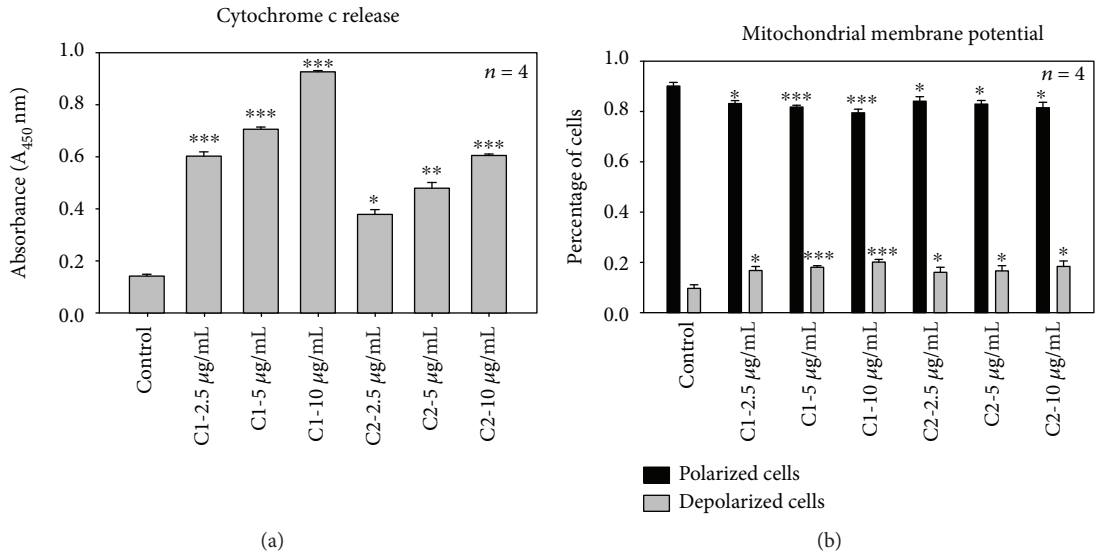

Figure 6: Effect of C1 and C2 on cytochrome c release and mitochondrial membrane potential. (a) Cytochrome c release. Cytochrome c release is an important measure of cellular damage. C1-treated cells showed significant ($***p < 0.001$) release of cytochrome c compared to C2 and control cells. The significant differences between the treated and control groups are shown as $***p < 0.001$, $**p < 0.01$, and $*p < 0.05$. (b) Mitochondrial membrane potential. Mitochondrial membrane potential evaluation using the flow cytometric analysis of JC-1 fluorometric stain. Percentages of polarized (black) and depolarized (grey) mitochondrial membrane potential were determined and compared to the percentage of the corresponding control cells. C1- and C2-treated cells showed a significant change in mitochondrial membrane potential ($***p < 0.001$).

death. Therefore, stimulation of apoptotic mechanisms in breast cancer cells could be an effective way to eradicate breast cancer. This study investigates the antitumor potential of two bioactive compounds C1 and C2 from *R. fairholmianus*. Our previous works described the antiproliferative activities of *Rubus* crude extracts on colorectal, breast, and lung cancer and melanoma cells [14–16]. However, the activities of isolated bioactive compounds from this species [17] against cancer cell lines have not been studied. Previously, many researchers made efforts to understand the effect of plant-derived compounds in induction of cancer cell death [7, 19, 20].

Our data illustrates that the compounds C1 and C2 have antiproliferative and apoptosis inducing effects on MCF-7 human breast adenocarcinoma cells. We pretreated MCF-7 cells with various concentrations of C1 and C2 for 24 h and counted the number of dead and viable cells in order to study the cell death. The cell number of MCF-7 cells was reduced significantly upon treatment with C1 and C2. The results of trypan blue dye exclusion assay demonstrated that both compounds reduced the viability of MCF-7 cells with C1 exhibiting prominent activity. Both compounds induced morphological variations, such as rounding up of cells. In addition, cells treated with compounds revealed loss of membrane integrity and cell detachment from the culture plate. The induced cell death has been categorized as apoptotic or necrotic [21, 22]. The major noticeable feature, which differentiate apoptosis from necrosis, is the presence of apoptotic bodies [23, 24]; these morphological features were observed in MCF-7 cells after treatment, whereas the WS1 fibroblast normal cells did not show any cytotoxicity and significant morphological changes upon treatment with bioactive compounds. This specific cell death induction was further confirmed as the apoptosis-inducing effect of C1 and C2 on MCF-7 cells.

The use of plant-based products is a novel approach in advanced cancer prevention and treatment. The plant-derived compounds require comparatively low cost and cause nontoxic effects with several molecular targets in chemoprevention of cancer [25, 26]. The inhibition of cell proliferation by C1 and C2 was determined using the metabolic ATP proliferative assay. This could either be the result of inhibition of cell growth or a direct cytotoxic effect. The cytoplasmic enzyme LDH will remain within the healthy cells with an intact membrane, but LDH will be released from the membrane-damaged cells. LDH has been established as a biomarker of cell death analysis. In the culture medium, LDH activity increases when cells are treated with cytotoxic agents and they induce apoptosis or necrosis [27–29]. The LDH cytotoxicity assay directly measures the toxicity by evaluating the LDH released upon the treatment with C1 and C2. Apoptosis and related cellular mechanisms display intense effects on cancer cell progression; those events are perfect target in several cancer therapies [30]. The role of natural products has been broadly examined for its involvement in cancerogenesis and metastasis [31].

Evaluation of apoptosis further confirmed that pretreatment with C1 and C2 induced cytochrome c release and drop in mitochondrial membrane potential. The loss in mitochondrial membrane integrity is related with intrinsic apoptosis [32]. When the death signal starts in a cell, the C terminal sequence of the receptor activates that targets the mitochondrial outer membrane and leads to permeabilization, which finally leads to the cell death [33]. The decrease of mitochondrial membrane potential suggests the initiation of the mitochondrial apoptotic pathway.

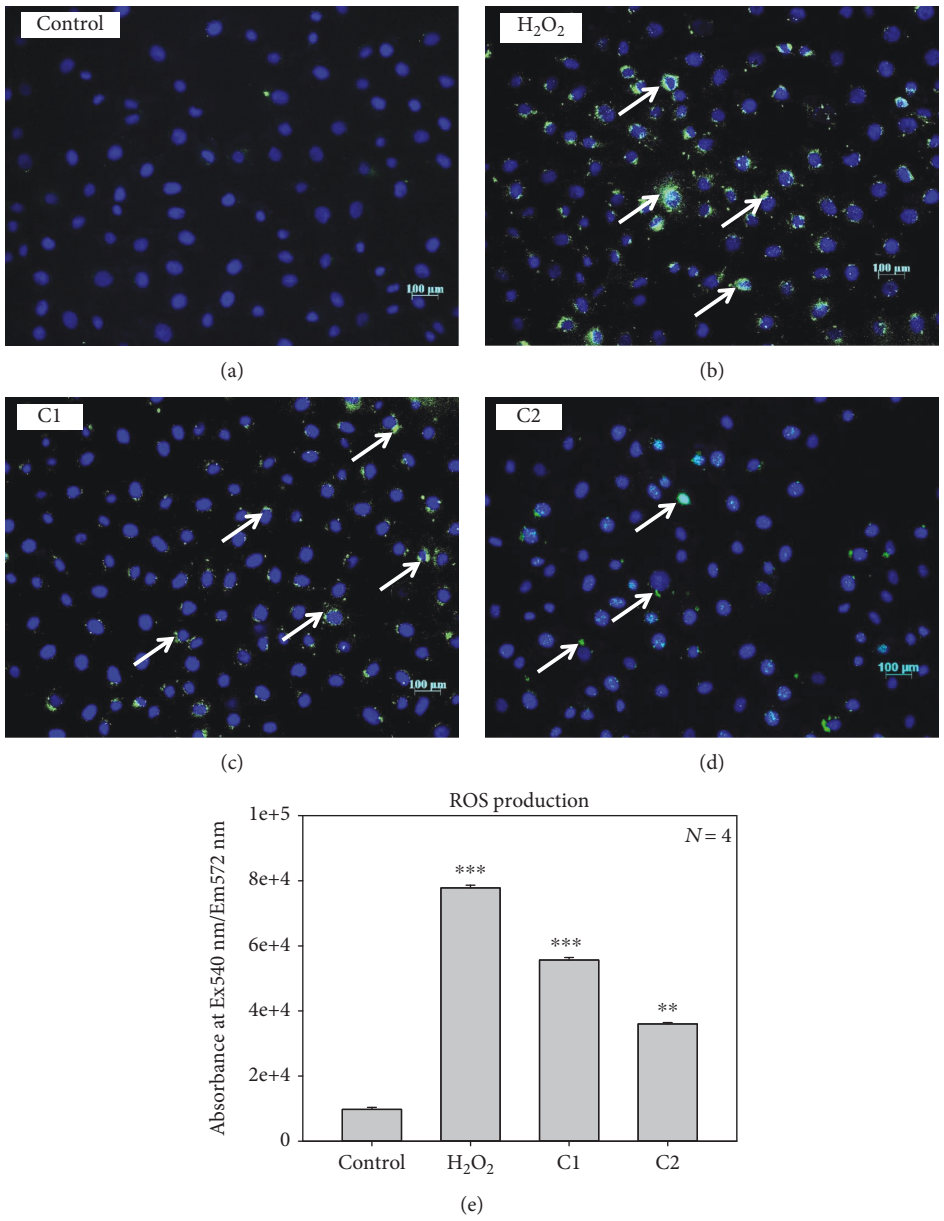

Figure 7: Effect of C1 and C2 on ROS production. ROS production were analysed by DCFH-DA staining in MCF-7 cells: (a) control; (b) positive control: H_2O_2; (c) C1: 4.69 μg/mL treated; (d) C2: 8.36 μg/mL treated. The green color indicates the fluorescence of detected ROS production and blue color indicates the nuclear stain-DAPI. C1-treated cells showed increased ROS production than C2-treated cells. (e) Quantitative ROS production analysis using ELISA showing significant ROS production upon treatment with C1 ($***p < 0.001$) and C2 ($**p < 0.01$).

Depolarization of mitochondrial membrane is due to the degeneration of mitochondrial membrane potential and interruption of the electron transport chain (ETC) gradient. The shift in mitochondrial polarization when exposed to the compounds could be the result of intact ETC [34]. The dose-dependent incubation with C1 and C2 depicted a continuous reduction of mitochondrial membrane potential.

The upregulation of Bax proteins may lead to cytochrome c release and thus caspase 9 upregulation [35]. The biochemical analysis of MCF-7 cells after the treatment with C1 and C2 confirmed such hypothesis. Hence, the viability, cytotoxicity, and proliferative results in conjunction with the mitochondrial dysfunction and cytochrome c release demonstrated the initiation of apoptotic pathway by C1 and C2.

ROS are either free radicals or reactive anions with oxygen ions and peroxides [36]. High level of ROS can destroy the integrity of the plasma membrane and cause DNA damage, cumulatively known as oxidative stress [37, 38]. Interestingly, we observed that ROS production in C1- and C2-treated MCF-7 cells was higher compared to that in untreated cells. The accumulation of ROS disturbs the redox control of cell cycle progression via phosphorylation and ubiquitination of cell cycle proteins, leading to aberrant

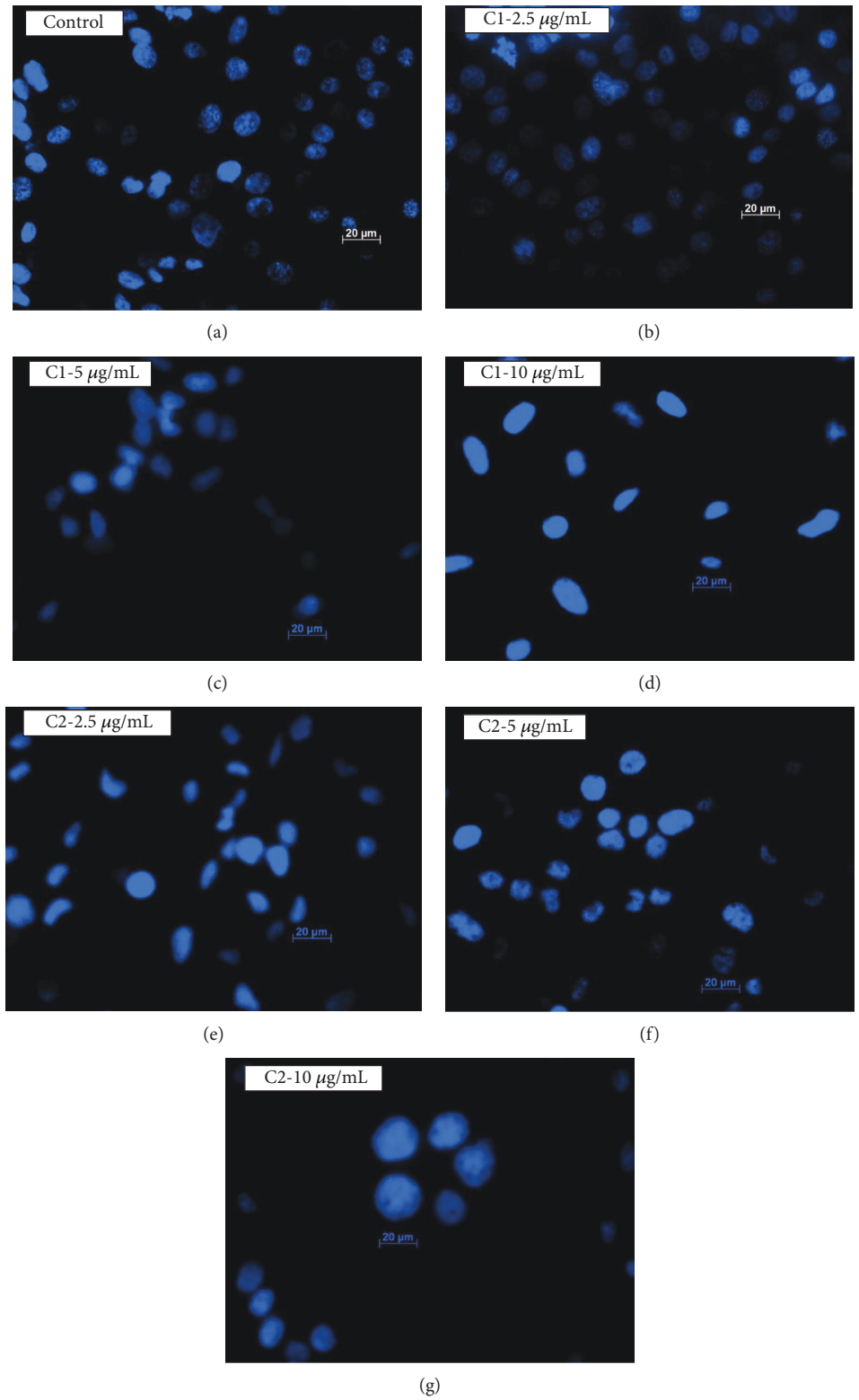

Figure 8: Hoechst staining. Nuclear damage was analysed by Hoechst stain. Control cells (a) showed intact nucleus without any damage; however, the cells treated with C1 and C2 (b–g) at different concentrations showed highly condensed chromatin granules, loss of nuclear shape which indicates the DNA damage. C1-treated cells showed higher nuclear damage.

cell proliferation and apoptosis [39]. Although ROS is dangerous to cells, the anticancer role of various treatments depends on their ability to stimulate controlled ROS production, which changes cellular redox balance leading to oxidative stress, damage to mitochondria, and consequent apoptosis induction [40]. Our results also supported this

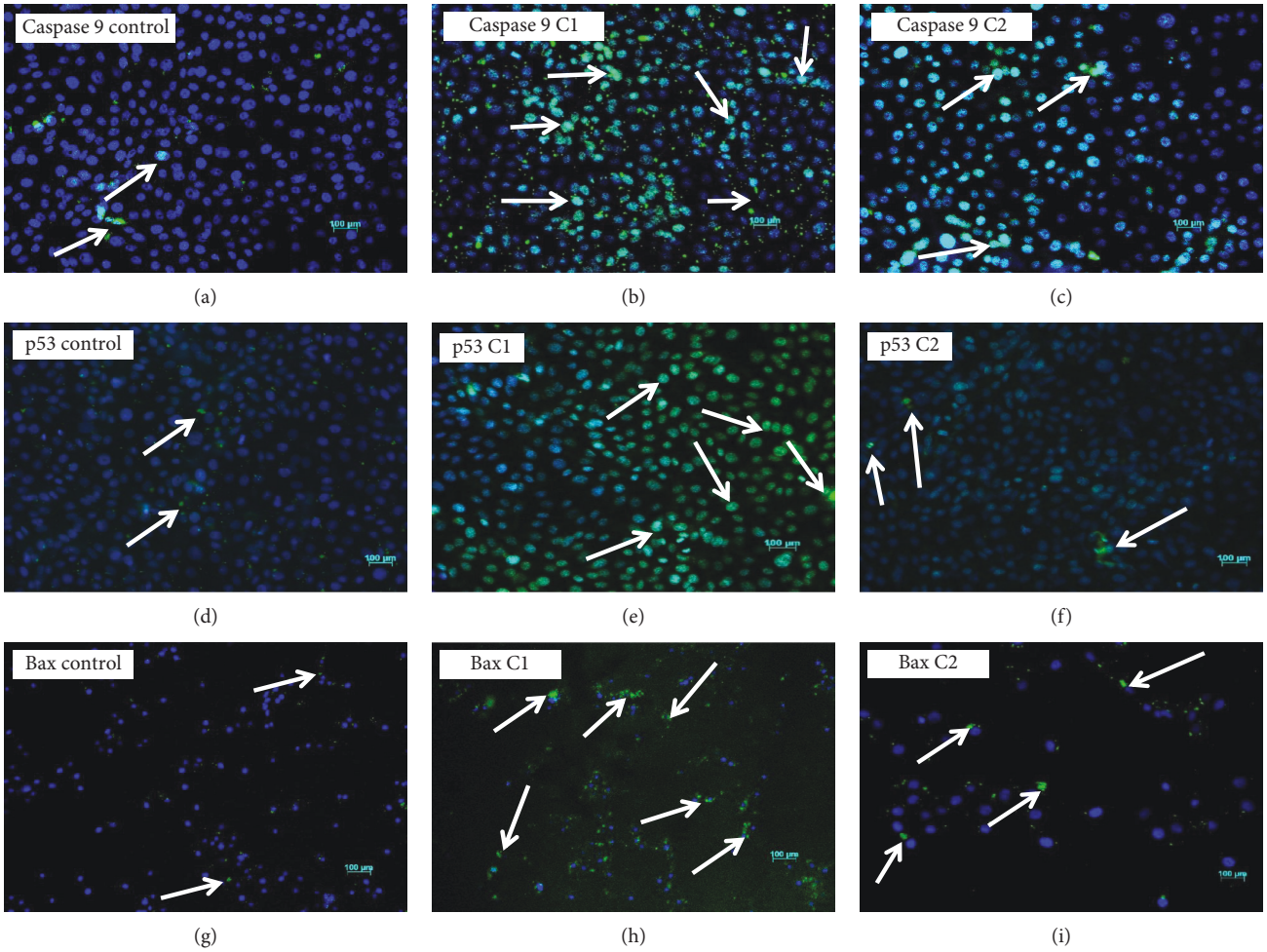

Figure 9: Immunofluorescence: caspase 9, p53, and Bax. (a) Control cells showing very few caspase 9-positive cells; (b) C1 4.69 μg/mL treated showing higher caspase 9-positive cells; (c) C2 8.36 μg/mL treated showing medium caspase 9-positive cells. (d) Control showing very few nuclear staining for active p53; (e) C1 4.69 μg/mL treated showing strong nuclear staining for active p53; (f) C2 8.36 μg/mL treated showing moderate nuclear staining for active p53. (g) Control showing weak staining for active Bax; (h) C1 4.69 μg/mL treated showing strong cytoplasmic staining for active Bax; (i) C2 8.36 μg/mL treated showing moderate cytoplasmic staining for active Bax. C1-treated cells showed increased expression of caspase 9, p53, and Bax proteins than C2-treated cells (×20, original magnification; arrow indicates the presence of proteins; nuclear stain: DAPI (blue color); p53 and Bax protein stain: FITC (green color)).

fact by mitochondrial destabilization, by the increased ROS production, which in turn regulated the expression of apoptotic proteins.

Several studies have revealed that mitochondria has a potential role in apoptosis [41, 42]. The mitochondrial membrane potential variations lead to the release of apoptogenic factors including cytochrome c. Cytochrome c forms an apoptosome with procaspase 9, apoptotic protease activating factor-1 (Apaf-1), and ATP. The apoptosome further activates downstream caspase signals [43, 44]. Initiator caspase 9 is activated during cytochrome c release [44], which can further activate executioner caspases and achieve apoptosis [45]. Treatment with C1 and C2 causes a dose-dependent activation of caspase 9; these results propose that C1 and C2 induced apoptosis via the mitochondrial intrinsic pathway. Mitochondrial molecules involved in ROS generation and pro- and antiapoptotic factor regulation have been reported to control apoptosis in several cell lines [46]. Studies show that ROS can facilitate mitochondrial membrane permeabilization [47]. The ROS produced in oxidation processes are essential to living organisms to produce the energy required to fuel biological processes. However, excessive production of ROS damages the cells because ROS destroy molecules such as DNA and proteins. Thus, ROS play an important role in the pathogenesis of various serious diseases, such as neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, and inflammation [48–51].

Deregulation of pro- and antiapoptotic factors disrupts mitochondrial role, leading to cytochrome c release to the cytoplasm and subsequent activation of caspase cascade [52]. In our experiments, treatment with C1 and C2 resulted in the expression of Bax and release of cytochrome c in MCF-7 cells. Bax possibly controls the cytochrome c release from mitochondria that occurs after the treatment. ROS also plays a crucial role in the modulation of proapoptotic Bax and antiapoptotic Bcl2, and studies reported that the translocation of Bax to mitochondria could change permeability [53, 54].

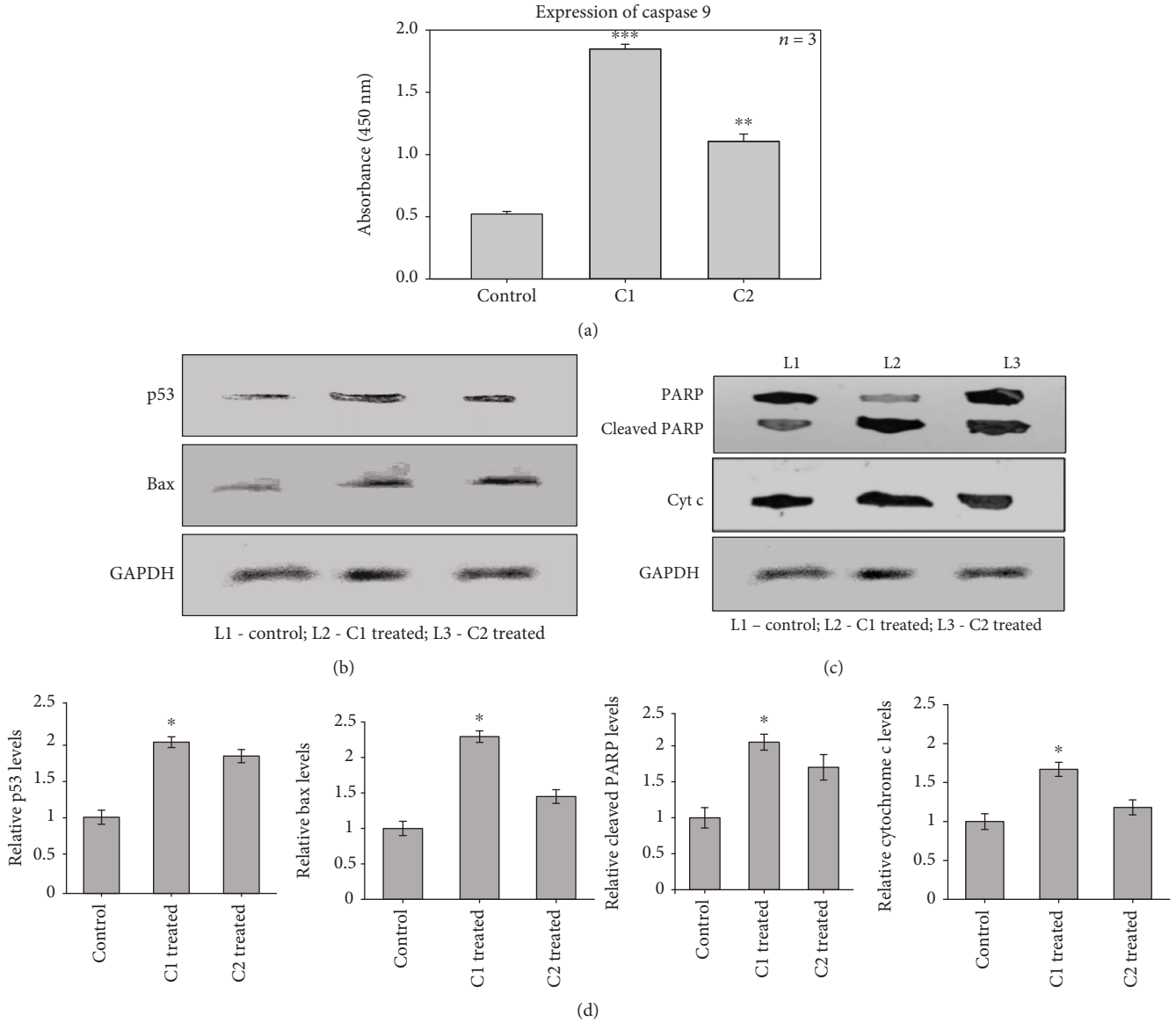

Figure 10: Expression of apoptotic proteins. (a) The effect of C1 and C2 on the expression of caspase 9 by ELISA. (b, c) The effect of C1 and C2 on the expression of p53, Bax, cleaved PARP, and cytochrome c by western blotting (L1: control; L2: C1 treated; L3: C2 treated). (d) The relative expression levels of p53, Bax, cytochrome c, and cleaved PARP. The results showing that apoptotic proteins such as caspase 9, p53, Bax, cytochrome c, and cleaved PARP levels were significantly increased in C1-treated groups than C2 and control cells.

Based on our results, it is possible that sufficient Bax exists in the mitochondrial membrane to induce cytochrome c release and intrinsic apoptosis after the treatment with C1 and C2.

p53 is a multifunctional tumor suppressor that regulates DNA repair, cell cycle arrest, apoptosis, and cell survival as well as oxidative stress. p53 responds to a wide range of cell death stimuli, which can induce apoptosis by activating gene expression or by permeabilizing the mitochondria. Increased ROS in cancer cells is also associated with the activation of a key signaling protein p53. p53 accumulates in the nucleus and controls the expression of proapoptotic members, Bax and PUMA. Once upregulation of PUMA occurs, PUMA binds to Bcl-xL, releasing p53 to activate Bax [55]. p53 can translocate to mitochondria, interact with the antiapoptotic Bcl-2 family of proteins, neutralise them, and cause apoptosis [56]. The present study showed the expression of p53 in MCF-7 cells when treated with C1 and C2; therefore, the induction of apoptosis in MCF-7 cells upon *Rubus* bioactive compound treatment is a major target for cancer therapy.

Relatively high PARP expression was found in triple-negative breast cancers (TNBC) [57]. The findings from this research suggest that inhibition of PARP could be a potential therapeutic approach in breast cancer and other tumor types. Cleavage of PARP to its inactive form is considered as an important event in cancer cell apoptosis [57]. PARP cleavage and higher expression of cleaved PARP in the C1- and C2-treated groups and the generation of intracellular ROS assured the induction of apoptosis. Generation of intracellular ROS plays a pivotal role in

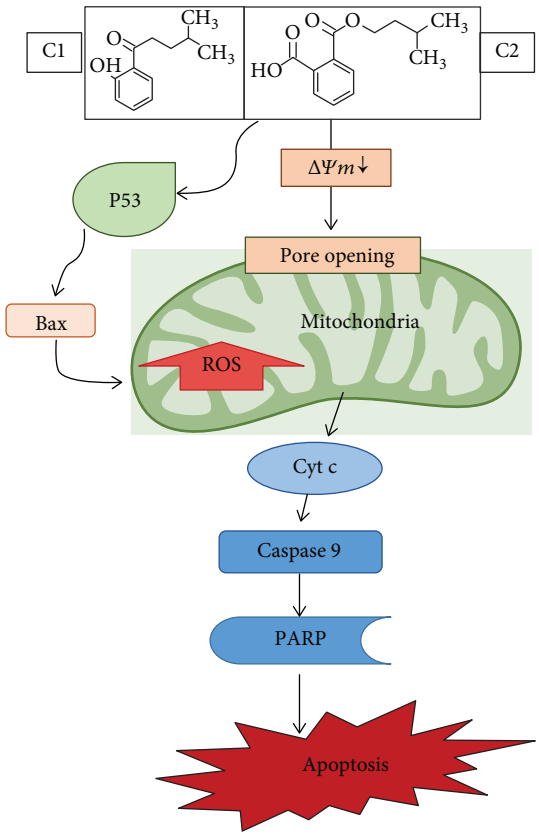

Figure 11: Proposed cell death mechanism. An illustration of proposed cell death induced by C1 and C2 in MCF-7 cells. Mitochondria mediated caspase-dependent intrinsic apoptotic pathway is upregulated. Increased expression of p53, caspase 9, cleaved PARP, and Bax proteins mediated the cell death.

apoptosis [37]. In this study, upregulation of Bax with higher expression of p53, caspase 9, cytochrome c, and PARP provides an insight that C1 and C2 could initiate a favourable signal transduction mechanism, which triggers MCF-7 cells to undergo apoptosis. It is implicated that caspase 3 and caspase 9 are the major signaling proteins in inducing apoptotic cascade. Cleavage and higher expression of downstream protein PARP also established the DNA-related damage in MCF-7 cells [58].

Several studies have shown that bioactive compounds such as polyphenols and flavonoids induce cell death via apoptosis [59–62] with numerous morphological and biochemical changes. Evidence suggests that berries have beneficial effects against numerous cancers; its anticancer potential has been related to a multitude of bioactive compounds, including polyphenols, stilbenoids, lignans, and triterpenoids. Reports show that the antiproliferative effects of berry compounds are partially mediated through their ability to repair damage from oxidative stress. Bioactive compounds from berries also regulate carcinogen and xenobiotic metabolizing enzymes, various transcription factors, and cellular signaling pathways and apoptosis. They may also potentially sensitize tumor cells to chemotherapeutic agents by inhibiting pathways that lead to treatment resistance [63].

Compounds 1-(2-hydroxyphenyl)-4-methylpentan-1-one (C1) and 2-[(3-methylbutoxy) carbonyl] benzoic acid (C2) were isolated from *R. fairholmianus* root extract by George et al. [17] which can be used as a promising lead compound for the development of potent drug for the prevention and treatment of malignant tumors. The studied compounds markedly suppressed the proliferation of MCF-7 cells and induced cytotoxicity, which favours the induction of apoptosis by cytochrome c release and altering mitochondrial membrane potential significantly. Taken together, the results from this study preliminarily demonstrated that the compounds isolated from *Rubus* species (C1 and C2) induce apoptosis in human breast cancer cells by causing accumulation of intracellular ROS, by activation of mitochondrial signaling pathway by showing higher expression of cleaved PARP, caspase 9, cytochrome c, and Bax apoptotic proteins. We provide insights on and perspectives for future development of effective therapeutic ROS-inducing anticancer agents from plant extracts. In this article, we present evidence on the role of ROS in inducing cancer cell death, which may be utilized to increase our understanding on ROS-associated signaling pathways in cancer chemotherapy. ROS-mediated cell death induction reported in this article will be verified more accurately in our future investigations analysing ROS in the presence of antioxidants.

The upregulation of apoptotic proteins (p53, Bax, cytochrome c, PARP, and caspase 9) were significantly observed after the treatment with *Rubus* bioactive compounds. The proposed mechanism by which ROS mediated cell death induction by C1 and C2 bioactive compounds from *Rubus* is illustrated in Figure 11. In conclusion, a novel mechanism of bioactive compounds from *Rubus* involves the induction of apoptotic cell death via caspase activation. The results of this study are consistent with the notion that bioactive compounds of *Rubus* are beneficial in preventing cancer cell proliferation. Hence, this article provides an outline of *Rubus* prooxidant and anticancer properties, with the special focus on C1 and C2, and discusses their possible use as breast cancer chemotherapeutic agents. Further studies are recommended to evaluate the effect of these compounds on other mode of cell death mechanisms.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors confirm that this article content has no conflict of interest.

Authors' Contributions

Dr. Blassan P. George designed the project, performed the laboratory experiments, and prepared the manuscript. Prof. Heidi Abrahamse coordinated, supervised this research work, and reviewed and corrected the manuscript.

Acknowledgments

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Supplementary Materials

Supplementary Figure 1: LDH cytotoxicity of C1- and C2-treated A549 and A375 cells. Lung cancer cells (A549-ATCC CCL185) were cultured in Roswell Park Memorial Institute 1640 medium complemented with 10% FBS, 0.5% penicillin-streptomycin, and 0.5% amphotericin B. The melanoma cells (A375-ATCC CRL1619) were cultured in Dulbecco's modified Eagle's media with 1.2 g/L sodium carbonate, 10% FBS, 10 mM nonessential amino acids, 0.5 mM sodium pyruvate, 2.5 mM L-glutamine, 1% penicillin-streptomycin, and 1% amphotericin B. Once the cells reached 80% confluence, they were seeded in a 3.5 cm^2-diameter culture plates at a concentration of 2×10^5 (A549) 5×10^5 (A375) cells for experimental purposes. The cultures were incubated at 37°C with 5% CO_2 and 85% humidity. The Cyto-Tox96 X assay (Anatech, Promega G 400) was used to evaluate the cytotoxic activity of C1 and C2 on A549 cells. The cytotoxicity assay results showed that both C1 and C2 significantly induced the release of LDH from A549 and A375 cells in a dose-dependent manner indicating its cytotoxicity; however, these bioactive compounds found to be more toxic towards A549 lung cancer cells compared to A375 melanoma cells. *(Supplementary Materials)*

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Review Article

ROS-Mediated Cancer Cell Killing through Dietary Phytochemicals

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Reactive oxygen species (ROS) promote carcinogenesis by inducing genetic mutations, activating oncogenes, and raising oxidative stress, which all influence cell proliferation, survival, and apoptosis. Cancer cells display redox imbalance due to increased ROS level compared to normal cells. This unique feature in cancer cells may, therefore, be exploited for targeted therapy. Over the past few decades, natural compounds have attracted attention as potential cancer therapies because of their ability to maintain cellular redox homeostasis with minimal toxicity. Preclinical studies show that bioactive dietary polyphenols exert antitumor effects by inducing ROS-mediated cytotoxicity in cancer cells. These bioactive compounds also regulate cell proliferation, survival, and apoptotic and antiapoptotic signalling pathways. In this review, we discuss (i) how ROS is generated and (ii) regulated and (iii) the cell signalling pathways affected by ROS. We also discuss (iv) the various dietary phytochemicals that have been implicated to have cancer therapeutic effects through their ROS-related functions.

1. Introduction

Reactive oxygen species (ROS) are highly reactive metabolic by-products that cause both deleterious and beneficial effects. Cellular ROS act as secondary messengers in signalling cascades that are critical for normal physiological functions such as differentiation and development [1, 2]. However, overproduction of ROS can cause damage to biomolecules such as DNA, lipids, carbohydrates, and proteins [3, 4], leading to loss of cell integrity and subsequently cell pathology (Figure 1). For example, ROS is now recognized to promote tumorigenesis, metastasis, and angiogenesis [5]. But then again, in cancer, excessive accumulation of ROS induces cell death [6]. Studies have shown that cancer cells have increased ROS level compared to normal cells due to high metabolic rate and mitochondrial dysfunction, which render increased susceptibility to oxidative stress [7, 8]. Thus, additional surge in ROS level is likely to cause cancer cells to reach their oxidative stress threshold sooner than normal cells, resulting in oxidative stress-induced cancer cell death [7, 8]. Therefore, it is not surprising that several natural dietary bioactive compounds that cause increased ROS levels have been shown to selectively target cancer cells [9]. For instance, dietary phytochemicals such as polyphenols, flavonoids, and stilbenes have the capacity to inhibit cancer cell proliferation and induce apoptosis and autophagy [10]. While most dietary bioactive compounds possess antioxidant capacity at low doses, high doses induce prooxidant activity that leads to cancer cell death. These compounds also influence mitochondrial functions by altering mitochondrial enzymes, oxidative phosphorylation, and mitochondrial pathways [11]. In this review, we focus on ROS regulation, ROS-mediated signalling pathways, and the contemporary use of dietary phytochemicals for cancer therapy.

2. ROS Regulation

ROS production is affected by both external factors such as tobacco smoke and ionizing radiation and intracellular factors such as the endoplasmic reticulum (ER), mitochondria,

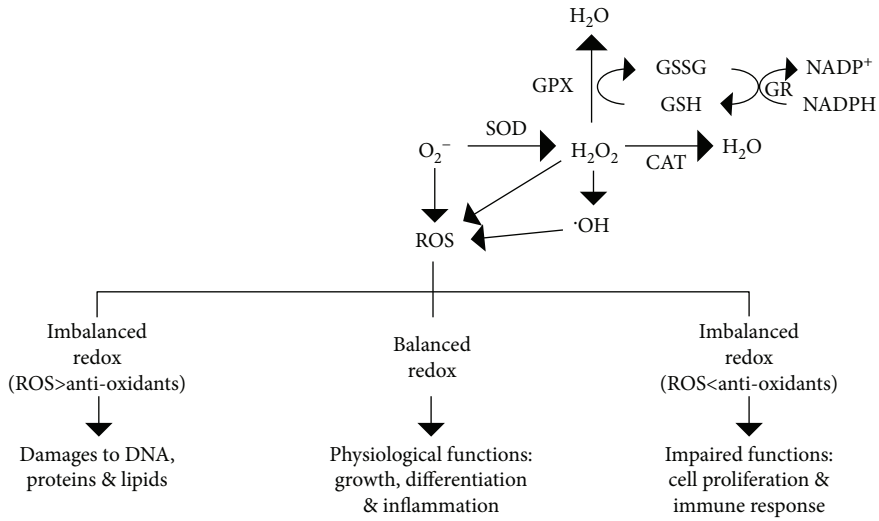

Figure 1: Intracellular redox homeostasis and imbalance and their effects on cellular functions. SOD: superoxide dismutase; CAT: catalase; OH: hydroxyl radical; GPX: glutathione peroxidase; GSSG: glutathione disulfide; GR: GSSG reductase; GSH: glutathione.

and peroxisomes [12] (Figure 2). Endogenous ROS are mainly produced in mitochondria during oxidative phosphorylation. Superoxide anions are generated through the electron transport chain complexes I and III localized in the inner mitochondrial membrane, and superoxide dismutase (SOD) converts superoxide ions into hydrogen peroxide (H_2O_2), which is subsequently catalyzed by glutathione peroxidase (GPX) to generate H_2O. Catalase (CAT) also converts H_2O_2 to water (Figure 1) [13]. Other intracellular enzymes such as NADPH oxidase, lipoxygenases, and xanthine oxidase are also capable of ROS production [14]. Although intracellular redox homeostasis is well controlled by the enzymatic antioxidants, SOD, GPX, and CAT, it is also regulated by nonenzymatic antioxidants such as ascorbic acid (vitamin C) and glutathione (GSH) [15] (Figure 2).

Besides these antioxidants, the transcription factor, nuclear factor erythroid 2- (NFE2-) related factor 2 (Nrf2), also contributes in controlling oxidative stress. Activation of Nrf2 requires inhibition of its negative regulator Keap1, which results in Nrf2 nuclear translocation [16]. This leads to the expression and production of the antioxidant enzymes, CAT, GPX, heme oxygenase-1 (HO-1), and peroxiredoxin (PRX), and maintenance of redox balance [16]. We note, however, that intracellular oxidative stress induces activation of hypoxia-inducible factors (HIFs), resulting in the transcription of genes that promote survival and proliferation of cancer cells [17].

3. ROS in Cancer Signalling Pathways

ROS serve a crucial role in the regulation of a number of cellular processes such as cell proliferation and differentiation and cell death. Therefore, it is critical that a delicate balance in ROS level is maintained. ROS level is regulated by redox homeostasis via ROS elimination through antioxidants. Within the threshold limit of redox homeostasis, a regulated ROS increase could serve as a signal for H_2O_2-mediated oxidation of protein cysteine residues, triggering specific cellular events such as proliferation [18]. Conversely, disturbance of redox homeostasis in the direction of ROS overload leads to deleterious outcomes such as irreversible oxidative DNA damage that could trigger cell death. It is now known that metabolically transformed and fast-growing cancer cells have higher ROS levels than neighboring normal cells, placing cancer cells at a greater risk of reaching the ROS threshold to induce apoptosis. This infers that promoting further ROS production in cancer cells may be utilized as a strategy to induce cancer cell death.

ROS play an important role in tumor initiation, promotion, and progression [19]. At levels below the ROS threshold, ROS activate oncogenes such as Ras and c-Myc [20] and induce p53-mediated DNA repair and survival [21] in cancer cells. At levels above the ROS threshold, ROS trigger apoptotic signals [6]. These cellular processes are controlled by ROS through its regulation of various signalling pathways (Figure 3), including the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK), the phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT), the inhibitor of kappa B (IκB) kinase (IKK)/nuclear factor κB (NFκB), and the protein kinase D (PKD) signalling pathways [22, 23]. For example, ROS-dependent ERK activation controls the expression of proapoptotic genes by phosphorylation of transcription factors [23, 24]. Conversely, ROS-induced JNK activation results in phosphorylation and downregulation of antiapoptotic proteins such as BCL-2 and BCL-X_L [25]. In response to ROS, IκB phosphorylation by IKK and subsequently ubiquitination lead to activation and translocation of NFκB into the nucleus to stimulate the expression of antiapoptotic genes [26]. ROS directly activates PI3K subsequently converting phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-triphosphate (PIP_3) and resulting in transcriptional inhibition of the AKT target genes, glycogen synthase kinase 3 (GSK3), forkhead box O (FOXO), and BCL-2-associated death

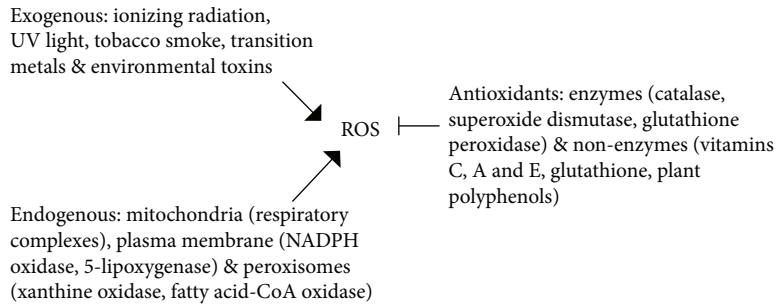

Figure 2: Exogenous and endogenous sources of ROS and enzymatic and nonenzymatic antioxidants.

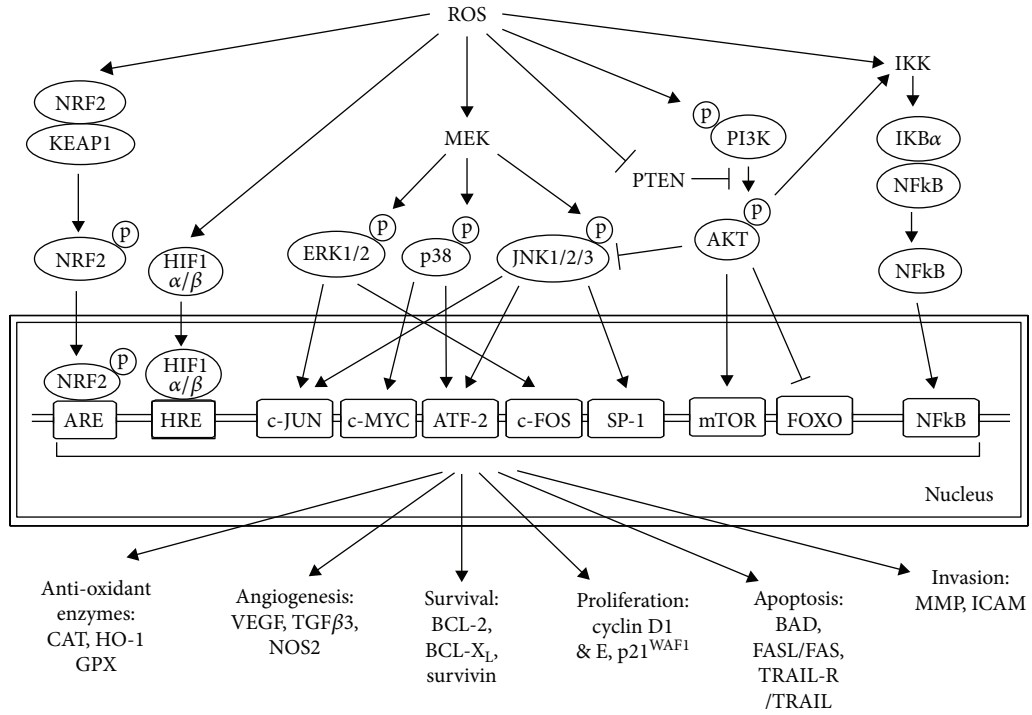

Figure 3: ROS-mediated intracellular cell signalling pathways. The indicated signalling pathways regulate molecules associated with angiogenesis, survival, proliferation, apoptosis, and invasion and the expression of antioxidant enzymes. NRF2: nuclear factor erythroid 2-related factor 2; KEAP1: Kelch-like ECH-associated protein 1; HIF1 α/β: hypoxia inducing factor 1 α/β; HRE: HIF-responsive elements; p38 MAPK: p38 mitogen-activated protein kinase; ERK: extracellular signal-related kinases; MEK: MAPK kinase; JNK; c-Jun N-terminal kinase; PTEN: phosphatase and tensin homolog; PI3K: phosphoinositide-3-kinase; AKT: protein kinase B; IKK: IκB kinase; NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells; FOXO: forkhead box protein O; mTOR1: mechanistic target of rapamycin 1; ATF2: activating transcription factor 2; CAT: catalase; HO-1: heme oxygenase-1; GPX: glutathione peroxidase; VEGF: vascular endothelial growth factor; TGFβ3: transforming growth factor beta 3; NOS2: nitric oxide synthase 2; BCL-2: B-cell lymphoma 2; BCL-X_L: B-cell lymphoma-extra large; BAD: BCL2-associated agonist of cell death; TRAIL: TNF-related apoptosis-inducing ligand; MMP: matrix metalloproteinase; ICAM: intercellular adhesion molecule-1.

promoter (BAD) and activation of mammalian target of rapamycin (mTOR1) [27].

ROS-mediated apoptosis can be initiated by mitochondrial intrinsic apoptotic signalling or by extrinsic apoptotic signalling through death receptor pathways (Figure 4). Increased production of ROS depolarizes the mitochondrial membrane, releasing cytochrome C from the mitochondria. Cytochrome C induces activation of caspase-9 by promoting nucleotide binding to apoptotic protein-activating factor 1 (APAF-1), which leads to activation of caspase-3 [28]. Antiapoptotic (BCL-2 and BCL-X_L) and proapoptotic (BAD, BAK, BAX, BID, and BIM) proteins also contribute to the formation of distinct channels for mitochondrial membrane permeabilization [29]. Elevated ROS levels have also been implicated in the activation of death receptors and in triggering caspase 8-mediated cleavage of caspase 3 [6]. In addition, ROS modulates the TRAIL- and Fas-mediated apoptosis through p53-mediated upregulation of death receptors. p53 regulates such apoptosis by controlling the expression of anti- and proapoptotic (e.g., PUMA and

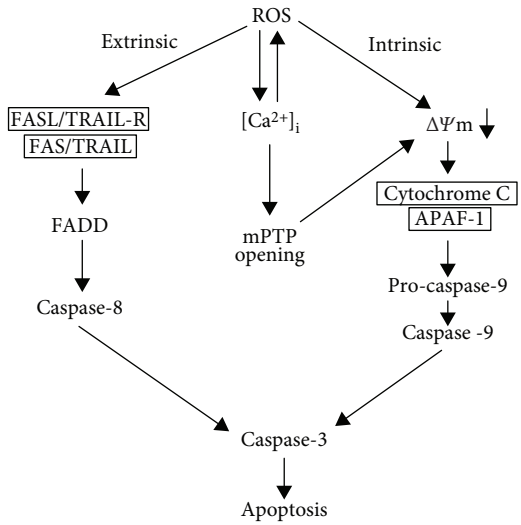

Figure 4: ROS-mediated extrinsic and intrinsic apoptotic pathways. TRAIL: TNF-related apoptosis-inducing ligand; FADD: Fas-associated death domain; $[Ca^{2+}]_i$: intracellular calcium concentration; mPTP: mitochondrial permeability transition pore; $\Delta\Psi$m: mitochondrial membrane potential.

NOXA) proteins [30, 31]. ROS further promotes apoptosis by inducing increased Ca^{2+}-mediated mitochondrial permeability transition pore opening [32].

4. Dietary Polyphenols

There is increasing claim that certain natural bioactive compounds can maintain redox homeostasis and hold promise as anticancer therapeutics due to their biocompatibility, biodegradability, comparatively less toxicity, and reduced side effects. The polyphenol bioactive compounds are secondary metabolites found in plants [33]. The most abundantly occurring plant polyphenols are phenolic acids and flavonoids which account for 30% and 60%, respectively, of dietary polyphenols [33]. Interestingly, they have both antioxidant and prooxidant properties that modulate cell proliferation and apoptotic pathways [34]. Some of the most common bioactive compounds that were suggested to have cancer therapeutic effects through their ROS-related activities are discussed below.

4.1. Quercetin. Quercetin (3,5,7,3′,4′-pentahydroxyflavone) is a flavonoid, present in numerous vegetables and fruits [34, 35]. Quercetin (Qu) displays neuroprotective, chemopreventive, and anticancer activities [36, 37], and these have been attributed to their anti- and prooxidative capacities. Qu efficiently scavenges mitochondrial superoxide anions (O_2^-) and subsequently generates semiquinone, Qu radicals, and H_2O_2 [11, 34, 38]. While, H_2O_2 is eliminated by peroxidase, semiquinone and Qu radicals alter intracellular ROS metabolism by depleting the intracellular GSH pool in a concentration-dependent manner [39–41] and inhibiting thioredoxin reductase activity [42]. *In vitro* and *in vivo* studies (Table 1) show that Qu promotes ROS-induced apoptosis, necrosis, and autophagy [43] at a range of 10-100 μM in a variety of cancers, including glioma [43], osteosarcoma [44], and cervical [45] and breast cancer [46]. Qu induces apoptosis through distinct mechanisms: (i) via the mitochondrial pathway through activation of caspase-3. Qu reduces the mitochondrial membrane potential (MMP), inducing cytochrome C release and subsequent activation of caspase-3. This mechanism was observed in MDA MB-231 breast cancer cells [47], U937 promonocytic leukemia cells [48], HL-60 promyelocytic leukemia cells [49], HepG2 hepatocellular carcinoma cells [50], and oral cancer cells [51]. (ii) Qu alters the expression of the antiapoptotic BCL-2 and BCL-X_L and proapoptotic BAX and BAD proteins [47, 48]. Leukemic cells treated with Qu showed upregulation of BAX and increased phosphorylation of BCL-2 [52]. Similar results were observed in osteosarcoma [44] and breast cancer cells [46]. (iii) Qu induces the expression of death receptor-(DR-) 5, enhancing TNF-related apoptosis-inducing ligand-(TRAIL-) induced apoptosis [53–55] either by accumulating death receptors in lipid rafts [56] or inhibiting survivin in the ERK signalling pathway [57]. In addition to its proapoptotic capacity, Qu also promotes cell cycle arrest [58] by modulating $p21^{WAF1}$, cyclin B, and $p27^{KIP1}$ in squamous cell carcinoma [59] and breast [60], lung [61], and hepatoma cancer cells [62].

4.2. Curcumin. Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is the principal polyphenol derived from turmeric (Curcuma longa). Various pharmacological activities have been attributed to curcumin, including its anti-inflammatory and anticarcinogenic properties which are triggered at 25 μM [63]. Its anticancer effect is currently being evaluated in clinical trials for a variety of cancers [64–66] (Table 2). In normal cells, curcumin acts as a potent antioxidant. It scavenges hydroxyl radicals, superoxide, nitric oxide, H_2O_2, and peroxynitrite [11, 67–69] and modulates the expression of SOD, HO-1, and GPX through an indirect mechanism [11, 70–72]. In contrast, curcumin's anticancer properties rely on its prooxidative capacity to induce apoptosis, likely via the mitochondria-mediated pathway [73–75]. Curcumin oxidizes thiols in the mitochondrial membrane, leading to mitochondrial permeability transition pore (mPTP) opening, mitochondrial swelling, mitochondrial depolarization, and inhibition of ATP synthesis, resulting in apoptosis [76]. Evidence shows that curcumin increases ROS levels, including superoxides, hydroxy radicals, and H_2O_2 [77–79]. Indeed, in human hepatoma cells, curcumin causes cell death by ROS-induced mitochondrial DNA damage and impairment of OXPHOS [80, 81]. Curcumin also activates TRAIL-induced apoptosis by ROS-mediated upregulation of DR5 in renal cancer cells and colon cancer cells [82, 83]. Curcumin further induces autophagy in colon cancer cells through ROS-dependent activation of the ERK1/2 and the p38 MAPK pathway [84]. In glioblastoma [85] and liver cancer [86], curcumin decreases cancer stem cell viability and proliferation by ROS-mediated inhibition of NFκB and signal transducer and activator of transcription 3 (STAT3). As with Qu, curcumin promotes cancer cell apoptosis by upregulating proapoptotic proteins (BAX, BIM, BAK, and NOXA) [87, 88] and downregulating antiapoptotic

Table 1: *In vivo* dosages and mechanistic effects of known natural bioactive compounds.

Compound	Animals	Cancer model	Dose	Mechanism
Quercetin	Male Sprague-Dawley rats	Glioma	100 mg/kg, every other day for 15 days, i.v	Autophagy and apoptosis [43]
	Female BALB/c mice	Colon & breast cancer	100 and 200 mg/kg for 36 days, i.p	Apoptosis [178]
	Male BALB/cA nude mice	Prostate cancer	20 mg/kg for 16 days, i.p	Antiangiogenesis [179]
	Female BALB/c/nude mice	Hepatic cancer	10 mg/kg for 7 days, i.p	Necrosis and antiproliferation [180]
	Female NOD.CB17-Prkdcscid/J lineage	Acute myelogenous leukemia	120 mg/kg, once every 4 days for 21 days, i.p	Apoptosis, autophagy, and cell cycle arrest [181]
Curcumin	Female BALB/c/nude mice	Colon cancer (multidrug resistance)	50 mg/kg, 2x/day for 14 days, peritumoral	Reduced expression of MDR1 and survivin [182]
	Male BALB/c/nude mice	Prostate cancer	25, 50, and 100 mg/kg, every 2 days for 30 days, abdominal cavity injection	Apoptosis [89]
	Female athymic nude mice	Breast cancer	45 mg/kg, 2x/week for 4 consecutive weeks, i.p	Antiproliferation [183]
Capsaicin	Female athymic nude mice	Pancreatic cancer	2.5 & 5 mg/kg, 5x/week, gavage	Activation of JNK and apoptosis [99]
	Female BALB/c nude mice	Colon cancer	1 & 3 mg/kg, 3 days once for 40 days, i.p	Apoptosis [95]
	Male BNX nu/nu mice	Prostate cancer	5 mg/kg, 3x/week for 4 weeks, gavage	Antiproliferation and apoptosis [184]
	Female BNX nu/nu	Breast cancer	5 mg/kg, 3x/week for 4 weeks, gavage	Reduced EGFR/HER2 activation and apoptosis [185]
ECGC	Female C3H/HeJ syngeneic mice	Squamous cell carcinoma	50 mg/kg, 5 days/week, i.p	Apoptosis [186]
	NOD/SCID mice	Myeloid leukemia	10 mM, oral drinking fluid	Antiproliferation [110]
	Female BALB/c mice	Bladder cancer	100 mg/kg for 4 weeks, i.p	Antiproliferation and migration [187]
	Male BALB/c/nude mice	Lung cancer	0.05% in drinking water for 21 days	Angiogenesis [188]
	Male BALB/c/nude mice	Adrenal pheochromocytoma	15 mg/kg, every other day for 15 days, i.p	Apoptosis [189]
PEITC	Male athymic nude mice	Glioblastoma	20 μmol/100 μl PBS for 21 days, gavage	Apoptosis [190]
	Male athymic mice	Prostate cancer	12 μmol/100 μl PBS for 5 days, oral	Apoptosis [191]
	Female BALB/c/nude mice	Lung cancer	25 mg/kg, 3x/week, i.p	Antiproliferation, reduced cancer stem cells [128]
	Female SCID/NOD mice	Breast cancer	81 mg/kg for 35 days, oral gavage	Apoptosis [192]
	Female athymic nude mice	Ovarian cancer	12 μmol for 42 days, oral gavage	EGFR-AKT pathway inhibition, antiproliferation, and apoptosis [193]
Piperine	Female BALB/c mice	Mouse 4T1 mammary carcinoma	2.5 and 5 mg/kg, every 3 days for 3 times, intratumoral	Cell cycle arrest and apoptosis [194]
	Male nude mice	Prostate cancer	100 mg/kg/day for 1 month, i.p 10 mg/kg for 1 month, gavage	Antiproliferation and apoptosis [195]
	Male albino Wistar rats	Hepatocellular carcinoma (diethylnitrosamine-induced)	5 mg/kg, 3x/week for 6 weeks, oral	Apoptosis [138]

Table 1: Continued.

Compound	Animals	Cancer model	Dose	Mechanism
Resveratrol	Male nude mice	Lung cancer	20 mg/kg, every other day for 25 days, i.p	Reduce metastasis [196]
	Male BALB/c/nude mice	Bladder cancer	20 mg/kg/day for 4 weeks, i.p	Decreased VEGF and FGF-2 level, cell cycle arrest, and apoptosis [197]
	Female athymic mice	Breast cancer	25 mg/kg/day for 3 weeks, i.p	Apoptosis [198]
	BALB/c/nude mice	Pancreatic cancer	20, 40, and 60 mg/kg, 5 days/week for 6 weeks, gavage	Inhibition of FOXO transcription factors and apoptosis [199]
	Male athymic nude mice	Prostate cancer	50 mg/kg, every other day for 2 weeks, gavage	Antiproliferation [200]

i.p: intraperitoneal; i.v: intravenous.

Table 2: Clinical trials of natural phytochemicals.

Bioactive compounds (Clinicaltrials.gov identifier)	Disease condition	Phase	Dosage	Study goal
Quercetin (NCT03476330)	Squamous cell carcinoma	II	4 g/day	Efficacy in reducing buccal micronuclei in patients with Fanconi anemia
Curcumin (NCT03769766)	Prostate cancer	III	500 mg, 2x/day	Effect on prostate cancer progression
(NCT00094445)	Pancreatic cancer	II	8 g/day	Effect in pancreatic cancer growth and the safety of treatment
(NCT01246973)	Radiation dermatitis	III	500 mg, 3x/day	Effect on dermatitis caused by radiation therapy in breast cancer patients
With piperine (NCT02598726)	Neoplasms	I	A dose escalation study	Optimal biological dose in cancer patients
Capsaicin (NCT02037464)	Prostate cancer	II	2 capsules/day for 6 months	Expression of Ki67 and p27 in a posttreatment biopsy
(NCT00003610)	Head & neck cancer, mucositis	III	4 lozenges/day up to 2 weeks after radiation therapy	Efficacy of lozenges in patients with mucositis caused by radiation therapy
Patch (Qutenza) (NCT03317613)	Cancer	II	Qutenza (8% capsaicin patch) for every 3 months	Efficacy in peripheric neuropathic pain in cancer patients
EGCG (NCT02891538)	Colon cancer	Early I	450 mg, 2x/day	Chemopreventive effects
(NCT01317953)	Small cell lung carcinoma	I	2×450 mg/day to 5×450 mg/day	Side effects and best dose
PEITC (NCT00691132)	Lung cancer	II	4x/day for 5 days in week 4	Effect in preventing lung cancer in smokers
(NCT01790204)	Oral cancer	I & II		Effect on oral cells with mutant p53
Nutri-PEITC jelly (NCT03034603)	Head & neck neoplasms		200 mg/day, 5 days/week for 3 months	Safety and efficacy
Resveratrol (NCT00256334)	Colon cancer	I	20 mg/day	Modulation of Wnt signalling *in vivo*
(NCT01476592)	Neuroendocrine tumor		5 g/day	Effect on Notch-1 signalling
SRT501 (NCT00920803)	Colorectal cancer	I	5 g/day	Safety and tolerability

proteins (BCL-2 and BCL-X_L) [89, 90]. In addition, curcumin can impede tumor angiogenesis by downregulating the expression of the vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) [91, 92].

4.3. Capsaicin. Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide), the major component of Capsicum [93], has been implicated to have anticarcinogenic properties [94–96]. However, the mechanisms by which capsaicin induces cancer cell death are still unclear. The proposed anticancer mechanisms of capsaicin include promotion of ROS accumulation, mitochondria-mediated apoptosis, cell cycle arrest, and impairment of endoplasmic reticulum (ER) calcium homeostasis [97]. Capsaicin induces a rapid rise of ROS level followed by a disruption of mitochondrial membrane potential and subsequent activation of downstream caspase-3 in human colon cancer [98], pancreatic cancer [99], glioma [100], and prostate cancer [101]. In transformed T-cells, capsaicin inhibits the plasma membrane NADH-oxidoreductase (PMOR) electron transport chain, causing an increase in ROS level and subsequent disruption of the mitochondrial membrane potential [102]. Capsaicin at 150 μM also blocks complexes I and III of the respiratory chain and decreases SOD activity in pancreatic cancer [103]. Interestingly, binding of capsaicin to the transient receptor potential vanilloid type 1 (TRPV1) results in an increase in intracellular calcium level and activation of the apoptotic pathway [104–106]. Besides its proapoptotic effects, capsaicin can also induce cell cycle arrest through inhibition of the cyclin-dependent kinases, Cdk2, Cdk4, and Cdk6 [107, 108].

4.4. Epigallocatechin-3-Gallate (EGCG). Epigallocatechin-3-gallate ((2R,3R)-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2H-1-benzopyran-3-yl 3,4,5-trihydroxybenzoate) is a prominent catechin polyphenol in green tea. EGCG has dual antioxidant and prooxidant roles. It produces ROS by autooxidation [109] and its ability to modulate ROS level accounts for its chemopreventive property. EGCG induces

apoptosis in various cancer cell types, including myeloid leukemia cells [110], human lymphoblastoid B cells [111], and hepatocarcinoma cells [112]. In pancreatic carcinoma [113] and lung cancer cells [114], EGCG-induced apoptosis occurs through inhibition of the PI3K/AKT signalling pathway. EGCG also decreases the mitochondrial membrane potential, increasing the intracellular free Ca^{2+} level and causing activation of the intrinsic apoptotic pathway. EGCG further decreases the expression of the antiapoptotic BCL-2, BCL-X_L, xIAP, and cIAP and increases the expression of the proapoptotic BAD, BAX, and FAS/CD95 [115]. In pancreatic [116] and bladder cancer cells [117], EGCG also induces G_0/G_1 cell cycle arrest through regulation of cyclin D1, Cdk4, Cdk6, $p21^{WAF1}$, and $p27^{KIP1}$ via the ERK, IKK, and PI3K signalling pathways. A combination of EGCG (10 μM) and curcumin (10 μM) inhibits breast cancer stem cell growth by inactivating the NFκB-STAT3 pathway [118].

4.5. PEITC and BITC. Phenethyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC) are abundant in cruciferous vegetables that have been implicated to have anticancer properties [119–122]. Epidemiological studies show that increased intake of dietary isothiocyanates (ITC) reduces cancer risk [123] and increases cancer patient survival [124]. Both PEITC and BITC induce ROS production in many cancer cells [125–127]. IC_{50} value of PEITC is at the range of 3-14 μM in various human cancer cells [128]. PEITC increases ROS level by decreasing intracellular GSH level, leading to mitochondrial dysfunction as observed in ovarian [126, 129] and non-small-cell lung cancer [128] cells but not in normal cells. PEITC-induced ROS production correlates with inhibition of complex III activity, inhibition of OXPHOS, and ATP depletion in prostate cancer [125]. PEITC also inhibits HO-1 and subsequently induces the ROS-mediated mitochondrial apoptotic pathway, which was noted in human chronic myeloid leukemia [130]. Conversely, BITC causes oxidative stress in pancreatic [131], glioma [122], and prostate cancer [132] cells by depleting SOD and GSH, which is accompanied by the induction of caspase-mediated apoptosis [121, 133]. BITC also activates the ERK/JNK/p38MAPK pathway in pancreatic cancer [134]. Both PEITC and BITC induce G_2/M cell cycle arrest by downregulating cyclin B1, Cdc2, and Cdc25C [135, 136].

4.6. Piperine. Piperine ([5-(1,3-benzodioxol-5-yl)-1-oxo-2,4-pentadienyl]piperidine) is the most abundant natural alkaloid found in long pepper (Piper longum L.). Recently, it was determined to be a promising anticancer compound [137]. Piperine suppresses tumor growth in vitro and in vivo by modulating the ROS-induced oxidative stress response pathway, cell cycle arrest, and ER stress. In hepatocellular carcinoma, piperine treatment initiates ROS-induced mitochondria-mediated apoptosis by inhibiting catalase activity [138]. In human oral squamous cells exposed to high concentrations of piperine, ROS elevation is associated with mitochondrial depolarization and activation of caspase-mediated apoptosis. Piperine also induces nuclear condensation and cell cycle arrest in these cells [139].

4.7. Resveratrol. Resveratrol (3,4′,5-trihydroxystilbene), a polyphenol that is found in grapes and berries, effectively prevents tumor initiation and progression by stimulating apoptosis at 10 to 100 μM [140] in prostate [141] and neuroblastoma cells [142]. Resveratrol has been shown to promote apoptosis by activating p53, ROS-dependent caspases, and death receptors for TRAIL and FasL [143]. Resveratrol-mediated apoptosis is mainly associated with the inhibition of the PI3K/AKT, MAPK, and NFκB pathways [144] and STAT3 [145]. Moreover, resveratrol suppresses the expression of antiapoptotic proteins such as survivin, xIAP, and BCL-X_L and increases BAX/caspase-3-associated apoptosis [146]. Resveratrol further binds to F_1-ATPase, inhibiting mitochondrial ATP synthesis [147, 148]. It triggers cell cycle arrest by upregulating $p21^{WAF1}$ and $p27^{KIP1}$ and downregulating cyclins D1, D2, and E and Cdks 2, 4, and 6 [149, 150].

4.8. Others. Peanuts, tomatoes, and carrots are rich in *p-Coumaric acid (p-CoA)*, an isomer of cinnamic acid [151]. In colon cancer cells, p-CoA triggers apoptosis by increasing ROS generation and mitochondrial depolarization, resulting in p53-mediated upregulation of BAX and downregulation of BCL-2 [151, 152]. In addition, p-CoA treatment of these cells in vitro and in vivo induces apoptosis mediated by the unfolded protein response [153].

The naturally occurring quinone compounds have potent cytotoxicity against cancer cells. In lung adenocarcinoma cells, *2-methoxy-1,4-naphthoquinone (MNQ) and 8-hydroxy-2-methoxy-1,4-naphthoquinone (HMNQ)* elicit ROS production and induce apoptosis via the JNK/p38 MAPK pathway [154–156].

Naringenin, a citrus flavonoid, triggers ROS-induced apoptosis and stimulates p38MAPK-mediated caspase activation [157, 158].

Gallic acid (3,4,5-trihydroxy-benzoic acid; GA), which is widely present in grapes and red wine, inhibits lung cancer cell growth by increasing ROS level and depleting GSH [159]. In prostate cancer cells, autooxidation of GA produces H_2O_2 and O_2^-, leading to mitochondria-dependent apoptosis [160]. GA also induces apoptosis via ROS-dependent activation of the ATM/p53 [161] and JNK pathways [162].

5. Limitations

Poor bioavailability is a major obstacle for natural bioactive compounds, especially for Qu, curcumin, and resveratrol, which are associated with poor absorption and fast metabolism in the liver and intestine. Pharmacokinetic profile analysis of Qu revealed that about 93% of the compound is metabolised after oral administration (10 mg/kg) in male Sprague-Dawley rats [163]. On the other hand, people taking high oral doses (10 or 12 g) of curcumin attained limited availability of this compound in the plasma and other tissues [164]. Similarly, oral bioavailability of resveratrol is low at less than 1% [165]. Thus, the cytotoxic concentration of these compounds appears to be difficult to achieve by oral administration in cancer patients [166]. Several strategies have been proposed to overcome the problem of low oral bioavailability. One approach is to use a combination of phytochemicals.

For example, a combination of piperine and curcumin [167] (in rats: 20 mg/kg piperine + 2 g/kg curcumin; in humans: 20 mg piperine + 2 g curcumin) or piperine and resveratrol [168] (in mice: 10 mg/kg piperine + 100 mg/kg resveratrol) showed increased bioavailability of curcumin and resveratrol, respectively. Other promising approaches include the use of novel formulations, synthetic analogues, prodrugs, and different drug delivery systems (e.g., via liposomes, phospholipid complexes, micelles, and nanoparticles). These methods could increase bioavailability as well as solubility and/or metabolic stability [169, 170]. Some studies have also shown that natural bioactive compounds may promote carcinogenesis by inducing ROS-mediated chromosome aberrations and DNA damage [80, 171, 172]. For example, an *in vivo* study showed that curcumin promotes lung cancer [173] and topical application of capsaicin causes skin cancer in mice [174], suggesting that these natural compounds must be carefully assessed for safety prior to clinical application.

As dietary phytochemicals lack mechanistic selectivity, these natural compounds display a variety of effects in different cancer cell types and thus the discrepancies in results among separate studies. Other possible reasons for divergent findings in different studies include changes or differences in (i) stability of the bioactive compounds in cell culture medium, for example, stability of Qu decreases at pH 7 or 8 [175]; (ii) release of bioactive compounds under different conditions, for example, the maximum release of curcumin occurs in phosphate buffered saline at pH 6.4 [176]; (iii) sensitivity of different cell types to bioactive compounds; (iv) cellular permeability of bioactive compounds; (v) presence or contamination by metal ions [177]; (vi) number of hydroxyl groups present in a molecule [177]; and (vii) *in vivo* biodistribution.

6. Conclusion

Natural phytochemicals have been associated with anticancer properties through their ability to modulate oxidative stress, cell cycle regulators, and proapoptotic, antiapoptotic, and survival signalling pathways. In preclinical and clinical trials, bioactive compounds show a promising and wide therapeutic window against various malignancies, including glioblastoma and breast, colon, and prostate cancers where phytochemical-induced cancer cell death was observed. However, certain attributes such as poor solubility and bioavailability of these bioactive compounds limit their clinical application. Thus, further studies are required to identify ways for effective biological delivery of these compounds in different cancer cell types. It is also critical that detailed studies are conducted in large cohorts to establish the pharmacokinetic profile of these compounds alone and in combination with other chemotherapeutic agents to determine dosage, tissue targets, and toxicity. Indeed, natural phytochemicals may serve as future therapy for specific types of cancer.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

SN wrote the draft. JR and KYL revised the manuscript.

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Research Article

Bloom Syndrome Protein Activates AKT and PRAS40 in Prostate Cancer Cells

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Purpose. Prostate cancer (PC) is a common malignant tumor and a leading cause of cancer-related death in men worldwide. In order to design new therapeutic interventions for PC, an understanding of the molecular events underlying PC tumorigenesis is required. Bloom syndrome protein (BLM) is a RecQ-like helicase, which helps maintain genetic stability. BLM dysfunction has been implicated in tumor development, most recently during PC tumorigenesis. However, the molecular basis for BLM-induced PC progression remains poorly characterized. In this study, we investigated whether BLM modulates the phosphorylation of an array of prooncogenic signaling pathways to promote PC progression. *Methods*. We analyzed differentially expressed proteins (DEPs) using iTRAQ technology. Site-directed knockout of BLM in PC-3 prostate cancer cells was performed using CRISPR/Cas9-mediated homologous recombination gene editing to confirm the effects of BLM on DEPs. PathScan® Antibody Array Kits were used to analyze the phosphorylation of nodal proteins in PC tissue. Immunohistochemistry and automated western blot (WES) analyses were used to validate these findings. *Results*. We found that silencing BLM in PC-3 cells significantly reduced their proliferative capacity. In addition, BLM downregulation significantly reduced levels of phosphorylated protein kinase B (AKT (Ser473)) and proline-rich AKT substrate of 40 kDa (PRAS40 (Thr246)), and this was accompanied by enhanced ROS (reactive oxygen species) levels. In addition, we found that AKT and PRAS40 inhibition reduced BLM, increased ROS levels, and induced PC cell apoptosis. *Conclusions*. We demonstrated that BLM activates AKT and PRAS40 to promote PC cell proliferation and survival. We further propose that ROS act in concert with BLM to facilitate PC oncogenesis, potentially via further enhancing AKT signaling and downregulating PTEN expression. Importantly, inhibiting the BLM-AKT-PRAS40 axis induced PC cell apoptosis. Thus, we highlight new avenues for novel anti-PC treatments.

1. Introduction

Prostate cancer (PC) is a common malignancy of prostate epithelial cells [1]. PC is the most common cancer affecting American males, with 221,000 newly diagnosed cases and 27,500 deaths reported in 2015 alone [2]. In China, the rising average age of the population in combination with lifestyle changes have contributed to a clear upward trend in PC incidence and mortality [3]. PC is highly hereditary, and genetic PC risk factors can be passed from parents to their children [4]. PC is also a complex disease, and these genetic variants interact with environmental factors and dietary habits [5]. Active surveillance, radical prostatectomy, and radiation therapy are common treatment choices for localized PC. Chemotherapy drugs which target signaling pathways with a known association to PC tumor progression, including mTOR, PI3K-Akt, MAPK, AMPK, and p53 signaling, are used to induce PC cancer cell death. This is exemplified by BEZ235, a phosphatidylinositol-3-kinase (PI3K)/mammalian target of rapamycin (mTOR) inhibitor that blocks AKT phosphorylation (Thr308/Ser473) and can prevent breast [6, 7], glioma [8], and non-small-cell lung cancer growth [9, 10]. Combining BEZ235 with abiraterone acetate, which blocks cytochrome P450 17 alpha-hydroxylase

to significantly reduce androgen production, improves therapeutic outcomes in PC [11]. However, PC therapy remains ineffective overall, and more effective alternative treatments are urgently required [12].

DNA helicases within the RecQ protein family are involved in genome maintenance. These proteins, which are highly conserved from bacteria to humans, aid in maintaining genetic stability [13, 14]. RecQ helicases in human cells include RECQ1, BLM, WRN, RECQ4, and RECQ5. Defects in the WRN helicase are linked to a form of progeria associated with accelerated aging phenotypes termed Werner syndrome (WS). In contrast, mutations in Bloom syndrome protein (BLM) can result in the autosomal recessive disease Bloom syndrome (BS) [15, 16]. Unlike WS patients, BS patients do not exhibit a progeria phenotype but instead are prone to develop multiple malignancies including breast, prostate, and lung cancers [17, 18]. RecQ helicases are highly expressed in tumor cells, and silencing of the WRN helicase by RNA interference (RNAi) facilitates the treatment of many cancer types [19, 20]. Studies have also shown that BLM is highly expressed in breast cancer tissue and represents a novel breast cancer biomarker [21, 22]. Nonsense mutations in the BLM gene increase the risk of PC [23], and BLM expression is associated with PC susceptibility in the Chinese population [24]. Previous work has shown that knockdown of BLM inhibits PC cell proliferation and promotes PC apoptosis. However, the mechanism by which BLM may contribute PC tumorigenesis remains undetermined.

Given the close relationship between the WRN and BLM helicases, and the fact that the silencing of both is used in the treatment of multiple forms of cancer, it stands to reason that certain shared pathways govern the link between these two proteins and cancer cell proliferation. Oxidative stress has been proposed as a fundamental driver both of aging and cancer [25, 26]; thus, the shared oxidative stress-response pathway is a likely common link given that reactive oxygen species- (ROS-) mediated damage can substantially disrupt cellular protein function and DNA integrity.

The aim of the present study was to dissect the role of BLM in PC progression. To achieve this, iTRAQ technology was used to examine differentially expressed proteins (DEPs) among PC, normal prostate, and benign prostatic hyperplasia tissue samples. We confirmed that BLM was significantly overexpressed in PC cells and that it regulated the phosphorylation of an array of important cellular kinases. BLM was then silenced using CRISPR/Cas9 [27–30] in PC-3 prostate cancer cells to observe its effects on PC cell health, proliferation, and signaling. We found that BLM deletion inhibits PC cell proliferation via downregulating pAKT (Ser473) and pRAS40 (Thr246), which was accompanied by enhanced ROS production. We further show that AKT and PRAS40 inhibition also inhibit BLM and increase ROS levels, indicating that these signaling pathways are regulated by BLM through a positive feedback loop. These results give novel molecular insights into the role of BLM during PC tumorigenesis and lay the foundation for targeting the BLM-AKT-PSRAS40 axis during PC treatment. They also highlight avenues for future research, identifying ROS mechanisms acting in concert with BLM to facilitate PC oncogenesis, as key potential targets for future combinatorial therapy approaches.

2. Materials and Methods

2.1. Tissue Specimens. A total of 43 patients were included in this study. Tissue specimens were collected at the Department of Urology of Guizhou Provincial People's Hospital with informed consent from the patients and approval from the ethics committee. All procedures were performed in accordance with the Declaration of Helsinki principles and relevant policies in China. In total, 10 patients in the PC group underwent radical prostatectomy. The prostatic lesion was collected and confirmed as PC by pathological diagnosis. 15 patients underwent laparoscopic radical cystectomy. The prostatic tissue was collected, and the absence of cancer cell infiltration was confirmed by laboratory analysis. 18 patients with benign prostatic hyperplasia were treated via transurethral resection of the prostate and were confirmed with benign prostatic hyperplasia by laboratory analysis.

2.2. Cell Lines and Culture Conditions. The human prostate cancer cell lines PC-3, VCAP, and LnCAP and the human prostatic hyperplasia cell line BPH-1 were purchased from ATCC. LnCAP cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. All other cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM glutamine. All cell lines were cultured at 37°C and 5% CO_2.

2.3. CRISPR/Cas9-Mediated Gene Editing. CRISPR/CAS9 vector backbones, donor vector backbones, and T7E1 primers were synthesized by Beijing CasGene Biotech Co. Ltd. T4 ligase was purchased from Thermo Fisher Scientific. T7 endonuclease I was purchased from Beijing ViewSolid Biotech Co. Ltd. Polymerase chain reaction (PCR) systems were purchased from Becton Dickinson Inc. We analyzed a panel of 5 sgRNAs and selected the most efficient sgRNA (sgRNA3: TCACTTGATGGCCCTATGGA). This was used to construct the expression vector via the following primer sequences: (5′-3′) F: GTGGGAACGAACTGCTTCAG and R: TCTTGGTGTTTCAGCCCAGT. The donor vector was constructed by designing homology arms based on the sequences upstream and downstream of the sgRNA3 nick (Supplementary Figures 1–5).

2.4. iTRAQ. Samples were prepared for iTRAQ experiments using the SDT lysis method. SDT lysis buffer was added to samples followed by 15 mins of sonication in a boiling water bath. Samples were centrifuged at 14,000*g* for 15 mins, and protein concentration in the supernatant was quantified via bicinchoninic acid (BCA) assay. Samples were stored at -80°C until use. Samples were further digested by filter-aided sample preparation (FASP), and protein concentrations were assessed in each fraction. For each sample, 100 μg of total peptide was labeled using the AB SCIEX iTRAQ Labeling Kit. The normal prostate group was labeled with tags 113 and 114; the benign prostatic hyperplasia group

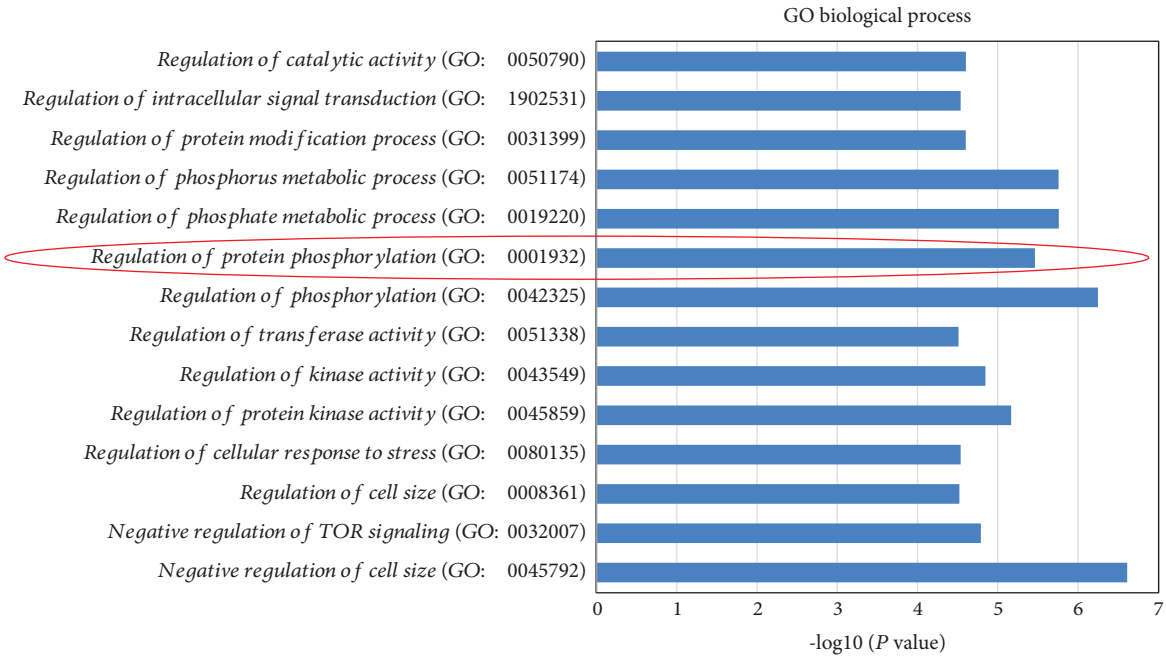


Figure 1: Enrichment of the DEPs in biological processes. The regulation of protein phosphorylation (GO: 0001932) was significantly enriched, indicating its involvement in the development and progression of PC ($P < 0.05$).

was labeled with tags 115, 116, and 117; and the PC group was labeled with tags 118, 119, and 121. Labeled peptides from each group were mixed and fractionated using an Agilent 1260 infinity II HPLC system, separated using an nanoliter flow rate Easy-nLC system, and analyzed using a Q Exactive Plus mass spectrometer. Identification and quantitative analysis were performed using the Mascot 2.5 and Proteome Discoverer 2.1 software. Differentially expressed proteins (DEPs) underwent Gene Ontology (GO) functional annotation (database version: go_201504.obo) and KEGG pathway annotation. The distributions of each GO class or KEGG pathway for these DEPs and for overall protein sets were compared by Fisher's exact tests to evaluate the significance of enrichment. Proteins which were differentially expressed among prostate cancer, normal prostate, and benign prostatic hyperplasia tissue samples were identified using the iTRAQ technology (Supplementary Table 1).

2.5. BLM-shRNA Vectors. We constructed a short hairpin RNA (shRNA) molecule targeting the Bloom helicase using the mammalian expression plasmid vector CMV-copGFP-T2A-Puro-H1-mcs. Successful construction of the vectors was validated by DNA sequencing prior to synthesis of the RNAi vectors by the Shanghai Gemma Medical Technology Dev. Co. Ltd. Sequences were as follows: shRNA-1: 5′-GCA GCG ATG TGA TTT GCA TCG TTC AAG AGA CGA TGC AAA TCA CAT CGC TGC TTT TTT G-3; shRNA-2: 5′-GCT TCA GCA GCG GAA CAT AAG TTC AAG AGA CTT ATG TTC CGC TGC TGA AGC TTT TTT G-3′. Both of these shRNAs silenced BLM expression, with respective efficiencies of 33% and 51% as confirmed in our previous studies (Supplementary Figure 5).

2.6. Antibody Array Detection. Arrays were performed using the PathScan® Antibody Array Kit (Cell Signaling Technology, 7323) according to the manufacturer's instructions. Loading concentrations were adjusted to 0.2-1.0 mg/mL. All array experiments were conducted in triplicate.

2.7. Reagents. The PRAS40 inhibitor GDC0068 (mw: 530.91, mf: $C_{24}H_{34}CL_3N_5O_2$), the AKT inhibitor BEZ235 (mw: 469.53, mf: $C_{30}H_{23}N_5O$), and the chemotherapeutic drug cisplatin (CDDP) (mw: 300.05, mf: $CL_2H_6N_2Pt$) were purchased from MedChemExpress.

2.8. Apoptosis Detection and CCK-8 Drug Sensitivity Assessment. Cells were seeded into 96-well plates at a density of 5,000 cells per well for CCK-8 assays. Adherent cells were treated with various concentrations of BEZ235, GDC-0068, and CDDP for 48 h in culture media. Next, the CCK-8 reagent was added to each well for 2 h, after which the absorbance at 450 nm was measured using a microplate reader.

Annexin V-FITC/PI staining was used to assess cellular apoptosis. Briefly, cells were seeded into 6-well plates at a density of 20,000 cells per well. Adherent cells were treated with BEZ235, GDC-0068, and CDDP in the culture media for 24 hrs. Then, staining was performed using an Annexin V-FITC/PI Cell Apoptosis Assay Kit according to the manufacturer's instructions. Stained cells were detected on a flow cytometer (CytoFLEX).

2.9. Intracellular ROS Detection. The nonfluorescent probe DCFH-DA was used to monitor ROS production. DCFH-DA diffuses into cells where it is deacetylated by esterases to form the nonfluorescent product DCFH. In the presence of ROS, DCFH reacts with ROS to form DCF, a fluorescent

Table 1: DEPs involved in the regulation of protein phosphorylation (GO: 0001932).

Accession Number	UniProtKB AC	Gene Name	Cancer/normal 118, 119, 121/113, 114	Cancer/normal *P* value	Cancer/BPH 118, 119, 121/115, 116, 117	Cancer/BPH *P* value
Upregulated proteins[a]						
Q9Y4K3	Q9Y4K3	TRAF6	1.470	0.002	1.246	0.004
Q96B36	Q96B36	AKT1S1	1.212	0.006	1.218	0.001
Q8TB45	Q8TB45	DEPTOR	1.446	0.002	1.221	0.002
Q59FU8	P25445	FAS	1.606	0.013	1.239	0.020
Q02750	Q02750	MAP2K1	1.225	0.004	1.227	0.000
P54132	P54132	BLM	1.258	0.001	1.215	0.001
P31749	P31749	AKT1	1.207	0.010	1.205	0.001
Downregulated proteins[b]						
A0A1V1FWL6	P49815	TSC2	0.706	0.001	0.786	0.002
A0A024R593	Q15173	PPP2R5B	0.719	0.002	0.777	0.004

[a]Proteins with fold changes > 1.2 ($P < 0.05$) are considered upregulated. [b]Proteins with fold changes < 0.8 ($P < 0.05$) are considered downregulated.

Table 2: Target pathways identified by KEGG enrichment.

Map name	Map ID	Input number	Input proteins	*P* value
Pathways in cancer	K05200	8	Q02750|P31749|P50150|P25445|P06753|Q9Y4K3|Q15147|P11166	$1.45E-08$
Thyroid hormone signaling pathway	K04919	5	P11166|Q02750|P31749|Q15147|P49815	$3.03E-07$
mTOR signaling pathway	K04150	5	Q02750|Q8TB45|P49815|P31749|Q96B36	$7.88E-07$
Adrenergic signaling in cardiomyocytes	K04261	5	Q15173|P31749|P06753|Q9BXT2|Q15147	$1.04E-06$
p53 signaling pathway	K04115	4	P25445|P27701|P31350|P49815	$1.32E-06$
PI3K-Akt signaling pathway	K04151	6	Q02750|P31749|P50150|P22105|Q15173|P49815	$2.55E-06$
Chagas disease (American trypanosomiasis)	K05142	4	P25445|P31749|Q9Y4K3|Q15147	$1.06E-05$
MAPK signaling pathway	K04010	5	P31749|P25445|Q02750|Q9BXT2|Q9Y4K3	$1.09E-05$
Cholinergic synapse	K04725	4	Q02750|P31749|P50150|Q15147	$1.32E-05$
Sphingolipid signaling pathway	K04071	4	Q15173|Q02750|P31749|Q15147	$1.44E-05$
AMPK signaling pathway	K04152	4	Q15173|P49815|P31749|Q96B36	$1.59E-05$
Dopaminergic synapse	K04728	4	Q15173|P31749|P50150|Q15147	$2.26E-05$
Phospholipase D signaling pathway	K04072	4	Q02750|P31749|Q15147|P49815	$2.91E-05$
Chemokine signaling pathway	K04062	4	Q02750|P31749|P50150|Q15147	$7.69E-05$

P value < 0.05, the number of target proteins involved in the pathway ≥4.

product, and the resulting fluorescence is proportional to ROS levels. For the measurement of intracellular ROS, cells were treated with H2DCF-DA (10 μM) for 30 min at 37°C in the dark, and washed twice with cold PBS. Intracellular ROS production was then analyzed using flow cytometry (excitation, 485 nm; emission, 530 nm).

2.10. Immunohistochemistry and Automated Western Blot Analysis. Anti-pAKT (Ser473, 60 kDa, isotype: rabbit), anti-pPRAS40 (Thr246, 40 kDa, source: rabbit), anti-AKT (60 kDa, isotype: rabbit), and anti-PRAS40 (40 kDa, source: rabbit) antibodies were purchased from Cell Signaling Technology. Anti-BLM antibodies (159 kDa, source: rabbit) were purchased from Abcam. Anti-β-actin antibodies (49 kDa) were purchased from Santa Cruz Biotechnology Inc. Antibodies were used at the following dilutions: BLM, 1 : 500; P-AKT, 1 : 50; and P-PRAS40, 1 : 200. Staining intensity criteria were as follows: 0 (-), 1 (weak), 2 (moderate), and 3 (strong). The positive staining rates were scored as follows: 0 (negative), 1 (1-25%), 2 (26%-50%), 3 (51-75%), and 4 (76%-100%). An automated western blot quantitative analyzer (ProteinSimple) was used to assess protein expression according to standard protocols (antibody dilution factor: 1 : 50). A grayscale analysis of the band intensities was then performed using Compass software.

2.11. Statistical Analysis. The distributions of each GO class or KEGG pathway among the DEPs and total proteins were compared using Fisher's exact test to assess the enrichment significance. One-sided or two-sided paired Student's *t*-tests

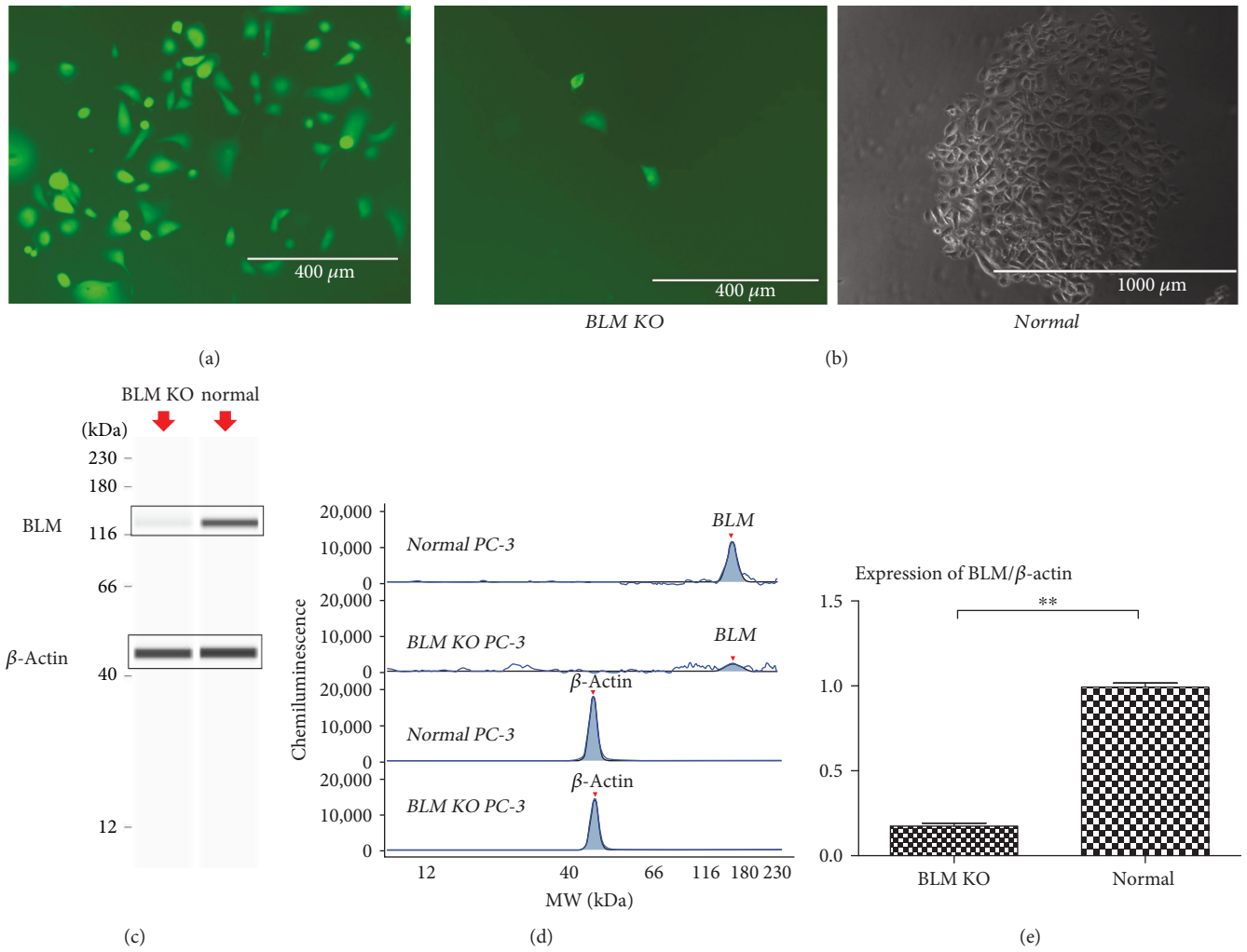


FIGURE 2: Site-directed knockout of the BLM gene in PC-3 cells. (a) PC-3 cells were observed under fluorescence microscopy following transfection with BLM CRISPR/Cas9 expression and donor vectors. (b) Monoclonal PC-3 cell clusters are shown after 3 weeks of culture. Normal PC-3 cells undergo monoclonal proliferation, while BLM KO PC-3 cells do not. (c) Expression of BLM in different PC-3 cell lines. Results were generated using the WES system. Band intensities represent protein expression. (d) Chemiluminescence was used to generate the peak area and molecular weight data by linear analysis using the WES system. (e) The BLM/β-actin ratio was significantly decreased in BLM KO PC-3 cells ($^{*}P < 0.01$ and $^{**}P < 0.01$).

were performed for single comparisons. All quantitative results were expressed as the mean ± SD. A P value < 0.05 was considered statistically significant.

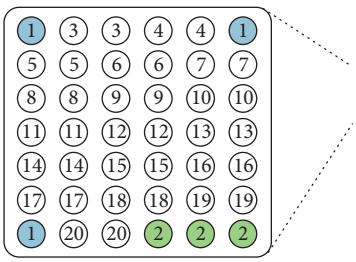
3. Results

3.1. iTRAQ-Mediated Analysis of DEPs in PC Samples. iTRAQ technology was used to uncover DEPs among prostate cancer, normal prostate, and benign prostatic hyperplasia tissues [31, 32]. A total of 21 DEPs (fold change >1.2 or <0.8, P value < 0.05) for which protein expression was up- or downregulated in cancerous tissue were identified (of which 17 proteins were upregulated and 4 were downregulated). Functional annotation was performed for these DEPs using the GO database. Whether DEPs were significantly enriched among specific biological processes (BPs) was analyzed using Fisher's exact test (Figure 1), revealing that protein phosphorylation (GO: 0001932) was significantly enriched. An analysis of the DEPs involved in the regulation of protein phosphorylation revealed high BLM expression in PC cancer tissue (Table 1). We also performed KEGG pathway annotation of these 21 DEPs and screened the target pathways following enrichment analysis ($P < 0.05$, ≥4 target proteins involved in the pathway) (Table 2). Among these, known oncogenic pathways including the mTOR [33], PI3K-Akt [34], MAPK, AMPK, [35], and p53 pathways were identified, indicating potential links to ROS production as related to tumor growth and alterations in cellular translational activity worthy of future investigation. In addition, all of these pathways have well-characterized links to cancer cell proliferation, differentiation, apoptosis, and DNA damage repair [36].

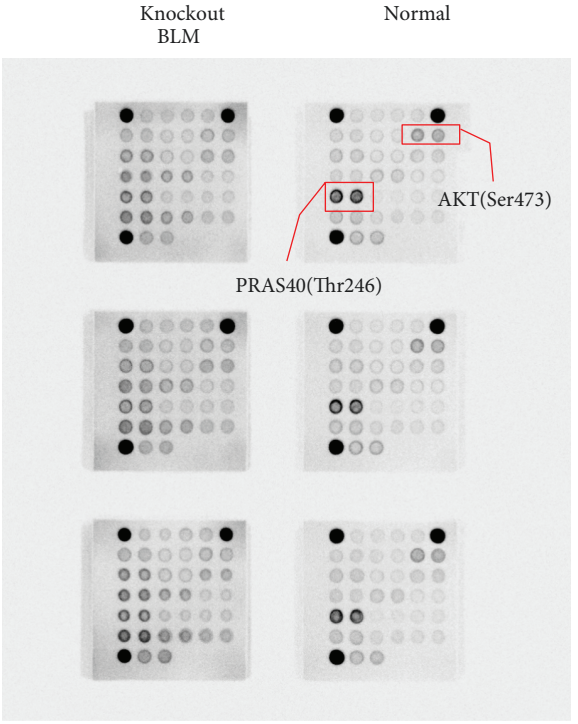
3.2. Site-Directed Knockout of BLM Inhibits PC-3 Cell Proliferation. To examine the role of BLM in PC progression, we generated a BLM knockout human PC-3 cell line using

Intracellular signaling

	Target	Phosphorylation site	Modification
1	Positive control	N/A	N/A
2	Negative control	N/A	N/A
3	ERK1/2	Tr202/Tyr204	Phosphorylation
4	Stat1	Tyr701	Phosphorylation
5	Stat3	Tyr705	Phosphorylation
6	Akt	Tr308	Phosphorylation
7	Akt	Ser473	Phosphorylation
8	AMPkα	Tr172	Phosphorylation
9	S6 ribosomal protein	Ser235/236	Phosphorylation
10	mTOR	Ser2448	Phosphorylation
11	HSP27	Ser78	Phosphorylation
12	Bad	Ser112	Phosphorylation
13	p70 S6 kinase	Tr389	Phosphorylation
14	PRAS40	Tr246	Phosphorylation
15	p53	Ser15	Phosphorylation
16	p38	Tr180/Tyr182	Phosphorylation
17	SAPK/JNK	Tr183/Tyr185	Phosphorylation
18	PARP	Asp214	Cleavage
19	Caspase-3	Asp175	Cleavage
20	GSK-3β	Ser9	Phosphorylation



(a)



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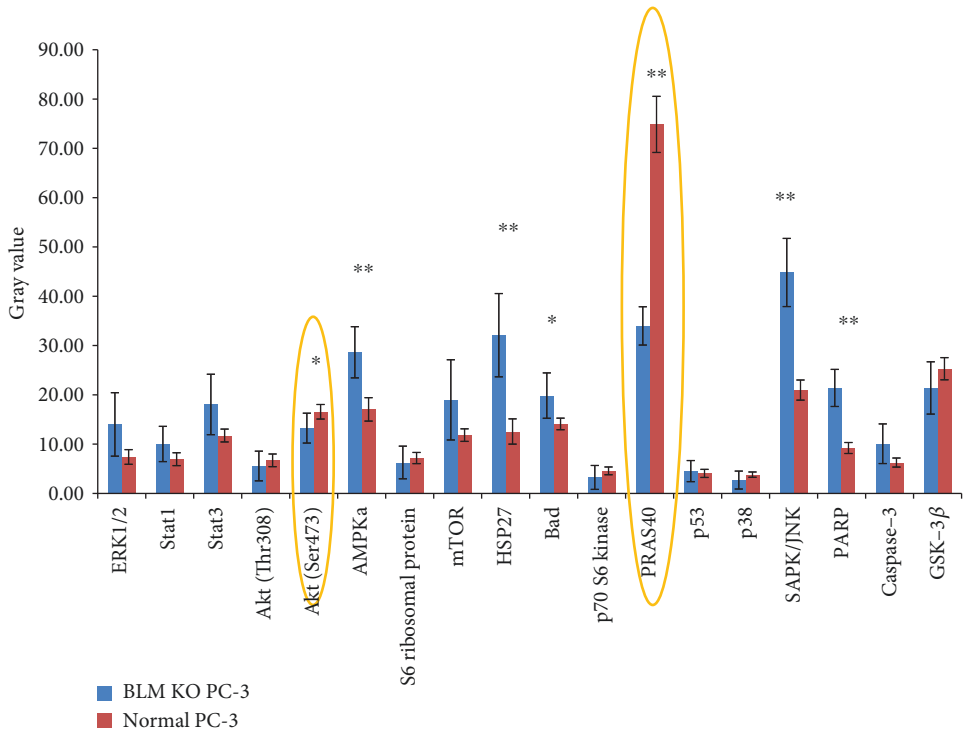
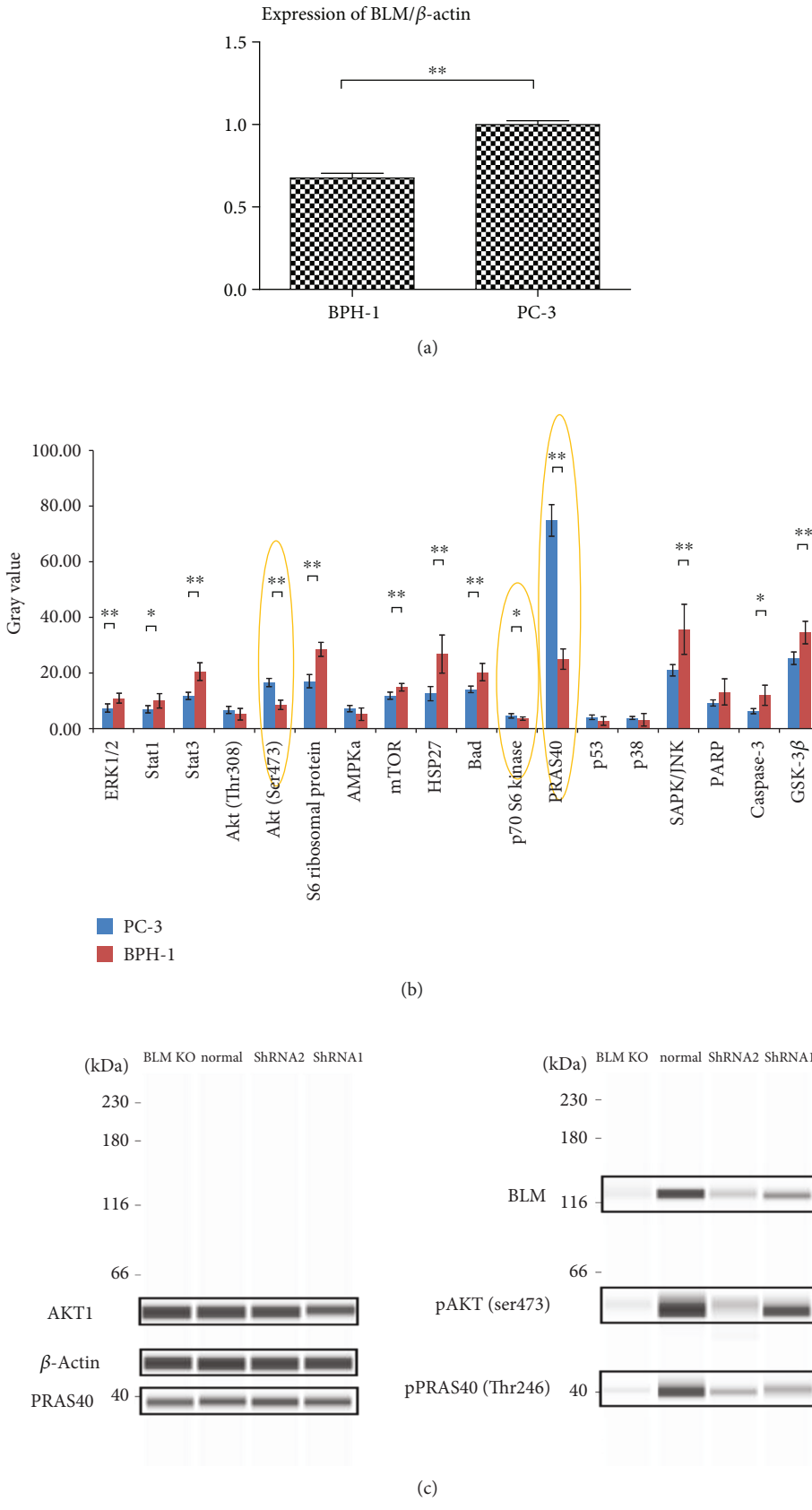


(c)

Figure 3: Selected phosphorylated nodal proteins in PC-3 and BLM KO PC-3 cells. (a) Phosphorylated nodal proteins investigated in this study. (b) Expression of phosphorylated signaling proteins in two different PC-3 cell lines. (c) Quantitation of AMPKα, HSP27, Bad, SAPK/JNK, and PARP expression levels revealed their upregulation in BLM KO PC-3 cells, while AKT (Ser473) and PRAS40 (Thr246) expression levels were significantly downregulated (mean ± SD; $^{*}P < 0.05$ and $^{**}P < 0.01$).



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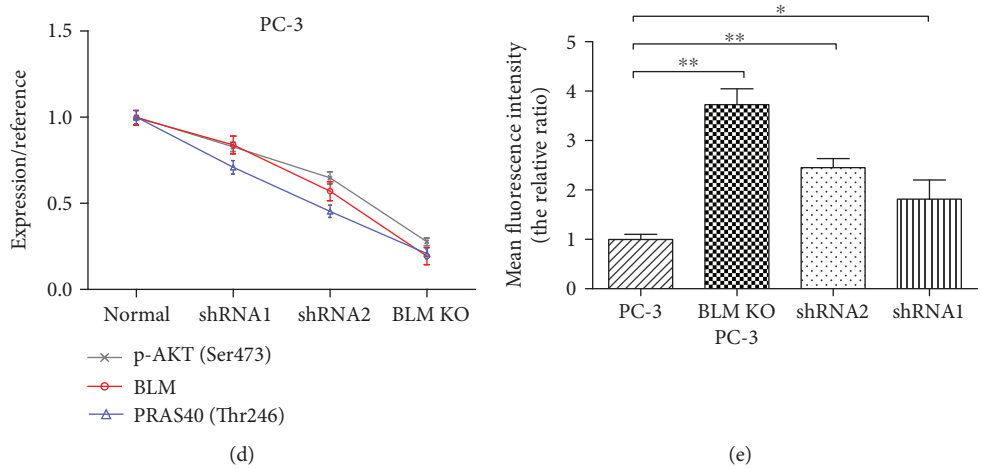
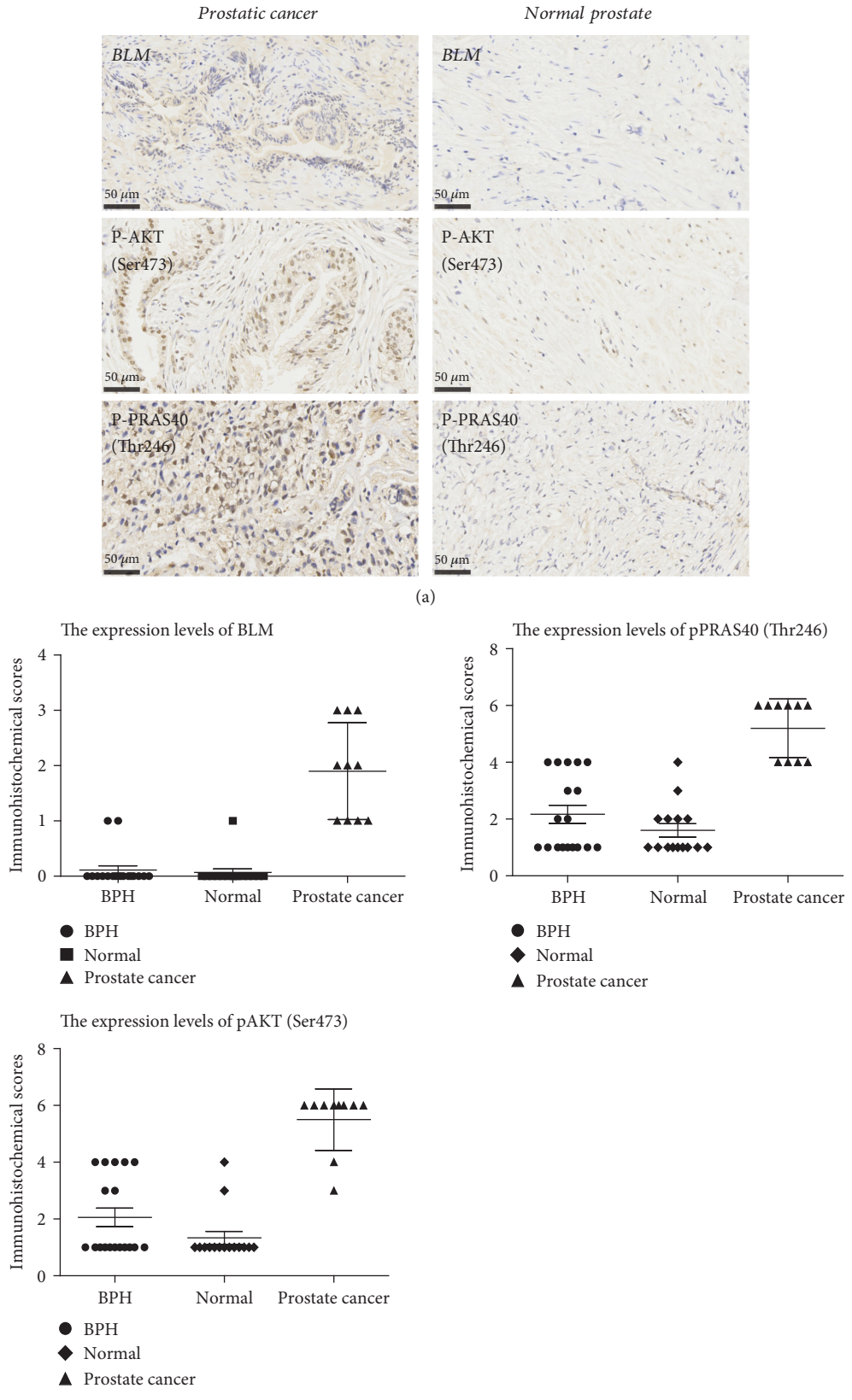


Figure 4: The correlation between BLM expression and phosphorylation of Akt1 and PRAS40. (a) BLM/β-actin expression in BPH-1 and PC-3 cells. BLM expression was lower in BPH-1 cells than in PC-3 cells. (b) Quantification of phosphorylated signaling proteins in PC-3 and BPH-1 cells. The levels of phosphorylated AKT (Ser473), p70 S6 kinase, and PRAS40 (Thr246) were lower in BPH-1 than in PC-3 cells. (c) BLM helicase, pAKT, and pPRAS40 expression in PC-3 cells was assessed by western blot and WES analysis. (d) Cells displayed significantly reduced BLM expression, as well as decreased levels of pAKT (Ser473) and pPRAS40 (Thr246). BLM expression values were normalized to β-actin, while pAKT and pPRAS40 (Thr246) expression values were normalized to total AKT1 and total PRAS40 expression, respectively. BLM expression was positively correlated with pAKT ($P < 0.01$) and pPRAS40 levels ($P < 0.05$). (e) Intracellular ROS levels were higher in BLM KO PC-3 cells as well as those treated with shRNA1 and shRNA2 as compared to those of PC-3 cells (mean ± SD; $^*P < 0.05$ and $^{**}P < 0.01$).

CRISPR/Cas9 [37]. We initially designed five Cas9/sgRNA expression vectors, and the activity of each was detected using T7 endonuclease I. Based on these screening assays, we determined that sgRNA3 was the most active. Next, donor vectors were designed based on the sequences upstream and downstream of sgRNA3. The expression vectors and donor vectors were cotransfected into PC-3 cells, and these cells underwent ampicillin selection (6 μg/mL for 6 days) 48 h posttransfection, followed by the selection for the donor vector using puromycin (0.6 μg/mL for 5 days). The donor vector expressed enhanced yellow fluorescent protein (eYFP) driven under control of the hEF-1α promoter, and as such, remaining cells were screened for green fluorescence (Figure 2(a)) confirming successful vector integration into the PC-3 cell genome in BLM KO cells.

Normal PC-3 cells can undergo monoclonal proliferation, and 3 weeks of cell growth results in the formation of readily visible monoclonal cell clusters. To rule out any off-target effects due to plasmid transfection, we cotransfected PC-3 cells with the plasmid backbone and selected for resistant cells which did not undergo gene recombination. These control cells formed a monoclonal cell mass after 3 weeks, indicating that plasmid transfection alone had no effect on the ability of PC-3 cells for monoclonal proliferation. However, monoclonal screening of the BLM KO PC-3 cells revealed a reduction in proliferative capacity. The cells proliferated slowly, aged, became vacuolated, and died (Figure 2(b)). Because these KO cells could not form monoclonal clusters, sufficient numbers of BLM KO PC-3 cells could only be obtained via repeated transfections. After collecting a sufficient number of BLM KO PC-3 cells, changes in BLM helicase expression between BLM KO and wild-type PC-3 cells were assayed using the automated western blot analysis system (WES) [38, 39]. We observed significantly lower BLM expression in the BLM KO PC-3 cells as compared to wild-type cells ($P < 0.01$) (Figures 2(c)–2(e)). Taken together, these data confirmed that knockout of BLM was successful and it impaired the proliferation and viability of PC-3 cells.

3.3. Screening for Significant Differences in Phosphorylated Proteins. Given the effects of BLM on PC-3 cell proliferation, we next assessed whether changes in BLM expression influenced phosphorylation states within prooncogenic signaling cascades. The PathScan® Antibody Array Kit was used to analyze WT and BLM KO PC-3 cells. Previous studies have shown that BLM is more highly expressed in PC tissue than in normal prostate and benign prostatic hyperplasia tissues and that BLM regulates protein phosphorylation [40–42] (Figure 3(a)). Our screening results showed that AMPKα, HSP27, Bad, SAPK/JNK, and PARP expression levels were significantly upregulated in BLM KO PC-3 cells as compared to normal PC-3 cells, while AKT and PRAS40 expression levels were significantly downregulated (Figures 3(b) and 3(c)). We also observed significantly higher BLM levels in PC-3 cells than in benign prostatic hyperplasia BPH-1 cells (Figure 4(a)). When the levels of phosphorylated proliferation-associated proteins were assessed in BPH-1 cells, the levels of pAKT (Ser473), p70 S6 kinase, and pRAS40 (Thr246) were significantly downregulated (Figure 4(b)). These data confirmed that BLM influences a wide range of oncogenic signaling pathways, most notably via AKT (Ser473) and PRAS40 (Thr246) phosphorylation. In order to verify the correlation between BLM expression and the levels of phosphorylated Akt1 and PRAS40, we employed a RNAi-based approach to reduce BLM expression, revealing



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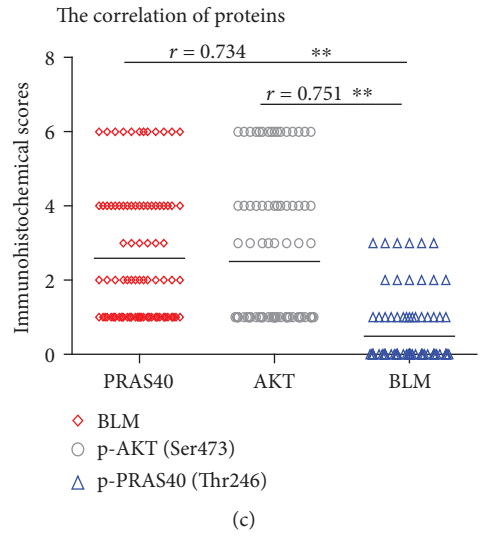


(c)

Figure 5: Immunohistochemical analysis of phosphoprotein levels. (a) Prostate cancer tissue: weak BLM staining intensity (10-30% positive staining), moderate pAKT (Ser473) staining (50-70% positive staining), and moderate pPRAS40 (Thr246) staining (50-70% positive staining). Normal prostate tissue: negative BLM staining (0% positive staining), moderate pAKT (Ser473) staining (10-30% positive staining), and moderate pPRAS40 (Thr246) staining (10-30% positive staining). (b) Immunohistochemical scores were calculated based on the sum of the staining intensity scores and positive staining rates. Interpretation criteria were as follows: staining intensity score: 0 (-), 1 (weak), 2 (moderate), and 3 (strong). Positive staining rates were scored as follows: 0 (negative), 1 (1-25%), 2 (26%-50%), 3 (51-75%), and 4 (76%-100%). pAKT (Ser473), pPRAS40 (Thr246), and BLM expression in prostate cancer tissue was higher than in other tissues. (c) BLM expression positively correlated with pAKT ($r = 0.751$, $P < 0.01$) and pPRAS40 (Thr246) expression ($r = 0.751$, $P < 0.01$).

that levels of phosphorylated Akt1 and PRAS40 were specifically linked to BLM downregulation (Figures 4(c) and 4(d)). Given that AKT (Ser473) and PRAS40 (Thr246) phosphorylation also influence tumor growth-related ROS production, we assessed the link between BLM expression and ROS production in these cells, revealing that ROS production was significantly increased upon BLM downregulation (Figure 4(e)).

3.4. Effects of AKT and PRAS40 Inhibition on PC Cells. We found that phosphorylated AKT and PRAS40 levels were lower when BLM expression was reduced in PC cell lines. We performed immunohistochemical staining to validate these finding in PC tissue samples. According to the cytoplasmic staining intensity and positive staining rates, pAKT (Ser473), pPRAS40 (Thr246), and BLM expression levels were higher in prostate cancer tissue than in non-PC tissues (Figure 5). To investigate the effects of AKT and PRAS40 activity in PC cell lines, PC-3, LnCAP, and VCAP cells were treated with BEZ235, a highly specific AKT inhibitor [43–45], and GDC-0068, a PRAS40 inhibitor [46, 47]. Cell viability was then assessed via a CCK-8 assay. Cisplatin (CDDP) was included in these experiments as a positive control. All three drugs reduced the proliferation of all the PC cell lines, with BEZ235 having the lowest half maximal inhibitory concentration (IC50), followed by that of GDC-0068 and that of CDDP (Figure 6(a)). Annexin V-FITC/PI staining was used in conjunction with flow cytometry to investigate whether BEZ235, GDC-0068, and CDDP induced apoptosis in these PC cell lines following 24 h of treatment. Relatively low concentrations of BEZ235 and GDC-0068 induced significant levels of apoptosis compared to CDDP (Figure 6(b)), confirming that they exert a strong apoptotic effect in PC cancer cells. Cisplatin is known to mediate ROS production in PC cells [48], and it is possible that similar mechanisms contributed to the observed induction of apoptosis mediated by these additional inhibitory compounds (Figure 6(c)).

3.5. Impact of AKT (Ser473) and PRAS40 (Thr246) Phosphorylation on BLM Expression. Our results so far have shown that BLM is required for AKT and PRAS40 phosphorylation. Given that BLM, pAKT, and pPRAS40 all displayed reduced expression in non-PC tissue, we assessed whether inhibiting AKT and PRAS40 could also influence BLM expression. For these experiments, PC-3 and LnCAP cells were treated with BEZ235 and GDC-0068 followed by assessment of BLM, pAKT, and pPRAS40 levels using WES (Figures 7 and 8). Treating cells with BEZ235 and GDC-0068 resulted in a significant decrease in BLM expression. Drug activity was confirmed by measuring downregulation of pAKT and pPRAS40 following their respective drug treatments. These findings suggested that BLM expression positively correlates with AKT and PRAS40 activity and that inhibiting these kinases downregulates BLM expression in PC cells. Increases and decreases in ROS serve as a double-edged sword, and both can mediate tumor occurrence and development. Therefore, it is feasible that altered BLM expression may contribute to this process in a similar way. We found that reductions in BLM expression in PC-3 cells were associated with increased ROS production and that these increases occurred in a dose-dependent manner (Figure 7(e)).

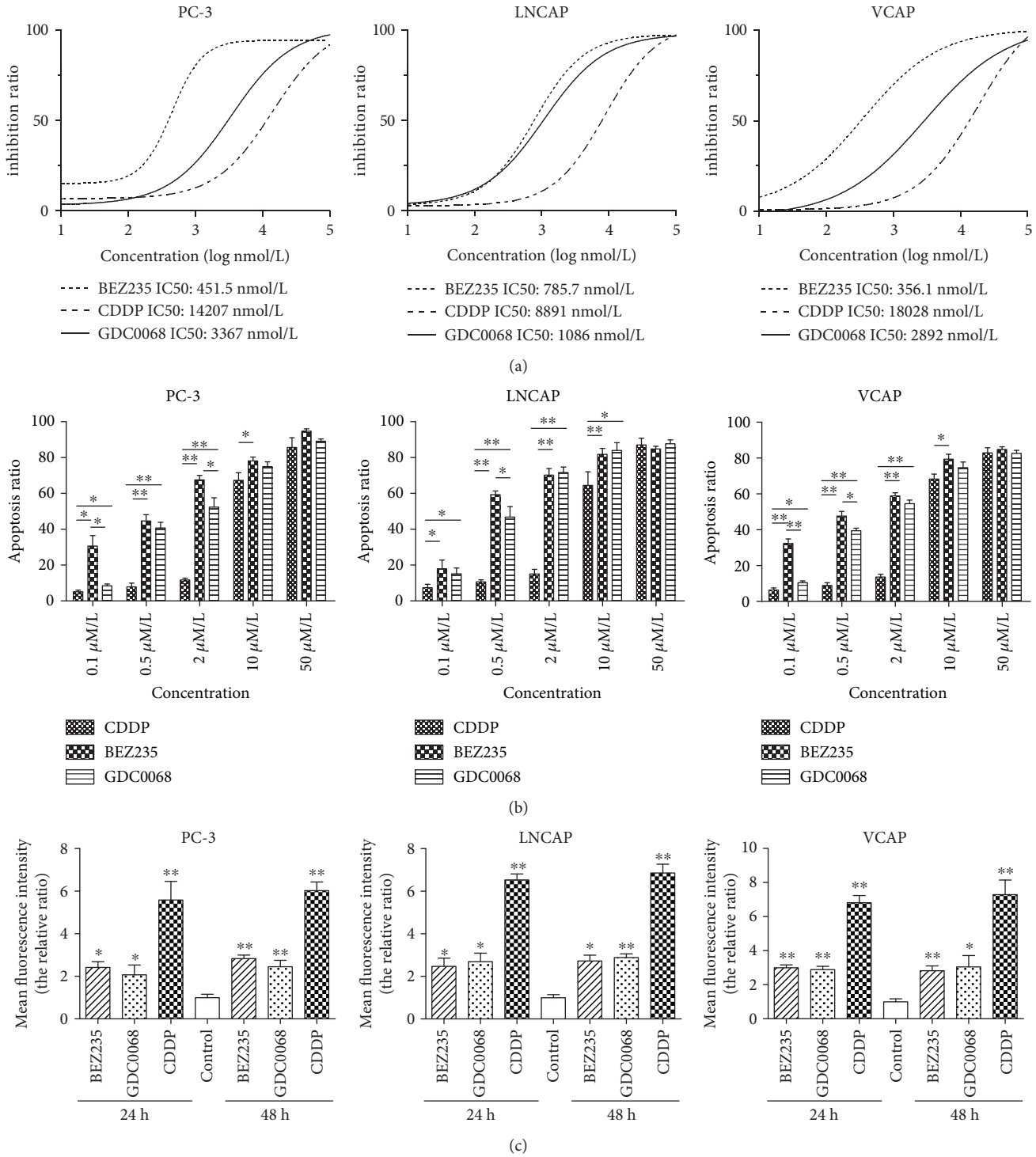
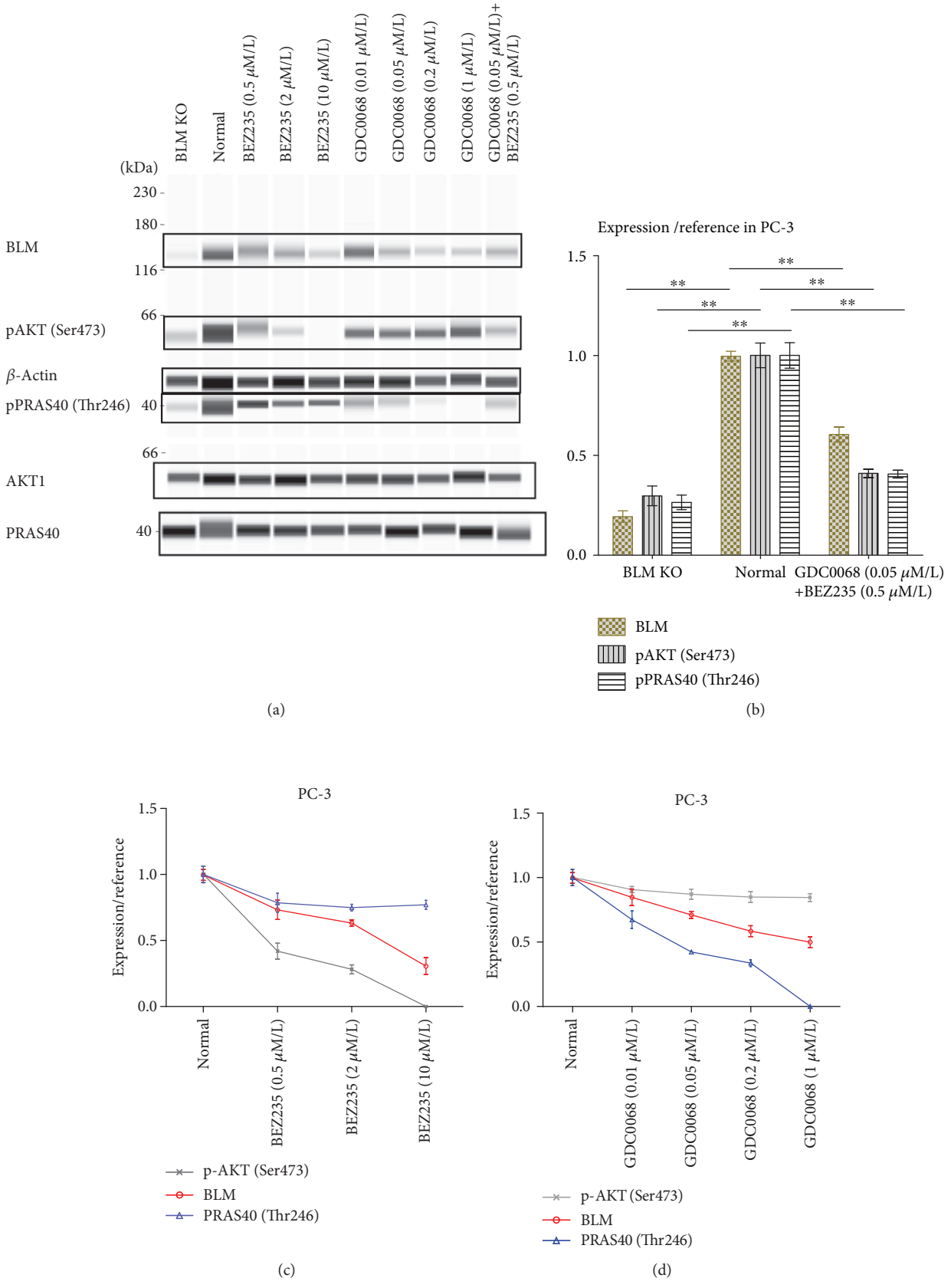


Figure 6: BEZ235 and GDC-0068 reduce proliferation and induce apoptosis more potently than CDDP in PC cells. (a) Inhibition ratios in prostate cancer cells following 48 h treatment: for PC-3 cells, BEZ235 IC50—451.5 ± 79.23 nmol/L, CDDP IC50—14207 ± 96.49 nmol/L, and GDC-0068 IC50—3367 ± 97.79 nmol/L; for LnCAP cells, BEZ235 IC50—785.7 ± 94.4 nmol/L, CDDP IC50—8891 ± 100.7 nmol/L, and GDC-0068 IC50—1086 ± 94.7 nmol/L; and for VCAP cells, BEZ235 IC50—356.1 ± 99.07 nmol/L, CDDP IC50—18028 ± 113.9 nmol/L, and GDC-0068 IC50—2892 ± 105.8 nmol/L ($P < 0.05$). (b) Apoptosis ratios in various prostate cancer cell lines after drug treatments. At a concentration of 0.5 μmol/L, BEZ235 and GDC-0068 induced apoptosis in ~40% of cancer cells. CDDP induced lower relative levels of apoptosis (~20%; 24 h treatment). (c) Cells were treated with BEZ235 (2 μmol/L), GDC-0068 (2 μmol/L), and CDDP (10 μmol/L) for 24 and 48 hours. GDC-0068, BEZ235, and CDDP significantly increased ROS production in tumor cells. Values represent the mean ± standard deviation (SD); $^{*}P < 0.05$ and $^{**}P < 0.01$.



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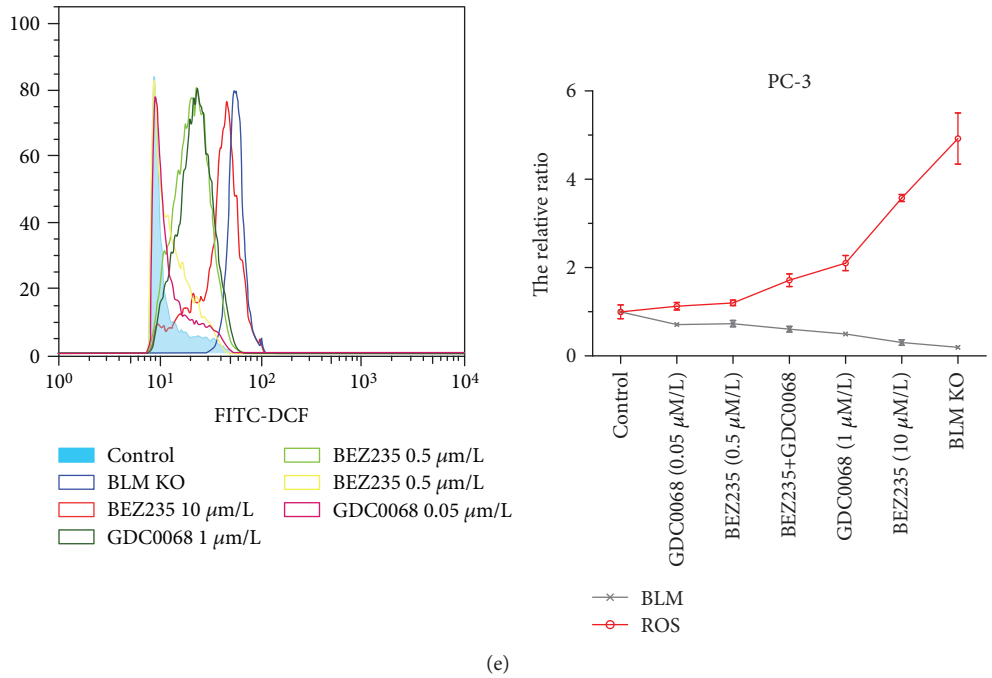

(e)

Figure 7: BLM, pAKT, and pPRAS40 expression in PC-3 cells. (a) BLM helicase, pAKT, and pPRAS40 expression in PC-3 cells as measured by western blot and WES analysis. (b) Cells treated with BEZ235 and GDC-0068 displayed significantly reduced BLM expression, while BLM knockout decreased pAKT (Ser473) and pPRAS40 (Thr246) levels. BLM expression values were normalized to β-actin, and pAKT and pPRAS40 (Thr246) expression values were normalized to total AKT1 and PRAS40, respectively. Data represent the mean ± SD ($^{*}P < 0.05$ and $^{**}P < 0.01$). BLM expression is positively correlated with (c) pAKT ($P < 0.05$) and (d) pPRAS40 levels ($P < 0.01$). (e) ROS production is negatively correlated with BLM expression ($P < 0.05$).

4. Discussion

As a result of the aging population and lifestyle changes, the incidence of PC continues to increase worldwide, lending an urgency to the requirement for new and effective PC treatments. Aging is a key mediator of cancer development, both for probabilistic reasons and potentially due to increased ROS production which is linked with both cancer and with so-called "inflamm-aging" processes [49]. In this study, through an iTRAQ-mediated analysis of PC clinical samples, we screened for differentially expressed proteins in human PC tissue, normal prostate tissue, and benign prostatic hyperplasia tissue in order to identify DEPs which may represent novel anti-PC targets. Our results showed that BLM expression was significantly increased in PC tissue, and the results of a GO term enrichment analysis revealed that BLM regulates protein phosphorylation in PC cells (GO: 0001932). We further determined that BLM downregulation reduced both pAKT and pPRAS40 levels, suggesting that their activity is dependent upon BLM expression. Importantly, we found that BLM downregulation, AKT inhibition, and PSRAS40 inhibition all resulted in enhanced ROS production, reduced PC cell proliferation, and increased apoptosis. This highlights AKT and PSRAS40 signaling as potential mediators of BLM-induced PC tumorigenesis, suggesting that this signaling axis could be a novel target for PC therapy.

BLM is a helicase implicated in homologous recombination, a key pathway involved in the repair of double-strand DNA breaks [40]. Helicases unwind DNA or RNA and are important for maintaining genome integrity during DNA replication and repair. Therapies that target DNA damage repair enzymes have recently shown promise as a chemotherapeutic strategy due to cancer-associated genomic instability [50, 51]. This led to the characterization of the first BLM helicase inhibitor (termed ML216); however, this compound lacked specificity and also inhibited the ability of closely related helicases to unwind DNA, leading to off-target effects [52, 53]. In this study, GO analysis annotated BLM expression in PC cells as a "regulatory process for protein phosphorylation," as BLM participates in the regulation of cyclin-dependent protein serine/threonine kinase activity. BLM expression is lowest in the G1 phase, significantly increases in the S phase, and peaks in the G2/M phase of the cell cycle [54, 55]. BLM binds to the spindle assembly checkpoint kinase, monopolar spindle 1 (MPS1), and is phosphorylated at Ser144. Phosphorylated BLM may then bind to Polo-like kinase 1 (PLK1) via its polo-box domain (PBD), further contributing to the maintenance of genomic stability in both healthy [56, 57] and tumor tissues [58–61]. Together, these data highlight the multifunctional roles of BLM and its ability to regulate multiple different oncogenic pathways.

Precision cancer medicine has emerged as a modern cancer treatment strategy and has enabled more accurate and efficient therapeutic treatment for individual cancer patients. Precision medicine employs tumor genomic profiling to inform treatment decisions [62–67]. This is the result of identifying numerous cancer drivers, alterations in their

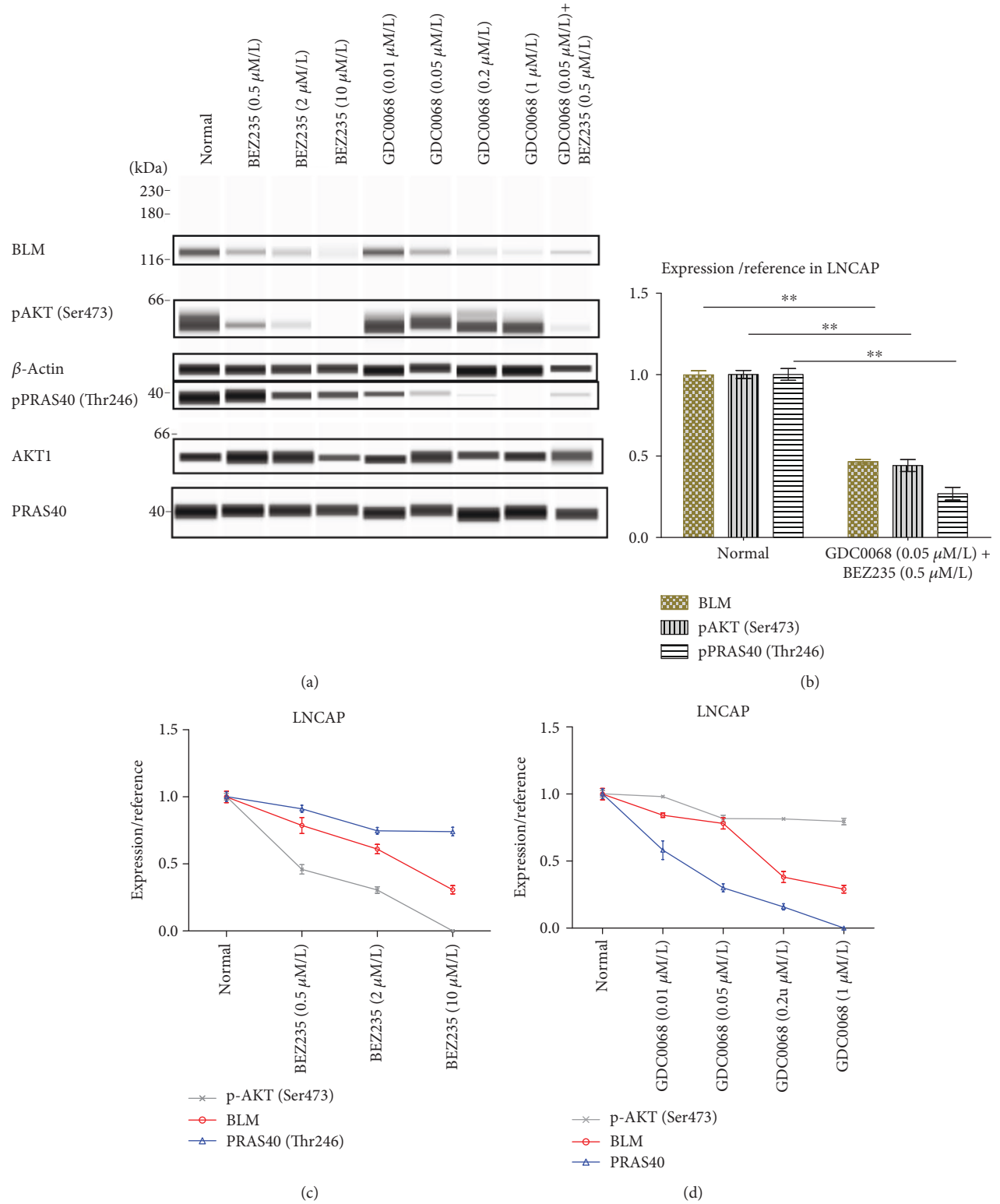

Figure 8: BLM, pAKT, and pPRAS40 expression in LNCAP cells. (a) BLM helicase, pAKT, and pPRAS40 expression in LNCAP cells as measured by western blot and WES analysis. (b) Cells treated with BEZ235 and GDC-0068 displayed significantly reduced BLM expression. BLM expression values were normalized to β-actin, and pAKT and pPRAS40 (Thr246) expression values were normalized to total AKT1 and PRAS40, respectively. Data represent the mean ± SD ($^{*}P < 0.05$ and $^{**}P < 0.01$). BLM expression is positively correlated with (c) pAKT ($P < 0.05$) and (d) pPRAS40 levels ($P < 0.05$).

activity, and therapies that target these alterations. PI3K is the major activator of AKT, and this PI3K signaling pathway is commonly mutated in PC, often through the downregulation of PTEN, PIK3CA/B amplification, or activating mutations of PIK3CA/B and AKT1 [68]. Interestingly, AKT was also identified in the present study. Indeed up to 49% of PC tumors are thought to have aberrant PI3K signaling, making it the 2nd most frequently altered pathway in PC [68–70]. However, PI3K monotherapies for PC are ineffective, most likely due to their lack of specificity and complex signaling feedback mechanisms [68, 69, 71–74]. Recently, clinical trials have been initiated for inhibitors of specific PI3K isoforms and may provide increased specificity. In addition, the combination of Ipatasertib and Abiraterone in PTEN-null prostate cancer has improved PC patient survival in phase II studies, demonstrating the efficacy of the reciprocal targeting of PTEN loss and PI3K [75].

In this study, we identified a novel reciprocal relationship in PC tissue between BLM-AKT and PRAS40 and demonstrated that BLM, AKT, and PSRAS40 inhibition was beneficial for PC treatment *in vitro*. Using CRISPR/Cas9-mediated homologous recombination to perform site-directed knockout of the BLM gene in PC-3 cells, we demonstrated that BLM KO leads to a reduction in PC cell proliferation. Similarly, prior studies have shown that BLM silencing using RNAi interference inhibited the proliferation, migration, and invasion of PC-3 cells [40]. Interestingly, PLK1 phosphorylates and inactivates PTEN and is regulated in part by BLM. BLM is also involved in the regulation of the G2/M cell cycle checkpoint and mitotic progression, and BLM expression levels fluctuate with the cell cycle and peak during the G2/M phase. In addition, phosphorylation of PRAS40 can activate 14-3-3, and the 14-3-3 protein can promote PLK1 catalytic activity to control mitotic progression and the G2/M checkpoint [76]. BLM may therefore use PLK1 as an intermediary to affect AKT (Ser473) and PRAS40 (Thr246) phosphorylation. In addition, since BLM expression is related to the cell cycle, increased BLM expression indicates that an increased proportion of cells are in the G2/M phase. Given the intense research into the discovery of novel potent and selective BLM inhibitors with improved physiochemical properties [52, 53, 77], future efforts to utilize BLM and AKT inhibitors in a combination therapy to treat PC tumors may represent a viable target for precision medicine.

While our primary focus in this study was to observe the differences in the regulation of BLM-mediated protein phosphorylation, ROS levels are known to be key players in oncogenic processes, with marked elevations in ROS levels in many tumor systems indicating the presence of a stressful redox environment in these cells [78]. Some of the DEPs identified in the present study are linked to ROS-related pathways in addition to their association with the regulation of protein phosphorylation. For example, ROS produced in response to the activation of growth factor signaling pathways can mediate the activation of key kinases in the mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and focal adhesion kinase families, thereby enhancing the migration and invasive potential of cancer cells [79]. Importantly, ROS have been shown to modulate signaling in order to promote AKT upregulation and PTEN downregulation both *in vitro* and *in vivo* [80].

We further confirmed that ROS production is negatively correlated with BLM expression. Multiple studies have shown that ROS-mediated damage can cause substantial disruptions in cellular protein function and DNA integrity. This is one of the reasons underlying the susceptibility of BS patients with BLM mutations to multiple malignancies. In addition, BLM expression positively correlates with AKT and PRAS40 activity in PC cells. Enhancing PI3K/AKT/mTOR pathway signaling plays a critical role in determining the lifespan and cellular senescence of mammalian cells. Since AKT and RAS40 were downregulated, this may be one of the reasons why BS patients do not exhibit a progeria phenotype. BLM, pAKT, and pPRAS40 all display increased expression in PC tissues, and ROS is similarly increased in this context [81]. ROS and BLM may cooperate with one another in order to promote tumorigenesis. As a promoter of DNA replication, the high BLM expression satisfies the high replicative demands of cancer cells, while the ability of BLM to maintain genomic stability confers survival advantages to these malignant cells. ROS production is associated with increased metabolic rates and DNA damage. Thus, the correlation between BLM and ROS production suggests that BLM is involved in repairing this ROS-mediated DNA damage.

5. Conclusions

Our results have revealed that BLM overexpression and subsequent changes in protein phosphorylation contribute to PC progression. BLM, pAKT, and pPRAS40 levels are significantly increased in PC tissue, and the expression levels of these proteins are closely related, with BLM inhibition leading to a loss of pAKT/PRAS40 and vice versa. While a large number of studies have implicated AKT and PRAS40 hyperactivity in promoting tumor cell proliferation, the correlation between these signaling molecules and BLM has not been previously reported. In addition, we have demonstrated that BLM, AKT, and PSRAS40 influence the proliferative capacity of PC cells and that their inhibition enhances ROS production and leads to PC-3 cell apoptosis. These novel results suggest that ROS-related mechanisms may contribute to PC pathogenesis. Combining BLM and AKT inhibitors with therapeutic modalities targeting ROS generation may facilitate the achievement of durable responses against PC as a consequence of these effective multimodal approaches.

Abbreviations

BLM: Bloom syndrome protein
WES: Automated western blot analysis system
iTRAQ: Isobaric tag for relative and absolute quantitation
TRAF6: TNF receptor-associated factor 6
AKT1S1: AKT serine/threonine kinase 1 substrate 1
DEPTOR: DEP domain-containing MTOR interacting protein
MAP2K1: Mitogen-activated protein kinase 1

TSC2: Tuberous sclerosis complex subunit 2
PPP2R5B: Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit beta isoform
PLK1: Polo-like kinase 1
14-3-3: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein theta
ROS: Reactive oxygen species.

Data Availability

Datasets used in the study are available from the corresponding author upon reasonable request.

Ethical Approval

Clinical samples were collected at the Department of Urology of Guizhou Provincial People's Hospital with the approval of the ethics committee. All procedures were performed in accordance with the Declaration of Helsinki and relevant policies in China.

Consent

Clinical samples were collected at the Department of Urology of Guizhou Provincial People's Hospital with the informed consent of the patients.

Conflicts of Interest

The authors declare no conflicts of interest related to this project.

Acknowledgments

We thank the members of the Department of Urology of Guizhou Provincial People's Hospital for their assistance in specimen collection. All cell lines were obtained from the ATCC. This work was funded by the National Natural Science Foundation of China (Grant No. 31860242), the Youth Fund of Guizhou Provincial People's Hospital (Grant No. GZSYQN (2015)13), and the Academician Workstation ((2015)4015).

Supplementary Materials

Figure 1: PC-3 cells were identified by STR authentication. Figure 2: it is proven by sequence analysis that sgRNA are correctly inserted into the expression vector. Figure 3: the construction of BLM helicase CRISPR/Cas9 donor vector. Figure 4: the target efficiency was analyzed by T7E1 enzyme digestion. Figure 5: expression of BLM helicase gene mRNA in PC-3 cells. Table1: the DEP list among prostate cancer, normal prostate, and benign prostatic hyperplasia tissues was identified using iTRAQ. *(Supplementary Materials)*

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Research Article

ROS Induced by KillerRed Targeting Mitochondria (mtKR) Enhances Apoptosis Caused by Radiation via Cyt c/Caspase-3 Pathway

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During radiotherapy, reactive oxygen species- (ROS-) induced apoptosis is one of the main mechanism of radiation. Based on KillerRed which can induce ROS burst in different cell substructures, here we hypothesized that KillerRed targeting mitochondria (mtKR) could induce ROS to enhance apoptosis by radiation. In this study, empty vector, mtKR, and mtmCherry plasmids were successfully constructed, and mitochondrial localization were detected in COS-7 and HeLa cells. After HeLa cells were transfected and irradiated by visible light and X-rays, ROS levels, mitochondrial membrane potential ($\Delta\psi_m$), ATPase activities, adenosine triphosphate (ATP) content, apoptosis, and the expressions of mRNA and protein were measured, respectively. Data demonstrated that the ROS levels significantly increased after light exposure, and adding extra radiation, voltage-dependent anion channel 1 (VDAC1) protein increased in the mitochondria, while Na^+-K^+ and Ca^{2+}-Mg^{2+} ATPase activities, ATP content, and $\Delta\psi_m$ significantly reduced. Additionally, the cell apoptotic rates dramatically increased, which referred to the increase of cytochrome c (Cyt c), caspase-9, and caspase-3 mRNA expressions, and Cyt c protein was released from the mitochondria into the cytoplasm; caspase-9 and -3 were activated. These results indicated that mtKR can increase the production of ROS, enhance mitochondrial dysfunction, and strengthen apoptosis by radiation via Cyt c/caspase-3 pathway.

1. Introduction

Mitochondria are essential organelles for cell survival, death, and signaling and are also one of the main production sites of reactive oxygen species (ROS) [1, 2]. In addition, mitochondria also play a prominent role in the regulation of apoptosis [3–5]. When ROS is produced in the mitochondria, adenosine triphosphate (ATP) is also produced. ROS can be generated endogenously during cellular respiration or in response to infection and can be induced exogenously by chemical and physical agents, such as radiation, UV, and cigarette smoke. Lower levels of ROS play a role in normal cellular function [6], while increased levels of ROS induce oxidative stress which is the cause or consequence of the damage to mitochondria and mitochondrial DNA (mtDNA) [7].

In addition, mitochondria are also a damaging target of ROS. Under normal physiological conditions, ROS resulting from mitochondria is removed by a cellular antioxidant defense system. However, once ROS is overproduced, it will lead to the accumulation of excess radicals that damage the mitochondria and cells [8]. The literatures suggest that oxidative damage has also played a key role in diseases such as diabetes, Parkinson's disease, Alzheimer's disease, and even in the progress of cancers [9–11]. And ROS may mediate the programmed cell death (PCD) at a moderately high concentration among different cell types [12, 13]. In apoptosis, external stimuli such as radiation and cytotoxic agents can result in the formation of pores at mitochondrial membranes. Disruption of mitochondrial membrane potential ($\Delta\psi_m$) is a major sign of mitochondrial dysfunction. Loss of the $\Delta\psi_m$ can result in a defective mitochondrial electron transport chain (ETC) and decrease metabolic oxygen consumption and ATP depletion [14]. Mitochondrial dysfunction results in the release of proapoptotic protein Cyt c and

activates caspases to induce apoptosis. Once mitochondrial permeability transition pore (MPTP) is activated by oxidative stress, the membrane depolarization will develop, and the uncoupling of oxidative phosphorylation and ATP depletion will be induced [15]. Nowadays, the strategy targeting mitochondrial dysfunction in cancer therapy has been the research hotspots [16].

Radiotherapy is a conventional mean for cancer treatment for several decades. There is a growing interest in understanding how the altered mitochondrial functions may be the target to improve the effects of radiotherapy [17]. The modified bioenergetic and biosynthetic states of mitochondria play an eminent role for cancer cells in response to radiation [18]. Radiotherapy causes death of cancer cells through apoptosis and autophagy induced by excessive production of ROS [19–21]. Therefore, how to induce enough ROS to target mitochondria is a crucial research topic. KillerRed can directly express in cells, and under appropriate light excitation, it can efficiently induce ROS to cause cell death [22–25]. KillerRed can be used for the inactivation of light-induced protein, killing specific cell populations *in vivo* and studying intracellular local oxidative stress [26–28]. Additionally, because of light-inducing inactivation of KillerRed, in some studies, KillerRed was replaced by another red fluorescence protein mCherry (no phototoxicity) to study intracellular localization.

In this study, the N-terminal mitochondrial-targeting sequence (MTS) of PTEN-induced putative kinase 1 (Pink1) was used to mediate downstream mCherry and KillerRed to express in mitochondria [29]. Under fluorescence microscope, the colocalization of mCherry (red) and mitochondrial tracker COX IV (green) in both African green monkey kidney cell COS-7 and human cervical cancer cell HeLa was observed. Furthermore, we explored mtKR-induced mitochondrial dysfunction and apoptosis by light and X-rays, and proapoptotic mechanisms via Cyt c/caspase-3 pathway, to provide a new idea for cancer radiotherapy.

2. Materials and Methods

2.1. The mtmCherry and mtKR Vectors. In this study, the DNAs of mCherry, KillerRed, and Pink1-MTS were amplified with PCR using Q5 High-Fidelity DNA Polymerase (NEB, Beverly, MA, USA), and plxsp-TetA-mCherry, plxsp-TetA-KillerRed (kindly given by Dr. Shen from Cancer Institute of New Jersey, USA), and pcDNA-DEST47 PINK1 C-GF plasmids (Addgene, Cambridge, MA, USA) were used as templates. The following primers were used: mCherry: 5′-GGAATTCGCCACCATGGTGAGCAAGGG-3′(F), 5′-CGGGATCCTTACTTGTACAGCTCGTCCATG-3′(R); KillerRed: 5′-GGAATTCATGGGTTCAGAGGGC-3′(F), 5′-CGGGATCCCTAGATCTCGTCG-3′(R); Pink1-MTS: 5′-AAGGAAAAAAGCGGCCGCAATGGCGGTGCGACAG-3′(F), 5′-CGAATTCCGGCCGCCCCAAGCCGTAG-3′(R). The schematic diagram was shown in Figure 1(a), and the PCR products of mCherry, KillerRed, and Pink1-MTS were shown in Figure 1(b). All of the resulting plasmids were sequenced to verify that the clones had the correct sequence.

2.2. Cell Transfection and Observation with Fluorescence Microscope. COS-7 and HeLa cells were obtained from the ATCC (American Type Culture Collection). Both cell lines were maintained at 37°C under humidified conditions and 5% CO_2 and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (MRC, Jiangsu, China). COS-7 and HeLa cells were seeded into a 6-well plate with coverslips at 2×10^5/well and routinely incubated for 8-12 h without light. The mtmCherry plasmids were transfected into the cells with Hieff Trans™ Liposomal Transfection Reagent (Shanghai YESEN Biotechnology Co., Ltd.). At 30 h post-transfection, the coverslips were taken out, and the cells were fixed in PBS with 4% paraformaldehyde for 10 min at room temperature (RT), permeabilized and blocked with sealing fluid (0.3% Triton X-100 and 2% BSA in PBS) for 1 h at RT. The cells were incubated with COX IV antibody diluted in sealing fluid overnight at 4°C, followed by incubation with secondary antibodies (green fluorescence) diluted in sealing fluid for 1 h at 37°C. The coverslips were mounted onto microscope slides; mCherry and COX IV expressions were observed under fluorescence microscope. The images were processed for analyzation.

2.3. ROS Detection. HeLa cells were transfected with empty vector and mtKR plasmids for 30 h and exposed to visible light for 10, 30, and 60 min, respectively, then at 10, 30, and 60 min after exposure, 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, MO, USA) was added into the cells. Finally, the mean fluorescence intensity (MFI) was detected by Cytation™ 3 Cell Imaging Multi-Mode Reader System (BioTek, Winooski, Vermont, USA). There were 6 replicate wells per group, and the experiment was performed in triplicate.

2.4. Detections of Na^+-K^+ and Ca^{2+}-Mg^{2+} ATPase Activities and ATP Content. At 12 h postlight exposure, HeLa cells were irradiated by 4 Gy X-rays with X-RAD 320iX machine (Precision X-ray, Inc., USA), at 24 h postirradiation, the cells were homogenized using homogenate medium (pH 7.4, 0.01 M Tris-HCl, 0.001 M EDTA-2Na, 0.01 M saccharose, and 0.8% NaCl) (Nanjing Jiancheng Bioengineering Institute, China), and protein concentrations were determined. Na^+-K^+ and Ca^{2+}-Mg^{2+} ATPases and ATP were measured using biochemical assay kits (Nanjing Jiancheng Bioengineering Institute, China) and a spectrophotometer (Beckman, USA) with 636 nm excitation wavelengths. There were 4 replicate wells per group, and the experiment was performed in triplicate.

2.5. Flow Cytometry (FCM). Rhodamine123 (Rh123, Sigma-Aldrich, St. Louis, MO, USA) was used to detect $\Delta\psi_m$, and Annexin V-FITC/PI kit (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was used to measure apoptotic rate. The collected cells were resuspended at 12 h postirradiation, then, Rh123 was added into the cells to yield final concentrations of 5 μM for detecting $\Delta\psi_m$ and stained with 10 μl Annexin V-FITC and PI for 15 min in the dark for detecting apoptotic rate. Then the $\Delta\psi_m$ and apoptotic rate were detected by FCM (Becton, Dickinson and Company,

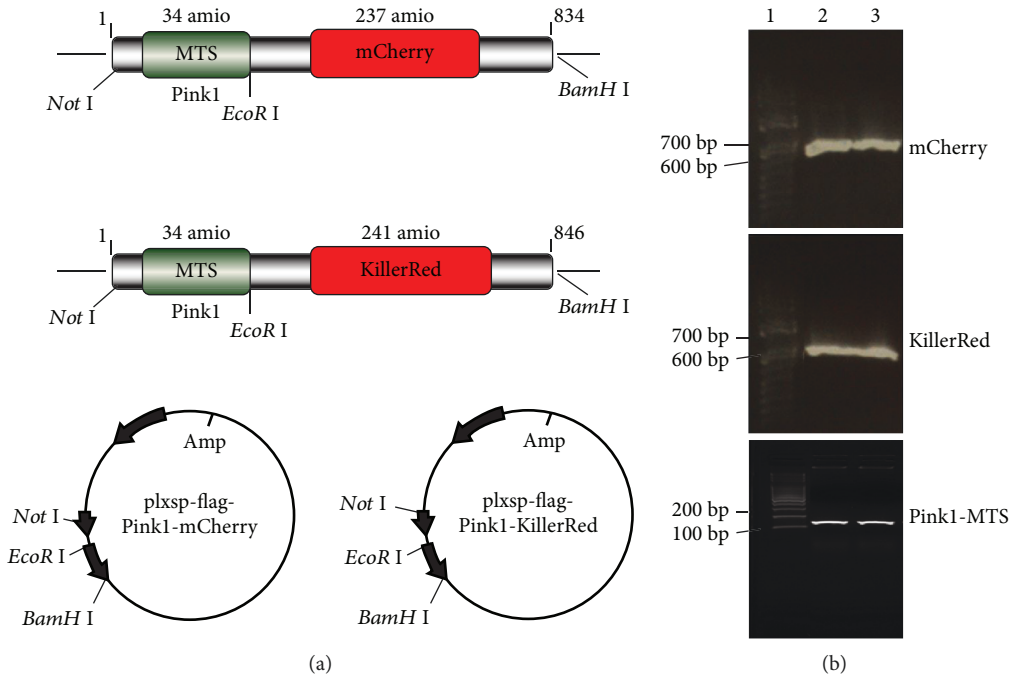


Figure 1: Development of vectors for mtmCherry and mtKR. (a) Schematic diagram of mtmCherry and mtKR vectors: Pink1-MTS was cloned into empty vector (plxsp-flag) (*Not* I and *EcoR* I sites); mCherry and KillerRed were cloned into plxsp-flag-Pink1-MTS (*EcoR* I and *BamH* I sites). (b) PCR products of mCherry, KillerRed, and Pink1-MTS. Lane 1 was 100 bp DNA Marker; lane 2 and 3 were PCR amplification products.

Franklin Lakes, NJ, USA). For each sample, at least 1×10^4 cells were collected. There were 4 replicate wells per group. The experiment was performed in triplicate.

2.6. Quantitative Real-Time PCR (qRT-PCR). Total RNA was extracted with TRIzol reagents (Invitrogen, Carlsbad, CA, USA), and the complementary DNA (cDNA) was synthesized using a high-capacity reverse transcription kit (Takara Bio Inc., Japan). The reverse transcription of 1 μg RNA was performed according to the protocol, and the reaction was incubated at 42°C for 60 min, then at 70°C for 2 min. GAPDH: 5′-ACCACAGTCCATGCCATCAC-3′(F), 5′-TCCACCACCCTGTTGCTGTA-3′(R); Cyt c: 5′-GGGCGAGAGCTATGTAATGCAAG-3′(F), 5′-TACAGCCAAAGCAGCAGCTCA-3′(R); caspase-9: 5′-GGACATCCAGCGGGCAGG-3′ (F), 5′-TCTAAGCAGGAGATGAACAAAGG-3′ (R); caspase-3: 5′-TTCAGGCCTGCCGTGGTACA-3′(F), 5′-CCAAGAATAATAACCAGGTGCT-3′(R). The qRT-PCR reaction was performed and analyzed (Bio-Rad, Hercules, CA, USA) according to SYBR® Premix Ex Taq ™ II kit (Takara Bio Inc., Japan) protocol.

2.7. Mitochondrial Protein Extraction. The cells were washed with 0.01 M PBS and collected at × 200 g for 5 min, added with 3 ml mitochondrial separation reagents (Beyotime® Biotechnology, Hangzhou, China) consisting of PMSF and put on ice for 10 min. The cell homogenate was transferred into glass homogenizer, performed for 30 min, and centrifuged at × 600 g at 4°C for 10 min. The suspension was transferred to another tube and centrifuged at × 11000 g at 4°C for 10 min. When the suspension was removed after centrifugation, the mitochondria were obtained. Then the mitochondrial proteins were extracted and quantitatively determined.

2.8. Western Blot. After the total proteins were extracted and quantitatively determined, 40 μg proteins were separated by SDS-PAGE (10% resolving gel, 5% stacking gel) and transferred to NC membrane (200 mA, 1.5 h; Merck Millipore, Billerica, MA, USA). After blocking with 5% nonfat dry milk, the membranes were incubated with diluting solution (1 : 200) of the primary antibodies including anti-VDAC1, anti-HSP60 and anti-Cyt c (Bioworld Technology Inc., USA), anti-caspase-9 (cleaved) and anti-caspase-3 (cleaved) (Cell Signaling Technology, Danvers, MA, USA), and anti-GAPDH (Santa Cruz, CA, USA), respectively, overnight at 4°C. After washing with TBST, the membranes were incubated with IgG-HRP-conjugated secondary antibody (ImmunoWay, Plano, TX, USA) at 1 : 1000 dilution for 1.5 h at RT. Finally, the membranes were identified using an enhanced chemiluminescence detection system (ECL detection kit, Santa Cruz, CA, USA). The films were scanned for the following gray scale ratio analysis.

2.9. Statistical Analysis. All the data were analyzed using SPSS, version 24.0 (SPSS Inc., Chicago, IL, USA). The results were presented as mean ± SD and subjected to one-way ANOVA followed by Student's t-test; $P < 0.05$ was considered as significant.

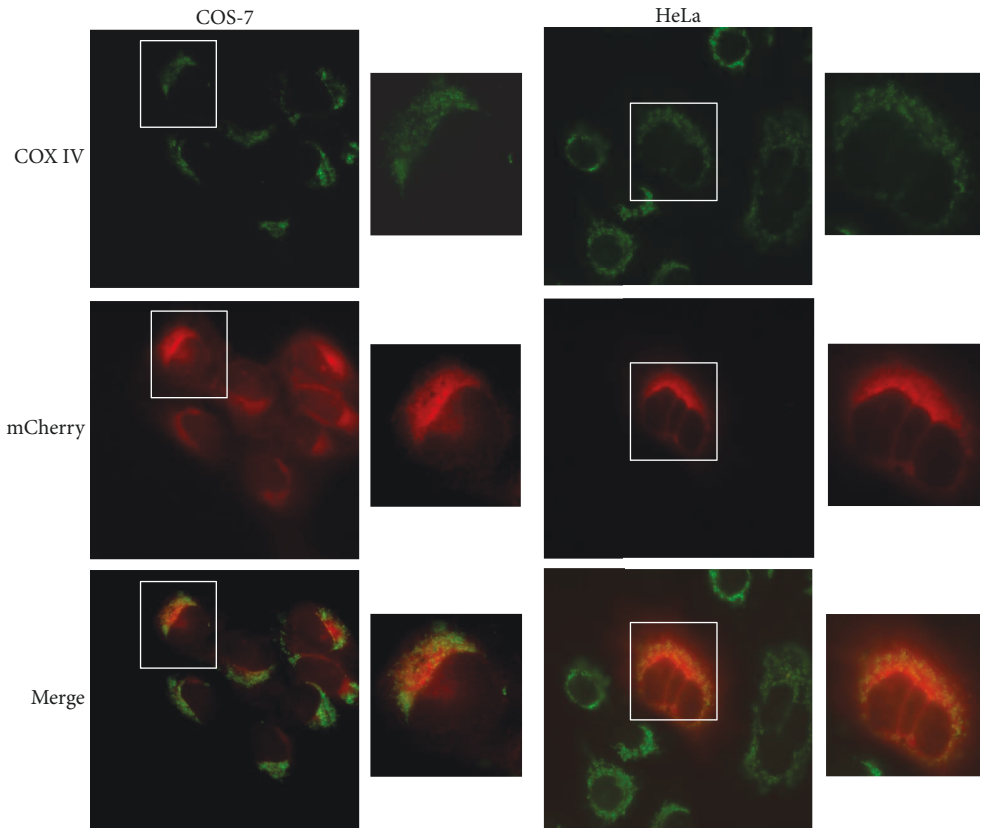


Figure 2: The mtmCherry colocalized the mitochondria with COX IV, ×200. COS-7 and HeLa cells were transfected with mtmCherry plasmids. At 30 h posttransfection, the cells were stained with COX IV, and COX IV (green) and mCherry (red) expressions were observed.

3. Results

3.1. The mtmCherry Protein to Localize Mitochondria. As shown in Figure 2, the fluorescence images clearly indicated that COX IV expressed in the mitochondria, and mCherry also specifically localized to the same sites. Hence, it demonstrated that Pink1-MTS sequence might mediate mCherry to localize mitochondria.

3.2. ROS Induced by mtKR Exposed to Visible Light. As shown in Figure 3(a), before light exposure, there were a large amount of red cells and very few green cells; after light exposure, red cells decreased and green cells increased, indicating ROS production. As shown in Figure 3(b), at 60 min post-10 or -30 min light exposure, MFIs reached for maximum value, but at 30 or 60 min post-60 min light exposure, MFIs reduced. Taken together, these results indicated that light exposure caused the inactivation of mtKR protein and the increase of ROS.

3.3. Mitochondrial Dysfunction Caused by mtKR and Irradiation. The Na^+-K^+ and Ca^{2+}-Mg^{2+} ATPase activities and ATP content significantly decreased after light exposure and irradiation (Figure 4(a)). Additionally, even though $\Delta\psi_m$ significantly reduced, it had similar change regularity as ATPase activities (Figure 4(b)). As shown in Figures 4(c) and 4(d), VDAC1 expressions in total and mitochondrial proteins were all increased, but after 4 Gy irradiation, VDAC1 decreased in total protein. Taken together, these results showed that mtKR-induced ROS and X-rays caused mitochondrial dysfunction, and MPTP was kept in opening status.

3.4. Changes of Apoptotic Rate Caused by mtKR and Irradiation via Cyt c/Caspase-3 Pathway. At 12 h postirradiation, early apoptotic rates caused by mtKR exposure to light were significantly increased, and 4 Gy X-rays also induced the increase of apoptotic rate (Figure 5(a)). As shown in Figure 5(b), at 24 h postirradiation, the mRNA expressions of Cyt c, caspase-9, and caspase-3 dramatically increased. And caspase-9 and -3 proteins were cleaved into active fragments in total proteins; Cyt c protein expression reduced in mitochondrial protein; however, it increased in total protein (Figures 5(c) and 5(d)).

4. Discussion

Radiotherapy is the major means of cancer treatments, and its major feature is the induction of toxic oxidative damage in targeted cancer cells. Under normal physiological condition, cells maintain a basal redox balance between prooxidative and antioxidative reactions [30]. During radiation, ROS generated from water by radiation energy deposition can oxidize DNAs, proteins, and lipids and target mitochondria to

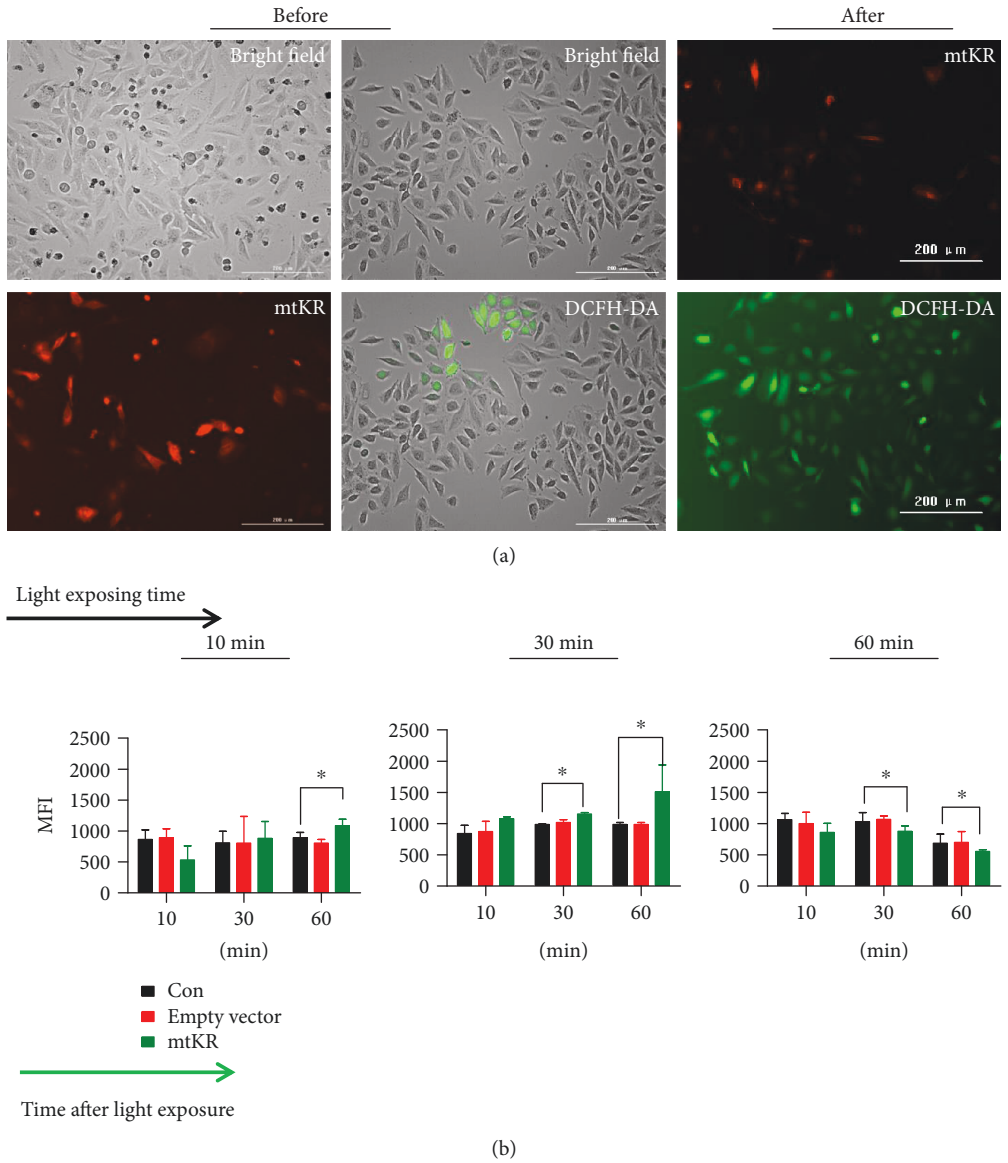


Figure 3: ROS changes measured by DCFH-DA staining. (a) The images of mtKR (red) before or after light exposure for 30 min in HeLa cells and cells stained by DCFH-DA (green), scale bars: 200 μm. (b) The changes of MFIs at different time postlight exposure for 10, 30, and 60 min, respectively. The bars represent the mean ± SD of triplicate measurements. $^*P < 0.05$ versus control.

cause mitochondrial dysfunction and final cell death. Moreover, the ROS has extremely short lifespan and a limited diffusion distance leading to low killing efficiency to tumor cells and unsatisfactory therapeutic effects [31–33]. In this study, based on KillerRed-induced ROS, we utilized Pink1-MTS to mediate mitochondrial localization, and our results also indicated our hypothesis. In addition, our data showed that mtKR might promote mitochondrial ROS burst. The nature of the cytotoxicity of KillerRed, a generator of ROS, therefore offers a significant opportunity to genetically investigate the mechanisms regulating cellular responses.

Mitochondrion is an ancient organelle generating approximately 90% of cellular ATP via oxidative phosphorylation [34]. Unlike normal cells, there is an abnormal redox status in cancer cells, which is unable to regulate redox homeostasis [35]. It is postulated that mitochondrial dysfunction in cancer cells would affect the relative cellular ATPase activities, ATP production, and subsequent apoptosis and migration processes. In the present study, the relative ATPase activity and ATP in HeLa cells transfected by mtKR plasmids were significantly decreased. Moreover, various evidences suggest that the mitochondrial dysfunction plays a key role in oxidative stress [36, 37], and ROS generation impairs mitochondrial electron transport chain [38]. The decline in $\Delta\psi_m$ is an earlier event in the process of cell death, and we also showed that mtKR-induced ROS can result in the loss of $\Delta\psi_m$. VDAC is the most abundant protein in the outer mitochondrial membrane, and the fact that VDAC plays a role in MTPT is undeniable, so it has long been considered to be a candidate for the outer membrane component of

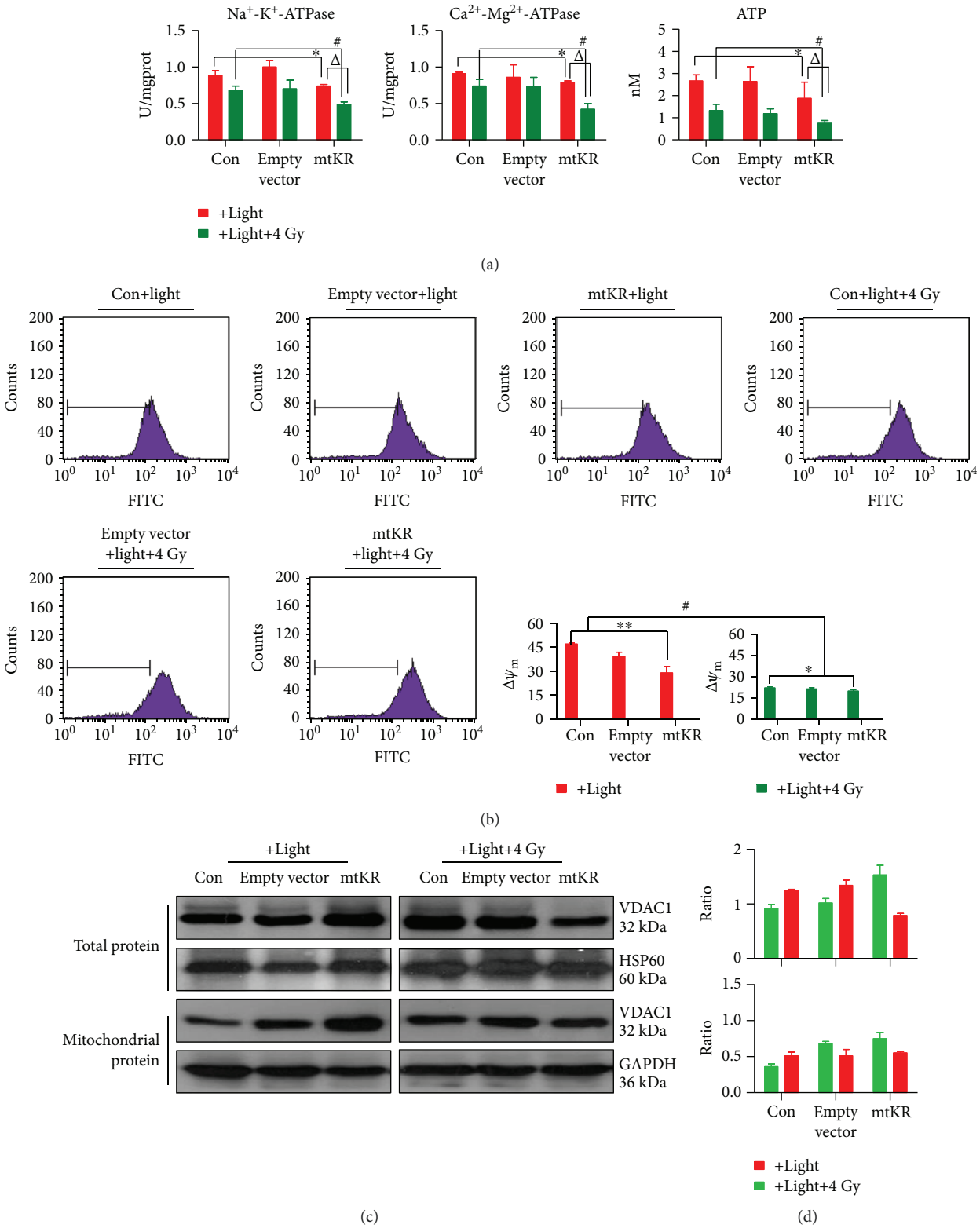
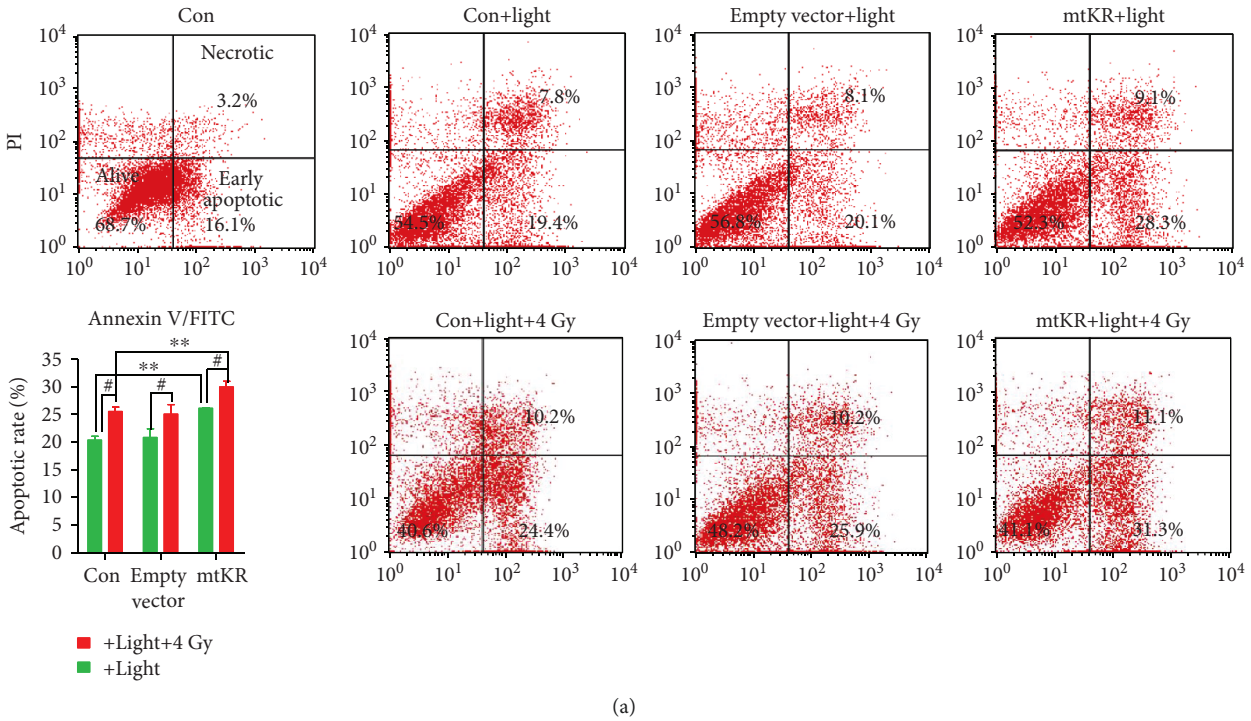
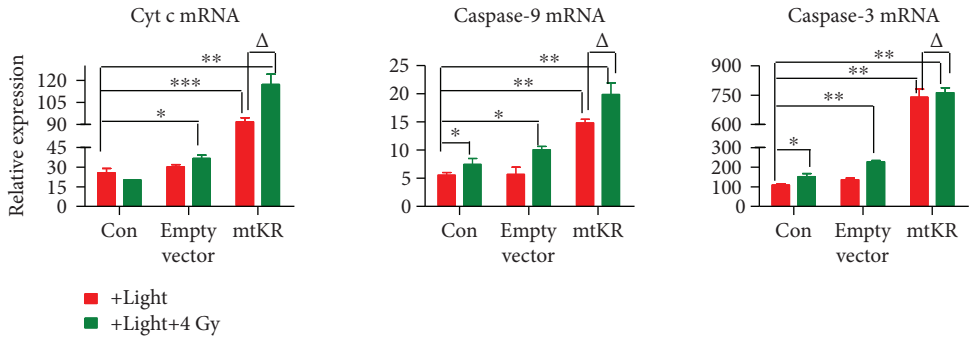


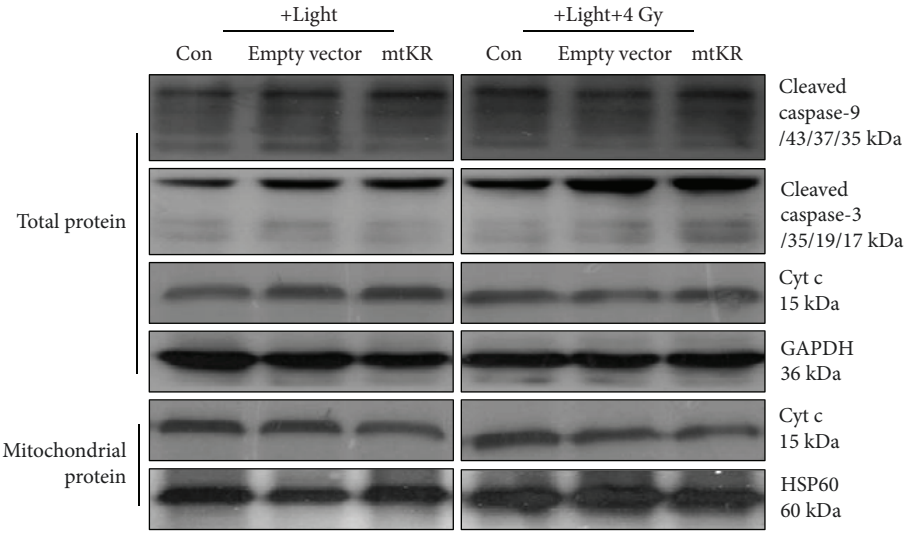
Figure 4: Mitochondrial dysfunctions caused by mtKR-induced ROS and X-rays. (a) The changes of Na^+-K^+ and Ca^{2+}-Mg^{2+} ATPase activities and ATP content by biochemical assay after light exposure and irradiation. (b) The FCM pictures of $\Delta\psi_m$. HeLa cells were stained by Rh123, followed by FCM analysis of the $\Delta\psi_m$. (c) Western blot analysis was performed to determine the protein levels of VDAC1 in total and mitochondrial protein. GAPDH and HSP60 proteins were used for loading control. (d) From top to bottom, the gray ratios of VDAC1/GAPDH and VDAC1/HSP60. The bars represent the mean ± SD of triplicate measurements. $^*P < 0.05$ versus control; $^\#P < 0.05$ versus 4 Gy irradiation, and $^\triangle P < 0.05$ versus light exposure.



(a)



(b)



(c)

Figure 5: Continued.

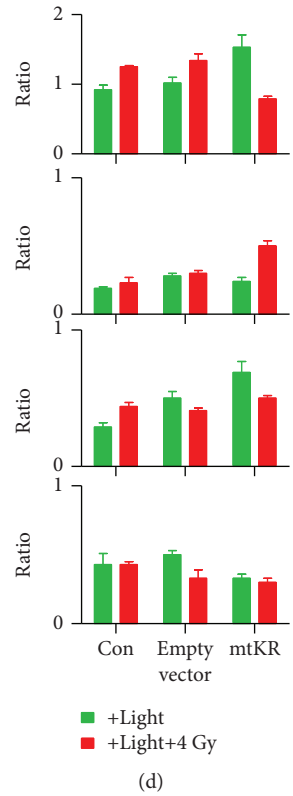


Figure 5: Apoptosis induced by mtKR and irradiation via Cyt c/caspase-3 pathway. (a) The FCM pictures of apoptosis and FCM analysis in HeLa cells stained by Annexin V/FITC and PI; the apoptotic population was defined as early apoptosis (lower right, green of FITC staining). (b) Cyt c, caspase-9, and caspase-3 mRNAs were detected by qRT-PCR. (c) Western blot was performed to determine the protein levels of Cyt c, caspase-9, and caspase-3 in total and mitochondrial proteins. GAPDH and HSP60 proteins were used for loading control. (d) From top to bottom, the gray ratios of cleaved caspase-9/GAPDH, cleaved caspase-3/GAPDH, Cyt c/GAPDH, and Cyt c/HSP60. The bars represent the mean ± SD of triplicate measurements. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$ versus 4 Gy irradiation, and $^{\triangle}P < 0.05$ versus light exposure.

the MPTP [39]. Recently, it has been shown that the opening of VDAC is a regulated process, and VDAC may exhibit some degree of specificity in the mitochondrial import/export of molecules (e.g., ATP, Ca^{2+}, and other ions) [40]. In some studies, VDAC1-deficient mitochondria isolated from a mutant yeast strain failed to exhibit the Bax/Bak-induced $\Delta\psi_m$ loss and Cyt c release that was observed with VDAC1-expressing control mitochondria [41]. Our results showed that VDAC1 protein expression significantly increased in total and mitochondrial proteins, indicating an opening status of the mitochondrial membrane. In addition, it is well known that radiation-induced increase in ROS causes DNA damage, cell cycle arrest, and activation of some transcription and apoptotic factors [42, 43]. Thus, the hypothesis that mtKR aggravates the mitochondrial dysfunction induced by radiation is understandable. Interestingly, our results also verify this hypothesis.

There are two major pathways in apoptosis [44]. One involves death receptors and is marked by Fas-mediated caspase-8 activation, and the other is the stress- or mitochondrial-mediated caspase-9 and -3 activation. Mitochondria are the major source of ROS production in cells, in turn, the most adversely affected organelles [45]. To better understand the mechanism that ROS leads to apoptosis, we demonstrated in this study that an acute burst of ROS in the mitochondria specifically resulted in the apoptosis, the subsequent Cyt c release and activation of caspase-9 and -3. Our results showed a promoting role on the apoptosis resulting from mtKR, which might be enhanced by radiation, and had impressive significance for tumor radiotherapy. The releases of Cyt c as well as other proteins from the mitochondria and cytosol appear to play a central role in the induction of the apoptotic cascade that ultimately leads to the programmed cell death [46]. To further explore the mechanisms, we analyzed the transcriptional levels and protein expressions of Cyt c, caspase-9, and caspase-3 at 24 h postirradiation, and their mRNA levels all increased. Interestingly, Cyt c protein expression increased in total protein however reduced in mitochondrial protein. Moreover, radiation could enhance these effects of mtKR, which indicated that Cyt c was released from the mitochondria. Under the downstream of mitochondrial apoptotic pathway, caspase-9 and -3 were activated (Figures 5(c) and 5(d)), which made us believe mtKR induced-ROS might act synergistically with radiation to induce the apoptosis via Cyt c/caspase-3 pathway.

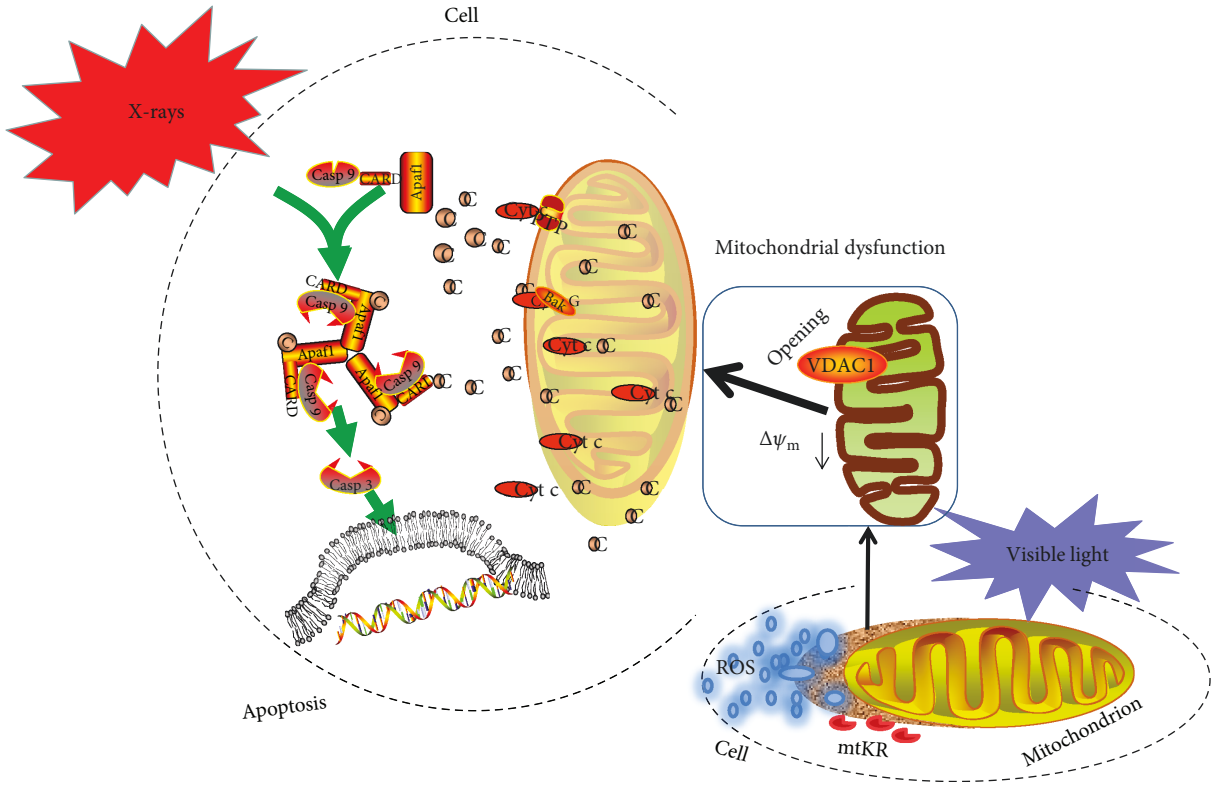


Figure 6: The proposed scheme of apoptosis induced by mtKR and radiation via Cyt c/caspase-3 pathway.

In conclusion, as illustrated in Figure 6, this present study demonstrates that the mitochondrial targeting characteristics of Pink-MTS and ROS increased by mtKR exposure to visible light in HeLa cells and then to impaired mitochondrial function. When ATPase activities and ATP content as well as $\Delta\psi_m$ reduced, and VDAC1 expression increased, the cell apoptosis also increased dependently on the Cyt c/caspase-3 pathway. Notably, mitochondrial dysfunction and final apoptosis enhanced by radiation has provided a new strategy for ROS sensitization in future clinical cancer therapy.

Data Availability

In these studies, all data were obtained by PCR technique, flow cytometry (FCM), biochemical assay, observation by fluorescence microscope, quantitative real-time PCR, and Western blot, and some pictures were plotted using different tools, such as BVTech plasmid software, Adobe Photoshop CS2 software, SPSS 24.0 version, GraphPad prism 6.0 software, and PPT of Microsoft office, Science-Slides, etc. The data (the schematic diagram of vector construction, the cell picture under fluorescence microscope, some pictures drawn with experiment results by GraphPad prism 6.0 software, FCM pictures, Western blot pictures, and the proposed scheme of conclusions) used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Xin Li and Fang Fang contributed equally in this work.

Acknowledgments

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