Sensitization of recombinant human tumor necrosis factor-related apoptosis-inducing ligand-resistant malignant melanomas by quercetin

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Malignant melanoma is the most commonly diagnosed skin cancer associated with a high rate of metastasis. Low-stage melanoma is easily treated, but metastatic malignant melanoma is an extremely treatment-resistant malignancy with low survival rates. The application of recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL) for the treatment of metastatic malignant melanoma holds considerable promise because of its selective proapoptotic activity towards cancer cells and not nontransformed cells. Unfortunately, the clinical utilization of rhTRAIL has been terminated due to the resistance of many cancer cells to undergo apoptosis in response to rhTRAIL. However, rhTRAIL-resistance can be abrogated through the cotreatment with compounds derived from 'Mother Nature' such as quercetin that can modulate cellular components responsible for rhTRAIL-resistance. Here, we show that rhTRAIL-resistant malignant melanomas are sensitized by quercetin. Quercetin action is manifested by the upregulation of rhTRAIL-binding receptors DR4 and DR5 on the surface of cancer cells and by increased rate of the proteasome-mediated degradation of the antiapoptotic protein FLIP. Our data provide for a new efficient and nontoxic treatment of malignant melanoma. *Melanoma Res* 28:277–285 Copyright © 2018 The Author(s). Published by Wolters Kluwer Health, Inc.

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Introduction

The frequency of malignant melanoma has been on the rise over the last 30 years. Although it is the least diagnosed of the skin cancers, it is associated with the highest rate of mortality. When the melanoma is localized to the epidermis, the survival rate is 98%. However, once the cancer metastasizes the 5-year survival rate decreases to 17% [1]. The decrease in survival rate correlates with the lack of effective treatment options for metastatic malignant melanoma. Current therapies (chemotherapy, radiation therapy, targeted therapy, and immunotherapy) are characterized by slow efficacy and only temporary antitumor properties due to acquired resistance. In addition, a high degree of negative side effects are associated with these therapies which deeply impacts the patient's quality of life and limits the optimum drug dose. As a result, there is no standard therapeutic regimen for metastatic malignant melanoma [2,3]. It is vital to invest in the development of novel therapeutics for metastatic malignant melanoma and increase the survival outcome for these patients.

The application of recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL) as a treatment for human cancer holds great promise due to its cancer-cell-specific induction of apoptosis while showing no harm to normal nontransformed cells [4,5]. rhTRAIL, the optimized form of the cytokine TRAIL, consists of only the biologically active C-domain of the endogenous molecule. rhTRAIL selectively induces apoptosis by interacting with membrane-bound extracellular receptors, death receptor (DR) 4 and DR5, which are more abundantly expressed on cancer cells compared with normal cells [6]. Upon rhTRAIL-binding, the extrinsic pathway of apoptosis is initiated characterized by the activation of caspase 8 and the subsequent activation of executioner caspases 3, 6, and 7. In addition, mediated by the caspase 8 cleavage of BID to tBID, the application of rhTRAIL can also result in the initiation of the intrinsic pathway of apoptosis independent of *p53*. Once activated, tBID will translocate to the mitochondria and stimulate the release of cytochrome *C* into the cytosol and ultimately results in the activation of caspase 9. Caspase 9 can then cleave and activate executioner caspases that carry out the hallmark events of apoptosis including DNA fragmentation and cytoplasmic condensation [7].

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Although the application of rhTRAIL as an anticancer therapeutic holds great promise, the clinical use has been limited due to the heterogeneity seen in rhTRAIL-sensitivity among cancers. This is especially prevalent in cases of advanced metastatic malignant melanoma. Nearly two-thirds of melanoma cells are resistant to rhTRAIL-induced apoptosis, including melanomas directly isolated from cancer patients [8,9]. Melanoma rhTRAIL-resistance can be attributed to a number of different causations. One of which is the decreased membrane expression of proapoptotic rhTRAIL-binding receptors DR4 and DR5 [10,11]. The lack of receptor expression may be associated with gene mutations or decreased transcription [12,13]. In addition, failure to transport the receptors to the membrane or constitutive endocytosis can lead to the decreased expression of DRs on cancer cells [14–16]. Moreover, the upregulation of intracellular antiapoptotic proteins plays an indispensable role in rhTRAIL-resistance. The ratio between proapoptotic and antiapoptotic Bcl-2 proteins takes part in regulating rhTRAIL-sensitivity with high levels of antiapoptotic proteins, Bcl-2 and Bcl-xL, correlating with rhTRAIL-resistance and the inactivation of the proapoptotic Bcl-2 proteins, Bax and Bak, rendering cells resistant to rhTRAIL [17–19]. Another mechanism of rhTRAIL-resistance is mediated through the overexpression of inhibitors of apoptosis proteins (IAPs) such as FLIP, XIAP, cIAP, and survivin that inhibit caspase activity [12,20]. It is essential to understand the molecular mechanisms of rhTRAIL-resistance and develop ways to overcome this resistance through combination therapy.

Epidemiological evidence shows that a plant-based diet is associated with a decrease incidence of various types of cancers. The benefit of which is most accredited to the natural phytochemicals such as flavonoids present. One of the most prevalent flavonoids with potent physiological activity is quercetin. Quercetin is found in a wide variety of sources ranging from onions and apples to red wine. As a pleiotropic molecule, quercetin exhibits its anticancer effects on a number of different pathways such as cell survival pathways, cell-cycle arrest, upregulation of tumor suppressor genes, downregulation of antiapoptotic proteins, and proapoptotic pathways [21–26]. Most noteworthy is the impact quercetin has on the expression of DR5. Several studies show that quercetin can upregulate DR5 on the membrane of cancer cells through enhanced transcription [27–31]. Moreover, it has been demonstrated that quercetin is able to downregulate a number of antiapoptotic proteins that promote rhTRAIL-resistance, specifically FLIP, Mcl-1, and survivin [26,32]. Overall, this evidence shows the potential of quercetin as a cotreatment for rhTRAIL. The ability of quercetin to sensitize rhTRAIL-resistant malignant melanomas has yet to be evaluated. Here, we test the combination rhTRAIL plus quercetin to overcome the intrinsic resistance of metastatic malignant melanomas to rhTRAIL.

Methods

Drugs and chemicals

rhTRAIL was produced according to well-defined and previously detailed protocols [6]. Quercetin dihydrate (Calbiochem, Temecula, California, USA) was dissolved in polyethylene glycol-400 (Fisher Scientific, Pittsburgh, Pennsylvania, USA). MG132 (Calbiochem) was dissolved in dimethyl sulfoxide. General caspase inhibitor Z-VAD-FMK (Calbiochem) was dissolved in dimethyl sulfoxide.

Cell culture

WM164 cells were maintained in DMEM and MeWo cells were maintained in RPMI, both supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution. Cells were incubated in a 90% humidified atmosphere with 5% CO_2 at 37°C.

Apoptosis assay

Apoptotic cells were identified using FITC-Annexin-V Kit Apoptosis Detection Kit I (BD Pharminogen, Sparks, Maryland, USA) and previously described methods [6].

Western blot analysis

Total cell lysates were analyzed for levels of poly(ADP-ribose) polymerase (PARP), caspase 8, caspase 3, BID, caspase 9, caspase 6, caspase 7, FLIP, DR4, and DR5 and the cytosolic cellular fraction was analyzed for cytochrome *C* (Cell Signaling, Danvers, Massachusetts, USA) using previously described western blotting techniques [6].

Death receptor membrane expression

The cell surface expression of DR4 and DR5 were determined according to previously described methods [6]. For the permeabilization experiments, after the first antibody incubation cells were fixed in 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.1% saponin for 5 min at room temperature. Cells were then incubated with anti-IgG1κ, DR4, or DR5 for 30 min in the saponin buffer and analyzed by fluorescence-activated cell sorting. To calculate the cytoplasmic DR expression, the permeablized cells representing the total DR expression was subtracted from the unpermeablized cells representing membrane-bound DRs.

Reverse transcription-PCR

Total RNA was extracted using TRIzol reagent (Ambion, Pittsburgh, Pennsylvania, USA) and treated with DNase according to manufacturer's protocol (Invitrogen deoxyribonuclease I, amplification grade). RT-PCR was performed following the manufacturer's protocol (Invitrogen SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase, Pittsburgh, Pennsylvania, USA). Human DR5 mRNA was amplified using the forward

primer 5′-GGGAGCCGCTCATGAGGAAGTTGG-3′ and the reverse primer 5′-GGCAAGTCTCTCTCCCAGCG TCTC-3′. For DR4, forward primer 5′-GAGCAACGCA GACTCGCT-3′and the reverse primer 5′-TCACTCCA AGGACACGGC-3′ were used. For FLIP, forward primer 5′-CTTGGCCAATTTGCCTGTAT-3′and the reverse primer 5′-CCCATGAACATCCTCCTGAT-3′ were used. For β-actin, the forward primer 5′-TGACGGGGTCACC CACACTGTGCC-3′and the reverse primer 5′-CTGCATC CTGTCGGCAATGCCAG-3′ were used. cDNA synthesis was performed at 60°C for 30 min using the Applied Biosystems GeneAmp PCR System 9700 (Pittsburgh, Pennsylvania, USA). The PCR cycling conditions (30 cycles) were as follows: denature for 2 min at 94°C, anneal for 30 s at 55°C for FLIP and DR4 and 65°C for DR5 and β-actin, extend for 1 min and 30 s at 68°C, and execute a final extension for 10 min at 68°C. Reaction products were analyzed on 1.2% agarose gels. The bands were visualized by ethidium bromide and an UV.

Statistical analysis

Student's *t*-test was used to determined significance. *P* values less than 0.05 were deemed significant.

Results

rhTRAIL-sensitivity

The in-vitro sensitivity of metastatic malignant melanoma cell lines MeWo and WM164 to rhTRAIL were tested by treating with increasing concentrations of rhTRAIL (5 ng/ml–1 μg/ml) for 72 h followed by Annexin-V and propidium iodide (PI) staining and fluorescence-activated cell sorting analysis (Fig. 1a–c). Both melanoma cell lines showed complete resistance to rhTRAIL-induced apoptosis. Treatment with rhTRAIL, even at the highest tested treatment concentration of 1 μg/ml, did not result in the formation of apoptotic cells as indicated by the lack of Annexin-V^+ and/or Annexin-V^+ and PI^+ cells as compared with the control ($P > 0.05$). The rhTRAIL-resistant status of the melanoma cell lines was confirmed through western blotting by probing with antibodies to several key components of the apoptotic cascade (Fig. 1d and e). The lack of PARP cleavage or activation of any proteins in the apoptotic pathway confirmed the rhTRAIL-resistance of both cell lines.

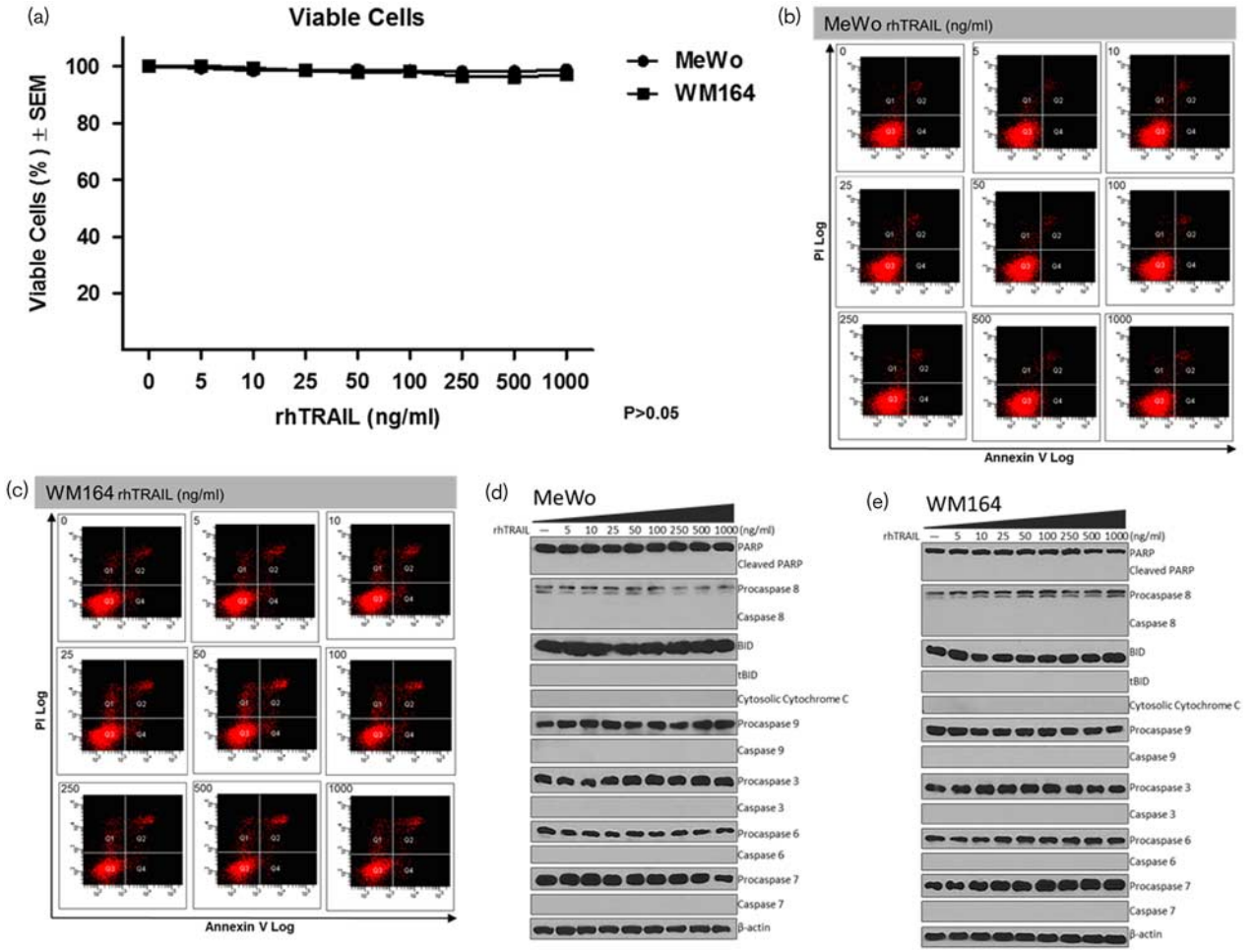
rhTRAIL plus quercetin apoptosis

To overcome the intrinsic rhTRAIL-resistance of MeWo and WM164, the combination treatment of rhTRAIL plus quercetin was employed. Melanoma cell lines were treated with single agent of rhTRAIL at 250 ng/ml, quercetin at 25 and 50 μmol/l and with the combination of the two agents. Post-treatment cells were collected and analyzed for induction of apoptosis through Annexin-V and PI staining and western blotting probing for key proteins in the apoptotic pathways (Fig. 2). Alone, quercetin induced minimal levels of apoptosis in the rhTRAIL-resistant melanoma cells. For MeWo, there was no significant induction of apoptosis when treating with both concentrations of quercetin marked by the lack of Annexin-V^+ and/or Annexin-V^+ and PI^+ cells as compared with the control ($P > 0.05$) (Fig. 2a and b). The lack of quercetin-induced apoptosis was confirmed by western blot where there was no PARP cleavage or activation of any proapoptotic proteins (Fig. 2d). However, in WM164, quercetin was able to induce minimal levels of apoptosis with the formation of $10.5 \pm 0.7\%$ apoptotic cells at 25 μmol/l and $19.1 \pm 1.6\%$ apoptotic cells at 50 μmol/l ($P < 0.05$) (Fig. 2a and c). Probing for apoptotic proteins did not reveal any PARP cleavage or protein activation in quercetin-treated WM164 cells as the low levels of apoptosis was not sufficient to be detected by western blotting (Fig. 2e). Furthermore, by combining rhTRAIL plus quercetin the minimal levels of apoptosis induced in the single agent treatments were dramatically increased in both melanoma lines. This was evident by the significantly higher formation of Annexin-V^+ and/or Annexin-V^+ and PI^+ cells as compared with any single agent treatments ($P < 0.05$) (Fig. 2a–c). The augmentation of apoptosis occurred dose-dependently with respect to quercetin. The ability of quercetin to sensitize rhTRAIL-resistant melanomas to undergo apoptosis was confirmed by western blotting (Fig. 2d and e). In both melanoma lines, PARP was cleaved in only the cotreatment group dose-dependently. By adding quercetin to the rhTRAIL-treatment, the once resistant cells were sensitized to activate the rhTRAIL-mediated extrinsic pathway of apoptosis as marked by the cleavage of procaspase 8 to caspase 8. In addition, through the cotreatment the intrinsic pathway of apoptosis was activated in WM164 but not in MeWo as noted by the cleavage of BID to tBID followed by the release of cytochrome *C* into the cytosol and the activation of caspase 9 from procaspase 9. Finally, through the cotreatment executioner caspases were activated. In both melanoma lines caspase 3 was activated through the cotreatment, caspase 6 in only the highest cotreatment in WM164 and caspase 7 in the highest cotreatment in both melanoma lines. The role of enhanced caspase activation in the sensitization of rhTRAIL-resistant malignant melanomas by quercetin was confirmed by the addition of the general caspase inhibitor Z-VAD-FMK (Fig. 2f). The inclusion of Z-VAD-FMK blocked the induction of apoptosis in the cotreatment group as indicted by the absence of PARP cleavage and activation of caspase 3.

Quercetin regulation of death receptors

The addition of quercetin to rhTRAIL was able to promote the activation of the rhTRAIL-mediated extrinsic apoptotic pathway evidenced by the activation of caspase 8 in only the cotreatment groups. To elucidate the mechanism of quercetin sensitization, the most apical part of the extrinsic apoptotic pathway, expression of rhTRAIL-binding receptors DR4 and DR5 were examined in response to quercetin

Fig. 1



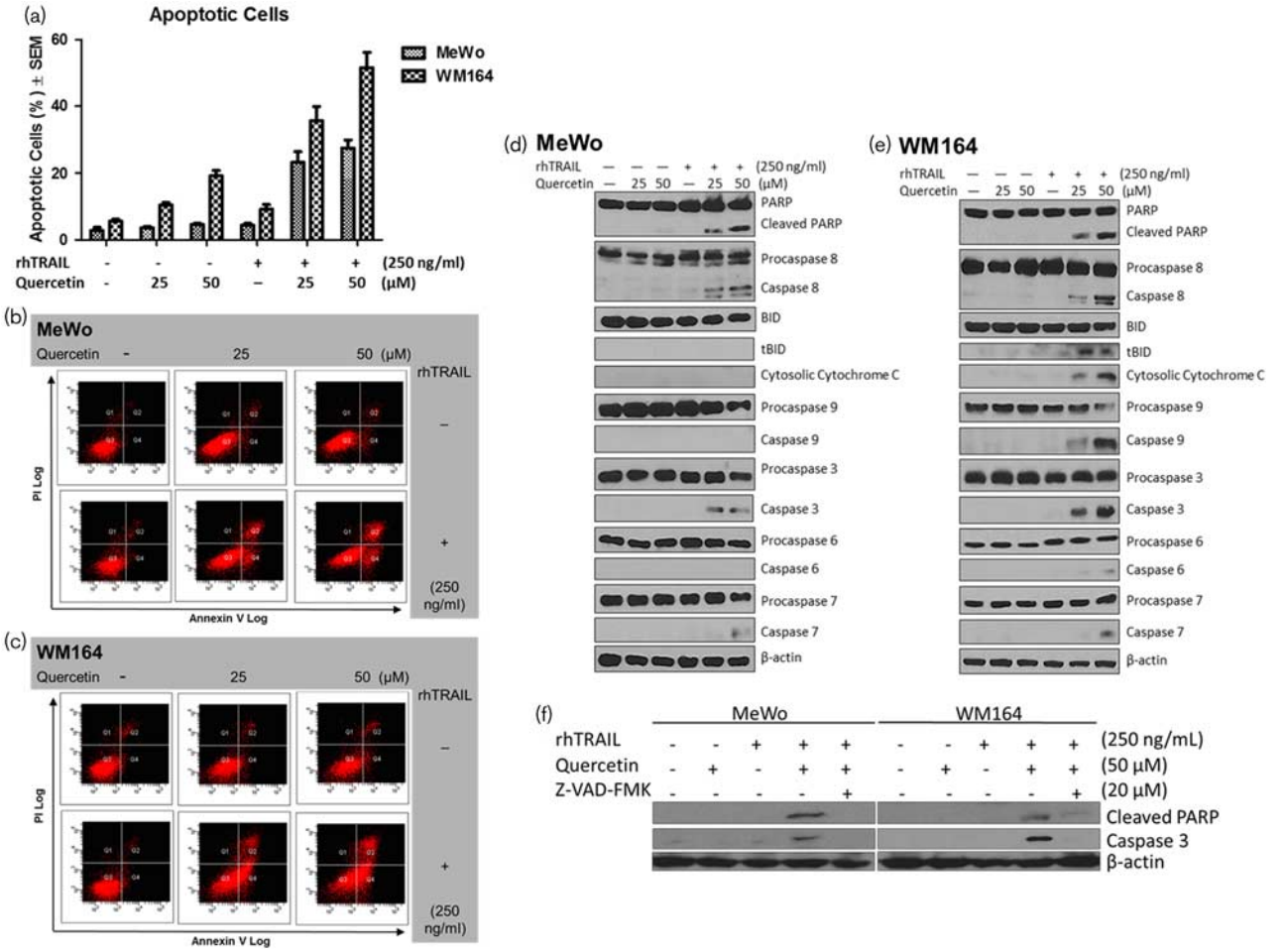
Recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL) sensitivity. Sensitivity of malignant melanomas MeWo and WM164 to rhTRAIL *in vitro*. (a) Average of three independent assays ± SEM. (b) Representative histogram of MeWo. (c) Representative histogram of WM164. Lower left quadrant: Viable cells (Annexin$^-$/PI$^-$), Lower right quadrant: early apoptotic cells (Annexin$^+$/PI$^-$), Upper right quadrant: late apoptotic cells (Annexin$^+$/PI$^+$). (d) MeWo and (e) WM164 ± rhTRAIL subjected to western blot analysis and probed with anti-PARP, caspase 8, BID, cytochrome C, caspase 9, caspase 3, caspase 6, and caspase 7. β-Actin was used as a loading control for each membrane. Representative β-actin is depicted.

treatment (Fig. 3). Quercetin was able to upregulate the membrane expression of DR4 in both melanoma cell lines dose-dependently (Fig. 3a). In response to quercetin treatment, MeWo upregulated DR4 on the cell membrane over four-fold at 25 μmol/l and over 11-fold at 50 μmol/l ($P < 0.001$). For WM164, DR4 was upregulated nearly five- and seven-fold at 25 and 50 μmol/l, respectively ($P < 0.001$). For DR5, the membrane expression was only upregulated in melanoma cell line WM164 dose-dependently with a quarter-fold increase at 25 μmol/l and a half-fold increase at 50 μmol/l ($P < 0.001$) (Fig. 3b). However, this increase did not occur in MeWo cells ($P > 0.05$). To further examine the effects of quercetin on the regulation of DR4 and DR5 the total protein expression and mRNA levels were analyzed post-treatment with quercetin (Fig. 3c–e). For DR4 there was no change in the total protein expression or the mRNA message in both melanoma lines. For DR5, there was no change in the total protein expression or the mRNA in MeWo. However, there was a dose-dependent upregulation of the total DR5 protein and DR5 mRNA in WM164. To explain the increase in the membrane expression of DR4 but the lack of increase in total protein and mRNA in response to quercetin treatment, the cytoplasmic levels of DR4 were measured (Fig. 3f and g). In both melanoma cell lines there was significant levels of DR4 within the cytoplasm. However, with the application of quercetin the cytoplasmic levels of DR4 decreased while the membrane levels increased ($P < 0.001$).

Quercetin regulation of FLIP

Also a player in the most apical part of the extrinsic apoptotic pathway, FLIP expression is a major regulatory

Fig. 2



Recombinant human tumor necrosis factor-related apoptosis-inducing ligand (rhTRAIL) plus quercetin-induced apoptosis. Combination treatment-induced apoptosis. (a) Average of three independent assays ± SEM. (b) Representative histogram of MeWo. (c) Representative histogram of WM164. Lower left quadrant: Viable cells (Annexin$^-$/PI$^-$), Lower right quadrant: early apoptotic cells (Annexin$^+$/PI$^-$), Upper right quadrant: late apoptotic cells (Annexin$^+$/PI$^+$). (d) MeWo and (e) WM164 ± rhTRAIL subjected to western blot analysis and probed with anti-PARP, caspase 8, BID, cytochrome *C*, caspase 9, caspase 3, caspase 6, and caspase 7. β-Actin was used as a loading control for each membrane. Representative β-actin is depicted. (f) Effects of general caspase inhibitor Z-VAD-FMK on induction of apoptosis.

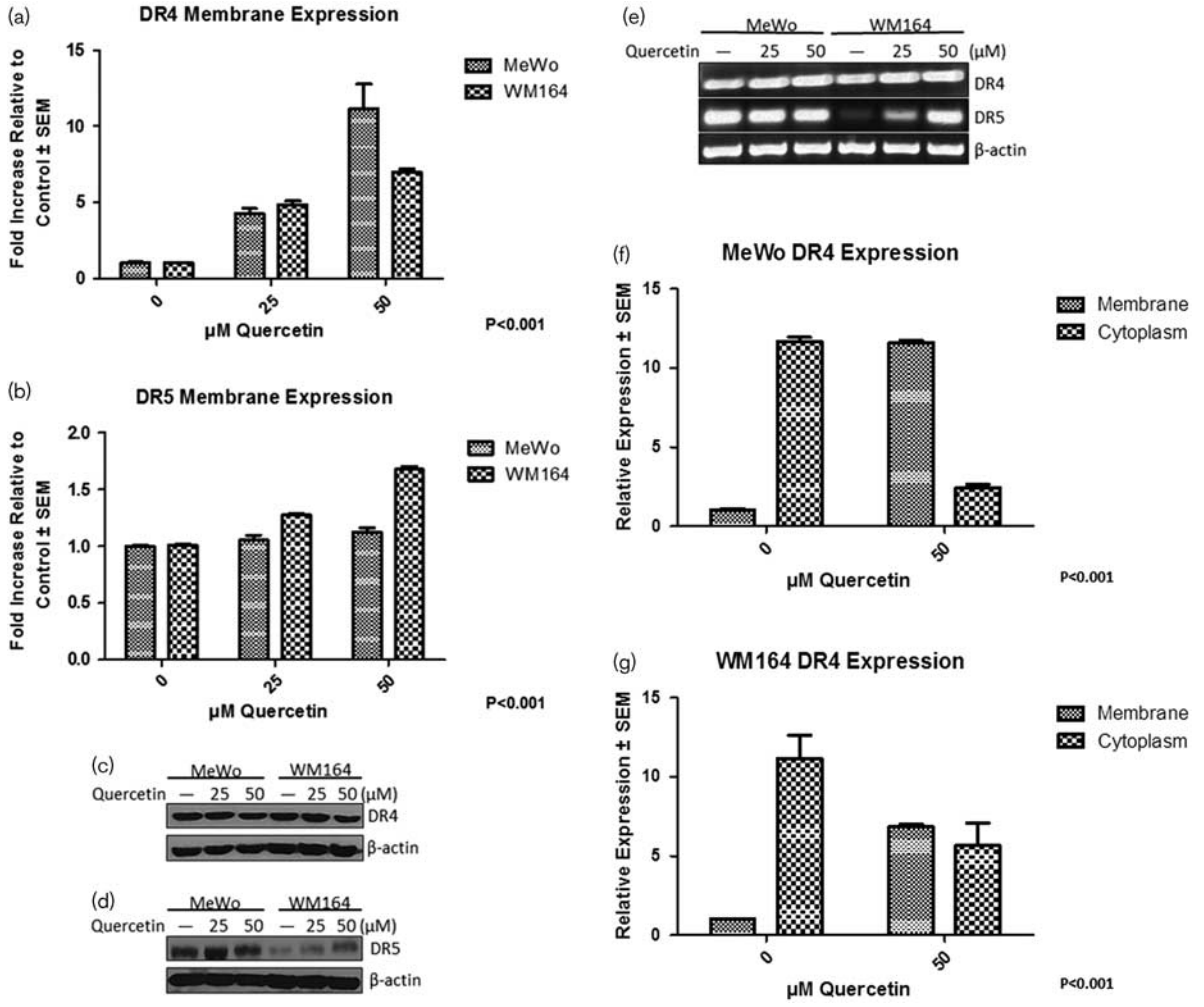
point for rhTRAIL-sensitivity. Here, we wished to see the potential regulation of FLIP by quercetin. Quercetin was able to dose-dependently downregulate the protein expression of FLIP in both melanoma cell lines (Fig. 4a). To see if quercetin transcriptional downregulates FLIP, the mRNA signal of FLIP in response to quercetin treatment was measured (Fig. 4b). Here, we found no change in the transcript signal in both cell lines. Another mechanism of protein downregulation is mediated through proteasomal degradation. To test if quercetin promotes the downregulation of FLIP mediated through the proteasome, the proteasome inhibitor MG132 was employed (Fig. 4c). By cotreating with quercetin and MG132 we were able to prevent the downregulation of FLIP and show that quercetin downregulates FLIP by promoting its proteasomal degradation.

Discussion

rhTRAIL selectively induce robust levels of apoptosis in cancer cells and makes it a prime candidate for the treatment of metastatic malignant melanoma. Unfortunately, the once promising molecule has been withdrawn from human trials due to the resistance exhibited by various cancers to undergo apoptosis when exposed to rhTRAIL. Understanding the molecular mechanisms of rhTRAIL-resistance is essential to formulating a combination therapy to sensitize resistant malignant melanomas. Here, we evaluate the plant-derived flavonoid quercetin as a potential cotreatment for rhTRAIL to overcome melanoma resistance.

Apoptosis induced by proapoptotic receptor agonists such as rhTRAIL is mediated through the binding to extracellular receptors, DR4 and/or DR5, and the direct

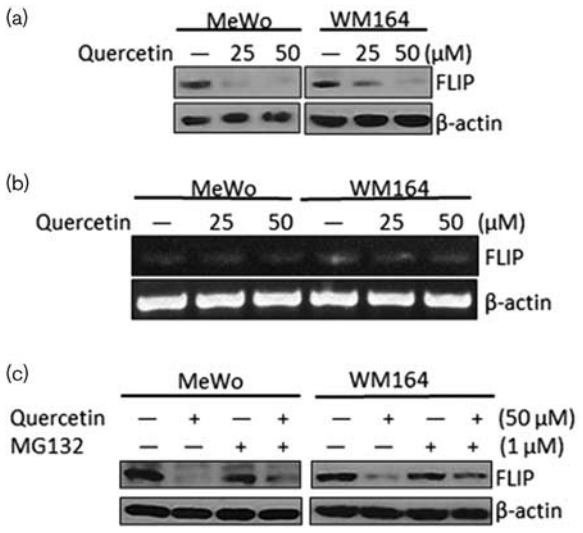
Fig. 3



Quercetin regulation of death receptors. Effects of quercetin on DR4 and DR5 expression. (a) Membrane expression of DR4. Fold increase relative to control ± SEM. (b) Membrane expression of DR5. Fold increase relative to control ± SEM. (c) Total DR4 protein. (d) Total DR5 protein. (e) DR4 and DR5 mRNA signal. (f) MeWo. (g) WM164. Membrane and cytoplasmic expression of DR4. Fold increase relative to control DR4 membrane expression ± SEM.

activation of the extrinsic apoptotic pathway along with the indirect activation of intrinsic pathway in a caspase-mediated fashion [33]. Melanoma cell lines MeWo and WM164, both derived from cases of metastatic malignant melanoma are among the melanomas resistant cell lines to rhTRAIL-induced apoptosis. Even following treatment with extremely high concentrations of rhTRAIL no apoptosis was observed. The lack of apoptotic Annexin-V^+ cells, cleaved PARP or activation of any key proteins in either the extrinsic or intrinsic pathways evidenced this. However, the addition of quercetin was able to negate the rhTRAIL-resistance of both MeWo and WM164. Treating at subcytotoxic concentrations of both single agents, the combination of rhTRAIL plus quercetin was able to induce apoptosis in both cell lines apparent by the significant formation of Annexin-V^+ cells, PARP cleavage and activation of executioner caspases 3, 6, and 7, dose-dependently with respect to quercetin. The combination treatment allowed for the activation of the extrinsic apoptotic pathway as noted by the formation of caspase 8. It is interesting to note that in WM164 and not in MeWo the intrinsic pathway was activated through the cotreatment mediated by formation of tBID followed by the release of cytochrome *C* from the mitochondria and the activation of caspase 9. The general caspase inhibitor Z-VAD-FMK was used to assess the indispensable role of enhanced caspase activity in the sensitization of rhTRAIL-resistant malignant melanomas through the addition of quercetin. Our data clearly show that caspase activation is required to mediate the apoptosis process and is only induced in the presence of quercetin and rhTRAIL.

Fig. 4



Quercetin regulation of FLIP. Effects of quercetin on FLIP expression. (a) Total FLIP protein. (b) FLIP mRNA signal. (c) Quercetin plus proteasome inhibitor MG132 on total FLIP protein.

Of the most significance is that the addition of quercetin to rhTRAIL allowed for the activation of rhTRAIL-mediated extrinsic apoptotic pathway. This is demonstrated by the cleavage of procaspase 8 to caspase 8, a marker for rhTRAIL-induced apoptosis [34]. The activation of caspase 8 in only the cotreatment group implies that quercetin plays a role in regulating cellular components responsible for controlling rhTRAIL-sensitivity. To understand the mechanism of the sensitization of rhTRAIL-resistant malignant melanomas by quercetin, the effects of quercetin on the most apical parts of the extrinsic pathway were evaluated. In addition, rhTRAIL interacts with two antagonist receptors, decoy receptor (DcR) 1 and DcR2. These antiapoptotic receptors lack an intracellular death domain and cannot transmit an apoptotic signal upon rhTRAIL-binding. Originally, the differential expression of proapoptotic compared with antiapoptotic receptors was thought to be the cause for differences in rhTRAIL-sensitivity. However, studies have shown that the distribution between proapoptotic and antiapoptotic receptors does not correlate with sensitivity [35]. It may be possible that DcR1 and DcR2 play a role in regulating rhTRAIL-sensitivity in malignant melanomas and quercetin may regulate their expression. However, because of the lack of scientific support in the literature for DcR expression predicting rhTRAIL-sensitivity or any role of quercetin in DcR expression, the effects of quercetin on DcR were not evaluated and focus was held on the functional components of the death-inducing signaling complex (DISC).

Multiple reports claim that low levels of DRs on the membrane of cancer cells confers rhTRAIL-resistance [14–16,36]. We have previously shown that rhTRAIL-resistant melanomas MeWo and WM164 have two-fold less membrane expression of both DR4 and DR5 compared with rhTRAIL-sensitive melanomas and the melanoma's benign counterpart melanocytes [37]. Nonetheless, the membrane expression of both DR4 and DR5 can be upregulated on melanoma cells via quercetin. The mechanism by which quercetin executes the upregulation in melanoma cells differs between DR4 and DR5. DR expression can be regulated transcriptionally or through post-translational modifications including protein glycosylation, trafficking and endocytosis [38]. Here, we show that quercetin promotes DR5 upregulation on the membrane of melanoma WM164 cells as a consequence of quercetin-stimulated gene transcription. This is illustrated by the correlation between the increased DR5 membrane expression and an increase in the total DR5 protein and mRNA levels, dose-dependently in response to quercetin. Several studies have shown that quercetin is capable of upregulating DR5 on the membrane of cancer cells mediated through the increased activity of transcription factors including *p53*, CHOP and SP1 [28,30,31,38]. However, the transcriptional regulation of DR5 by quercetin has never been evaluated in melanoma cells. The specific cellular targets responsible for the upregulation of DR5 by quercetin are unknown and warrant additional study. However, a *p53*-mediated mechanism can be excluded because WM164 has an inactivating Y220C mutation in the *p53* gene yet experienced DR5 upregulation [39,40].

Conversely, the robust upregulation of DR4 on the membrane of the melanoma cells was not by means of enhanced transcription. This is evident by the absence of an increase in the total protein and mRNA of DR4 in response to quercetin treatment. Previous studies have shown that total protein and mRNA levels do not correlate with the functional membrane expression of DRs. Studies show that some melanoma cells, despite the presence of mRNA, lack DR4 membrane expression but have high levels of the receptor within the cytoplasm [13]. In addition, immunohistochemistry staining of DR4 reveals that the receptor can be localized to the trans-Golgi network in melanoma cells [41]. The translocation of DRs from the trans-Golgi network to the plasma membrane is a complex system regulated by cargo transport proteins such as Arf and ARAP1. Malfunctions in this pathway can result in DR surface deficiency and increased localization to the cytoplasm [38]. Here, we show that both melanoma lines have substantial levels of DR4 within the cytoplasmic portion of the cell which substantially decreases upon quercetin treatment. We hypothesized that quercetin promotes the vesicular movement of DR4 from the cytoplasm or the trans-Golgi network to the cellular membrane. The ability of quercetin to promote the DR4 upregulation on the cancer cell membrane is a novel finding. The quercetin-mediated upregulation of DR4 has never been described and

warrants thorough examination of the exact mechanism by which quercetin promotes this movement.

What is interesting to note is that although rhTRAIL-resistant malignant melanomas, MeWo and WM164, possess less DRs than their sensitive melanoma counterparts, they still express some quantities of DR4 and DR5. However, the rhTRAIL-resistant melanomas do not express the optimum level of DRs required to overcome the apoptotic threshold of the cancer cell. To overcome this threshold a certain amount of caspase 8 must be activated and this is mediated by the binding of rhTRAIL to DR4 or DR5. Without sufficient DR membrane expression there can be no induction of apoptosis. Through the addition of quercetin the concentration of DRs can be increased allowing for enough caspase 8 to be activated to overcome the apoptotic threshold [42].

Moreover, the binding of rhTRAIL to DR4 and/or DR5 results in the trimerization of the receptors leading to the assembly of the intracellular DISC. At the DISC, the adaptor protein, Fas-associated death domain, acts as a bridge between the DR complex and the death effector domain of the initiator caspase, procaspase 8. Induced proximity results in the autoproteolytic cleavage of procaspase 8 into its active form, caspase 8. However, the antiapoptotic protein FLIP will compete for Fas-associated death domain binding, decreasing the formation of caspase 8 and impeding the proapoptotic signal generated by rhTRAIL-binding. This is a result of the homology between FLIP and procaspase 8, both possessing a death effector domain, yet FLIP lacks caspase activity [43]. The ratio between caspase 8 and FLIP is a major regulator of rhTRAIL-sensitivity [20,44]. Although quercetin has no direct effect on procaspase 8 levels, we were able to show downregulation of FLIP in the presence of quercetin which resulted in an increase in the concentration of the proapoptotic procaspase 8. Increase in procaspase 8 resulted in the sensitization of previously resistant melanoma cancer cells to rhTRAIL. FLIP levels are maintained by a balance between transcription and degradation mediated by the ubiquitin-proteasome degradation system [45]. Here, we show that quercetin promotes the downregulation of FLIP mediated through degradation by the proteasome. This is apparent by the inhibition of the quercetin-mediated FLIP downregulation when a proteasome inhibitor is applied. Additional studies have also shown that quercetin is able to downregulate FLIP mediated by the proteasome however this has never been shown in a melanoma model [31,46].

Here, we provide insight into the molecular mechanisms by which rhTRAIL-induced apoptosis can be modulated by quercetin in advanced metastatic malignant melanomas. The application of rhTRAIL is far superior to other therapeutics due the selectivity of rhTRAIL for only cancer cells while exhibiting no harm to healthy cells. This selectively will result in potent antitumor activity with minimal side effects for the patient. However, the presentation of resistance limits the application of rhTRAIL. Nonetheless, resistance can be overcome through the cotreatment of rhTRAIL with quercetin. Quercetin effect is translated by the combined action of the upregulation of both DR4 and DR5 on the cell membrane and the downregulation of the antiapoptotic protein FLIP. As a result of the diverse effects of quercetin, its addition led to the successful sensitization of rhTRAIL-resistant malignant melanomas through cotreatment with rhTRAIL. In the end, the multiple targeted properties of quercetin make it a prime candidate for rhTRAIL cotreatment compared with compounds that only affect a singular cellular component.

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Conflicts of interest

There are no conflicts of interest.

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