Roscovitine sensitizes breast cancer cells to TRAIL-induced apoptosis through a pleiotropic mechanism

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The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/APO2L) is a member of the TNF gene superfamily that induces apoptosis upon engagement of cognate death receptors. While TRAIL is relatively non-toxic to normal cells, it selectively induces apoptosis in many transformed cells. Nevertheless, breast tumor cells are particularly resistant to the effects of TRAIL. Here we report that, in combination with the cyclin-dependent kinase inhibitor roscovitine, exposure to TRAIL induced marked apoptosis in the majority of TRAIL-resistant breast cancer cell lines examined. Roscovitine facilitated TRAIL death-inducing signaling complex formation and the activation of caspase-8. The cFLIP_L and cFLIP_S FLICE-inhibitory proteins were significantly down-regulated following exposure to roscovitine and, indeed, the knockdown of cFLIP isoforms by siRNA sensitized breast tumor cells to TRAIL-induced apoptosis. In addition, we demonstrate that roscovitine strongly suppressed Mcl-1 expression and up-regulated E2F1 protein levels in breast tumor cells. Significantly, the silencing of Mcl-1 by siRNA sensitized breast tumor cells to TRAIL-induced apoptosis. Furthermore, the knockdown of E2F1 protein by siRNA reduced the sensitizing effect of roscovitine in TRAIL-induced apoptosis. In summary, our results reveal a pleiotropic mechanism for the pro-apoptotic influence of roscovitine, highlighting its potential as an antitumor agent in breast cancer in combination with TRAIL.

Keywords: apoptosis, roscovitine, CDK, TRAIL, DISC, FLIP, Mcl-1, E2F1

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Introduction

The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/APO-2L), a member of the TNF gene superfamily, induces apoptosis upon binding to the death domain (DD)-containing receptors TRAIL-R1/DR4 and TRAIL-R2/DR5 [1]. However, TRAIL can also bind to the decoy receptors TRAIL-R3/DcR1 and TRAIL-R4/DcR2, which do not transduce apoptotic signals [2]. Since

TRAIL induces apoptosis selectively in transformed cells of diverse origin but not in most normal cells *in vitro*, it is an attractive candidate for antitumor therapies [2–4]. However, some cancer cells show either partial or complete resistance to the apoptotic effects of TRAIL [3], although it appears that in certain instances this resistance can be overcome by combining TRAIL with chemotherapeutic drugs [5–7].

In the western world, breast cancer is the most common neoplasia amongst women, emphasizing the importance of developing effective treatments. Recently, different combined strategies have been studied to improve the effects of chemo- and radiotherapy. Indeed, we and others have reported that interferon-gamma, DNA-damaging drugs and ionizing radiation can sensitize breast cancer cells to TRAIL-induced apoptosis [8–10].

Upon binding to its proapoptotic receptors TRAIL induces the formation of the death-inducing signaling complex (DISC), recruiting the dual adaptor Fas-associated death domain (FADD) molecule through its DD. In turn, this complex recruits the initiator caspase-8 through

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Abbreviations: tumor necrosis factor-related apoptosis-inducing ligand (TRAIL); tumor necrosis factor (TNF); death-inducing signaling complex (DISC); Fas-associated death domain (FADD); cyclin-dependent kinase (CDK); FLICE-inhibitory protein (FLIP); benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (z-VAD.FMK); poly (ADP-ribose) polymerase (PARP)

its death effector domain (DED) [11], which is activated at the DISC by oligomerization. The processing and activation of caspase-8 at the DISC stimulates an apoptotic cascade that provokes cell death. The apoptotic signal from the DISC may be inhibited by the cellular FLICE-inhibitory protein (FLIP) [12]. In most cells, two alternatively spliced isoforms of cFLIP exist: a caspase-8 homologue cFLIP_L that lacks the amino acids critical for proteolytic caspase activity; and cFLIP_S, which is comprised of the two death effector domains alone [12]. Although the role of cFLIP in apoptotic signaling remains controversial, there is strong evidence that it displays antiapoptotic activity [13–15]. The expression of cFLIP varies in a cell type-specific manner and it fluctuates in response to various stimuli. While it can be transcriptionally controlled by the nuclear factor- κ B (NF- κ B) pathway [16], altered rates of proteasomal degradation also regulate its protein activity [17], making it a versatile inhibitor of the apoptotic responses mediated by death receptors.

Cyclin-dependent kinases (CDKs) are serine/threonine kinases that play a crucial role in regulating both the cell cycle and transcription, through the phosphorylation of transcription factors and tumor suppressor proteins involved in DNA replication and cell division [18]. Therefore, CDKs are attractive therapeutic targets for cancer therapy. Roscovitine (CYC202, Seliciclib) is a purine analogue that competes with ATP for binding to the active site of CDKs and it displays a potent *in vitro* activity against CDK1, CDK2, CDK5, CDK7 and CDK9 [19]. The growth of a wide range of tumor cell types is inhibited by roscovitine *in vitro* and, likewise, it inhibits the growth of human tumor xenografts in nude mice [20, 21]. Roscovitine causes not only cell cycle arrest but also apoptosis in cancer cells [22] and, accordingly, it is currently being evaluated in phase II clinical trials [23].

While the mechanism by which CDK inhibitors induce apoptosis remains unclear, the CDK inhibitor flavopiridol, a semisynthetic flavone, reduces the levels of antiapoptotic proteins such as XIAP, Mcl-1 or cFLIP [24–26] and up-regulates the transcription factor E2F1, known to be involved in apoptosis [27]. Roscovitine may also down-regulate Mcl-1 or XIAP [28] and it could sensitize glioma cells to TRAIL-induced apoptosis by reducing the levels of survivin and XIAP. However, the down-regulation of these factors may not be sufficient to trigger the activation of caspases [6].

Here we report that TRAIL-resistant human breast cancer cell lines can be sensitized to TRAIL-induced apoptosis by exposure to roscovitine. The molecular mechanisms underlying the effects of roscovitine involve an increase in the formation of the TRAIL DISC, the down-regulation of cFLIP and Mcl-1, and the up-regulation of the transcrip-

tion factor E2F1.

Results

Roscovitine sensitizes human breast tumor cell lines to TRAIL-induced apoptosis

To analyze whether roscovitine sensitizes breast tumor cells to the apoptotic ligand TRAIL, the breast tumor cell line MDA-MB231 was treated with different concentrations of roscovitine to determine the sub-toxic dose capable of sensitizing them to TRAIL-induced apoptosis (Figure 1A). Subsequently, six more breast tumor cell lines were tested to determine whether similar doses also induced roscovitine sensitization to TRAIL-induced apoptosis (Figure 1B). In all the cell lines tested, roscovitine alone induced little cell death at the concentration indicated. However, if the cells were exposed to roscovitine for 7 h before adding TRAIL overnight, apoptotic cell death was significantly increased in all the cell lines tested, even in the highly resistant BT474 and SKBr3 cell lines that over-express the ErbB2/Her-2/neu oncogene. Proteolytic processing of the caspase substrate poly (ADP-ribose) polymerase (PARP), a hallmark of apoptosis, was observed in the cells treated with roscovitine and TRAIL (Figure 2A). These results demonstrate that the combined treatment with roscovitine and TRAIL induces significant apoptotic cell death in these breast tumor cell lines.

Roscovitine enhances TRAIL-induced activation of caspase-8 without affecting the cell surface expression of TRAIL receptors

To elucidate the mechanism underlying the sensitization to TRAIL-induced apoptosis promoted by roscovitine in breast tumor cells, we examined different biochemical events that occur upon TRAIL binding to its receptors at the cell surface. TRAIL initiates apoptosis by inducing the recruitment of the adapter molecule FADD to the apoptotic TRAIL receptors and the subsequent engagement and activation of procaspase-8 [29, 30]. We determined whether caspase-8 was activated in MDA-MB231 cells by analyzing the processing of the pro-caspase to the 43/41 kDa intermediate proteolytic fragments, and through the generation of the mature p18 caspase-8 subunits upon TRAIL stimulation. Accordingly, TRAIL-induced activation of caspase-8 was clearly enhanced by pre-treatment with roscovitine (Figure 2A).

Several treatments have been shown to up-regulate the expression of the TRAILR1 (DR4) or TRAILR2 (DR5) death receptors, resulting in enhanced TRAIL-induced apoptosis [31]. We therefore examined the effect of roscovitine on the surface expression of TRAIL death and decoy receptors by flow cytometry (Supplementary information,

Figure 1 Roscovitine sensitizes breast tumor cell lines to TRAIL-induced apoptosis. **(A)** MDA-MB231 cells were incubated with the indicated concentrations of roscovitine for 7 h prior to the addition of TRAIL. Apoptosis was measured 15 h after the addition of TRAIL (500 ng/ml) as the percentage of cells with sub-G1 DNA content, as described in Materials and Methods. Error bars represent S.D. from three independent experiments. ****** $P < 0.0001$. **(B)** Different breast tumor cell lines were incubated in the presence or absence of roscovitine (20 μM) and subsequently exposed to TRAIL (500 ng/ml) in the same culture media. Alternatively, the MDA-MB435-S and MCF-7 cells were treated with 25 and 50 ng/ml TRAIL, respectively. In all cell lines with the exception of MCF-7, apoptosis was measured as in (A). In MCF-7 cells apoptosis was determined by measuring PS externalization by flow cytometry as described in Materials and Methods.

Figure S1). MDA-MB231 cells only express TRAIL-R2 and R4 on the cell surface and, furthermore, the expression of these TRAIL receptors at the cell membrane was not significantly altered by exposure to roscovitine (Supple-

mentary information, Figure S1). Hence, roscovitine does not appear to sensitize MDA-MB231 cells to TRAIL-induced apoptosis by modulating the surface expression of TRAIL receptors.

Figure 2 Roscovitine enhances the caspase-8 activation and PARP cleavage induced by TRAIL without affecting the expression of TRAIL receptor at the cell membrane. MDA-MB231 cells were incubated with roscovitine (20 μ M) for 7 h prior to the addition of TRAIL (500 ng/ml) for 15 h. Caspase-8 activation and PARP-1 cleavage were assessed by immunoblotting.

Recruitment of caspase-8 and FADD to the TRAIL DISC is augmented in MDA-MB231 cells exposed to roscovitine

TRAIL binding to its receptors provokes the formation of the DISC, which contains FADD and caspase-8, the main proximal initiators of apoptosis. Therefore, roscovitine may render cells more susceptible to apoptosis by enhancing

the formation of the TRAIL DISC. Using biotinylated TRAIL (TRAIL-b), we could monitor DISC formation by precipitating it from MDA-MB231 cell lysates with streptavidin-agarose beads. We found an increase in the recruitment of procaspase-8 and FADD into the DISC of roscovitine-treated cells (Figure 3). Furthermore, there was an increase in the cleavage of procaspase-8 to the p43/41 intermediate fragments and to the p18 subunit following exposure of the cells to roscovitine. The levels of cFLIP_L and cFLIP_S recruited to the TRAIL DISC were slightly lower in the cells treated with roscovitine (Figure 3, compare lanes 3-4, 5-6 and 7-8). In addition, in both the control and pre-treated cells, cFLIP_L was cleaved by caspase-8 to a 43 kDa fragment (FLIP_C), which probably represents the product obtained by removal of the C-terminal p10 fragment. Thus, we observed an increase in the caspase-8/FLIP ratio in the DISC of roscovitine-treated cells, suggesting that this ratio may influence the sensitivity to TRAIL-induced apoptosis as indicated previously [32].

Roscovitine treatment induces the redistribution of TRAIL DISC components to lipid rafts and enhances the redistribution after TRAIL treatment

As we indicated previously, the induction of apoptosis by TRAIL can be regulated at different levels. Indeed, it was recently shown that lipid rafts may also be involved in signaling through TRAIL and other death receptors [33,

Figure 3 The recruitment of caspase 8 and FADD to the TRAIL DISC is enhanced after roscovitine treatment. MDA-MB231 cells incubated in the presence or absence of roscovitine (20 μ M, 15 h) were treated with biotinylated-TRAIL (TRAIL-b, 1 μ g/ml) for the times indicated. TRAIL receptor complexes were collected with streptavidin-conjugated agarose beads and analyzed by Western blotting for the TRAIL DISC components FADD, caspase-8, FLIP_L and FLIP_S. Unstimulated receptor controls (u/s) represent the addition of biotinylated-TRAIL to an equivalent volume of lysate isolated from unstimulated cells. Lysates are included as a positive control for the expression of these proteins in MDA-MB231 cells. The data shown are representative of three independent experiments.

34]. Accordingly, the redistribution of death receptors to lipid rafts facilitates the emission of signals that induce apoptosis. To determine whether lipid rafts are involved in the roscovitine sensitization to TRAIL apoptosis, MDA-MB231 cells were incubated with roscovitine for 15 h, treated with TRAIL for 5 min, and lipid rafts were then isolated by sucrose density gradient centrifugation as described in Materials and Methods (Figure 4A). Roscovitine treatment caused a redistribution of procaspase-8, FADD and TRAIL R2 to rafts (I fraction), and, although exposure to TRAIL alone also induced a redistribution of these proteins into rafts, pre-treatment with roscovitine enhanced this redistribution. However, the disruption of lipid rafts using methyl- β -cyclodextrin (MBCD) or Imipramine (Supplementary information, Figure S2), did not prevent the sensitization of tumor cells to TRAIL apoptosis promoted by roscovitine, suggesting that the localization to lipid rafts does not play a significant role in roscovitine sensitization.

Treatment with roscovitine impairs cFLIP_L and cFLIP_S mRNA and protein expression

Several studies have suggested that a decrease in cFLIP, a short-lived protein, sensitizes cells to death receptor-induced apoptosis. Although this is somewhat controversial [13, 35], the antiapoptotic activity of this protein is clearly supported by data obtained from cells stably over-expressing cFLIP, from mice deficient in cFLIP and from the selective knockdown of cFLIP [13, 14].

When protein synthesis was inhibited in breast tumor cells with cycloheximide (CHX) before exposure to TRAIL (Figure 5A), all tumor cell lines tested were sensitized to the effects of TRAIL. Hence, short-lived inhibitory proteins

Figure 4 Roscovitine redistributes the TRAIL receptor-2 into lipid rafts. MDA-MB231 cells maintained in the presence or absence of roscovitine (20 μ M) for 15 h were stimulated with TRAIL-b (500 ng/ml) for 5 min. The cells were then lysed in buffer containing 1% Triton; the detergent-soluble (S) and insoluble (I) fractions were isolated as described in Materials and Methods, and the expression of the indicated proteins was analyzed by western blotting.

appear to play an important role in preventing TRAIL-induced apoptosis. Thus, we investigated whether roscovitine treatment affected the levels of cFLIP_L and cFLIP_S protein in MDA-MB231 cells. The protein levels of both cFLIP_L and cFLIP_S decreased in these cells following exposure to roscovitine (Figure 5B). The main decrease was found after 15 h of treatment and partial recovery of cFLIP_L was observed after 24 h of treatment. To determine whether caspases were required for the roscovitine-mediated decrease in cFLIP expression, MDA-MB231 cells were treated with the pancaspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (z-VAD.FMK) for 1 h prior to the addition of roscovitine (20 μ M). Inhibition of caspase activity did not prevent the decline of either cFLIP_L or cFLIP_S levels upon roscovitine treatment, indicating that a caspase-independent mechanism was involved in reducing cFLIP expression (Figure 5C).

Post-translational regulation of cFLIP protein can occur through proteasome-mediated degradation [17, 24, 36]. Hence, we determined whether the reduction of cFLIP after roscovitine treatment was due to protein degradation through the proteasome machinery. In the presence of the proteasome inhibitors MG132 or epoxomicin, there was an increase in the accumulation of cFLIP protein (Figure 5D). Nevertheless, despite the inhibition of cFLIP degradation by the proteasome, roscovitine treatment still caused a decrease in the levels of cFLIP protein when compared with cells exposed to proteasome inhibitor alone, suggesting that roscovitine may down-regulate cFLIP at the mRNA level. Indeed, roscovitine diminishes the endogenous levels of cFLIP_L and cFLIP_S mRNA in MDA-MB231 cells (Figure 5E and 5F). Moreover, there was a correlation between the cFLIP protein and mRNA levels following roscovitine treatment (Figure 5B and 5E) which suggests that roscovitine mainly down-regulates cFLIP at the mRNA level. However, we cannot completely exclude that treatment with roscovitine is also affecting cFLIP protein degradation by the ubiquitin/proteasome pathway.

Knockdown of endogenous cFLIP_L and cFLIP_S with siRNA oligonucleotides promotes apoptosis induced by TRAIL in breast tumor cells

Our data clearly show that roscovitine decreases cFLIP expression and sensitizes breast tumor cells to TRAIL-induced apoptosis. Thus, we tested whether the specific silencing of cFLIP expression by siRNA has a similar effect on TRAIL-induced apoptosis. In MDA-MB231 cells, the levels of both cFLIP_L and cFLIP_S were substantially reduced by a specific siRNA (Figure 6A), whereas a similar concentration of a scrambled control siRNA did not modify cFLIP protein expression. Transfection with cFLIP siRNA had no effect on the expression of procaspase-8, a struc-

Figure 5 Exposure to roscovitine reduces cFLIP protein and mRNA levels. **(A)** Different breast tumor cell lines were incubated for 1 h in the presence or absence of cycloheximide (CHX, 5 μ g/ml) and then treated with TRAIL at 500 ng/ml (BT-474, SKBr-3, EVSA-T, MDA-MB231), 50 ng/ml (MCF-7) or 25 ng/ml (MDA-MB435S), for a further 15 h period. Apoptosis was assessed as in Figure 1B. **(B)** Cells were incubated with roscovitine (20 μ M) or CHX (5 μ g/ml) for the times indicated. Levels of cFLIP protein were determined by western blotting as described in Materials and Methods. Densitometric analysis of cFLIP levels is presented as percentages of control (time 0), using tubulin levels as an internal control for normalization. **(C)** Cells were treated with roscovitine (20 μ M) for 15 h, and then exposed to TRAIL 500 ng/ml for 7 h in the presence or absence of Z-VAD (50 μ M) added 1 h before TRAIL. The cells were then processed as in (A) to detect cFLIP protein levels. **(D)** MDA-MB231 cells were treated with roscovitine (20 μ M) for 24 h in the presence or absence of the proteasome inhibitors MG132 (25 μ M) or epoxomicin (100 nM). Proteasome inhibitor was added 30 min before roscovitine and the cFLIP protein levels were analyzed by western blotting as in (A). **(E)** Cells were treated as in (B); the total RNA was isolated and cFLIP_L and cFLIP_S mRNA transcripts were analyzed by RT-PCR as described in Materials and Methods. PCR amplification of β -actin was used as a control of the input RNA. **(F)** MDA-MB231 cells were treated with roscovitine (20 μ M) for 15 h and cFLIP_L and cFLIP_S mRNA levels were determined by real time RT-PCR as described in Materials and Methods. Results are the average and range of two independent experiments in triplicate.

turally related proapoptotic protease (Figure 6A). Most importantly, silencing of cFLIP expression resulted in a clear sensitization to TRAIL-induced apoptosis (Figure 6B). These data correlated well with the effects of roscovitine and support the hypothesis that down-regulation of cFLIP expression by this CDK inhibitor is critical for the sensitization to TRAIL-induced apoptosis in breast tumor cells. To further substantiate the role of cFLIP in the sensitization observed, we generated MDA-MB231 cells over-expressing cFLIP_L and determined the effect of roscovitine treatment on apoptosis by TRAIL. Results shown in Figure 6C demonstrate that cells over-expressing cFLIP_L were clearly more resistant to roscovitine-induced

sensitization to TRAIL apoptosis than cells expressing normal cFLIP levels.

Down-regulation of antiapoptotic Mcl-1 protein expression by roscovitine contributes to sensitization to TRAIL-induced apoptosis

Roscovitine alters the expression of different proteins involved in apoptosis [6, 28] and these modifications are associated with the induction of apoptosis by CDK inhibitors. On the other hand, it is known that the antiapoptotic Bcl-2 family member Mcl-1 protects cells from apoptosis and that it is up-regulated in different types of cancer [37, 38]. Furthermore, Mcl-1 down-regulation induces apoptosis

Figure 6 Knockdown of endogenous cFLIP_L and cFLIP_S enhances the apoptosis induced by TRAIL in MDA-MB231 cells. **(A)** MDA-MB231 cells were transfected with either a siRNA oligonucleotide targeting both cFLIP isoforms, or a scrambled RNA oligonucleotide, as described in Materials and Methods. After 24 h, the cells were harvested for immunoblot analysis to verify protein knockdown using Tubulin as a protein loading control. The results are representative of three independent experiments. **(B)** MDA-MB231 cells were transfected as in (A), and after 24 h TRAIL (500 ng/ml) was added to the cultures. Apoptosis was measured 24 h after the addition of TRAIL as described in Figure 1. Error bars represent S.D. from three independent experiments. ** $P < 0.0001$. **(C)** Mock-transfected and MDA-MB231 cells transfected with the pCR3.V64-Met-Flag-FLIP_L vector were treated with roscovitine (R) (20 μ M) for 7 h prior to the addition of TRAIL (T) (500 ng/ml). Apoptosis was measured 15 h after the addition of TRAIL as the percentage of cells with sub-G1 DNA content, as described in Materials and Methods. The inset shows western blot analysis of FLIP_L expression in mock and FLIP_L transfected cells. GAPDH was used as an internal protein loading control. Error bars represent S.D. from three independent experiments. * $P < 0.005$.

[39] or sensitizes tumor cells to the apoptosis induced by different stimuli, including TRAIL [40–42]. Exposure to roscovitine markedly down-regulated Mcl-1 protein levels in MDA-MB231 cells (Figure 7A, top panel), suggesting that the decline in Mcl-1 may be a key event by which roscovitine sensitizes cells to TRAIL-induced apoptosis. In this respect, knockdown of Mcl-1 expression by siRNA (Figure 7A, bottom panels) significantly sensitized MDA-MB231 cells to TRAIL-induced apoptosis.

E2F1 plays a significant role in roscovitine sensitization to TRAIL-induced apoptosis

The transcription factor E2F1 is involved in the induction of apoptosis and its over-expression in various cell types sensitizes cells to apoptosis induced by ionizing radiation, chemotherapeutic drugs or death receptors [43, 44]. Here we show that roscovitine clearly up-regulates E2F1 protein levels (Figure 7B) in breast tumor cells. Interestingly, E2F1 has also been associated with the regulation of different apoptotic proteins including Mcl-1 and cFLIP_s [44, 45]. By transfecting small specific interference RNAs, we disrupted E2F1 expression in MDA-MB231 cells to determine if the up-regulation of E2F1 induced by roscovitine affects

Mcl-1 and/or cFLIP expression. The transfected cells were exposed to roscovitine (for 15 h) 24 h after siRNA transfection and the levels of the different apoptotic proteins were evaluated by immunoblotting. Knockdown of E2F1 did not alter the response of MDA-MB231 to roscovitine in terms of the expression of Mcl-1, cFLIP, XIAP, BID or Caspase-2 (Supplementary information, Figure S3), BAX and Apaf-1 (not shown) protein. However, although these protein levels remained unperturbed, there was a reversion of the roscovitine sensitization to TRAIL apoptosis when E2F1 was silenced (Figure 7C), demonstrating the importance of E2F1 in this sensitization process.

Discussion

Antitumor therapy based on the apoptosis-inducing properties of TRAIL and agonistic TRAIL receptor antibodies is currently under consideration [46]. However, despite the fact that TRAIL induces selective cell death in human tumor cells, sparing most untransformed cells, resistance to TRAIL is not uncommon in certain tumor cell lines. Furthermore, TRAIL might also promote cell migration and invasion in some apoptosis-resistant cells

Figure 7 Down-regulation of Mcl-1 and up-regulation of E2F1 protein levels after roscovitine treatment in MDA-MB231 cells. MDA-MB231 cells were treated with roscovitine (20 μ M) for the times indicated and then Mcl-1 (**A**) or E2F1 (**B**) protein levels were analyzed by immunoblotting. The results are representative of at least three independent experiments. In **A** (bottom panel, left) cells were transfected with either a siRNA oligonucleotide targeting Mcl-1, or a scrambled RNA oligonucleotide, as described in Materials and Methods. After 48 h, the cells were collected and Mcl-1 protein expression was analyzed by western blotting. In **A** (bottom panel, right) cells were siRNA transfected as in the left panel, and after 48 h TRAIL was added to the cultures. Apoptosis was measured 15 h after the addition of TRAIL as described in Figure 1. Error bars represent S.D. from three independent experiments. * $P < 0.005$. (**C**) Cells were transfected and treated with roscovitine as in Supplementary information, Figure S3 following prior treatment with TRAIL for 2 h. Apoptosis was determined as the percentage of subG1 cells. Error bars represent S.D. from three independent experiments. * $P < 0.005$.

[47]. Hence, sensitization of cells to TRAIL-induced apoptosis through different strategies would augment the therapeutic potential of TRAIL against its capacity to stimulate invasion, resolving the potential risk to patients with TRAIL-resistant cancers. Combination strategies have been implemented to facilitate TRAIL apoptotic signaling [48], and we have been investigating the resistance of human breast tumor cells to TRAIL and on how to overcome it. We previously indicated that the formation of the DISC is a common target for different sensitizing regimes [24, 49]. Indeed, CDK inhibitors have been reported to down-regulate the expression of antiapoptotic proteins and to up-regulate the levels of pro-apoptotic regulatory proteins [24–27, 50]. The apoptosis-inducing potential of the CDK inhibitor roscovitine led us to investigate its effects on the resistance of breast tumor cells to TRAIL. We found that roscovitine sensitizes different breast tumor cell lines to TRAIL, including the highly resistant cell lines BT474 and SKBr3 that over-express the ErbB2 receptor. Indeed, roscovitine sensitizes breast tumor cell lines to TRAIL irrespective of their p53 status (see Table 1). While roscovitine did not alter surface expression of TRAIL receptors, the step(s) involved in the sensitization to TRAIL may reside downstream of ligand binding.

A DISC is formed upon TRAIL binding to its pro-apoptotic receptors [11]. In breast tumor cells treated with roscovitine, an increase in the recruitment of procaspase-8 and FADD to the TRAIL DISC was observed, together with the complete activation of caspase-8. Whether or not roscovitine treatment induces post-translational modifications of FADD or TRAIL receptors that affect the binding affinities of the DISC components [51] remains to be ascertained. In this respect, it is noteworthy that treatments such as glucose deprivation, inhibition of CDKs and histone deacetylase (HDAC) inhibitors, all seem to enhance the formation of the TRAIL DISC in the absence of TRAIL receptor up-regulation [24, 49, 52]. The localization of death receptors and other DISC components into lipid rafts may play a role in mediating sensitization to apoptotic stimuli [33, 53]. Indeed, roscovitine itself caused a redistribution of DISC proteins into lipid rafts fractions and facilitated that induced by TRAIL in breast tumor cells. However, the disruption

of either cholesterol or ceramide-enriched lipid rafts by specific inhibitors did not prevent roscovitine-induced sensitization to TRAIL in breast tumor cells. Hence, the redistribution of DISC components to membrane lipid rafts is not absolutely necessary to induce apoptosis by TRAIL in these cells. In this respect, the treatment of cells with MBCD failed to block CD95-mediated apoptosis in the B-lymphoblastoid cell line SKW6.4 [35]. Furthermore, it has been demonstrated that acid sphingomyelinase-deficient hepatocytes and mice are protected from CD95-induced apoptosis or death. In contrast, acid sphingomyelinase-deficient and wild-type thymocytes, or Concanavalin- or lipopolysaccharide-pre-stimulated lymphocytes, were equally sensitive to CD95-induced apoptosis [54]. Together these results suggest that the requirement for death receptor localization in lipid rafts to activate apoptosis may be cell type dependent.

Since a protein synthesis inhibitor could restore the sensitivity of various breast tumor cell lines to TRAIL-induced apoptosis, short-lived inhibitory proteins are likely to be important to prevent TRAIL-induced signaling and apoptosis. We analyzed the expression of several antiapoptotic proteins such as cFLIP, Mcl-1 and XIAP, known to have a short half-life [55–57]. In cells treated with a sensitizing dose of roscovitine cFLIP_L, cFLIP_S and Mcl-1 were markedly down-regulated. Moreover, the down-regulation of cFLIP protein was associated with a decrease in the mRNA levels for both cFLIP_L and cFLIP_S in cells treated with roscovitine. These results are in agreement with other data indicating that CDK inhibitors regulate transcription through the potent inhibition of positive transcription elongation factor b (P-TEFb), comprised of CDK9 and cyclin T1, thereby controlling the elongation phase of transcription by RNA polymerase II [58]. However, whereas flavopiridol has been shown to reduce global mRNA levels, at the doses used here roscovitine mostly affects a subset of cellular genes resulting in down-regulation of several short-lived proteins, including Mcl-1 [23, 59, 60]. In contrast, despite reports that roscovitine treatment down-regulates XIAP expression in glioma cells [6], we did not observe a decrease in XIAP protein levels in breast tumor cells treated with roscovitine. This is consistent with our previous results indicating that specific siRNA knockdown of XIAP did not sensitize breast tumor cells to TRAIL-induced apoptosis [24].

The importance of cFLIP down-regulation by roscovitine in the sensitization process was supported by the finding that siRNA silencing of cFLIP was sufficient to sensitize breast tumor cells to TRAIL-induced apoptosis. Competition between FADD and cFLIP_L for the DD of TRAIL receptor DR5 [61] could also modulate DISC formation. The decrease in cFLIP_L levels upon roscovitine treatment observed in our work could favor binding of FADD and pro-

Table 1 p53 status in breast tumor cell lines

	p53
MDA-MB231	mutated
MCF-7	normal
MDA-MB435-S	mutated
SKBr-3	mutated
BT-474	mutated

caspase-8 to the TRAIL DISC. The increased recruitment of FADD and caspase-8 to the TRAIL DISC observed in roscovitine-treated cells, together with a decrease in cFLIP levels, resulted in an elevation of the caspase-8/cFLIP ratio in the DISC of roscovitine-treated cells, which should facilitate caspase-8 activation and promote apoptosis [32]. In breast tumor cells, TRAIL has been reported to induce apoptosis through a mitochondrial pathway, involving the translocation of truncated Bid and Bax to the mitochondria followed by the release of cytochrome *c* from this organelle [62]. The anti-apoptotic Bcl-2 family member Mcl-1 has recently been reported to bind truncated Bid and to prevent death-receptor mediated apoptosis [40, 63]. Furthermore, Mcl-1 down-regulation by siRNA has also been demonstrated to restore the sensitivity of tumor cells to TRAIL-induced apoptosis [26]. Our results also demonstrate that silencing of Mcl-1 expression by siRNA partly sensitizes breast tumor cells to TRAIL-induced apoptosis. Based on these evidence, it seems very likely that Mcl-1 down-regulation upon exposure to roscovitine could cooperate with cFLIP depletion to facilitate TRAIL-induced release of apoptotic factors from the mitochondria in breast tumor cells.

Our data show that treatment with a sensitizing dose of roscovitine caused a marked increase in E2F1 protein expression in breast tumor cells. The role played by E2F1 in the sensitization process was evaluated by siRNA silencing, indicating that the knockdown of E2F1 expression significantly reduced roscovitine-induced sensitization to TRAIL. A previous report has demonstrated that over-expression of E2F1 in human lung adenocarcinoma cell lines caused the down-regulation of cFLIPs expression and sensitized these cells to death receptor-induced apoptosis [44]. Furthermore, E2F1 directly represses Mcl-1 expression by binding to the Mcl-1 gene promoter. Together, these data suggest that the roscovitine-induced depletion of both cFLIP and Mcl-1 in breast tumor cells may be mediated by E2F1 up-regulation. However, we failed to observe reversal of cFLIP and Mcl-1 down-regulation in cells that were depleted of E2F1 by siRNA silencing. Clustering of the death receptor CD95/Fas and formation of the DISC in the absence of ligand have been also reported in cells over-expressing E2F1 [44]. Whether the increased recruitment of procaspase-8 and FADD to the TRAIL DISC observed in roscovitine-treated breast tumor cells is mediated by up-regulation of E2F1 is an issue that requires further study.

In conclusion, roscovitine can induce apoptosis in different tumor cell lines and causes regression of human xenograft tumors in mice [20]. Since it also seems less toxic for normal cells than other CDK inhibitors [28], it might be a good candidate to use in combined strategies against breast cancer. In fact, treatment with CDK inhibitors sensitizes

tumor cells to apoptosis induced by different anti-tumor agents [64, 65]. In the present study, we show that roscovitine treatment sensitizes human breast tumor cell lines to TRAIL-induced apoptosis, independent of the p53 and ErbB-2 receptor status, through a pleiotropic mechanism that involves increased DISC formation, cFLIP and Mcl-1 down-regulation, and E2F1 up-regulation. The present findings provide further support for the general strategy of combining TRAIL and CDK inhibitors in anti-cancer regimens against TRAIL-resistant tumor cells.

Materials and Methods

Reagents and antibodies

Roscovitine, MBCD, Imipramine, streptavidin-agarose beads, and MG 132 were obtained from SIGMA Chemical Corp. (St Louis, MO). Soluble human His-tagged recombinant TRAIL and biotin-labeled recombinant TRAIL (bTRAIL) were generated in our laboratory as described [66]. Anti-human TRAIL-receptor R1, R2, R3 and R4 antibodies and anti-cFLIP monoclonal antibody (NF6) were from Alexis Corp. (San Diego, CA). Anti-caspase 8 was a gift from Dr Gerald Cohen (Leicester University, UK). Bax, XIAP FADD and RIP antibodies were from BD Bioscience (Erembodegem, Belgium). The PARP polyclonal antiserum was from Roche Molecular Biochemicals (Germany). The monoclonal antibody to alpha-tubulin was purchased from Sigma Chemical Corp. Antibodies against GAPDH, Mcl-1 (S-19), E2F1 (KH95), DR5 (N19) and Caspase-2-L were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Horseradish peroxidase or FITC conjugated, goat anti-mouse and goat anti-rabbit secondary antibodies were obtained from DAKO (Cambridge, UK). zVAD-fmk was from Bachem AG (Bachem, Bubendorf, Switzerland).

Cell culture

The human tumor cell lines MDA-MB231 and EVSA-T were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 40 µg/ml gentamycin. MDA-MB468, SKBr-3, MDA-MB435-S and BT-474 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 40 µg/ml gentamycin. MDA-MB435-S was also supplemented with insulin (10 µg/ml). The cells were maintained at 37 °C in a humidified 5% CO₂, 95% air incubator. A stable cell line over-expressing cFLIP_L was generated upon transfection of MDA-MB231 cells with pCR3. V64-Met-Flag-FLIP_L (a kind donation of Dr J Tschopp, University of Lausanne) by electroporation. Mock-transfected cells and cells over-expressing FLIP_L were selected in culture medium with 1 mg/ml G418 (Sigma Chemical Co.) and analyzed for the expression of cFLIP_L by Western blot.

Analysis of apoptosis

Hypodiploid apoptotic cells were detected by flow cytometry according to published procedures [10]. Phosphatidylserine (PS) exposure on the surface of apoptotic cells was detected by flow cytometry after staining with Annexin-V-FLUOS (Roche Molecular Biochemicals).

Immunoblot analysis of proteins

The assay for measurements of cytochrome *c* and Bax was

performed as described previously [49]. Proteins were resolved on SDS-polyacrylamide minigels and visualized as described previously [10].

Analysis of TRAIL receptors by flow cytometry

MDA-MB231 cells were detached with RPMI 1640/EDTA, washed in ice-cold PBS, and resuspended in PBS. Cells were then labeled with anti-TRAIL receptor antibodies (5 µg/ml) or no antibody (negative control), and then incubated with goat anti-mouse FITC-conjugated antibody F(ab')₂ fragment. Labeled cells were analyzed by flow cytometry using the CellQuest software (Becton Dickinson, Mountain View, CA).

Isolation of the TRAIL DISC

DISC precipitation was performed using biotin-tagged recombinant TRAIL (bio-TRAIL) [66]. MDA-MB231 cells were incubated for 15 h in the presence or absence of 20 µmol/l roscovitine and they were then exposed to bio-TRAIL for the times indicated in Figure 3. DISC formation was determined as reported previously [66].

Reverse transcriptase (RT) and PCR assays

Total RNA was isolated from MDA-MB231 cells with the Trizol reagent (Life Technologies, Inc., Grand Island, NY) as recommended by the supplier. Total RNA (2 µg) was used as a template for cDNA synthesis using a RT-PCR kit (Perkin-Elmer). PCRs were carried out using specific primers for cFLIP_L and cFLIP_S as described previously [24].

Real-time RT-PCR

MDA-MB231 cells were treated with roscovitine (20 µM) for 15 h. Total RNA was isolated from cells as described before. Total RNA (1 µg) was used as a template for cDNA synthesis using a RT-PCR kit (Perkin-Elmer) with the supplied random hexamers and under the conditions described by the manufacturer. mRNA expression was analyzed in triplicate by quantitative RT-PCR on the ABI Prism 7500 sequence detection system using predesigned Assay-on-demand primers and probes (Applied Biosystems). mRNA expression was determined by the comparative cycle threshold (Ct) method ($\Delta\Delta C_t$). Hypoxanthine-guanine phosphoribosyltransferase was used as an internal control and mRNA expression levels of cFLIP_L and cFLIP_S were given as fraction of mRNA levels in control cells.

TRAIL stimulation and isolation of lipid rafts

Sixty million cells were left untreated or stimulated for the indicated time points with 1 µg/ml TRAIL-biotinylated after pre-incubation with Roscovitine (20 µM, 15 h) where indicated. Treated samples were routinely collected to subsequently evaluate cell viability as described [67]. Lipid raft extraction was performed according to standard protocols [68]. Briefly, the cell pellet was dissolved in 750 µl of 1% Triton X-100/25 mM MES/150 mM NaCl buffer at 4 °C. After homogenization, the cell lysates were subjected to sucrose gradient centrifugation at 45 000 rpm for 16 h in an SW60 rotor (Beckman Instruments). Twelve 375-µl fractions were collected from the top to the bottom of each gradient. For cholesterol depletion studies, cells were treated with 10 mM MBCD for 30 min at 37 °C in serum-free medium before lipid raft isolation.

Statistical analysis

All data are presented as the mean ± SE of at least three in-

dependent experiments. The differences among different groups were determined by the Student's *t* test. *P* < 0.05 was considered significant.

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