Tyrosinase overexpression promotes ATM-dependent p53 phosphorylation by quercetin and sensitizes melanoma cells to dacarbazine

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Abstract. Dacarbazine (DTIC) has been used for the treatment of melanoma for decades. However, monotherapy with this chemotherapeutic agent results only in moderate response rates. To improve tumor response to DTIC current clinical trials in melanoma focus on combining a novel targeted agent with chemotherapy. Here, we demonstrate that tyrosinase which is commonly overexpressed in melanoma activates the bioflavonoid quercetin (Qct) and promotes an ataxia telangiectasia mutated (ATM)-dependent DNA damage response. This response sensitizes melanoma cells that overexpress tyrosinase to DTIC. In DB-1 melanoma cells that overexpress tyrosinase (Tyr\textsuperscript{+} cells), the threshold for phosphorylation of ATM and p53 at serine 15 was observed at a low dose of Qct (25 \textmu M) when compared to the mock transfected pcDNA3 cells, which required a higher dose (75 \textmu M). Both pcDNA3 and Tyr\textsuperscript{+} DB-1 cells demonstrated similar increases in phosphorylation of p53 at other serine sites, but in the Tyr\textsuperscript{+} cells, DNApk expression was found to be reduced compared to control cells, indicating a shift towards an ATM-mediated response. The DB-1 control cells were resistant to DTIC, but were sensitized to apoptosis with high dose Qct, while Tyr\textsuperscript{+} cells were sensitized to DTIC with low or high dose Qct. Qct also sensitized SK Mel 5 (p53 wildtype) and 28 (p53 mutant) cells to DTIC. However, when SK Mel 5 cells were transiently transfected with tyrosinase and treated with Qct plus DTIC, SK Mel 5 cells demonstrated a more than additive induction of apoptosis. Therefore, this study demonstrates that tyrosinase overexpression promotes an ATM-dependent p53 phosphorylation by Qct treatment and sensitizes melanoma cells to dacarbazine. In conclusion, these results suggest that Qct or Qct analogues may significantly improve DTIC response rates in tumors that express tyrosinase.

Keywords: Tyrosinase, melanoma, p53, quercetin, ATM, DTIC

1. Introduction

The incidence of melanoma increases by 5–7% yearly and the death rate from melanoma has doubled over the last 3 decades [1]. The median survival of pa-
tients with advanced melanoma varies between 6 and 9 months, and only 1–2% have long-term complete response after treatment [2]. Dacarbazine or 5-3,3-dimethyltriazeno-imidazole-4-carboxamide (DTIC), is a DNA-binding agent which is approved for the treatment of melanoma and is still considered as the reference treatment for melanoma in several randomised clinical trials [3] with response rates ranging from 11 to 25% [4]. Therefore, continuous efforts have been made to combine various single active agents with DTIC for potential additive or synergistic effects to gain higher response rates.

Quercetin (Qct) is a bioflavonoid recognized for its anticancer properties because of its ability to modulate cell proliferation, survival and differentiation and target key molecules responsible for tumor cell viability [5]. Qct also exhibits inhibitory effects on tumor growth through cytotoxic mechanisms and is reported to induce DNA damage at high concentrations [6]. The cytotoxicity of Qct can also result from its pro-oxidant property which is from the oxidation of Qct into electrophilic o-quinone compounds by enzymes such as tyrosinase [7]. Tyrosinase is a catalytic enzyme expressed in the cells of neurocrest origin and will oxidize quinone compounds during various metabolic reactions [8]. Tyrosinase is commonly overexpressed in melanoma cells [7] and is used as a tumor marker [9]. Furthermore, its expression varies with different melanoma cell lines [10].

The ability of tyrosinase expression to selectively sensitize tumors to pro-drugs has been proposed and validated [11]. Other investigators [12,13] further demonstrated that tyrosinase oxidizes Qct which then binds to glutathione (GSH) forming toxic adducts. Earlier studies by our group revealed that Qct oxidation caused a decline in the bioreductive potential in melanoma cells that overexpress tyrosinase (Tyr+) and activates Qct. Activation of Qct will promote cytotoxicity by the induction of reactive quinones and oxygen species [14].

The mechanism of p53 induction by Qct after tyrosinase activation is unclear. However, Qct and ROS have been shown to induce phosphorylation of the protein, Ataxia telangiectasia mutated (ATM) on serine 1981 and thereby cause subsequent phosphorylation of histone H2AX on serine 139 [15]. ATM is a protein kinase that is activated in a rapid manner in response to DNA double-strand breaks (DSBs). The activation of ATM occurs through an autophosphorylation site on serine 1981, which leads to dissociation of the inactive ATM dimer (or higher-order multimer) into single protein molecules with kinase activity [16]. Other members of the phosphoinositide 3′-kinase related protein kinases (PIKKs) like the Rad3-related (ATR), and/or DNA dependent protein kinase (DNA pk) are also known to phosphorylate H2AX, a sensitive indicator of chromatin-associated DSBs at serine 139. Phosphorylation of ATM leads to the phosphorylation of p53 and can lead to DNA repair or apoptosis. Many studies have demonstrated Qct exhibits a hormesis effect. That is, lower doses of Qct can lead to antioxidant and antiapoptotic effects, while higher doses are prooxidative and proapoptotic [17,18]. Here we demonstrate that Qct induces differential cellular responses depending on the dose of treatment and level of tyrosinase expression. In DB-1 melanoma cells that overexpress tyrosinase (Tyr+), the threshold for ATM phosphorylation by Qct exposure was found to be reduced. We also found that Qct treatment phosphorylates p53 at different sites independent of ATM activation in these cells. Based on these results, the primary objective of this study was focused on the evaluation of the therapeutic potential of combining Qct with DTIC to sensitize melanoma cells to apoptosis. The rationale is that tyrosinase is overexpressed in melanoma tumors and activates Qct. Activation of Qct will promote cytotoxicity in tyrosinase expressing cells providing selectivity and enhanced DNA damage that will complement DTIC.

2. Materials and methods

2.1. Reagents

Quercetin (3,3′,4′,5,7-pentahydroxy flavone), dimethyl sulfoxide (DMSO), α-minimum essential medium (α-MEM), and dacarbazine were purchased from Sigma, (St. Louis, MO, USA). For western blotting, antibodies for tyrosinase, Bax and p53 were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). Antibodies for phosphorylated p53 (ser 20, 15, 37, 392), H2AX, ATM, cleaved caspase-3, -9 and PARP were from Cell Signaling (Danvers, MA, USA). Antibody for MDM2 (Ab-3) was purchased from Oncogene (San Diego, CA, USA). Antibodies for phosphorylated p53 (ser 20, 15, 37, 392), H2AX, ATM, cleaved caspase-3, -9 and PARP were from Cell Signaling (Danvers, MA, USA). Antibody for MDM2 (Ab-3) was purchased from Oncogene (San Diego, CA, USA). DB-1 cells were melanoma cells developed from lymph node biopsies from metastatic patients and was provided by Dr. David Berd, Thomas Jefferson University, Philadelphia [19] and adapted to growth in culture. Experiments were carried out in cells of passages 6–8. Sterile DMSO (0.1%) dissolved in α-MEM complete medium was used as vehicle. Quercetin was prepared in sterile filtered DMSO.
(0.1%) and dissolved in α-MEM complete medium supplemented with 10% FBS.

2.2. Dacarbazine treatment

DTIC was dissolved in DMSO by lowering the pH with HCl and was then dissolved in α-MEM complete medium and sterile filtered after adjusting the pH to 7.4. The drug was light activated for 1 h as described [20,21]. The mock transfected controls and Tyr+ cells were treated with 25 and 75 µM quercetin for 24 h, then the medium was removed and cells were washed with PBS and subsequently treated with different concentrations of DTIC (200, 400, 600, 800 µg/ml) for 48 h.

2.3. Expression plasmid construction

pRHOHT2 was a kind gift from Dr. Shibahara, Tohoku University School of Medicine, Japan [22]. Expression construct of human tyrosinase (pcDNA3/tyrosinase) was generated by digesting plasmid pRHOHT2 with restriction enzyme SalI/XbaI to isolate the full length human tyrosinase cDNA. The insert cDNA was then cloned at the XhoI/XbaI in the mammalian expression vector pcDNA3.

2.4. Cell culture and transfection

Stable clones over expressing tyrosinase (Tyr+) were generated from DB-1 cells as previously described [14]. DB-1 cells were transfected with 6 µg of tyrosinase DNA using lipofectamine 2000 (Invitrogen Corp., CA, USA) in serum free OPTIMEM medium. Transfectants were maintained in a selection medium of α-MEM complete medium containing neomycin. Control cells were mock transfected with empty vector pcDNA3 and selected similarly. SK Mel 5 and SK Mel 28 cell lines were obtained from American Tissue Culture Collections (Rockville, MD, USA). SK Mel 5 are wildtype and SK Mel 28 are mutant for p53 (145L/R) [23]. Transient transfection was carried out with 6 µg DNA using lipofectamine 2000 (Invitrogen Corp., CA, USA) in serum free OPTIMEM medium for 5 h followed by leaving the complex in α-MEM complete medium with 10% FBS for atleast 18 h. Tyrosinase over expression was confirmed by Western blotting analysis before further experiments were carried out.

2.5. Western blotting

Western blotting was carried out in 7 and 10% NUPAGE gels (Invitrogen Corp., CA, USA). Western detection was carried out using CDP star from Tropix, Applied Biosystems (Chicago, IL, USA). A primary goat polyclonal antibody at 1:100 dilution raised against a peptide mapping to the carboxyl terminus of human tyrosinase and a secondary antibody rabbit antibody ALP (1:10000) were used to detect tyrosinase expression. Antibodies to cleaved caspase-3, -9, PARP, phospho p53 (serines 15, 20, 37 and 392), ATM, H2AX, were used in the dilution of 1:1000. p53 and Bax was used in a dilution of 1:200. Anti-rabbit was used as a secondary at a dilution of 1:10000 for phospho antibodies of p53, H2AX, DNApk, Bax, cleaved caspase-3, -9 and PARP whereas, anti-mouse for p53, phospho ATM and GAPDH at a dilution 1:10000.

2.6. ROS measurement

About 1 × 10⁵ cells were plated in a 24 well culture plate and treated with Qct (25 µM) for 24 h in α-MEM complete medium. The cells were suspended in 1 ml of the medium and treated with 20 µl of 10 µM hydroethidine, incubated for 30 min and read by flow cytometry to measure ROS production.

2.7. Annexin V-FITC staining

The mock transfected controls and tyrosinase transfected (Tyr+) cells grown in antibiotic selection medium were treated with Qct for 24 h followed by dacarbazine for 48 h. Apoptotic analysis was carried out with Annexin V-FITC apoptosis detection kit (R&D systems, MN, USA) as per manufacturer’s instructions. Approximately 5 × 10⁵ cells were resuspended in 100 µl of manufacturer-supplied 1× binding buffer and mixed with 1 µl of Annexin V-FITC and 10 µl of propidium iodide. After 15 min incubation in the dark at room temperature, 400 µl of 1× binding buffer was added and the cells were analyzed using a BD FACS flow cytometer.

3. Statistical analysis

The data are expressed as mean ± SEM. Statistical analyses were performed with SPSS version 7.5 statistical package. For comparison between the experimental groups one-way ANOVA followed by post hoc or Students t-test was used. A value of p < 0.05 was used as a criterion for statistical significance.
4. Results

DB-1 melanoma cells stably overexpressing tyrosinase were constructed as described in the methods and tyrosinase expression was confirmed (Fig. 1(a) and (c)). As previously described [14], Tyr\(^+\) cells were found to exhibit increased NQO1 and p53 expression (Fig. 1(a) and (c)) and treatment with Qct for 24 h markedly increased total p53 in the mock transfected control cells (Fig. 1(b) compare lanes 1 with 2; Fig. 1(d)) and Tyr\(^+\) cells (Fig. 1(b) compare lanes 3 with 4; Fig. 1(d)). The greatest p53 accumulation was in the Tyr\(^+\) cells (Fig. 1(b), lane 4; Fig. 1(d)).

Quercetin has been reported to induce phosphorylation of ATM which in turn phosphorylates several downstream target substrates such as p53 and H2AX [24]. Studies investigating Qct utilize a wide range of doses [18,25,26], therefore we chose to investigate two different doses of Qct (25 and 75 µM) on ATM phosphorylation. In mock transfected cells, only high dose Qct (75 µM) induced ATM phosphorylation at serine 1981 and p53 at serine 15 (Fig. 2(a), lane 3; Fig. 2(c)). In contrast, in the Tyr\(^+\) cells a lower dose of Qct (25 µM) was sufficient to induce ATM phosphorylation.
Fig. 2. Effect of Qct treatment on DNA damage response proteins. Western blot of mock (pcDNA3) and tyrosinase (Tyr⁺) transfected cells after treatment with vehicle (DMSO), Qct 25 and 75 µM for 24 h. (a) Total p53, phosphorylation of ATM at serine 1981 and p53 at serine 15. (b) DNApk expression and p53 phosphorylation at serine 37, 20 and 392. (c, d) Mean ± SEM of relative fold expression of proteins observed in western blots when compared to mock transfected controls treated with DMSO for 24 h. The values are mean ± SEM of three to five different individual experiments. *p < 0.05 vs. to untreated control.

(Fig. 2(a), lanes 5 and 6; Fig. 2(c)). The phosphorylation of p53 in the serine 15 position was also induced by low dose Qct in these overexpressors (Fig. 2(a), lane 5; Fig. 2(c)), confirming ATM activation by low dose Qct. Phosphorylation of p53 at serine 20 was slightly induced by low dose Qct but was markedly increased by high dose Qct in both the cell lines (Fig. 2(b) and (d)).

We next investigated phosphorylation of p53 at serine 37 (DNApk dependent) and serine 392. Mock transfected cells demonstrated increased phosphorylation of p53 at serine 392 and 37 positions after treat-
Fig. 2. (Continued).

Fig. 3. Effect of low and high dose quercetin exposure on apoptosis-associated proteins. Western blot of mock (pcDNA3) and tyrosinase (Tyr\(^+\)) transfected cells after treatment with vehicle (DMSO), Qct 25 or 75 \(\mu\)M for 24 h. (a) MDM2, H2AX and Bax (b) cleaved caspase-3, -9 and PARP levels. (c, d) Mean \(\pm\) SEM of relative fold expression of proteins observed in western blots when compared to mock transfected controls treated with DMSO for 24 h. The values are mean \(\pm\) SEM of three to five different individual experiments. \(^*\) \(p<0.05\) vs. to untreated control.

The molecules downstream of p53 activation were also investigated. The expression of Bax correlated with the levels of total p53 as Bax expression was induced in a much higher degree in the Tyr\(^+\) cells compared to the controls (Fig. 3(a) and (c)). Basal levels of Bax and after treatment with low dose Qct
were significantly higher in these cells than the control cells (Fig. 3(a) comparing lanes 1, 2 with 4, 5; Fig. 3(c)). Consistent with the Bax expression, a decline in MDM2 expression was observed in the Tyr⁺ cells with Qct treatment (Fig. 3(a) and (c)). Cleaved caspases-3, -9 and PARP levels (Fig. 3(b) and (d)) also correlated well with the level of Bax. These results demonstrate that tyrosinase primes cells for apoptosis and a lower dose of Qct is sufficient to activate proapoptotic pathways in the Tyr⁺ cells.

H2AX is a part of the histone family of proteins that participate in nucleosomal organization of chromatin. In response to DNA double-strand breaks, histone H2AX is phosphorylated at serine 139 (γ-H2AX) and serves as a good indicator of DNA damage. Increases in γ-H2AX have been shown to function downstream of ATM activation in the cellular repair response. γ-H2AX was found to be increased in the Tyr⁺ cells even under basal conditions and was induced in both the cell lines by both low and high dose Qct (Fig. 3(a) and (c)).

Because oxidized Qct can induce ROS and can cause phosphorylation of several DNA damage responsive proteins, we further investigated if ROS levels in these
Fig. 4. Role of Qct on ROS production in Tyr+ cells. Flow cytometry of cells using HE dye in mock (pcDNA3) and tyrosinase (Tyr+) transfected cells. Percent of HE stained cells shown. Results are mean ± SEM of three individual experiments. *p < 0.05 vs. corresponding vehicle. †p < 0.05 vs. mock transfected control groups.

As melanoma cells are resistant to chemotherapy, we further investigated whether Qct could sensitize these cells to DTIC. We first elucidated the role of DTIC on mock transfected cells at various concentrations (0–800 µg/ml) as described previously [21]. These cells were subjected to DTIC treatment for 48 h and the cells were assessed for annexin-V FITC staining. Apoptosis determined by annexin V FITC staining was not observed until a concentration of 400–600 µg of DTIC (Fig. 5(a)). Then both the mock and Tyr+ transfected cells were treated with DTIC (0–800 µg/ml) for 48 h in the presence or absence of low and high dose Qct (Fig. 5(b)). The mock transfected cells were resistant to DTIC, but when treated with low dose Qct demonstrated a dose dependent increase in DTIC-induced apoptosis compared to DTIC alone (Fig. 5(b)). High dose Qct sensitized control cells to apoptosis, but it was independent of DTIC concentration. The Tyr+ cells demonstrated apoptosis induced by DTIC alone and combination treatment with low or high dose Qct and DTIC resulted in high levels of apoptosis which were DTIC-dose dependent. Therefore, the induction of apoptosis was consistent with the p53 levels as high dose Qct sensitized both cell lines and low dose Qct was most effective in the Tyr+ cells. The pattern of Annexin V FITC and PI staining for DTIC (600 µg/ml) with and without Qct is shown in Fig. 5(c). Cell counts in the upper and lower right hand quadrants were considered positive for apoptosis.

To confirm that these observations were not limited to the DB-1 cell lines, SK Mel 5 and 28 (p53 wild-type and mutant, respectively) were treated with low and high doses of Qct for 24 h. SK Mel 5 showed an induction of p53 and p53-inducible proteins (Fig. 6(a) and (b)), while in SK Mel 28 cells the induction of p53 and its transgenes were absent (Fig. 6(c) and (d)). When these cell lines were transiently transfected with tyrosinase, a basal increase in p53 was only observed in SK Mel 5 cells (Fig. 6(e) and (f)). The transiently transfected cells were then treated with Qct and DTIC. In the SK Mel 5 and 28 cells, treatment with low and high doses of Qct induced apoptosis (Fig. 7(a) and (b)). However, in SK Mel 5, transfection with tyrosinase resulted in a more than additive induction of apoptosis in the high dose treatment and a shift from necrosis (upper left quadrant) to apoptosis (upper and lower right quadrants). This was also observed in the DB-1 cells (Fig. 5(c)). In contrast, there was no ef-
Fig. 5. Impact of Qct and Dacarbazine treatment on apoptosis in DB-1 melanoma cell lines. (a) The percentage of apoptotic cells after DTIC treatment in mock transfected control cells. Results are mean ± SEM of duplicate set of experiments. (b) The percentage of apoptotic cells after DTIC with or without low or high dose Qct in mock and tyrosinase (Tyr+) transfected cells. (c) Early apoptotic cells can be visualized in the lower right quadrant, while late apoptotic cells are shown in the upper right.

fect of tyrosinase in the SK Mel 28 cells, confirming that the effect of tyrosinase is p53 mediated. However, Qct still sensitized the p53 mutant cells, although to a lesser degree.

The degree of apoptosis in both SK Mel cells was less than the DB-1 cells and was observed only with the high dose Qct treatment. However, with transient transfection the transfection efficiency varies between 20–60% and would likely account for the lower degree of apoptosis and lack of an effect at the low dose. Alternatively, SK Mel 5 cells are less sensitive to DTIC and may have a higher threshold for p53 activation. Nonetheless, a more than additive induction of apoptosis was observed in the SK Mel 5 Tyr+ cells treated with DTIC and Qct.

5. Discussion

This study explored the potential for tyrosinase to activate Qct and sensitize tumors to DTIC. The rationale was that tyrosinase is overexpressed in melanoma cells and can therefore specifically increase the cytotoxicity of quercetin. We have found that Qct sensitized tumors to DTIC, but in the presence of tyrosinase the sensitization was greater and mediated by p53. Activation of ATM and phosphorylation of p53 at serine 15 by Qct appear to be the mechanism of tyrosinase-mediated sensitization in the Tyr+ cells. A summary of the response of Tyr+ cells to Qct is shown in Table 1. A lower threshold of ATM phosphorylation and minimal induction of DNApk with Qct treatment suggests a dominant role of ATM dependent DNA damage response pathway in these cells. Although quercetin has been shown to directly activate ATM [15], ROS may also play a role. Previous studies have demonstrated that tyrosinase activated Qct can form adducts [27] and deplete GSH [14]. The increased ROS observed in our study could also explain the induction of ATM and γ-H2AX as ROS can also activate ATM and H2AX [28]. Studies by Heiss et al. [29] illustrated a positive and mutual feedback between ATM activation and increased ROS levels upon resveratrol exposure in p53 positive cancer cells. Additionally this mutual action could have also contributed to the ATM-dependent phosphorylation of histone H2AX. This is supported by studies by Ye et al. [15] who reported that Qct induces ATM-dependent phosphorylation of H2AX.
Fig. 5. (Continued).

(c)

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Fig. 6. Effect of Qct treatment on p53 and p53 related proteins in SK Mel 5 and 28 melanoma cell lines. Western blot in (a) SK Mel 5, p53 wild type and (c) SK Mel 28, p53 mutant melanoma cell lines treated with DMSO, 25 and 75 µM of Qct for 24 h. (e) Expression of tyrosinase and p53 in SK Mel 5 and 28 melanoma cell lines transiently transfected with pcDNA3 and tyrosinase. (b, d, f) Mean ± SEM of relative fold expression of proteins observed in western blots when compared to mock transfected controls treated with DMSO for 24 h. The values are mean ± SEM of three to five different individual experiments. *p < 0.05 vs. to untreated control.
Phosphorylation of p53 plays a key role in regulating its stability and its transcriptional activity in response to DNA damage [30]. Phosphorylation of p53 at serine 392 position in particular is believed to enhance the specific DNA binding of p53 in vitro [31]. As Qct can exhibit a modulatory role on the expression, stability and activity of p53, this could possibly explain the increased p53 expression observed in this...
Table 1

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<thead>
<tr>
<th>Protein</th>
<th>25 µM Qct</th>
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<td>pcDNA3 cells</td>
<td>Tyr+ cells</td>
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<tr>
<td>ATM Ser 1981</td>
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| p53 Ser 20 | ++ | ++ | ++++ | +++
| p53 Ser 392 | +++ | ++ | ++++ | +++
| DNApk      | + | – | +++ | +
| p53 Ser 37 | ++ | ++ | ++ | ++ |
| MDM2       | – | – | ++ | –
| Bax        | ++ | +++ | ++++ | +
| H2AX       | ++ | +++ | ++++ | +++
| Caspase-3  | – | ++++ | +++ | ++++ |
| Caspase-9  | – | ++ | + | +++ |
| PARP       | – | ++ | +++ | +++ |

Notes: The “+” values are based on relative fold expression of proteins rounded to the nearest whole number observed in western blots when compared to mock transfected controls treated with DMSO for 24 h. The values are mean ± SEM of three to five different individual experiments. Each “+” indicates a one fold increase when compared to control cells.
The phosphorylation of p53 by DNApk at serine 37 at the high dose (Fig. 2(b), compare lanes 3 and 6). The overexpressors showed minimal DNApk activity and sumoylation [33]. This mainly involves modifications to MDM2 which may be involved in its auto-ubiquitination and degradation and thus serves as a negative regulator of p53 [34]. Furthermore, phosphorylation at serine 15 has been reported to disrupt the interaction of p53 with the MDM2 protein, leading to stabilization of p53 and an increase in its transcriptional activity [35,36]. This also correlates to the increased p53 levels in the overexpressors after Qct treatment. The phosphorylation of p53 at serine 15 and serine 37 in vitro has also been reported to impair the ability of MDM2 to inhibit p53-dependent trans-activation [35] and this could have contributed to the decline in the MDM2 activity in the Tyr+ cells. p53 also can activate Bax in mitochondria to antagonize the anti-apoptotic ability of Bcl-2 and Bcl-XL [37]. In this study, the Bax activity was further enhanced by 25 and 75 µM Qct treatment especially in the Tyr+ cells, indicating the positive role for tyrosinase in the up-regulation of Bax. This is supported by our earlier studies where we reported increased Bax mRNA in melanoma cells under basal conditions as well as after 25 µM quercetin treatment [14].

DNApk is an important DNA repair protein and can affect the cellular response to DTIC derivatives [38]. We found induction of DNApk in the mock transfected cells, while the induction was minimal in the Tyr+ cells. DNApk is mainly involved in joining non-homologous sequences (NHEJ) and is mainly regulated by the DNApk/Ku complex, Xrcc4, DNA ligase IV, Rad50, and nuclease such as Mre11 and Fen1 [39]. In this study, we tried to establish a relationship between serine 37 phosphorylation and DNApk. The overexpressors showed minimal DNApk activity as well as reduced phosphorylation of p53 at serine 37 at the high dose (Fig. 2(b), compare lanes 3 and 6). The phosphorylation of p53 by DNApk at serine 37 as well as on serine 15 has been demonstrated in vitro [40] and the observed decrease in phosphorylation of p53 at serine 37 could be due to decreased DNApk expression in the overexpressors. There are also other kinases like ATR which have been shown to phosphorylate serines 15 and 37 of p53 [36]. Quercetin has also been shown to inhibit DNApk activity by competing with ATP in a competitive manner [41]. In addition, in melanoma cells that express high levels of tyrosinase, quercetin binds to GSH and can form quinone methide glutathionyl adducts [14,27]. Glutathionyl conjugates are also reported to bind with DNApk and inhibit its kinase activity [42]. Regardless, the decrease in DNApk led us to conclude that Qct plays an important role in the activation of ATM in the Tyr+ cells whereas, in the controls DNA damage activates ATM as well as DNApk dependent pathways.

Studies using the SK Mel 28 cells indicated that quercetin can sensitize mutant p53 cells to DTIC, although the most potent sensitization is in the cells with wild type p53. This is an important finding because a large percentage of melanoma tumors express mutant p53. Previously published data from our lab indicate that p53 levels correlate with sensitivity to apoptosis [14]. However, here we demonstrate that in cells with mutant p53, Qct activation works through other mechanisms, which are independent of tyrosinase. Many other studies demonstrate various anti-cancer effects of Qct treatment which would benefit cancer therapy and we believe that Qct is likely working through these alternative mechanisms [43].

The activation of Qct by tyrosinase in wildtype p53 cells will complement DTIC cytotoxicity. DTIC is an alkylating agent, which is believed to exert its anti-tumor activities by methylation of nucleic acids or causing direct DNA damage thereby resulting in growth arrest and subsequent cell death [44]. O6-methyl guanine produced by dacarbazine would allow the formation of DNA replication forks and thereby lead to the formation of O6-methyl guanine thymine mispair [45]. This could cause apoptosis in cells subjected to DTIC treatment as suggested by Sanada et al. [46]. O6-methyl guanine is also reported to trigger apoptosis by p53 stabilization and Fas/CD95/Apo-1 upregulation [47]. In this study we used light-activated DTIC, but the exact component of light-activated damage is still unclear [21]. Other studies report that non or light-activated DTIC can also cause apoptotic events. For example, non-activated DTIC was reported to upregulate p21 levels and activate cleaved caspase-3 and PARP levels in FEMX-1 cell lines [44]. As observed here and in our earlier studies [14], Qct can cause cell death in these Tyr+ cells through the up-regulation of p53 and caspase...
cleavage, which would complement or sensitize cells to DTIC-mediated toxicity regardless of the activation method. However, the full potential of quercetin-modified DTIC treatment will have to be determined by in vivo studies, but the mechanisms reported here would complement any mechanism of DTIC damage that has been reported.

It is also important to discuss the clinical applicability of this treatment. Many pro-drugs which are activated by tyrosinase provide a level of specificity to tumors [11]. Additionally, many viral vectors have been constructed to be induced using a tyrosinase responsive promoter [48]. The delivery of Qct as a “prodrug” can be performed by oral administration or through i.v. or i.p. administration [49,50]. Quercetin is relatively non-toxic and up to 2 gms can be delivered [51,52]. We have found activation of ATM and p53 in the Tyr+ cells at Qct concentrations of 25 µM. Blood levels close to this can be achieved orally and can certainly be achieved by i.v. administration. Preliminary data from our lab have shown comparable blood levels at 21 µM by i.p. administration (data not shown). Other studies also support this observation. With that being said, it will be important to investigate this effect in vivo using tumors with and without tyrosinase. Although these types of studies are extensive, they may provide further information regarding the clinical application of this treatment, especially as to the route of Qct administration. In addition, the level of endogenous tyrosinase may play a role in the level of tumor cell sensitivity. However, it is important to note that Qct can induce melanogenesis. This has been demonstrated in both cells [53] and in a model of human skin [54]. Therefore, longer-term administration could potentially increase the effectiveness of Qct treatment.

6. Conclusion

Although DTIC has been used for the treatment of melanoma for decades, its modest treatment effect encourages studies on novel combination therapies. In this study, we demonstrate that Qct sensitizes Tyr+ cells to DTIC. Quercetin plays an important role in the activation of ATM in the tyrosinase overexpressors. Moreover in the Tyr+ overexpressors low dose Qct is sufficient for p53 mediated apoptosis, while a higher dose may sensitize both the cell types. Many tumors express mutant p53 and Qct sensitizes these cells, although to a lesser extent. Therefore, Qct or Qct analogues may be used as a combination drug to improve the efficacy of conventional cytotoxic agents to treat the tumors that express tyrosinase.

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