CCT327 enhances TRAIL-induced apoptosis through the induction of death receptors and downregulation of cell survival proteins in TRAIL-resistant human leukemia cells

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Abstract. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has potential application in cancer therapy and it has the ability to selectively kill cancer cells without affecting normal cells. However, the development of resistance to TRAIL in cancer cells cannot be avoided. This study investigated the effects of 2-(5-methylselenophen-2-yl)-6,7-methylenedioxyquinolin-4-one (CCT327), an analogue of quinolin-4-one, on the sensitization of cancer cells to TRAIL and on TRAIL-induced apoptosis in TRAIL-resistant human leukemia cells (HL60-TR). We found that CCT327 enhanced TRAIL-induced apoptosis through upregulation of death receptors DR4 and DR5. In addition to upregulating DRs (death receptors), CCT327 suppressed the expression of decoy receptor DcR1. CCT327 significantly downregulated the expression of FLICE inhibitory protein (cFLIP) and other antiapoptotic proteins. We also demonstrated that CCT327 could activate p38 and JNK. Moreover, CCT327-induced induction of DR5 and DR4 was mediated by reactive oxygen species (ROS), and N-acetylcysteine (NAC) blocked the induction of DRs by CCT327. Taken together, these results showed that CCT327 combined with TRAIL treatment may provide an effective therapeutic strategy for cancer.

Introduction

The tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily, and is an attractive anticancer agent. The ability of TRAIL includes the selective killing of a variety of cancer cells without affecting normal cells (1-4). TRAIL is one of the most promising experimental cancer therapeutic drugs and is presently being tested

Abbreviations: CCT327, 2-(5-methylselenophen-2-yl)-6,7-methylenedioxyquinolin-4-one; 2PQs, 6,7-substituted 2-phenylquinolin-4-ones; TRAIL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; PI, propidium iodide; NAC, N-acetylcysteine; DR, death receptor; DcR, decoy receptor; OPG, osteoprotegerin; FADD, Fas-associated protein with death domain; DISC, death-inducing signaling complex; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; FLICE, FADD-like interleukin-1β-converting enzyme; c-FLIP, FLICE inhibitory protein; MAPKs, mitogen-activated protein kinases; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide; ECL, enhanced chemiluminescence; PBS, phosphate-buffered saline; RPMI-1640, Roswell Park Memorial Institute-1640

Key words: TRAIL, CCT327, human leukemia cells, ROS, death receptor
in clinical trials (5-8). To date, five different TRAIL receptors have been identified: death receptor DR4 (TRAIL-R1) and DR5 (TRAIL-R2), decoy receptor DcR1 and DcR2 and osteoprotegerin (OPG). DR4 and DR5 are able to transduce an apoptotic signal, whereas the other three (DcR1, DcR2 and OPG) are decoy receptors to impede TRAIL-induced apoptosis, and play a dominant-negative role by competing with DR4 and DR5 for interaction with TRAIL (9-12).

The binding of TRAIL to two closely related receptors, DR4 and DR5, leads to recruitment of the adaptor protein, Fas-associated protein with death domain (FADD) and initiator caspase-8 to form the death-inducing signaling complex (DISC). This process leads to the cleavage and activation of caspase-8, which in turn activates the downstream caspase cascade, such as caspase-9 and -3 in the presence or absence of mitochondrial amplification machinery (13-18). However, human cancer cell lines and primary tumor cells are found to develop resistance to TRAIL through intrinsic or acquired resistance mechanisms. This resistance is mediated through deregulation of apoptotic-related signaling molecules, such as downregulation of DR4, DR5, caspase-8, or Bax and enhanced expression of antiapoptotic molecules such as survivin, or overexpression of the Bcl-2 family proteins (19-21). FLICE causes the activation of caspase-8, and FLICE-like inhibitors such as cFLIP have been reported to bind to caspase-8 and impede the activation of downstream incidents leading to apoptosis, including TRAIL-mediated apoptosis (22-25). Consequently, the relationship between tumors and TRAIL has caused a large interest in understanding the effector mechanisms and the search for novel compounds which can resensitize tumor cells to TRAIL-induced apoptosis.

Reactive oxygen species (ROS), such as superoxide, H$_2$O$_2$ and hydroxyl radicals, trigger a variety of cellular responses leading to cell growth, differentiation, or cell death (26-31). Mitogen-activated protein kinases (MAPKs), such as stress activated protein kinase/c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 are principal mediators of the ROS-induced signaling pathway (31-35). In addition, when MAPKs are activated, this triggers diverse signaling cascades resulting in cell proliferation, differentiation or cell death in various tumor cells (30,34-39).

Several 6,7-substituted 2-phenylquinolin-4-ones (2PQs) have been synthesized and identified as novel antimitotic agents (40). Recently, novel 2-selenophenyl quinolin-4-ones have been synthesized and identified as novel antimitotic agents (40). Recombinant soluble human TRAIL was purchased from PeproTech (Rocky Hill, NJ, USA). Primary antibodies against caspase-3, caspase-8 and caspase-9, PARP, Bcl-2, survivin, JNK, phospho-JNK, ERK1/2, phospho-ERK1/2, p38 and phospho-p38 were purchased from Cell Signaling Technology (Danvers, MA, USA). DcR1 and DcR2 antibodies were purchased from ProSci Inc. (Poway, CA, USA). Antibodies against Bax, FLIP$_S$/FLIP$_I$ and Bid were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary DR4 and DR5 were purchased from Abcam Inc. (Cambridge, MA, USA) and Novus Biologicals (Littleton, CO, USA), respectively. Secondary antibodies, HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG, were obtained from Millipore (Billerica, MA, USA). Cell culture materials were obtained from Invitrogen Corp. (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and antibodies for β-actin were purchased from Sigma (St. Louis, MO, USA). PD98059, SB203580 and SP600125 were obtained from Calbiochem (San Diego, CA, USA).

**Materials and methods.**

**Chemicals and reagents.** CCT327 was synthesized in our laboratory (Fig. 1A). Recombinant soluble human TRAIL was purchased from PeproTech (Rocky Hill, NJ, USA). Primary antibodies against caspase-3, caspase-8 and caspase-9, PARP, Bcl-2, survivin, JNK, phospho-JNK, ERK1/2, phospho-ERK1/2, p38 and phospho-p38 were purchased from Cell Signaling Technology (Danvers, MA, USA). DcR1 and DcR2 antibodies were purchased from ProSci Inc. (Poway, CA, USA). Antibodies against Bax, FLIP$_S$/FLIP$_I$ and Bid were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary DR4 and DR5 were purchased from Abcam Inc. (Cambridge, MA, USA) and Novus Biologicals (Littleton, CO, USA), respectively. Secondary antibodies, HRP-conjugated goat anti-mouse IgG and goat anti-rabbit IgG, were obtained from Millipore (Billerica, MA, USA). Cell culture materials were obtained from Invitrogen Corp. (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and antibodies for β-actin were purchased from Sigma (St. Louis, MO, USA). PD98059, SB203580 and SP600125 were obtained from Calbiochem (San Diego, CA, USA).

**Cell lines and cell cultures.** The human leukemia cancer cell line used in this study was HL60 (CCL 240) from the parental cell line obtained from the American Type Culture Collection (Manassas, VA, USA). HL60 cells derived from a human acute promyelocytic leukemia are usually sensitive to chemotherapeutic drugs and TRAIL. TRAIL-resistant HL60 cells (HL60-TR) were selected by exposure of HL60 cells to escalating doses of TRAIL (10 ng/ml, 20 ng/ml, 50 ng/ml, 100 ng/ml, 500 ng/ml, 1 µg/ml, 5 µg/ml and 10 µg/ml) for 2 to 3 days. After each exposure, surviving cells were recovered and cultured in fresh medium for 3 days and then treated with the subsequent dose (42). HL60-TR cells were routinely maintained in RPMI-1640 (Invitrogen). Medium was supplemented with 2 mM L-glutamine, 100 µg streptomycin, 100 U penicillin and 10% fetal bovine serum (FBS) (Invitrogen). Cells were grown in a humidified incubator at 37°C under 5% CO$_2$ in air.

**Cytotoxicity assay.** In brief, cells were seeded on a 24-well plate (1x10$^4$ cells/well) overnight and then treated with different concentrations of CCT327 and TRAIL as indicated in the figure legends and then incubated for 48 h. Following treatments, 50 µl of MTT (stock concentration 2 mg/ml) was added to each well and incubated for 2 h under 5% CO$_2$ at 37°C. The cell viability was measured by MTT, which is converted by succinate dehydrogenase in the mitochondria of viable cells to form a purple formazan dye by metabolically viable cells. The formazan dye was dissolved in dimethyl sulfoxide (DMSO). To measure the absorbance, an enzyme-linked immunosorbent assay (ELISA) reader was used at OD 570 nm.
Flow cytometric analysis. To determine the effect of CCT327 plus TRAIL on the cell cycle distribution, treated and untreated cells were stained with PI as mentioned earlier. Briefly, 5x10^5 cells were treated with CCT327 plus TRAIL for 48 h at 37°C and subjected to PI staining. Cells were collected by trypsinization, fixed with 70% (v/v) ethanol at 4°C for 30 min and washed with phosphate-buffered saline (PBS). After centrifugation, cells were resuspended in 500 µl of PI solution comprising Triton X-100 (0.1%, v/v), RNase (100 mg/ml) and PI (80 mg/ml) and then analyzed with FACScan and the Cell Quest software (Becton-Dickinson, Mountain View, CA, USA) (43).

Western blotting. HL60-TR cells on 100-mm culture dishes (1x10^6 cells/dish) were treated with various agents as indicated in the figure legends and were then incubated for 48 h. Cells were harvested and the protein fraction was extracted by adding 50 µl of Gold lysis buffer (50 mM Tris-HCl, pH 7.4; 1 mM phenylmethylsulfonyl fluoride; 1 mM NaF; 1% NP-40; 150 mM NaCl; 1 mM EGTA and 10 mg/ml leupeptin) to the cell pellets. Lysate protein was measured by the Lowry protein assay (Bio-Rad Laboratories, Berkeley, CA, USA). Proteins between 50 and 100 µg were used for the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane.
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The blotted membrane was blocked with 5% skim milk for 1 h at room temperature and probed with the primary antibody overnight at 4°C. Finally, HRP-conjugated appropriated secondary antibodies were used for 1 h.

Statistical analysis. One-way analysis of variance (ANOVA) was used for the comparison of more than 2 mean values. The results represent at least two to three independent experiments and are shown as averages ± SEM. Results with a P-value <0.05 were considered statistically significant (P<0.05, P<0.01, P<0.001 as indicated in the figure legends).

Results

CCT327 sensitizes HL60-TR cells to TRAIL-mediated apoptosis. We first examined the sensitivity of HL60 and HL60-TR cells to TRAIL. HL60 and HL60-TR cells were treated with increasing doses of recombinant TRAIL and were then assessed for cell viability using the MTT method. The dose-response of HL60 and HL60-TR cells to TRAIL is shown in Fig. 1B. The HL60 cells were found to be highly sensitive to TRAIL whereas the HL60-TR cells were completely resistant (Fig. 1B). Therefore, the HL60-TR cells were used for a detailed investigation of the resistance mechanisms.

We next aimed to ascertain whether CCT327 enhances TRAIL-induced apoptosis in HL60-TR cells. HL60-TR cells were treated with CCT327 (0-600 nM) and then exposed to TRAIL (0-200 ng/ml) for 48 h. Treatment with TRAIL had no effect on cell viability. However, combination treatment with CCT327 and TRAIL significantly enhanced TRAIL-induced cytotoxicity (Fig. 1C). To further confirm the effect of CCT327 on TRAIL-induced apoptosis, we also investigated the distribution of cells by PI staining. The percentage of apoptotic cells in the sub-G1 peak as evidenced by the increase in subdiploid fraction of treated cells was measured by flow cytometry. We found that TRAIL-induced apoptosis was increased from 9.7 to 50.8% in the HL60-TR cells (Fig. 1D). Thus, our results indicated that CCT327 converted the TRAIL-resistant HL60-TR cells to TRAIL-sensitive cells.

Activation of caspases is an important hallmark of apoptosis induced by most agents. We next investigated whether the effect of CCT327 on TRAIL-induced cell death was through activation of caspase-8, -9 and -3 and PARP cleavage. Treatment with 300 nM CCT327 alone had little effect on the cleavage of procaspase-3, -8, -9 and PARP (Fig. 1E). Moreover, TRAIL alone did not induce processing of any caspases. Co-treatment with CCT327 and TRAIL effectively induced activation of all three caspases, thus leading to enhanced PARP cleavage. These results suggest that CCT327 can enhance TRAIL-induced apoptosis. The activation of caspases by CCT327 was essential for the stimulation of TRAIL-mediated apoptosis.

CCT327 inhibits the expression of antiapoptotic proteins. Several antiapoptotic proteins are known to suppress TRAIL-induced apoptosis. The mechanism by which CCT327 enhances TRAIL-induced apoptosis was next investigated. HL60-TR cells were exposed to 300 nM CCT327 for different times and were then examined for the expression of cFLIP L and cFLIP S (long and short), Bcl-2, Bcl-xL and survivin. CCT327 suppressed expression of the antiapoptotic proteins such as Bcl-2 and both the short and long forms of cFLIP (Fig. 2A). It had no effect on the expression of survivin. Expression of Bcl-xL was not distinct. Our results suggest that downregulation of antiapoptotic proteins is another mechanism by which CCT327 sensitizes TRAIL-induced apoptosis.

CCT327 regulates the expression of proapoptotic proteins. Whether CCT327 affects the expression of proapoptotic proteins was next examined. CCT327 caused the cleavage of bid protein and enhanced the expression of proapoptotic protein bax (Fig. 2B). Induction of bid and bax by CCT327 suggests that these proteins may disrupt mitochondrial homeostasis, which further contributes to the enhancement of the apoptotic effects of TRAIL.

CCT327 induces the expression of DR4 and DR5 in HL60-TR cells. To understand how CCT327 enhances TRAIL-induced apoptosis, we investigated its effect on DR4 and DR5 in HL60-TR cells. Treatment of HL60-TR cells with 300 nM CCT327 induced expression of DR4 and DR5 in a time-dependent manner (Fig. 3A, left). Treatment with different concentrations of CCT327 to HL60-TR cells for 48 h induced both DR4 and DR5 in a dose-dependent manner (Fig. 3A, right). Fig. 3B shows that treatment with TRAIL alone had
no effect on DR4 and DR5. However, combination treatment with CCT327 (300 nM) and TRAIL (100 ng/ml) significantly enhanced the expression of DR4 and DR5 in HL60-TR cells. These data show that CCT327 regulated DR4 and DR5 that both play a major role in TRAIL-induced apoptosis. This is another mechanism by which CCT327 enhanced the proapoptotic effects of TRAIL in HL60-TR cells.

CCT327 downregulates decoy receptors. Decoy receptors compete with the death receptors for ligand binding and thereby inhibit ligand-induced apoptosis (9-12). Therefore, we next examined whether CCT327 modulates the expression of DcRs. We found that CCT327 decreased the expression of DcR1, but did not influence the level of DcR2 (Fig. 3C). Thus, CCT327 may potentiate TRAIL-induced apoptosis by inhibition of DcR1.

CCT327-induced upregulation of TRAIL receptors is dependent on ROS. Several studies have reported that TRAIL-induced apoptosis is regulated by ROS (47-51). We attempted to ascertain whether CCT327-induced TRAIL receptors are also regulated by ROS. Our data showed that pretreatment of HL60-TR cells with the ROS scavenger N-acetylcysteine (NAC) reduced the CCT327-induced upregulation of both DR5 and DR4 expression in a dose-dependent manner (Fig. 4A). This suggests that ROS is involved in the induction of TRAIL receptors by CCT327. Next, we examined whether ROS is needed for potentiation of TRAIL-induced apoptosis by CCT327. As shown in Fig. 4B, we found that pretreatment with NAC abolished the effect of CCT327 on TRAIL-induced cleavage of PARP. These results show that CCT327 potentiated TRAIL-induced apoptosis through ROS.
CCT327-induced upregulation of TRAIL receptors is mediated through the activation of MAPKs. MAPKs, including ERK1/2, p38 and JNK, have been reported to mediate induction of TRAIL receptors (47,49). Therefore, we ascertained whether CCT327 activates ERK1/2, p38 and JNK. Cells were pretreated with CCT327 for different times and were then examined for phosphorylated ERK, JNK and p38. We found that CCT327 activated ERK1/2 in a time-dependent manner (Fig. 5A). No activation of JNK was noted. In addition, activation of p38 was observed (Fig. 5A). Our result showed that induction of TRAIL receptors by CCT327 required ERK1/2 and p38. Next, we also determined whether these MAPKs have any effect on CCT327-induced TRAIL receptors. Cells were pretreated with 20 µM of the ERK1/2 inhibitor, PD98059; 20 µM of the JNK inhibitor, SP600125; and 10 µM of the p38 inhibitor, SB202190, respectively (52). Both the ERK1/2 inhibitor (Fig. 5B) and the p38 inhibitor (Fig. 5D) suppressed the CCT327-induced upregulation of DR4 and DR5. No effect of the JNK inhibitor was observed on CCT327-induced TRAIL receptors. Cells were pretreated with 20 µM of the ERK1/2 inhibitor (PD98059), 20 µM of the JNK inhibitor (SP600125) and 10 µM of the p38 inhibitor (SB202190), respectively (52). Both the ERK1/2 inhibitor (Fig. 5B) and the p38 inhibitor (Fig. 5D) suppressed the CCT327-induced upregulation of DR4 and DR5. No effect of the JNK inhibitor was observed on CCT327-induced DR4 and DR5 expression. Upregulation of TRAIL receptors by CCT327 was reversed by inhibitors of ERK1/2 and p38. Thus, the activation of ERK1/2 and p38 is consistent with the results obtained with the effect of their inhibitors on the CCT327-induced expression of TRAIL receptors.

Discussion

TRAIL is the only cytokine that is being explored as an anticancer agent among all the apoptosis-inducing cytokines. The unique property of triggering apoptosis in a variety of human cancer cells while sparing normal cells makes TRAIL a highly promising cancer therapeutic agent (2,3). Both TRAIL and the agonistic antibodies against the receptor are presently in phase II clinical trial (53). TRAIL induces apoptosis through recognizing and binding to its cognate death receptors, DR4 and DR5 (also named as TRAIL-R1 and TRAIL-R2), on the cell surface. Upon ligand stimulation, DRs (Fas or death receptor 4/5, DR4/5) recruit FADD and the initiator caspases, caspase-8 or caspase-10, resulting in the formation of DISC, thereby inducing death signaling and the apoptosis pathway (9,10). However, TRAIL resistance is a major limitation to its clinical application as a cancer therapeutic agent. Nevertheless, a previous study demonstrated that the resistance of cancer cells to TRAIL is one of the major roadblocks to the development of this therapy (21). Thus, efforts to identify agents that activate DRs or block antiapoptotic effectors may improve therapeutic design.

In our previous report, we introduced and described a novel compound, CCT327, which has been shown to induce
apoptosis in human leukemia cancer cells (41). Research has shown convincing data that the upregulation of DR4 or DR5 could sensitize TRAIL-resistant cells to TRAIL-induced cell death (49-52). We showed that CCT327 sensitized TRAIL-induced apoptosis through modulation of death receptors. Our results also indicate that DR4 and DR5 are involved in the reversal of TRAIL-resistance by CCT327.

Research has shown that resistance to TRAIL can be due to several mechanisms, including overexpression of antiapoptotic proteins and decoy receptors (21). CCT327 decreased the expression of DcR1, but it did not influence the level of DcR2. In addition to the induction of DcR1, we also found that CCT327 downregulated expression of antiapoptotic proteins including cFLIP (long and short), Bcl-2, Bcl-xl and survivin. The effect was most pronounced on cFLIPL and Bcl-2. Recently, c-FLIPs was shown to be correlated with TRAIL resistance in various tumor types, and c-FLIP downregulation has been implicated in chemotherapy-sensitized TRAIL-induced apoptosis (22,55). Several studies have shown that Bcl-2 blocks apoptosis by maintaining mitochondrial function (56). Taken together, our results indicate that c-FLIP’s and Bcl-2 downregulation contributes to CCT327-facilitated TRAIL-mediated apoptosis.

ROS trigger a variety of cellular responses leading to cell growth, differentiation or cell death (54). ROS generation has been proposed to be involved in death receptor upregulation by cancer chemopreventive agents (51,52). In the present study, we found that induction of ROS was critical for the sensitization of cells to TRAIL by CCT327. Our data revealed that the mechanism by which CCT327 induces DR upregulation is through production of ROS. The antioxidant NAC abolished the upregulation of DR by CCT327. The effect of CCT327 on TRAIL-induced apoptosis was also neutralized by the antioxidant. This reversal was apparently due to inhibition of induction of TRAIL receptors. An important downstream mediator of ROS-induced signaling is the MAPKs (31,34). MAPKs, including ERK1/2, p38 and JNK, have been reported to mediate induction of TRAIL receptors (47,49). Recent studies have shown that activation of ERK, JNK or p38 is also associated with TRAIL-induced-apoptosis via upregulation of DR4/5 (49,50). CCT327 activated ERK1/2 p38 in a time-dependent manner. We questioned whether the activation of p38 and ERK1/2 was the cause or a downstream effect of the upregulation of the TRAIL receptors. Both ERK1/2 inhibitor (Fig. 5b) and p38 inhibitor (Fig. 5d) suppressed the CCT327-induced upregulation of DR4 and DR5. Notably, the presence of the JNK inhibitor had no effect on CCT327-induced DR4 and DR5 expression. CCT327 induced the expression of TRAIL receptors dependent on MAPK, particularly ERK1/2 and p38.

Overall, we demonstrated that CCT327 can sensitize TRAIL-induced apoptosis through the upregulation of DRs mediated by JNK and p38 and the downregulation of cFLIP and other antiapoptotic proteins. CCT327 has potential for application in the treatment of cancer by TRAIL, particularly for tumors that develop resistance to TRAIL.

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References


