Research Article

The Effect of Hydroxybenzoate Lithium Complexes in Inducing Apoptosis in HT-1080 Human Fibrosarcoma Cells

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There has been a growing interest in the beneficial effects of simple phenolic acids and their ability to exhibit various biological activities. The aim of this study was to assess *in vitro* biological activities of 2-, 3-, and 4-hydroxybenzoate lithium (HBLi) complexes on HT-1080 human fibrosarcoma cells by methods of using a metabolic activity assay, immunochemical and morphological techniques. Results showed that HBLi complexes exert their cytotoxic activities in a concentration- and chemical structure-dependent manner in the following order: 4-HBLi > 3-HBLi > 2-HBLi. Flow cytometry displayed evidence of apoptosis induced by 3-HBLi (21.8%) and 4-HBLi (33.2%). These results were verified by SEM, which revealed the formation of apoptotic bodies. In addition, these 3-HBLi and 4-HBLi caused an increase in HT-1080 cell cycle arrest in G0/G1 phase when compared to the controls (25% and 30.6%, resp.) when cells were treated with 6 mM for 24 hours. Immunochemical studies related to the molecular mechanism of apoptosis indicated that HBLi complexes downregulated the expression of Bcl-2 and upregulated Bax, p53, and caspases-3 in a concentration-dependent manner. HBLi complexes lowered Bcl-2/Bax ratios and induced the expression of p53 and caspase-3. These results suggest that HBLi complexes may exert their apoptotic effects through mitochondrial-mediated, caspase-dependent, apoptotic mechanisms.

1. Introduction

Hydroxybenzoic acids (HBA) are a group of molecules that belong to simple phenolic acids, a major class of plant secondary metabolites widely distributed in plants. The parent molecule in this group is benzoic acid, encompassing three substituted hydroxyl groups analogous at *ortho*-, *meta*-, and *para*- positions, or C1, C2, and C3, respectively. These compounds exert various biological activities, including antioxidant, anticancer, and antimicrobial properties [1–3]. In addition, they exhibit different physicochemical properties and interactions with the primary metabolites and display various biological properties [4–6]. HBAs readily interact with metal ions to form hydroxybenzoate (HB) complexes, which are more biologically effective than HBAs or the free metal ions. Zinc salicylate, for example, was found to be more

potent than salicylic acid and acetylsalicylic acid in terms of its ability to inhibit cell proliferation and induce apoptosis in cancer cells [7, 8]. Metal complexes, particularly transition element metals, interact with DNA noncovalently producing changes in the structure of the DNA, thereby interfering with replication and transcription processes [9, 10]. Interest in the therapeutic use of medicinal inorganic chemistry has led to the development of different anticancer agents, including cisplatin, carboplatin, and bis(thiosemicarbazone) [11, 12].

It has long been appreciated that transition organometallic complexes modulate molecular apoptotic pathways of cancer cells. Similarly, monovalent (Na^+) and divalent (Ca^{2+}) 2-HB complexes were also found to exhibit these biological effects but at concentrations between 2 and 20 mM [6, 13, 14]. Generally, each organometallic complex has its own chemical/biochemical mode of interaction, particularly in terms of

FIGURE 1: The chemical structure of HBLi complexes.

interactions with DNA. Therefore, HBLi complexes should be considered as distinct compounds and cannot be conflated with other complexes. Given the significant activity of 2-HB metal ion complexes, we examined the apoptotic effects of 2-, 3-, and 4-HBLi complexes on human HT-1080 fibrosarcoma cells. These HBLi complexes have the same molecular formula but different positions of the hydroxyl group (Figure 1).

2. Materials and Methods

2.1. Cell Culture. HT-1080 fibrosarcoma cell line (American Type Culture Collection; Rockville, MD, USA) was cultured at 37°C in a humidified atmosphere containing 5% CO₂. An optimal Dulbecco's Minimum Essential Medium (DMEM, Gibco, USA) was supplemented with 10% fetal bovine serum (FBS) and 1% gentamicin-streptomycin (GIBCO BRL), 1% L-glutamine, 0.1% hepes buffer (1 M), 0.1% sodium pyruvate (104 1M), and 0.1% ascorbic acid. HT-1080 cells were cultured for 3 days (based on the growth curve of HT-1080 cells), giving approximately 70% confluence and then further for another 24 h and 72 h with different concentrations of HBLi complexes.

2.2. Cell Metabolic Activity. HT-1080 cells were seeded at 5×10^4 cells per well in a 96-well plate for 3 days under optimal growth conditions. The medium was replaced with fresh medium with or without increasing concentrations of HBLi complexes (0.1–8 mM) for up to 72 h. Cell metabolic activity was measured using *meta*-(4,5-dimethylthiazol-*para*-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent. The assay detects the ability of the cells to reduce tetrazolium salts to coloured insoluble formazan by using the mitochondrial enzyme succinate dehydrogenase. 10 μ L MTT solution was added in each well and incubated at 37°C for 1 h then followed by the addition of 180 μ L DMSO to dissolve the formazan crystals. The absorption was then read colorimetrically at 575 nm. The results shown are the composite of two separate experiments, and each was conducted in triplicate.

2.3. Annexin V-FITC. The effect of hydroxybenzoate lithium complexes on HT-1080 cells was measured by Annexin V-FITC apoptotic assay (Bender Medsystems, Vienna, Austria). 5×10^5 cells were treated with an increasing concentration of HBLi complexes for up to 72 h at 37°C in 25 cm² flask. Cells were trypsinized with 2 mL trypsin, and the cell concentration was adjusted to 10⁶ cells/mL. 0.5 mL of the cell suspension was seeded in Eppendorf tubes, and 10 μ L Media Binding Reagent was added in each tube before 1.25 μ L

Annexin V-FITC was added. This was followed by incubation of cells at room temperature for 15 min before removing the medium, gentle resuspension of cells, and labelling with 10 μ L propidium iodide. The samples were then immediately analyzed by flow cytometry.

2.4. Scanning Electron Microscopy. HT-1080 cells were seeded into 12-well plates containing microscopic slide cover slips at a density of 15×10^3 cells and incubated for 48 h with 2 mM or 6 mM HBLi complexes. Cells were fixed for 1 h with 0.8% glutaraldehyde, 0.6% osmium tetroxide, 2 mM CaCl₂, and 0.2 M sucrose in 0.1 M cacodylate buffer pH 7.4. HT-1080 cells were then washed several times with PBS buffer and dehydrated using a sequence of alcohol concentrations (30%, 50%, 70%, and 90% each for 5 min and 100% for 10 min twice). The dehydrated HT-1080 cells were then dried to the critical point in Blazers CPD 030 using CO₂; cells were mounted onto 12 mm "Philips type" aluminium stubs using silver paint was then gold sputter coated in an Edwards S150B sputter coater. Finally, the samples were imaged using a Philips XL20 SEM.

2.5. Immunoblot Analysis. HT-1080 cells were seeded in six-well plates at 3×10^4 initial density and cultured in DMEM medium. Cells were allowed to grow for 48 h in the presence of 0.2 or 6 mM of 2-HBLi, 3-HBLi, or 4-HBLi under optimal culture conditions and were washed with cold PBS (10 mM, pH 7.4) to remove any remaining medium. This step was followed by adding 200 μ L of 2x sample buffer (250 mM Tris-HCl pH 6.8, 4% SDS, 0.006% bromophenol blue, 2% β -mercaptoethanol; Pharmacia, Uppsala, Sweden) to each well, and the cells were then harvested using a cell scraper. HT-1080 cells were treated with lysate buffer after which they were transferred to a 1.5 mL Eppendorf tube, heated at 100°C for 10 min, cooled to room temperature, and centrifuged at 12000 $\times$ g for 5 min. The supernatant was further centrifuged at 4°C and 16000 $\times$ g for 5 min to obtain a clear solution of protein mixture, which was used to measure the expression of p53, Bcl-2, Bax, and caspase-3 by Western blotting. β -Actin was used as the internal standard. Total cell lysate protein concentrations were determined according to a reported method [15].

Thirty micrograms (22 μ L) of protein samples and 10 μ L of molecular marker were loaded on to 4–12% bis-tris acrylamide gel in NuPAGE MOPS (*meta*-(N-morpholino) propanesulphonic acid) SDS running buffer (Invitrogen, Life Technologies, Scotland, UK). After running the gel at 200 V for 30–50 min, resolved proteins were transferred on to a nitrocellulose membrane (Sigma). Membranes were incubated first with an appropriate primary antibody (p53, Bcl-2,

FIGURE 2: Time- and concentration-response curves of treated HT-1080 cells with HBLi complexes. The data was derived from MTT cell viability assay after HT-1080 cells cultured with 0–8 mM HBLi complexes for 24 h, 48 h, and 72 h under optimal conditions. (a) Cell viability after treatment with 2-HBLi, (b) cell viability after treatment with 3-HBLi, (c) cell viability after treatment with 4-HBLi, (d) comparison of HT-1080 cell viability after treatment with 2-, 3-, or 4-HBLi at 72 h, and (e) an example of the cell density for treated HT-1080 with 0, 2, and 6 mM 4-HBLi, 72 h.

Bax, or β -actin as a loading control and internal standard), then with peroxidase conjugated anti-mouse IgG antibody (Sigma). They were then washed and developed using a chemiluminescent reagent (Amersham, UK) and then were exposed to photographic films. The protein bands intensities were scanned and quantified using a densitometer.

2.6. Statistical Analysis. Data obtained in these experiments were evaluated using equal variance and paired Student's *t*-test. In addition, Pearson correlation coefficients were calculated, along with other statistical analyses, using Graphpad Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA).

FIGURE 3: The apoptotic effect of 3-HBLi and 4-HBLi on HT-1080 cells by annexin-V/propidium iodide after treatment for 72 h under optimal culture conditions ($n = 3$).

3. Results

3.1. Cytotoxic Effects of HBLi Complexes on HT-1080 Fibrosarcoma Cells. To assess the cytotoxic effect of HBLi complexes, HT-1080 human fibrosarcoma cells were cultured with increasing concentrations of 2-, 3-, and 4-HBLi complexes for 24 h, 48 h, and 72 h under optimal conditions. The results of the MTT assays showed a concentration- and time-dependent decrease in cell metabolic activity (Figures 2(a)–2(e)). The cytotoxic effect was also dependent on the HBLi analogue. 4-HBLi induced the highest antiproliferative activity compared to 2-HBLi and 3-HBLi (Figure 2). These results suggest an association between the molecular structure and cytotoxicity. 3-HBLi and 4-HBLi reduced HT-1080 cell proliferation by 7.75% and 51.16%, respectively, compared to 2-HBLi at 4 mM. At higher concentrations (i.e., 6 mM and 8 mM), both 3- and 4-HBLi complexes exerted more cytotoxic effects on HT-1080 cells (Figures 2(a)–2(d)).

3.2. Immunochemical and Morphological Assessment of Apoptosis Induced by HBLi Complexes. Figure 3 shows the results that were obtained from the annexin-V/propidium iodide plots for treated HT-1080 cells with 2 mM and 6 mM 3-HBLi or 4-HBLi for 72 h. Both 3-HBLi and 4-HBLi induced apoptosis in a concentration-dependent manner. 3-HBLi induced apoptosis (early and late) by at least 18.3% at 2 mM (Figure 3). The induction of apoptosis increased to 32.3% when HT-1080 cells were treated with 6 mM 3-HBLi. Furthermore, 4-HBLi induced apoptosis when HT-1080 cells were treated with 6 mM (Figure 3).

In order to confirm apoptotic cell death, HT-1080 cells were also assessed morphologically by SEM after exposure to 2 mM and 6 mM 3-HBLi or 4-HBLi for 48 h. Figure 4 demonstrates evidence of apoptotic effects of both complexes. Untreated HT-1080 cells showed a flat monolayer structure and retained attachment to the surface as well as pseudopodia attachment to each other. These features were not evident in cells treated with 3-HBLi and 4-HBLi and instead were replaced by the appearance of apoptotic bodies (Figure 4).

3.3. The Effects of HBLi Complexes on the Cell Cycle Distribution of HT-1080 Cells. The effect of 3-HBLi and 4-HBLi on dividing HT-1080 cell cycle phases were analysed based on the DNA contents after culturing for 24 h. Treatment of HT-1080 with these complexes did not induce a significant increase in the cell population at the sub-G1 phase at this time point. However, there was a significant increase in G0/G1 phase following exposure to 3-HBLi and 4-HBLi ($P = 0.0204$ and $P = 0.0135$, respectively; Figure 5). In the case of 4-HBLi, there was a clear concentration-dependent increase in G0/G1 ($P = 0.0315$). The arrest of HT-1080 cells in G0/G1 resulted in a suppression of cell progression to the S-phase. As a result of 3- or 4-HBLi treatment, DNA content at S-phase decreased in a concentration-dependent manner. At 6 mM concentration, 4-HBLi (36.0%) induced a significant reduction in S-phase by approximately 1.9-fold compared to 3-HBLi (18.9%). These results indicate that 3- and 4-HBLi exert an antiproliferative activity that is to some extent dependent upon the chemical structure (Figure 5).

3.4. The Effects of HBLi Complexes Pro- and Antiapoptotic Gene Expression. In order to assess the response of treated HT-1080 cells with HBLi complexes for 48 h, pro- and anti-apoptotic proteins was analysed using Western blot analysis. The expression of β -actin was used as a loading control. Figure 6(a) shows that HBLi complexes decreased the expression of Bcl-2 in HT-1080 cells between 19 and 68% compared to the untreated cells. The level of Bcl-2 decreased depending upon the concentration and chemical structure of the HBLi complex that was used. The trend of Bcl-2 downregulation followed the following order: 2-HBLi (19% and 36%) < 3-HBLi (40% and 60%) < 4-HBLi (53% and 68%) at 2 mM and 6 mM, respectively (Figure 6(a)). In contrast, the treatment of HT-1080 cells with 2 mM or 6 mM HBLi complexes showed a concentration-dependent increase in the expression of Bax (Figure 6(b)). Western plots analysis of Bax indicated that 2 mM and 6 mM 2-HBLi increased their expression by 26% and 29%, respectively. At the same concentrations, 3-HBLi further increased Bax expression between 36% and 44%. In addition, 4-HBLi upregulated Bax by 45% and 51% when HT-1080 cells exposed to 2 mM and 6 mM compared to control sample. In addition, the assignment of Bcl-2/Bax ratio by Western blotting (Figure 6(c)) showed that a low ratio was characteristic for the sensitivity of HT-1080 cell population to HBLi complexes. The ratios decreased in a concentration- and chemical structure-dependent manner in the following order: 4-HBLi > 3-HBLi > 2-HBLi. At 2 mM concentration, 4-HBLi was significantly higher than 3-HBLi ($P = 0.015$) and 4-HBLi ($P = 0.0002$) while at 6 mM 4-HBLi was significantly higher than 3-HBLi ($P = 0.0053$) and 2-HBLi ($P = 0.0016$).

Furthermore, the proapoptotic protein p53 (Figure 6(d)) increased in the range of 29–57% and in a concentration-dependent manner. p53 was upregulated by 29% and 31% when HT-1080 cells were treated with 2 mM and 6 mM 4-HBLi, respectively. The upregulation of p53, induced by 2 mM and 6 mM 3-HBLi, was further increased by 40% and 44% when treated cells were cultured for 48 h.

In addition, 4-HBLi upregulated p53 by 53% and 57% compared to control samples (Figure 6(d)). Caspase-3 was

FIGURE 4: Morphological evidence of apoptosis in treated HT-1080 cells with 3-HBLi and 4-HBLi grown for 48 h. (+) refers to normal HT-1080 cells, whereas cells retain their monolayer flat structure and pseudopodia attachments. (*) refers to apoptotic HT-1080 cells.

FIGURE 5: The effect of HBLi on HT-1080 cell's cycle. Cells were cultured at optimal conditions and treated separately with 2 and 6 mM 3-HBLi or 4-HBLi for 24 h.

also assessed immunochemically in treated HT-1080 cells. As shown in Figure 6(e), there is a concentration-dependent association between caspases-3 activation and HBLi complexes exposure. 2 mM and 6 mM 4-HBLi induced caspases-3 activation by 13% and 26%, respectively. Furthermore, 3-HBLi upregulated caspase-3 expression by 29% and 42% at both concentrations, respectively, compared to the control sample. In addition, exposing HT-1080 cells to 2 mM and 6 mM 4-HBLi caused further upregulation of caspase-3 by 35% and 43%, respectively. Computing the expression of Bcl-2/Bax ratios with p53 showed a negative correlation of $r = -0.5758$ ($P = 0.0009$) and $r = -0.7353$ ($P = 0.0001$), respectively (Figure 7).

4. Discussion

The most common HBA is salicylic acid, chemically known as 2-HBA, the precursor of 2-acetylbenzoic acid (ABA), or aspirin. These two drugs belong to a group of compounds collectively known as nonsteroidal anti-inflammatory drugs (NSAIDs), a class of agents that were first identified in 1897 [16]. These drugs possess simple chemical structures and two functional groups that can readily interact with primary

metabolites of the biological system. 2-substituted hydroxyl group at C2-position forms intramolecular H-bonding with carboxylic group while 3- and 4-hydroxyl groups display inductive and resonance effects on carboxylic group. As a result, 2-HBA is more acidic than 3-HBA and 4-HBA. Although, the acidity of HBLi complexes is less than HBA counterparts, the objective of this study was to determine whether 2-HBLi exerts different cytotoxicity compared to 2HBCa [14] and whether the position of the hydroxyl group at C3 and C4 makes a difference when considering the cytotoxic effects HT-1080 cells. In this respect, the reactivity of these molecules derives from their functional groups (mainly carboxylic), which is influenced by substituted hydroxyl groups at 2-, 3-, and 4-positions. Our previous research indicated that 4-HBZn selectively inhibited COX-2 and its transcription compared to ASA [6]. These complexes exhibited different cytotoxic and apoptotic effects in HT-1080 cells, suggesting different environments of interaction, and hence exhibit different efficacy. HBLi complexes induced antiproliferative effects in HT-1080 cells with the order of potency as followed: 4-HBLi > 3-HBLi > 2-HBLi complexes and in a concentration-dependent manner. In addition, HBLi complexes induced an arrest in G0/G1 of the cell cycle with no evidence of a G2 arrest. It may be possible that HBLi complexes exert their biological activities by direct interaction with the DNA. Research in this area indicated that organometallic complexes, particularly transition metal ion group, interact with DNA noncovalently producing changes in the structure of the DNA, thereby interfering with cell cycle and induce apoptosis [7, 17–22]. HBLi complexes inducing apoptosis, which was detected by annexin V labelling and confirmed morphologically by SEM, in concentration- and structure-dependent manners. Both the appearance of apoptotic bodies and the translocation of phosphatidylserine on the outer plasma membrane are common events during the induction of apoptosis that can be triggered by anticancer compounds *via* the extrinsic or intrinsic pathways [23–26]. The signalling of these pathway-dependent events involves the modulation of proapoptotic and antiapoptotic proteins, and their expression can be altered by anticancer drugs [25, 27]. In this respect, the molecular changes in the HBLi-treated HT-1080 cells included the upregulation of Bax and downregulation of Bcl-2 proteins to reduce Bcl-2/Bax ratios. Bax enhances apoptosis *via* the intrinsic pathway, which involves cytochrome c that regulates the expression of caspases-9 and then caspase-3 [23, 28, 29]. Other

FIGURE 6: Immunoblotting analysis of (a) Bcl-2, (b) Bax, (c) ratios of Bax/Bcl-2, (d) p53, and (e) caspase-3 protein expressions in treated HT-1080 cells cultured with different concentrations of 2-HBLi, 3-HBLi, and 4-HBLi for 48 h. β -Actin antibody was used as an internal standard. Immunoblotting of each protein was conducted on three independent lysates. The values for optical density (OD) were calculated by normalizing relative optical density values to that of the control. Thereby OD for control is 100% while treatment is either below or above 100%. Normalized values were calculated as ratios of $(OD_{\text{control}} - OD_{\text{treatment}})/OD_{\text{control}}$.

organometallic complexes, such as HB Na^+ , Ca^{2+} , and Zn^{2+} also modulated Bcl-2 and Bax protein expression [7, 30, 31]. In addition, HBLi complexes also induced p53 and caspase-3 in a concentration-dependent manner. The induction of p53 plays a vital role in regulating apoptosis and the cell cycle [32, 33]. Therefore, a loss of p53 enhances survival of cells with DNA damage and helps cancer progression [32–34]. The upregulation of p53 and caspase-3 in HBLi-treated HT-1080 cells is likely to agree with the decreasing Bcl-2/Bax ratios, as indicated from the negative correlation.

5. Conclusion

These results demonstrate that HBLi complexes exert apoptotic effects in a concentration- and chemical structure-dependent manner. The cellular modulation of HT-1080 cells by HBLi complexes shows an association between apoptosis induction and reduction in Bcl-2/Bax ratios, suggesting that it is a potential molecular marker for predicting response to HBLi complexes. Furthermore, although HBLi complexes exert cytotoxic effects in HT-1080 cells at relatively high

FIGURE 7: Pearson correlation coefficient pattern between Bcl-2/Bax ratios, p53, and caspases-3, respectively.

concentrations, they are more potent mole for mole than aspirin so the results here provide some important impetus for future research. Indeed, here we provide the first evidence that HBLi complexes may have therapeutic potential as anticancer agents.

Conflict of Interests

Authors declare that there is no any conflict of interests.

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