Quercetin-mediated Mcl-1 and survivin downregulation restores TRAIL-induced apoptosis in non-Hodgkin's lymphoma B cells

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ABSTRACT

Background
Non-Hodgkin's B-cell lymphomas account for approximately 70% of B-cell lymphomas. While its incidence is dramatically increasing worldwide, the disease is still associated with high morbidity due to ineffectiveness of conventional therapies, creating an urgent need for novel therapeutic approaches. Unconventional compounds, including polyphenols and the cytokine TRAIL, are being extensively studied for their capacity to restore apoptosis in a large number of tumors, including lymphomas.

Design and Methods
Molecular mechanisms of TRAIL-resistance and reactivation of the apoptotic machinery by quercetin in non-Hodgkin's lymphoma cell lines were determined by Hoescht, flow cytometry, Western blot, qPCR, by use of siRNA or pharmacological inhibitors of the mitochondrial pathway and by immunoprecipitation followed by post-translational modification analysis.

Results
Results demonstrate that quercetin, a natural flavonoid, restores TRAIL-induced cell death in resistant transformed follicular lymphoma B-cell lines, despite high Bcl-2 expression levels due to the chromosomal translocation t(14;18). Quercetin rescues mitochondrial activation by inducing the proteasomal degradation of Mcl-1 and by inhibiting survivin expression at the mRNA level, irrespective of p53. Restoration of the TRAIL pathway requires Bax and Bak but is independent of enhanced TRAIL DISC formation.

Conclusions
We demonstrate that inactivation of survivin and Mcl-1 expression by quercetin is sufficient to restore TRAIL sensitivity in resistant non–Hodgkin's lymphoma B cells. Our results suggest, therefore, that combining quercetin with TRAIL treatments may be useful in the treatment of non–Hodgkin's lymphoma.

Key words: follicular lymphoma, diffused large B-cell lymphoma, quercetin, TRAIL, Mcl-1, survivin, apoptosis; proteasome, p53, bax, caspases.

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Introduction

Follicular lymphomas (FL) are indolent non-Hodgkin's lymphomas that in many cases respond to first-line therapy. However, the majority of patients experience recurrent relapse leading to death.[1,2] FL are associated with Bcl-2 overexpression and chromosomal translocation t(14;18),[3] leading to dysregulated apoptosis. As Bcl-2 is an important negative regulator of the mitochondrial pathway, novel therapeutic approaches circumventing the mitochondrial block may prove useful to treat these patients.[4] Unconventional anti-tumor compounds, including polyphenols and the cytokine TRAIL, could meet these objectives.[5]

Apo2L/TRAIL is a promising anti-tumor drug since it can trigger apoptosis selectively in cancer cells. Binding of TRAIL to its cognate receptors, TRAIL-R1 or TRAIL-R2, induces the formation of a molecular platform called the DISC (Death-Inducing Signaling Complex) through homotypic interactions, enabling the recruitment of the adaptor protein FADD, which in turn allows the recruitment of caspase-8 and -10.[6] Formation of the TRAIL DISC brings together caspase monomers in close proximity, promoting their activation and subsequent release to the cytosol, inducing caspase-3 activation through proteolytic cleavage, and execution of the apoptotic program.[7]

Cell dismantling heavily relies on the amount of caspase-8 that is activated within the DISC.[8] Two main apoptotic signaling pathways have so far been described based on caspase-8 and mitochondrial activation. In type I cells, caspase-3 is directly processed by the active caspase-8 that originates from the TRAIL DISC, while caspase-3 activation in type II cells requires the mitochondrial amplification loop leading to the activation of caspase-9.[9] In the latter situation, caspase-8 cleaves Bid, a BH3-only protein that targets the intrinsic pathway through Bax and Bak, allowing the formation of the apoptosome, another molecular platform, in which caspase-9 is activated. Mitochondrial block in type II cells induced by Bcl-2 or Bcl-xL overexpression, or by a deficiency in Bax and/or Bak expression, impedes caspase-3 activation and thus protects tumor cells from TRAIL-induced apoptosis.[10-12]

At the membrane level, TRAIL-induced cell death can also be tightly controlled by two antagonistic receptors, namely TRAIL-R3 and TRAIL-R4. These receptors can selectively compromise TRAIL-induced apoptosis.[13] We have demonstrated that TRAIL-R4 can interact with TRAIL-R2 within the TRAIL DISC, where it impairs caspase-8 activation.[14] Restoration of cell sensitivity to TRAIL can, however, be obtained in a large panel of tumor types by conventional or non-conventional anti-tumor drugs, including polyphenols.[5]

We demonstrate here that two lymphoma cell lines exhibit resistance to TRAIL-induced cell death due to endogenous elevated expression of several anti-apoptotic proteins, including Mcl-1, survivin, Bcl-2 or TRAIL-R4. Interestingly, the tested cell lines, which are characterized by a robust inhibition of the mitochondrial pathway, become sensitive to apoptosis after sequential stimulation with non-cytotoxic concentrations of quercetin and TRAIL. Quercetin rescues TRAIL-induced cell death through Mcl-1-mediated proteasomal degradation and inhibition of survivin expression at the mRNA level. Our results uncover a novel molecular mechanism by which quercetin exerts synergistic activity with TRAIL.

Design and Methods

TRAIL production and antibodies

His-tagged recombinant soluble human TRAIL was produced and used as previously described.[15] For Western blot analysis, antibodies against TRAIL-R1, TRAIL-R2 and TRAIL-R4 were purchased from Chemicon (Millipore, Molsheim, France). Anti-FADD and anti-Bid were obtained from Transduction Laboratories (BD biosciences, Le Pont de Claix, France). Anti-caspase-8 and -10 were from Medical & Biological Laboratories (Clinisciences, Montrouge, France). Antibodies against survivin, phospho-MDM2 and cleaved fragments of caspase-3 were from Cell Signaling (Millipore). Anti-caspase-2 (C-20), Bcl-2, cytochrome c, Bax (2D2), Mcl-1 (S-19) and MDM2 antibodies were purchased from Santa Cruz Biotechnology (Tebu-bio, Le Perray en Yvelines, France). Anti-Bak (ab-1), anti-caspase-9 and anti-FLIP (NF6) antibodies were purchased from EMD Biosciences (Darmstadt, Germany), Upstate (Millipore, Molsheim, France) and Alexis (Coger, Paris, France), respectively. Anti-Bcl-xL antibody was from Calbiochem (VWR, Fontenay-sous-Bois, France), anti-COXII from Molecular probes (Invitrogen, Cergy Pontoise, France), anti-p53 from Ancell (Coger, Paris, France) and anti-actin from Sigma-Aldrich (Lyon, France). For flow cytometry experiments, the antibodies directed against TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4 (wB-K32, B-L27, wB-B44 and wB-P30 clones, respectively) were kindly provided by Diaclone (Besançon, France). The secondary antibody was an Alexa-488 coupled-goat anti-mouse from Molecular Probes (Invitrogen). 3,3'-dihexyloxacarbocyanine (DiOC6) was purchased from Sigma-Aldrich.

Cell culture and treatments

VAL, RL and SUDHL4 cell lines (human B-cell lymphomas) were cultured in RPMI 1640 medium (Lonza, Levallois-Perret, France) containing ultraglutamine, 10% fetal bovine serum and penicillin/streptomycin/amphotericin B. These cell lines were grown in 5% CO_2 at 37°C.

Quercetin (>98% pure) was obtained from Sigma-Aldrich. A 24 mg/ml stock solution was prepared in DMSO. For sequential treatments, cells were treated for 24 h with 20 μM quercetin in complete medium before being treated with His-TRAIL (500 ng/ml) for the indicated times. Control cells were treated with DMSO alone. Caspases inhibitors (20 μM) were added 30 min prior to TRAIL. The pan caspase inhibitor (z-VAD-fmk), caspase-8 inhibitor (z-IETD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) were purchased from Alexis. The Bax channel blocker (Santa Cruz Biotechnology) was used at 5 μM, 1 h prior to TRAIL stimulation.

Measurement of cell viability

In 96-well plates, 5.10^4 cells were incubated for 24 h with increasing concentrations of His-TRAIL (from 0 to 25 000 ng/mL) or staurosporin (from 0 to 1000 nM) (Sigma-Aldrich). Cell viability was assessed by the AlamarBlue® method, according to the manufacturer's specifications (Invitrogen).

Quantification of apoptosis

After treatments, cells were washed twice with PBS and stained with annexin V-FITC, according to the manufacturer's protocol (BD Pharmingen). After staining with annexin V for 15 min at room temperature, the percentage of annexin V-positive cells was analyzed by flow cytometry.

Immunoprecipitation of the TRAIL DISC

For DISC analysis, 30.10^6 cells were stimulated with 5 μg of His-TRAIL in 1 mL of complete medium, for the indicated times at 37°C. Cells were then washed with cold PBS and lysed in 1 mL

of lysis buffer containing 1% NP40, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 10% glycerol. Lysates were pre-cleared with Sepharose 6B (Sigma-Aldrich) for 1 h at 4°C with gentle shaking, and immunoprecipitated at 4°C overnight with G-protein Sepharose beads (Amersham Biosciences, Les Ullis, France) in the presence of 4 μg of anti-TRAIL-R2 antibody. Beads were then washed four times, and immunoprecipitates were eluted in lysis buffer (Tris-HCl 63 mM, SDS 2%, phenol red 0.03%, glycerol 10%, DTT 100 mM, pH 6.8), boiled for 5 min and processed for immunoblotting.

Activation of Bax and Bak by immunoprecipitation

After treatments, cells were lysed in CHAPS buffer (10 mM HEPES ph7.4; 150 mM NaCl; 1% CHAPS) for 30 min on ice, and lysates were pre-cleared with G-coupled sepharose beads for 1 h at 4°C. Then, the conformationally active form of Bax or Bak was immunoprecipitated with 4 μg of anti-Bax (clone 6A7, BD Biosciences) or anti-Bak (clone NT, Millipore) antibodies, overnight at 4°C on a rotating wheel. The immunoprecipitated proteins, as well as whole cell lysates, were then analyzed by Western blot.

Western blot analysis

Immunoprecipitates or cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Non-specific binding sites were blocked by incubation in PBS containing Tween 20 (0.05%) and fat-free dry milk (5%). Membranes were incubated with specific primary antibody, overnight at 4°C, followed by HRP-conjugated secondary antibody, at room temperature for 1 h. Immunoblots were then developed by the enhanced chemiluminescence (ECL) reagent kit from Santa Cruz Biotechnology, according to the manufacturer's protocol.

Measurement of cytochrome c release

After treatment, cells were washed in PBS and resuspended in a permeabilization buffer containing 400 μg/mL digitonin, 75 mM KCl, 1mM NaH2PO4, 8 mM Na2HPO4 and 250 mM sucrose, and were kept on ice for 10 min. After centrifugation (5 min at 16,000 g), supernatants were collected as the cytosolic fraction. Pellets were then lysed in buffer containing 1% Triton-X100 for 30 min on ice. After centrifugation (15 min at 16,000 g), supernatants were collected as the total extracts that contain mitochondria.

Measurement of mitochondrial membrane potential

Cells were stimulated or not with His-TRAIL (500 ng/mL) or staurosporine (1 μM) for 16 or 6 h. After treatment, cells were collected, resuspended in PBS and then stained for 20 min at 37°C with 50 nM DiOC6, mitochondrial membrane potential (MMP)-sensitive fluorescent dye. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (CCP, Sigma) was used as positive control to quickly collapse MMP. Fluorescence related to MMP was measured by flow cytometry at 525 nm. Each measurement was conducted on 8,000 events and analyzed on Cell Quest software.

Gene silencing using small interfering RNA

For siRNA-mediated gene knockdown, 4.10^6 cells were transfected by nucleoporation with the Amaxa nucleofector (Köln, Germany). VAL and RL cells were resuspended in 100 μL Nucleofector solution V containing 200 pm siRNA, and electroporated with the program N-016 (VAL) or X-001 (RL). Then, cells were cultured in complete medium for 48 h before treatments with TRAIL and/or quercetin. Akt and TRAIL-R4 siRNAs were from Eurogentec (Angers, France) and have been previously described.[16,17] Mcl-1, c-FLIP, Bid, Bax and Bak SiGenome SMARTpool technology siRNAs (set of 4) were purchased from Thermo Scientific (Dharmacon Division).

Real-time PCR assay

RNA was extracted from treated cells with the RNeasy Mini Kit from Qiagen (Valencia, CA, USA). cDNAs were synthesized from total RNA using M-MLV Reverse Transcriptase (Promega). Real-time PCR was performed in triplicate using syber green PCR master Mix from Applied Biosystems (Foster City, CA, USA) and analyzed in a 7500 Fast Detection System (Applied Biosystems). The oligonucleotides used in this study were designed and synthesized (Eurogentec) as follows: caspase-10 sense GAAGAGAACAGTGTGGGGTG, antisense GAGGTTTCCGTCTTGCTGTA; Mcl-1 sense CGTTGTCTCGAGTGATGATCCA, antisense TCACAATCCTGCCCCAGTTT; survivin sense GCCGAGGCTGGCTTCA, antisense GAAGAAACACTGGGCCAAGTCT.

Statistical analysis

With the exception of the experiment using AlamarBlue (Figure 1A), which was analyzed by ANOVA with Bonferroni post-testing, all other quantitative experiments were analyzed using Student's t-test. All statistical analyses were performed using Prism version 5.0a software (GraphPad Software, San Diego, CA, USA). Group comparisons were considered significant for two-tailed *P* values *<0.05, **<0.01 and ***<0.001.

Results

VAL and RL B-cell lines display strong resistance to TRAIL-induced apoptosis

The non-Hodgkin's B-lymphoma cell lines VAL, RL and SUDHL4 exhibit differential sensitivity to TRAIL-induced cell death (Figure 1A). Follicular lymphoma VAL and RL cells were nearly completely insensitive to TRAIL-induced killing, while the viability of SUDHL4 cells, defined as a diffused large B-cell lymphoma, decreased after TRAIL stimulation in a dose dependent manner (Figure 1A). Analysis of caspase activation by Western blotting after TRAIL stimulation showed that caspase-3 was fully cleaved in the sensitive SUDHL4 cell line, but only partly processed in the resistant VAL and RL cells (Figure 1B). Strikingly, although the sensitive cell line SUDHL4, contrary to VAL and RL cells, was nearly devoid of caspase-10 (Figure 1B), activation of caspase-8, caspase-9, caspase-2 and cleavage of Bid appeared to occur to a similar extent in the three lymphoma cell lines (Figure 1B). Importantly, Bax and Bak were not significantly activated upon TRAIL stimulation in VAL and RL cells (Figure 1C). Likewise, cytochrome c was not released from mitochondria (Figure 1D), contrary to SUDHL4 cells. Therefore, since caspase-9 has been demonstrated to be a direct target of caspase-8,[18] these data suggest that activation of caspase-9 and caspase-2 in VAL and RL cells may directly result from caspase-8 activation, but not from mitochondria. In line with this hypothesis, TRAIL stimulation in these resistant cells induced no loss of mitochondrial potential (MMP) (Figure 1E) and caspase-9 cleavage was inhibited by caspase-8 inhibitors (*data not shown*). Moreover, VAL and RL cells were refractory to CCP- or staurosporin-induced MMP loss (Figure 1E) and were consequently resistant to apoptosis-induced by staurosporin, while MMP dropped substantially in SUDHL4 cells under similar conditions (Figure 1E), leading to apoptosis (*Online Supplementary Figure S1*).

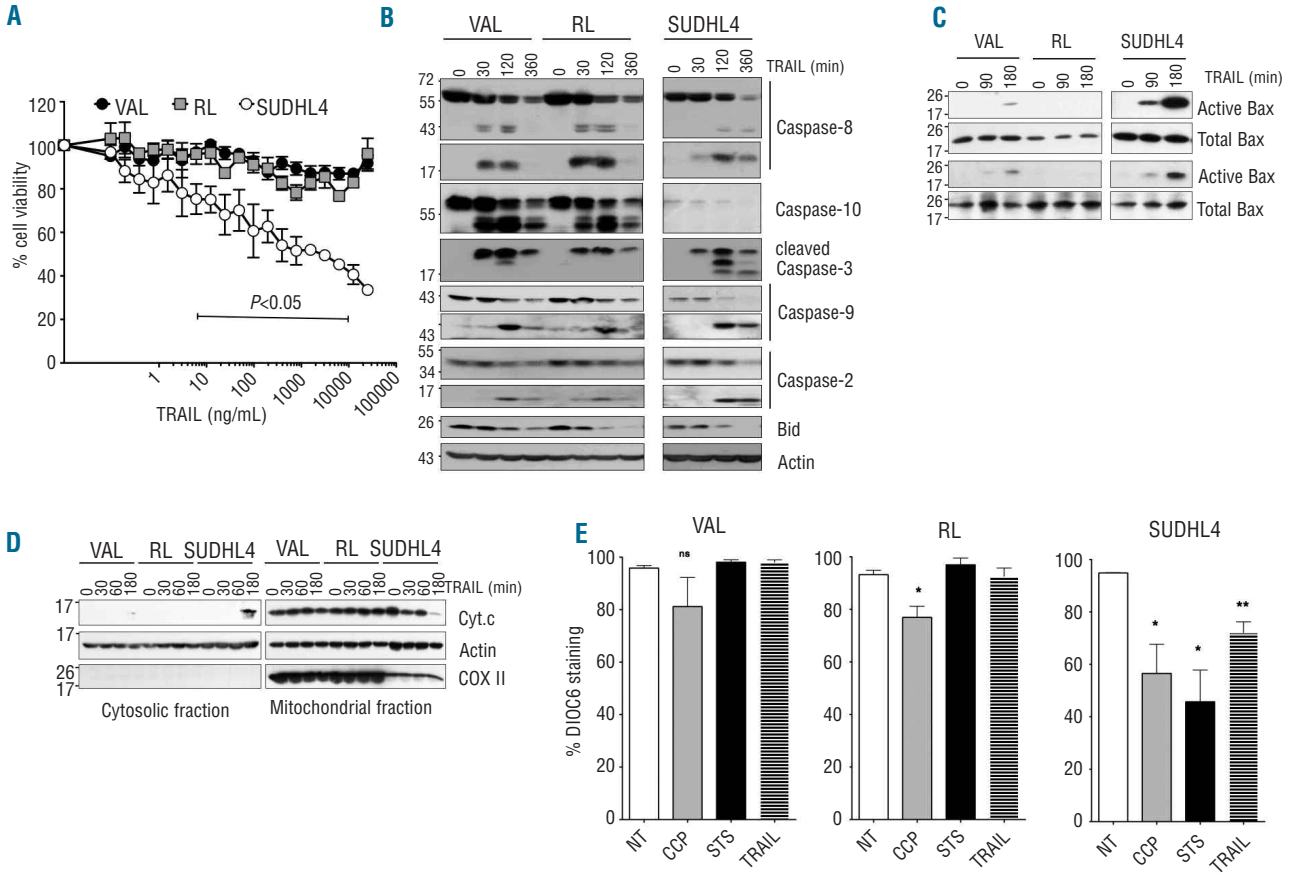

Figure 1. VAL and RL non-Hodgkin's B-cell lymphomas are resistant to TRAIL-induced cell death because of a defect in the mitochondrial pathway of apoptosis. (A) Sensitivity to TRAIL-induced cell death of the non-Hodgkin's B-lymphoma cell lines VAL, RL and SUDHL4. Cells were treated with different concentrations of His-TRAIL for 24 h. Cell viability was measured by the AlamarBlue method. Data presented are means plus or minus SD (n=3; $P<0.05$ for SUDHL4 as compared to VAL or RL cell lines). (B) Analysis of caspase activation and Bid by Western blot after treatment with His-TRAIL (500 ng/mL) for the indicated times. (C) TRAIL-induced Bax and Bak activation. After treatment with TRAIL at 500 ng/mL for the indicated times, the active forms of Bax or Bak were immunoprecipitated and analyzed by Western blot. (D) TRAIL-induced cytochrome c release from the mitochondria to the cytosol. VAL, RL and SUDHL4 cells were treated with His-TRAIL (500 ng/mL) for the indicated times. Cytosolic and mitochondrial fractions were analyzed by Western blot to detect the presence of cytochrome c. COXII was used as a mitochondrial marker. (E) Cells were left untreated (NT), treated with His-TRAIL (500 ng/mL) or staurosporine (1 μM) for 16 or 6 h, respectively, then incubated with the MMP-sensitive fluorescent dye $DiOC_6$ for 20 min, and fluorescence related to MMP was measured by flow cytometry. CCP was used to elicit rapid disruption of MMP (mitochondrial membrane potential), as revealed by the decrease in $DiOC_6$ fluorescence in SUDHL4 cells. Data presented are means plus or minus SD; n=3; *$P<0.05$ or **$P<0.01$ or ***$P<0.001$ respective to NT; ns: not statistically relevant.

Resistance to TRAIL-induced apoptosis in VAL and RL cells is multifactorial

Owing to the chromosomal translocation t(14;18), follicular B-cell lymphomas express high levels of Bcl-2 (*Online Supplementary Figure S2A*). We have recently shown, in addition, that besides Bcl-2, these lymphoma cell lines express different levels of TRAIL receptors,[19] TRAIL-R4 in particular (*Online Supplementary Figure S2B*). Inactivation of Bcl-2 by use of a specific siRNA targeting Bcl-2 (*Online Supplementary Figure S3A*), significantly restored apoptosis induced by TRAIL in VAL and RL cells (*Online Supplementary Figure S2C*). Likewise, siRNA-mediated targeted inhibition of TRAIL-R4 expression in VAL cells (*Online Supplementary Figure S3B*) significantly restored sensitivity to TRAIL (*Online Supplementary Figure S2C*). Conversely, inhibition of TRAIL-R4 expression in RL cells, which express low levels of TRAIL-R4 (*Online Supplementary Figure S3B*), failed to restore TRAIL-induced cell death (*Online Supplementary Figure S2C*). Strikingly, SUDHL4 and VAL cells exhibited differential sensitivity to TRAIL-induced cell death, despite comparable expression levels of TRAIL-R4 (*Online Supplementary Figure S2B*). The differential behavior did not result from mutations in TRAIL-R4 in SUDHL4 cells as demonstrated by DNA sequence analysis (*data not shown*). Therefore, in order to understand why the follicular B-cell lines VAL and RL fail to engage the apoptotic machinery upon TRAIL stimulation, we have focused our attention on several additional key anti-apoptotic proteins including c-FLIP, Mcl-1 or survivin (*Online Supplementary Figure S2A*). We have recently proposed that TRAIL-R4 and c-FLIP may cooperate to inhibit TRAIL-induced apoptosis.[17] In line with this hypothesis, c-FLIP long and short were both expressed to a much higher extent in the resistant cells as compared to the sensitive cell line SUDHL4 (*Online Supplementary Figure S2A*). Consistently, inhibition of c-FLIP expression by use

of specific siRNA (*Online Supplementary Figure S3C*) partially but significantly restored TRAIL-induced cell death in both resistant cell lines (*Online Supplementary Figure S2C*), while ectopic expression of c-FLIP long in SUDHL4 inhibited TRAIL-induced cell death (*Online Supplementary Figure S4*). Besides TRAIL-R4 and c-FLIP, mitochondrial- or post-mitochondrial apoptotic inhibitors may play a role in controlling caspase-9 and caspase-3 activation in these resistant cells. For instance, we have found that Mcl-1 and survivin were expressed at higher levels in VAL and RL cells as compared to the sensitive cell line SUDHL4 (*Online Supplementary Figure S2A*), while other inhibitors such as Bcl-xL (*data not shown*) or XIAP (*Online Supplementary Figure S2A*) were expressed at similar levels. Moreover, Mcl-1 expression appeared to increase in a time-dependent manner upon TRAIL stimulation in both resistant cells, but not in SUDHL4 cells (*Online Supplementary Figure S2A*). These results prompted us to check whether inhibition of Mcl-1 or survivin expression (*Online Supplementary Figure S2D*) could restore TRAIL-induced apoptosis in VAL and RL cells. Indeed, siRNA targeting of either survivin or Mcl-1 significantly restored TRAIL-induced cell death in these cells (*Online Supplementary Figure S2C*). Altogether, these results highlight that VAL and RL cell resistance to TRAIL-induced cell death is a multimodal process, which takes place at the membrane-, the mitochondrial- and at the post-mitochondrial level.

Quercetin overcomes cell resistance to TRAIL-induced cell death

We next assessed the ability of quercetin to restore TRAIL-induced cell death in these resistant cells, as this flavonoid has previously been demonstrated to target survivin,[20] and to synergize with TRAIL in various tumor cell types.[5] Remarkably, pre-treatment with 20 μM quercetin for 24 h significantly overcame TRAIL resistance in these B-lymphoma cell lines, in a caspase-dependent manner, as demonstrated by the use of the pan-caspase inhibitor zVAD (Figure 2A). This flavonoid restored full caspase-3 activation (Figure 2B). Engagement of the apoptotic machinery required both caspase-8 and caspase-9, as specific inhibitors of these initiator caspases similarly abrogated TRAIL-induced apoptosis (Figure 2C).

Quercetin restores TRAIL sensitivity regardless of caspase-10 upregulation and recruitment to the DISC

To understand the molecular mechanisms involved in the restoration of apoptosis induced by TRAIL, after quercetin stimulation, we first evaluated whether this flavonoid might regulate TRAIL receptor expression or enhance TRAIL-DISC formation. Flow cytometry analysis demonstrated that quercetin pre-treatment induced no change in the expression of any of the TRAIL receptors in VAL or RL cells (*Online Supplementary Figure S5*). TRAIL-DISC formation was also not significantly affected by quercetin stimulation, with the exception of TRAIL-R1, FADD and caspase-8 and -10 whose recruitment and activation within the DISC appeared to be slightly enhanced in VAL cells but less so in RL cells (*Online Supplementary Figure S6A*). Interestingly, quercetin induced a strong increase in caspase-10 expression in these cells, associated with an enhanced caspase-10 processing upon quercetin/TRAIL stimulation as compared to TRAIL alone (*Online Supplementary Figure S6B*). As measured by qPCR analysis, quercetin-mediated caspase-10 upregulation was controlled at the mRNA level (*Online Supplementary Figure S6C*). However, caspase-10 itself did not appear to be essential for the restoration of TRAIL-induced apoptosis by the quercetin, as inactivation of this initiator caspase, using a specific caspase-10 targeting siRNA, failed to compromise the efficacy of the combined treatment (*Online Supplementary Figure S6D and E*). Altogether, these results indicate that quercetin-mediated TRAIL sensitization is independent of caspase-10 and most likely independent of regulation of TRAIL DISC formation.

Sensitization mainly requires mitochondrial activation

Since enhanced TRAIL DISC formation does not appear to be essential for quercetin-mediated TRAIL-induced cell death restoration, we focused our attention on the mitochondrial pathway. Fractionation experiments to analyze cytochrome c release were performed from cells pre-treated or not with quercetin and stimulated with TRAIL, for the indicated periods of time. Stimulation with quercetin enhanced cytochrome c release after TRAIL stimulation in VAL cells and induced cytochrome c release in RL cells (Figure 3A). To determine whether reactivation of the mitochondrial pathway required Bid, its expression was knocked-down using a Bid targeting siRNA (*Online Supplementary Figure S3E*). Inactivation of Bid significantly inhibited TRAIL-induced cell death after quercetin stimulation (Figure 3B), suggesting that the mitochondrial amplification loop, through Bax and/or Bak activation was required. In agreement with this hypothesis, Bax channel blockers were found to inhibit TRAIL-induced cell death after quercetin pre-treatment (Figure 3C). Moreover, while inactivation of Bax or Bak alone was insufficient to fully inhibit TRAIL-induced apoptosis after quercetin pre-treatment, combined Bax and Bak knockdown (*Online Supplementary Figure S3F*) completely abrogated the synergy (Figure 3D).

Quercetin reactivates the mitochondrial pathway through Mcl-1 and survivin downregulation, irrespective of p53

To clarify the molecular events required to bypass the mitochondrial block in VAL and RL cells upon quercetin stimulation, we next assessed the expression levels of some anti-apoptotic proteins, including Bcl-2 family members, by Western blot analysis. While no change in Bcl-2, Bcl-xL or XIAP protein expression was found after quercetin treatment, the flavonoid induced the depletion of both Mcl-1 and survivin in RL and VAL (Figure 4A). In agreement with previous findings,[21] we found that Mcl-1 expression was induced upon TRAIL stimulation in both resistant cell lines, but remarkably, TRAIL-mediated Mcl-1 upregulation was completely abrogated by quercetin (Figure 4A). Quercetin-mediated survivin and Mcl-1 downregulation occurred in a caspase-independent manner (*data not shown*). Since p53 is known to be a negative regulator of Mcl-1 and survivin,[22,23] we first checked whether this transcription factor might be involved in the regulation of the expression levels of these proteins upon quercetin stimulation. As shown by Western blot analysis, quercetin induced p53 upregulation and a decrease in the expression of the p53 inhibitor MDM2 (Figure 4B). Interestingly, as evidenced by qPCR, survivin mRNA expression levels were reduced by more than 40% in quercetin stimulated cells as compared to non-stimulated cells, whereas Mcl-1 mRNA levels increased upon stimu-

lation (Figure 4C). These results prompt us to assess whether p53 may promote restoration of TRAIL sensitivity through inhibition of survivin expression. However, inactivation of p53, using specific siRNAs, had no impact on quercetin-mediated survivin or Mcl-1 expression inhibition (Figure 4D) and failed to inhibit the synergistic apoptotic activity of the combination TRAIL and quercetin (Figure 4E). Moreover, using the proteasome inhibitor MG132, we could demonstrate that inhibition of Mcl-1 expression levels, but not survivin, following quercetin treatment occurred through proteasomal degradation (Figure 4F). Accordingly, Mcl-1 was strongly ubiquitinated upon quercetin treatment (Figure 4G).

Altogether, our results demonstrate that quercetin restores TRAIL-induced apoptosis in resistant NHL-B cell lines, at least in part through inhibition of Mcl-1 and survivin expression.

Discussion

In this study, we demonstrate that quercetin synergizes with TRAIL to trigger apoptosis in FL transformed resistant B-cell lines, despite strong mitochondrial inhibition due to high Bcl-2, Mcl-1 and survivin expression. Quercetin has been reported to synergize with TRAIL,[24-29] but the molecular mechanisms underlying this sensitization remain largely unknown. At the proximal level, quercetin-mediated sensitization to TRAIL has been correlated with TRAIL-R2 stabilization,[24] increased TRAIL-R2 expression at the cell surface,[25,29] enhanced TRAIL DISC formation[27] and even c-FLIP downregulation.[25] In our cellular models, regulation of proximal events is unlikely to explain the synergy since quercetin induced no change in TRAIL receptor or c-FLIP expression and only modest differences in TRAIL-R1, FADD, caspase-8 and caspase-10 recruitment within the DISC. As compared to conventional chemotherapeutic drugs, such as cisplatin or 5FU, which induce a robust increase in caspase-8 recruitment and activation within the TRAIL DISC in VAL cells,[17] quercetin only weakly enhanced initiator caspase-8/10 or TRAIL-R1 recruitment. Moreover, caspase-10 upregulation was not essential to the restoration of quercetin-mediated TRAIL sensitivity in both resistant cell lines. Yet, we cannot definitely exclude the possibility that the slight increase in caspase-8 or TRAIL-R1 recruitment within the TRAIL DISC might, to some extent, contribute to the restoration of the TRAIL signaling pathway. Discrepancies regarding the implication of TRAIL proximal events in restoring TRAIL-induced cell death by quercetin may merely reflect differences in drug concentrations. At this point, it should be emphasized that the concentrations of quercetin used in our study, 20 μM, are lower than those used in most studies (50-200 μM).[24-26] Besides, cell specificities may also give rise to discrepant results. Likewise, the mitochondrial pathway is strongly inhibited in resistant B-lymphoma cell lines, yet quercetin achieves restoration of the TRAIL apoptotic machinery.

Our findings rather suggest that the main target is the mitochondria, since quercetin treatment enhanced cytochrome c release upon TRAIL stimulation, whereas inactivation of Bid or Bax/Bak using siRNA, or inhibition of the mitochondrial pathway using a Bax channel blocker, efficiently abrogated the synergy. Quercetin-induced restoration of the mitochondrial apoptotic potential was associated with a dysregulation of Mcl-1 and survivin expression. It has been proposed that survivin acts mainly at the post-mitochondrial level, through its ability to inhibit Smac release from the mitochondria, stabilizing

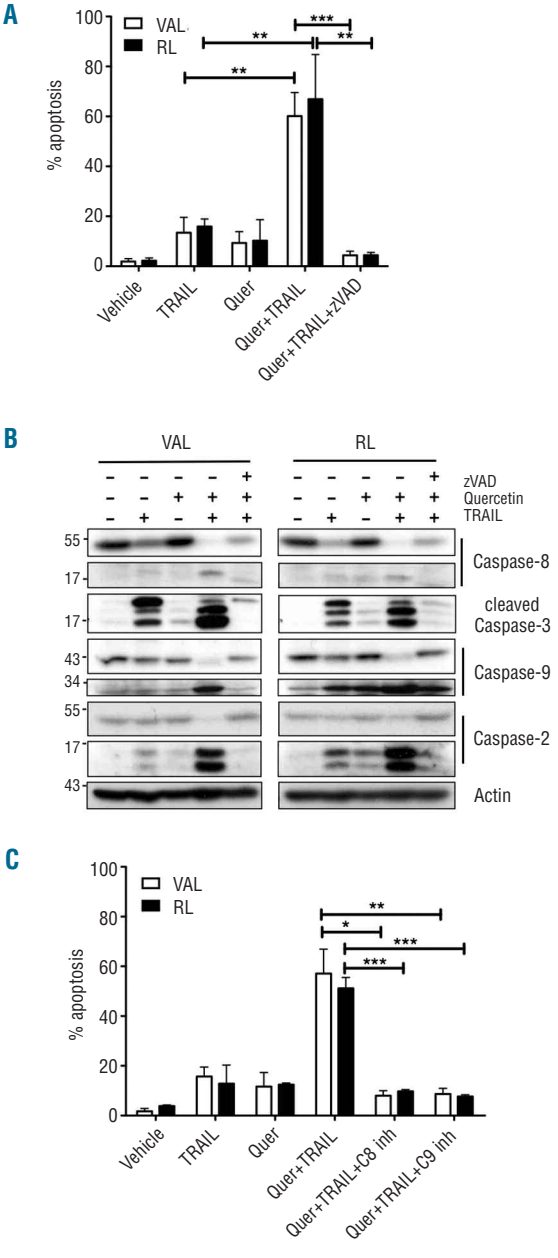

Figure 2. Quercetin sensitizes VAL and RL resistant non-Hodgkin's lymphoma B-cell lines to TRAIL-induced apoptosis. (A) VAL and RL cells were treated with 20 μM quercetin (Quer) for 24 h prior to TRAIL (500 ng/mL for 3 h). The pan caspases inhibitor zVAD-fmk (20 μM) was added 30 min before treatment with TRAIL. Apoptosis was measured by annexin V staining. (B) Western blot analysis of caspase activation upon treatment with quercetin (20 μM, 24 h), followed by TRAIL (500 ng/mL, 6 h) and/or zVAD-fmk (20 μM, 30 min before TRAIL). (C) Quantification of apoptosis by annexin V staining after treatment with quercetin and TRAIL as described in (A). Caspase-8 inhibitor (z-IETD-fmk) and caspase-9 inhibitor (z-LEHD-fmk) were used at 20 μM, 30 min before TRAIL stimulation. (A) and (C) Data are means plus or minus SD; n=3; $^{**}P<0.01$ or $^{***}P<0.001$ respective to quercetin alone or to quercetin+TRAIL in the presence of caspase-inhibitors.

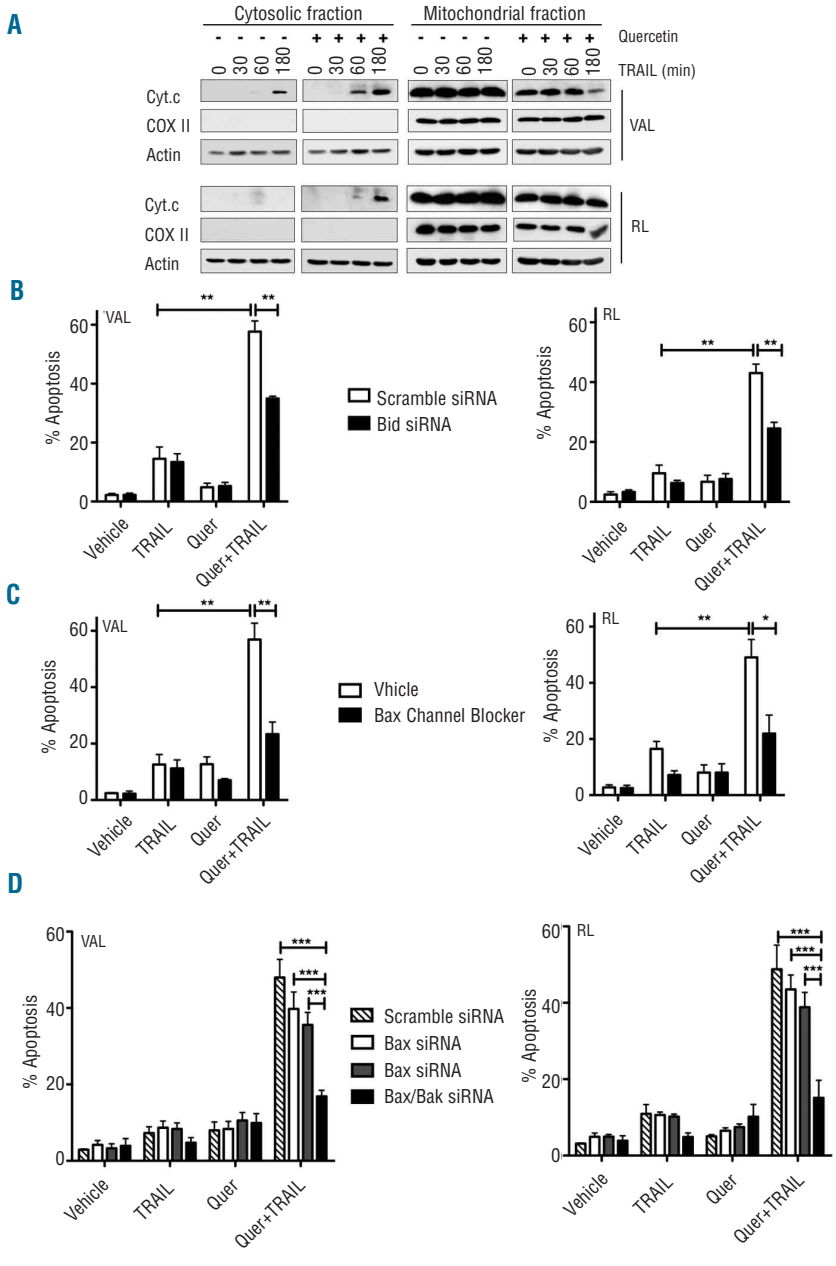

Figure 3. Sensitization to TRAIL by quercetin requires the mitochondrial pathway of apoptosis. (A) TRAIL-induced cytochrome c release from the mitochondria to the cytosol after quercetin pre-treatment. VAL and RL cells were treated with quercetin (20 μM) for 24 h, followed by TRAIL (500 ng/mL) for the indicated times. Cytosolic and mitochondrial fractions were analyzed by Western blot for the detection of cytochrome c. COXII was used as a mitochondrial marker. (B) Effect of siRNA-mediated knockdown of Bid on the efficiency of quercetin and TRAIL combined treatment. Twenty-four hours after electroporation with a specific siRNA or a control siRNA (scramble), VAL and RL cells were treated with quercetin (20 μM) for 24 h, followed by TRAIL (500 ng/mL) for 3 h. (C) Effect of Bax channel formation on the efficacy of the combined quercetin and TRAIL treatment. VAL and RL cells were treated with quercetin (20 μM) for 24 h. Bax Channel Blocker was added at 5 μM, 1 h before stimulation with TRAIL (500 ng/mL for 3 h). (D) Effect of siRNA-mediated knockdown of Bax and/or Bak on the efficiency of the combined treatment with quercetin and TRAIL. Cells were treated as in (B). (B-D) Apoptosis was measured by flow cytometry after annexin V staining. (B-D) Data are means plus or minus SD; n=3; **P*<0.05; ***P*<0.01 or ****P*<0.001 respective to TRAIL alone or to target siRNA as compared to scramble; ns: not statistically relevant.

XIAP and leading to inhibition of caspase-9 and -3 activation.[30] Survivin expression has been demonstrated to be negatively regulated by a large number of transcription factors or signaling pathways, including p53, Akt or proteosomal degradation.[20,22,29] In VAL and RL cells, neither p53 nor the proteasome or Akt (*Online Supplementary Figure S7*) appear to be required to repress survivin expression upon quercetin stimulation. Further studies will be required to clarify how survivin expression is repressed upon quercetin stimulation.

Importantly, our results highlight a novel regulatory event controlling the restoration of TRAIL apoptotic signaling activity by quercetin. To our knowledge, we are the first to report that quercetin affords restoration of TRAIL-induced cell death in aggressive B-lymphoma cell lines through Mcl-1-mediated proteasomal degradation. This Bcl-2 family member is known to sequester BH3-only proteins including Bid and Bim[31-33] but also Bak,[34] affording high levels of protection against mitochondrial depolarization, cytochrome c release and activation of caspase-9. Mcl-1 has thus been proposed to protect cells from TRAIL-induced cell death by inhibiting Bak and Bid, the inhibition of which impacts on Bax activation.[32,35] This assumption is in agreement with our findings as inactivation of Bak or Bax alone by siRNA was not sufficient to inhibit apoptosis induced by the combination of quercetin and TRAIL, while simultaneous inhibition of Bax and Bak was required to impair the synergy. Interestingly, quercetin-mediated Mcl-1 proteasomal degradation was associated with an increase in Mcl-1 ubiquitination. Keeping in mind that quercetin has been extensively used in the past as a heat shock protein inhibitor,[36] it is interesting to note that HSP70 has recently been demonstrated to impair the association of the ubiquitin ligase Mule with

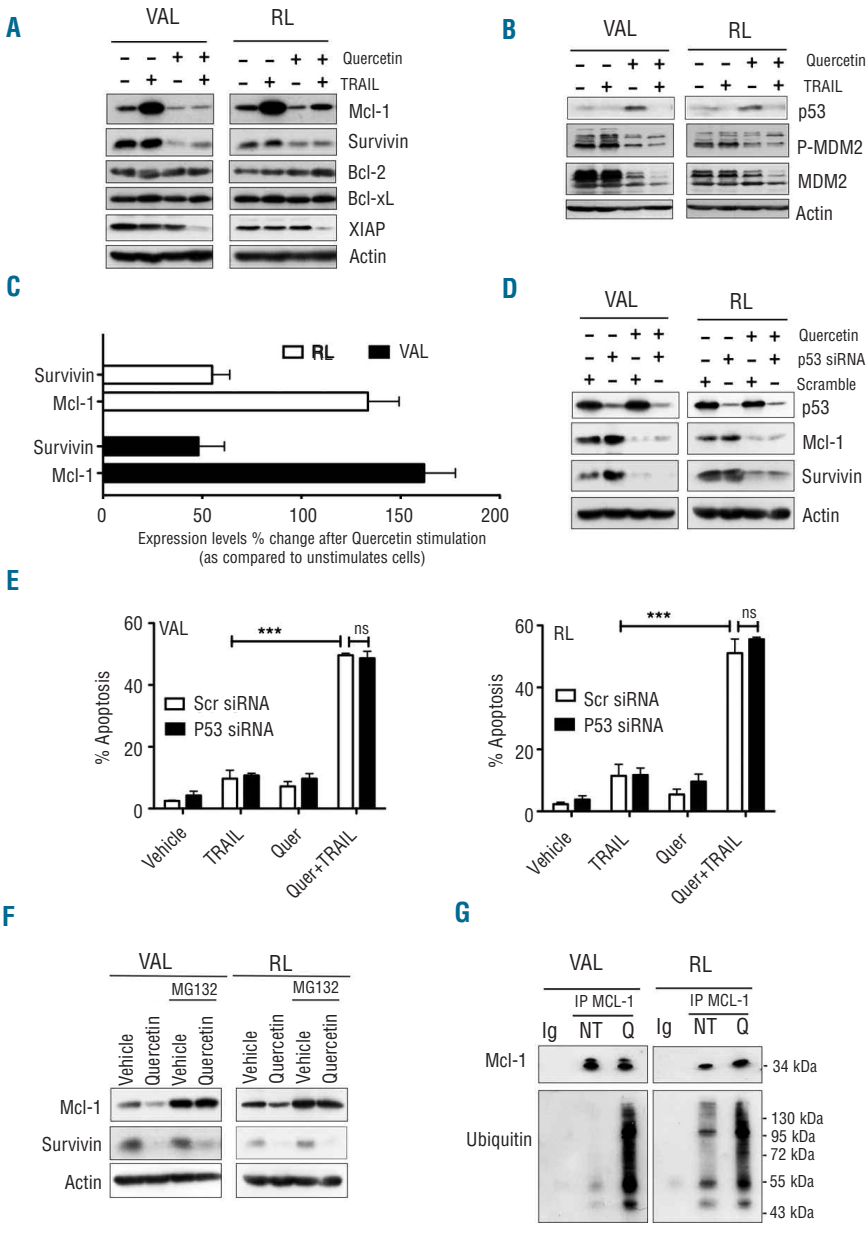

Figure 4. Quercetin inhibits Mcl-1 through ubiquitin-dependant proteasomal degradation and downregulates survivin at the mRNA level, independently of p53. (A-B) Western blot analysis of Mcl-1, survivin, p53, phospho-MDM2 and total MDM2 expression after treatment with quercetin (20 μM, 24 h), followed by TRAIL (500 ng/mL, 6 h). (C) Relative expression of Mcl-1 or survivin mRNA by qPCR after treatment with quercetin (20 μM, 24 h). Results correspond to the fold change mRNA expression (%) compared with cells treated with DMSO, and were normalized to L32 levels. (D) Effect of siRNA-mediated knockdown of p53 on Mcl-1 and survivin after TRAIL and/or quercetin stimulation. Twenty-four hours after electroporation with a specific siRNA or a control siRNA (scramble), VAL and RL cells were treated or not with quercetin (20 μM) for 24 h, followed by TRAIL (500 ng/mL) for 3 h or left untreated and p53, survivin and Mcl-1 expression was evaluated by Western blotting. (E) Effect of quercetin- and TRAIL-induced apoptosis in the absence of p53 was measured by flow cytometry after annexin V staining. (F) Impact of proteasome inhibition on Mcl-1 and survivin protein levels. Cells were treated with quercetin (20 μM) or DMSO (vehicle), in the presence of the proteasome inhibitor MG132 (1 μM) for 24 h. (G) Quercetin-mediated ubiquitination of Mcl-1. Cells were treated with quercetin (Q) or vehicle (NT) in the presence of MG132 for 24 h as previously described. Mcl-1 was immunoprecipitated and ubiquitin residues were detected by Western blot analysis. Immunoglobulin (Ig) was used as a negative control for immunoprecipitation. Data presented panel (E) are means plus or minus SD (n=3; ***$P<0.001$ respective to TRAIL alone or to quercetin+TRAIL in the presence or the absence of p53 siRNA; ns: not statistically relevant).

Mcl-1, leading to Mcl-1 stabilization and to inhibition of Bax activation.[37] Our findings are particularly important since it has recently been found that Mcl-1 expression in mantle cell lymphoma was associated with high-grade morphology and a proliferative state.[38] Quercetin's ability to induce Mcl-1 degradation possibly represents a very important mechanism enabling restoration of the mitochondrial apoptotic pathway induced by TRAIL in human lymphomas. These findings could also apply to some leukemias, since it has been recently demonstrated that quercetin alone, at higher concentrations, could induce tumor-selective apoptosis through Mcl-1 downregulation and Bax activation.[39] Therefore, therapeutic strategies associating TRAIL and quercetin to eradicate tumors and to overcome cell resistance may be close at hand,[5] since quercetin and TRAIL, when applied either alone or in combination, exhibited no toxicity towards normal lymph nodes or tonsil cells (*Online Supplementary Figure S8*). Considering that these compounds alone exhibit limited side effects, and are extremely well tolerated in humans as demonstrated in clinical trials,[40,41] our results suggest that combining TRAIL with the naturally occurring flavonoid quercetin could represent an attractive therapeutic approach for NHL.

Authorship and Disclosures

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References

1. Lossos IS, Alizadeh AA, Diehn M, Warnke R, Thorstenson Y, Oefner PJ, et al. Transformation of follicular lymphoma to diffuse large-cell lymphoma: alternative patterns with increased or decreased expression of c-myc and its regulated genes. Proc Natl Acad Sci USA. 2002;99(13):8886-91.
2. de Vos S, Hofmann WK, Grogan TM, Krug U, Schrage M, Miller TP, et al. Gene expression profile of serial samples of transformed B-cell lymphomas. Laboratory investigation; a journal of technical methods and pathology. 2003;83(2):271-85.
3. Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science. 1984;226(4678):1097-9.
4. Fulda S. Inhibitor of apoptosis proteins in hematological malignancies. Leukemia. 2009;23(3):467-76.
5. Jacquemin G, Shirley S, Micheau O. Combining naturally occurring polyphenols with TNF-related apoptosis-inducing ligand: a promising approach to kill resistant cancer cells? Cell Mol Life Sci. 2010;67(18):3115-30.
6. Merino D, Lalaoui N, Morizot A, Solary E, Micheau O. TRAIL in cancer therapy: present and future challenges. Expert opinion on therapeutic targets. 2007;11(10):1299-314.
7. Micheau O, Merino D. Controlling TRAIL-mediated caspase-3 activation. Leukemia. 2004;18(10):1578-80.
8. Peter ME. The TRAIL DISCussion: It is FADD and caspase-8! Cell Death Differ. 2000;7(9):759-60.
9. Ashkenazi A, Holland P, Eckhardt SG. Ligand-based targeting of apoptosis in cancer: the potential of recombinant human apoptosis ligand 2/Tumor necrosis factor-related apoptosis-inducing ligand (rhApo2L/TRAIL). J Clin Oncol. 2008;26 (21):3621-30.
10. Ndozangue-Touriguine O, Sebbagh M, Merino D, Micheau O, Bertoglio J, Breard J. A mitochondrial block and expression of XIAP lead to resistance to TRAIL-induced apoptosis during progression to metastasis of a colon carcinoma. Oncogene. 2008;27 (46):6012-22.
11. LeBlanc H, Lawrence D, Varfolomeev E, Totpal K, Morlan J, Schow P, et al. Tumor-cell resistance to death receptor--induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. Nat Med. 2002;8(3):274-81.
12. Han J, Goldstein LA, Gastman BR, Rabinovitz A, Wang GQ, Fang B, et al. Differential involvement of Bax and Bak in TRAIL-mediated apoptosis of leukemic T cells. Leukemia. 2004;18(10):1671-80.
13. Riccioni R, Pasquini L, Mariani G, Saulle E, Rossini A, Diverio D, et al. TRAIL decoy receptors mediate resistance of acute myeloid leukemia cells to TRAIL. Haematologica. 2005;90(5):612-24.
14. Merino D, Lalaoui N, Morizot A, Schneider P, Solary E, Micheau O. Differential inhibition of TRAIL-mediated DR5-DISC formation by decoy receptors 1 and 2. Mol Cell Biol. 2006;26(19):7046-55.
15. Schneider P. Production of recombinant TRAIL and TRAIL receptor: Fc chimeric proteins. Methods Enzymol. 2000;322:325-45.
16. Jacquel A, Benikhlef N, Paggetti J, Lalaoui N, Guery L, Dufour EK, et al. CSF-1-induced oscillations in PI3K/AKT are required for caspase activation in monocytes undergoing differentiation into macrophages. Blood. 2009;114(7):3633-41.
17. Morizot A, Merino D, Lalaoui N, Jacquemin G, Granci V, Iessi E, et al. Chemotherapy overcomes TRAIL-R4-mediated TRAIL resistance at the DISC level. Cell Death Differ. 2011;18(4):700-11.
18. McDonnell MA, Abedin MJ, Melendez M, Platikanova TN, Ecklund JR, Ahmed K, et al. Phosphorylation of murine caspase-9 by the protein kinase casein kinase 2 regulates its cleavage by caspase-8. J Biol Chem. 2008; 283(29):20149-58.
19. Travert M, Ame-Thomas P, Pangault C, Morizot A, Micheau O, Semana G, et al. CD40 ligand protects from TRAIL-induced apoptosis in follicular lymphomas through NF-kappaB activation and up-regulation of c-FLIP and Bcl-xL. J Immunol. 2008;181 (2):1001-11.
20. Siegelin MD, Reuss DE, Habel A, Rami A, von Deimling A. Quercetin promotes degradation of survivin and thereby enhances death-receptor-mediated apoptosis in glioma cells. Neuro Oncol. 2009;11(2):122-31.
21. Son JK, Varadarajan S, Bratton SB. TRAIL-activated stress kinases suppress apoptosis through transcriptional upregulation of MCL-1. Cell Death Differ. 2010;17(8):1288-301.
22. Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M. Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. J Biol Chem. 2002;277(5):3247-57.
23. Pietrzak M, Puzianowska-Kuznicka M. p53-dependent repression of the human MCL-1 gene encoding an anti-apoptotic member of the BCL-2 family: the role of Sp1 and of basic transcription factor binding sites in the MCL-1 promoter. Biol Chem. 2008;389(4): 383-93.
24. Jung YH, Heo J, Lee YJ, Kwon TK, Kim YH. Quercetin enhances TRAIL-induced apoptosis in prostate cancer cells via increased protein stability of death receptor 5. Life Sci. 2010;86(9-10):351-7.
25. Kim JY, Kim EH, Park SS, Lim JH, Kwon TK, Choi KS. Quercetin sensitizes human hepatoma cells to TRAIL-induced apoptosis via Sp1-mediated DR5 up-regulation and proteasome-mediated c-FLIPS down-regulation. J Cell Biochem. 2008;105(6):1386-98.
26. Kim YH, Lee DH, Jeong JH, Guo ZS, Lee YJ. Quercetin augments TRAIL-induced apoptotic death: involvement of the ERK signal transduction pathway. Biochem Pharmacol. 2008;75(10):1946-58.
27. Psahoulia FH, Drosopoulos KG, Doubravska L, Andera L, Pintzas A. Quercetin enhances TRAIL-mediated apoptosis in colon cancer cells by inducing the accumulation of death receptors in lipid rafts. Mol Cancer Ther. 2007;6(9):2591-9.
28. Russo M, Nigro P, Rosiello R, D'Arienzo R, Russo GL. Quercetin enhances CD95- and TRAIL-induced apoptosis in leukemia cell lines. Leukemia. 2007;21(5):1130-3.
29. Chen W, Wang X, Zhuang J, Zhang L, Lin Y. Induction of death receptor 5 and suppression of survivin contribute to sensitization of TRAIL-induced cytotoxicity by quercetin in non-small cell lung cancer cells. Carcinogenesis. 2007;28(10):2114-21.
30. Altieri DC. Survivin and IAP proteins in cell-death mechanisms. Biochem J. 2010;430(2): 199-205.
31. Clohessy JG, Zhuang J, de Boer J, Gil-Gomez G, Brady HJ. Mcl-1 interacts with truncated Bid and inhibits its induction of cytochrome c release and its role in receptor-mediated apoptosis. J Biol Chem. 2006;281 (9):5750-9.
32. Gillissen B, Wendt J, Richter A, Muer A, Overkamp T, Gebhardt N, et al. Endogenous Bak inhibitors Mcl-1 and Bcl-xL: differential impact on TRAIL resistance in Bax-deficient carcinoma. J Cell Biol. 2010;188(6):851-62.
33. Han J, Goldstein LA, Gastman BR, Rabinowich H. Interrelated roles for Mcl-1 and BIM in regulation of TRAIL-mediated mitochondrial apoptosis. J Biol Chem. 2006; 281(15):10153-63.
34. Willis SN, Chen L, Dewson G, Wei A, Naik E, Fletcher JI, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev. 2005;19(11):1294-305.
35. Chen S, Dai Y, Harada H, Dent P, Grant S. Mcl-1 down-regulation potentiates ABT-737 lethality by cooperatively inducing Bak activation and Bax translocation. Cancer Res. 2007;67(2):782-91.
36. Elia G, Santoro MG. Regulation of heat shock protein synthesis by quercetin in human erythroleukaemia cells. Biochem J. 1994;300 (Pt 1):201-9.
37. Stankiewicz AR, Livingstone AM, Mohseni N, Mosser DD. Regulation of heat-induced apoptosis by Mcl-1 degradation and its inhibition by Hsp70. Cell Death Differ. 2009;16 (4):638-47.
38. Khoury JD, Medeiros LJ, Rassidakis GZ, McDonnell TJ, Abruzzo LV, Lai R. Expression of Mcl-1 in mantle cell lymphoma is associated with high-grade morphology, a high proliferative state, and p53 overexpression. J Pathol. 2003;199(1):90-7.
39. Cheng S, Gao N, Zhang Z, Chen G, Budhraja A, Ke Z, et al. Quercetin induces tumor-selective apoptosis through downregulation of Mcl-1 and activation of Bax. Clin Cancer Res. 2010;16(23):5679-91.
40. Herbst RS, Mendolson DS, Ebbinghaus S, Gordon MS, O'Dwyer P, Lieberman G, et al. A phase I safety and pharmacokinetic (PK) study of recombinant Apo2L/TRAIL, an apoptosis-inducing protein in patients with advanced cancer. J Clin Oncol (Meeting Abstracts). 2006;24(18_suppl):3013.
41. Ferry DR, Smith A, Malkhandi J, Fyfe DW, deTakats PG, Anderson D, et al. Phase I clinical trial of the flavonoid quercetin: pharmacokinetics and evidence for in vivo tyrosine kinase inhibition. Clin Cancer Res. 1996;2 (4):659-68.