

Quercetin-mediated Mcl-1 and survivin downregulation restores TRAIL-induced apoptosis in non-hodgkin 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, urging 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 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 :** We demonstrate here that quercetin, a natural flavonoid, restores TRAIL-induced cell death in resistant transformed follicular lymphoma B cell lines, despite high Bcl-2 expression levels owing 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 :** Altogether, 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 for therapy of non-Hodgkin's lymphoma.

Introduction:

Follicular lymphomas (FL) are indolent non-Hodgkin 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 owing to its ability to 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, enabling 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 been described so far, 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'-dihexyloxycarbocyanine (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 % CO₂ 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 hours 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 hour prior to TRAIL stimulation.

Measurement of cell viability:

In 96-well plates, $5 \cdot 10^4$ cells were incubated for 24 hours 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 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, $30 \cdot 10^6$ cells were stimulated with 5 μ g 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 precleared with Sepharose 6B (Sigma-Aldrich) for 1 hour 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 μ g 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 minutes 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 precleared with G-coupled sepharose beads for 1h at 4°C. Then, the conformationally active form of Bax or Bak was immunoprecipitated with 4 μ g 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. Nonspecific 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 1h. 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 ug/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 (MMP)

Cells were stimulated or not with His-TRAIL (500 ng/ml) or staurosporine (1 μM) for 16 or 6 hours. After treatment, cells were collected, resuspended in PBS and then stained for 20 min at 37°C with 50 nM DiOC₆, a 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, 4.10⁶ cells were transfected by nucleoporation with the Amaxa nucleofactor (Köln, Germany). VAL and RL cells were resuspended in 100 μL Nucleofactor 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 hours 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). 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) and analyzed in a 7500 Fast detection System (Applied Biosystems). The oligonucleotides used in this study were designed and synthesized (Eurogentec) as follow: Caspase-10 sense GAAGAGAACAGTGTGGGGTG, antisense GAGGTTTCCGTCTTGCTGTA; Mcl-1 sense CGTTGTCTCGAGTGATGATCCA, antisense TCACAATCCTGCCCCAGTTT; Survivin sense GCCGAGGCTGGCTTCA, antisense GAAGAAACACTGGGCCAAGTCT.

Statistics

With the exception of the experiment using AlamarBlue, Figure 1A, which was analyzed by ANOVA with Bonferroni posttesting, all other quantitative experiments were analyzed using Student *t* test. All statistical analyses were performed using Prism version 5.0a software (GraphPad Software, San Diego, CA.). Group comparisons were deemed significant for 2-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 caspases activation by western blotting after TRAIL stimulation indicated 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 1 E) and caspase-9 cleavage was inhibited by caspase-8 inhibitors (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 (Supplementary Figure 1).

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 (Figure 2A). 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 (Figure 2B). Inactivation of Bcl-2 by use of a specific siRNA targeting Bcl-2 (Supplementary Figure 2A), significantly restored apoptosis induced by TRAIL in VAL and RL cells (Figure 2C).

Likewise, siRNA-mediated targeted inhibition of TRAIL-R4 expression in VAL cells (Supplementary Figure 2B) significantly restored sensitivity to TRAIL (Figure 2C). Conversely, inhibition of TRAIL-R4 expression in RL cells, which express low levels of TRAIL-R4 (Supplementary Figure 2B), failed to restore TRAIL-induced cell death (Figure 2C). Strikingly, SUDHL4 and VAL cells exhibited differential sensitivity to TRAIL-induced cell death, despite comparable expression levels of TRAIL-R4 (Figure 2B). The differential behavior did not result from mutations in TRAIL-R4 in SUDHL4 cells as demonstrated by DNA sequence analysis (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 (Figure 2A). 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 (Figure 2B). Consistently, inhibition of c-FLIP expression by use of specific siRNA (Supplementary Figure 2C) partially but significantly restored TRAIL-induced cell death in both resistant cell lines (Figure 2C), while ectopic expression of c-FLIP long in SUDHL4 inhibited TRAIL-induced cell death (Supplementary Figure 3). 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 (Figure 2B), while other inhibitors such as Bcl-xL (not shown) or XIAP (Figure 2B) 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 (Figure 2B). These results prompted us to check whether inhibition of Mcl-1 or survivin expression

(Supplementary Figure 2D) could restore TRAIL-induced apoptosis in VAL and RL cells. Indeed siRNA targeting of either survivin or Mcl-1 restored significantly TRAIL-induced cell death in these cells (Figure 2C). 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, pretreatment with 20 μ M quercetin for 24 hours 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 3A). This flavonoid restored full caspase-3 activation (Figure 3B). 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 3C).

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 pretreatment induced no change in the expression of any of the

TRAIL receptors in VAL or RL cells (Supplementary Figure 4). 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, (Figure 4A). 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 (Figure 4B). As measured by qPCR analysis, quercetin-mediated caspase-10 up-regulation was controlled at the mRNA level (Figure 4C). However, caspase-10 itself appeared to be dispensable 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 (Figure 4D and E). Altogether these results indicate that quercetin-mediated TRAIL sensitization is independent of caspase-10 and most likely independent of TRAIL DISC formation regulation.

Sensitization mainly requires mitochondrial activation

Since enhanced TRAIL DISC formation appears to be dispensable 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 pretreated 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 5A). To determine whether reactivation of the mitochondrial pathway required Bid, its expression was knocked-down using a Bid targeting

siRNA (Supplementary Figure 2E). Inactivation of Bid significantly inhibited TRAIL-induced cell death after quercetin stimulation (Figure 5B), 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 pretreatment (Figure 5C). Moreover, while inactivation of Bax or Bak alone was insufficient to fully inhibit TRAIL-induced apoptosis after quercetin pretreatment, combined Bax and Bak knockdown (Supplementary Figure 2F) completely abrogated the synergy (Figure 5D).

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

To elucidate 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 6A). In agreement with previous findings (21), we have found that Mcl-1 expression was induced upon TRAIL stimulation in both resistant cell lines, but remarkably, TRAIL-mediated Mcl-1 up-regulation was completely abrogated by quercetin (Figure 6A). Quercetin-mediated survivin and Mcl-1 down-regulation occurred in a caspase-independent manner (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 6B). 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 stimulation (Figure 6C). 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 6D) and failed to inhibit the synergistic apoptotic activity of the combination TRAIL and quercetin (Figure 6E). 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 6F). Accordingly, Mcl-1 was strongly ubiquitinated upon quercetin treatment (Figure 6 G).

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 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 up-regulation was dispensable to restore quercetin-mediated TRAIL sensitivity in both resistant cell lines. Yet, we cannot definitely exclude 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 μ M, are lower than those used in most studies (50 to 200 μ M) (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.

Rather, our findings 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. Survivin has been proposed to act mainly at the post-mitochondrial level, through its ability to inhibit Smac release from the mitochondria, stabilizing 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 (Supplementary Figure 5) appear to be required to repress survivin expression upon quercetin stimulation. Further studies will be required to elucidate 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 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 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 demonstrated recently 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), as quercetin and TRAIL, when applied either alone or in combination, exhibited no toxicity towards normal lymph nodes or tonsils cells (Supplementary Figure 6). 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: OM was the principal investigator and takes primary responsibility for the paper. GJ, VG, ASG, NL, AyM, EI, AM performed the laboratory work of this study. GJ TG and OM co-ordinated the research. GJ, CG, TG and OM wrote the paper. The authors reported no potential conflicts of interests.

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Figure legends

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 hours. 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 analysed 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 analysed 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 μ M) for 16 or 6 hours, respectively, then incubated with the MMP-sensitive fluorescent dye DiOC₆ 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 DiOC₆ 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 stands for not statistically relevant).

Figure 2. Bcl-2, Mcl-1 and survivin account for the resistance to TRAIL of VAL and RL lymphoma B cell lines. (A) Western blot analysis of antiapoptotic proteins upon stimulation with His-TRAIL (500 ng/mL) for the indicated times. (B) Expression of TRAIL receptors on VAL, RL and SUDHL4 cells, at the membrane level, was measured by flow cytometry (unfilled peaks). Shaded peaks correspond to the isotype control antibody staining. (C) Effect of siRNA-mediated knockdown of Mcl-1, survivin, Bcl-2, c-FLIP or TRAIL-R4 on the sensitivity to TRAIL-induced apoptosis. 48 hours after electroporation with a specific siRNA or a control siRNA (scramble),

VAL and RL cells were treated with TRAIL at 500 ng/mL for 3 hours. Apoptosis was measured by flow cytometry after annexin V staining. Data presented are means plus or minus SD (n =3; *P < 0.05 ; **P<0.01 or ***P<0.001 respective to scramble siRNA).

Figure 3. 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 μ M quercetin (Quer) for 24 hours prior to TRAIL (500 ng/mL for 3 hours). The pan caspases inhibitor zVAD-fmk (20 μ M) 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 μ M, 24 hours), followed by TRAIL (500 ng/mL, 6 hours) and/or zVAD-fmk (20 μ M, 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 μ M, 30 min before TRAIL stimulation. Data presented panels (A) and (C) 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 4. Quercetin induces caspase-10 upregulation and recruitment to the DISC, but sensitization to TRAIL occurs independently of caspase-10. (A) Analysis of TRAIL-induced DISC formation. VAL and RL cells were treated with quercetin (20 μ M, 24 hours) and stimulated with TRAIL (5 μ g/mL) for the indicated times. After cell lysis, the DISC was immunoprecipitated using an antibody against TRAIL-R2 and the DISC-associated proteins were analysed by western blotting. Data are representative of three independent experiments. (B) Western blot analysis of caspase-10 expression after treatment with quercetin (20 μ M, 24h), followed by TRAIL (500 ng/mL, 6 hours) and/or zVAD-fmk (20 μ M, 30 min before TRAIL). (C) Relative expression of caspase-10 mRNA by qPCR after treatment with quercetin (20 μ M, 24 hours). Results correspond to % fold change mRNA expression compared with cells treated with DMSO, and were normalized to L32 levels. (D-E) Effect of siRNA-mediated caspase-10 knockdown on the efficiency of the combined treatment with quercetin and TRAIL. 24 hours after electroporation with a specific siRNA or a control siRNA (scramble), VAL and RL cells were treated with quercetin (20 μ M) for 24 hours, followed by TRAIL (500 ng/mL) for 3 hours. Apoptosis was measured by flow cytometry after annexin V staining. Efficiency of the caspase-10 siRNA was evaluated by western blotting. Data presented panels (D) and (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 Caspase-10 siRNA; ns stands for not statistically relevant).

Figure 5. 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 μ M) for 24 hours, followed by TRAIL (500 ng/mL) for the indicated times. Cytosolic and mitochondrial fractions were analysed 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. 24 hours after electroporation with a specific siRNA or a control siRNA (scramble), VAL and RL cells were treated with quercetin (20 μ M) for 24 hours, followed by TRAIL (500 ng/mL) for 3 hours. (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 μ M) for 24 hours. Bax Channel Blocker was added at 5 μ M, 1 hour before

stimulation with TRAIL (500 ng/mL for 3 hours). (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-C-D) Apoptosis was measured by flow cytometry after annexin V staining. Data presented panels (B-D) 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 stands for not statistically relevant).

Figure 6. 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 μ M, 24 hours), followed by TRAIL (500 ng/mL, 6 hours) and/or zVAD-fmk (20 μ M, 30 min before TRAIL). (C) Relative expression of Mcl-1 or survivin mRNA by qPCR after treatment with quercetin (20 μ M, 24 hours). 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. 24 hours after electroporation with a specific siRNA or a control siRNA (scramble), VAL and RL cells were treated or not with quercetin (20 μ M) for 24 hours, followed by TRAIL (500 ng/mL) for 3 hours 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 μ M) or DMSO (vehicle), in the presence of the proteasome inhibitor MG132 (1 μ M) for 24 hours. (G) Quercetin-mediated ubiquitination of Mcl-1. Cells were treated with quercetin (Q) or vehicle (NT) in the presence of MG132 for 24 hours 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. (F) 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 stands for not statistically relevant).