WEE1 Inhibition Sensitizes Basal Breast Cancer Cells to TRAIL-Induced Apoptosis

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Abstract

TRAIL is a member of the TNF superfamily and has been shown to induce apoptosis in many cancer cell lines but not in normal cells. Breast cancers can be divided into different subgroups on the basis of the expression of estrogen and progesterone receptors, HER-2 amplification, or the lack of these three markers (known as triple-negative or basal-type breast cancer). Our group and others have shown previously that triple-negative breast cancer cell lines are sensitive to TRAIL whereas others are relatively resistant. In an earlier study, we reported that inhibition of WEE1, a cell-cycle checkpoint regulator, causes increased cell death in breast cancer cell lines. In this study, we tested the effects of WEE1 inhibition on TRAIL-mediated apoptosis in breast cancer cell lines. Pretreatment with WEE1 inhibitor or knockdown of WEE1 increased the toxicity of TRAIL in the basal/triple-negative breast cancer cell lines compared with WEE1 inhibitor or TRAIL treatment alone. The enhanced cell death is attributed to increased surface expression of death receptors, increased caspase activation which could be blocked by the pan-caspase inhibitor, Z-VAD-FMK, thereby rescuing cells from caspase-mediated apoptosis. The cell death was initiated primarily by caspase-8 because knockdown of caspase-8 and not of any other initiator caspases (i.e., caspase-2, -9, or -10) rescued cells from WEE1 inhibitor–sensitized TRAIL-induced cell death. Taken together, the data suggest that the combination of WEE1 inhibitor and TRAIL could provide a novel combination for the treatment of basal/triple-negative breast cancer. Mol Cancer Res; 1–11. ©2011 AACR.

Introduction

Breast cancer is a heterogeneous group of diseases. Approximately 60% to 70% of breast cancers express estrogen receptors (ER) and/or progesterone receptors (PR), and approximately 20% to 30% of breast cancers have amplified HER-2 and thus express high levels of the HER-2 protein (1, 2). In approximately 15% to 20% of patients with breast cancer, the tumors do not express ER or PR and do not have amplification of HER-2 (1). These are categorized as triple-negative breast cancer (TNBC). Molecular classification by expression profiling of primary breast cancers and breast cancer cell lines has determined that the majority of these triple-negative tumors share expression profiles with basal epithelial cells of the breast duct and hence are referred to as basal-like tumors (3–6). Currently, the mainstay of treatment for these tumors is chemotherapy, and patients with these tumors have a poor prognosis (1). Thus, identification of novel, molecularly targeted therapies for triple-negative/basal-like breast cancer would be of great benefit.

Studies have shown that TNBCs are defective in double-stranded DNA repair and, like many other tumor cells, lack the G₁–S checkpoints (7, 8). WEE1, a tyrosine kinase, serves as a critical component of the response to double-stranded DNA breaks by phosphorylating CDC2, thereby activating the G₂–M checkpoint and allowing the cells to repair the damaged DNA (9, 10). Therefore breast cancer cells, like other cancer cells, are susceptible to death induced by G₂–M checkpoint abrogators (11). Previously, we have identified through an RNA interference (RNAi) screen of the human tyrosine kinase that silencing of WEE1 in breast cancer cells resulted in DNA damage, cell-cycle arrest in S-phase, and caspase-mediated cell death (12).

TRAIL may have potential use in cancer therapy because of its ability to kill selectively cancer cells over normal cells (13–15). TRAIL binds to its receptors, TRAIL-R1 (TR1, also called DR4) or TRAIL-R2 (TR2, also called DR5) at the cell surface which leads to the recruitment of the adaptor molecule FADD and pro-caspase-8 forming the death-inducing signaling complex (DISC; ref. 16). Pro-caspase-8...
is cleaved to its active form at the DISC which then cleaves and activates the downstream executioner caspases-3 and -7 (16). Active caspase-8 can also cleave the BH3 protein BID which results in activation of the intrinsic mitochondrial pathway of apoptosis and the activation of caspase-9 (17). Work done in animals has shown that TRAIL mediates regression of cancer xenographs without affecting normal tissues (14). Human phase I studies have shown that TRAIL agonists are safe and phase II trials are now in progress (18).

Initial reports of TRAIL-mediated apoptosis in breast cancer cell lines have shown that while TRAIL could induce apoptosis in the MB231 breast cancer cell line, the majority of cell lines tested were highly resistant (13, 19–21). The data from several groups, including ours, have shown that most TNBC/basal cell lines and a few of the HER-2–amplified cell lines are sensitive to TRAIL-induced apoptosis, but all ER–positive (ER+) breast cancer cells are resistant to TRAIL–induced apoptosis (22–24). Studies in the literature have investigated the combination of a wide range of chemotherapeutic drugs and radiation with TRAIL agonists to potentiate cell death and/or overcome resistance in breast cancer cells (24, 25).

In this study, we find that loss of WEE1 function enhances TRAIL-mediated apoptosis in TNBC/basal breast cancer cells. The enhanced apoptosis is mediated by increased caspase activation and dependent on the initiator caspase, caspase-8.

Materials and Methods

Cell culture

The MDA-MB231 (MB231), HCC38, BT549, BT474, MCF7, Hs578T, and SKBR3 cell lines were obtained from American Type Culture Collection and BT20 and HCC1937 were obtained from Reinhard Ebner (Avalon Pharmaceuticals). American Type Culture Collection cell lines are authenticated by short tandem repeat analysis. On receiving the cell lines, they were immediately cultured and expanded to prepare frozen ampoule stocks. MB231 cells were grown in RPMI-1640 medium supplemented with 5% FBS, (R5); all other cells were grown in RPMI-1640 medium supplemented with 10% FBS (R10). All cell lines were passaged for no more than 2 to 3 months before establishing new cultures from the early-passage frozen ampules. MB231 breast cancer cells were resistant to TRAIL-induced apoptosis, but all ER–positive (ER+) breast cancer cell lines have shown that while TRAIL could induce apoptosis in the MB231 breast cancer cell line, the majority of cell lines tested were highly resistant (13, 19–21). The data from several groups, including ours, have shown that most TNBC/basal cell lines and a few of the HER-2–amplified cell lines are sensitive to TRAIL-induced apoptosis, but all ER–positive (ER+) breast cancer cells are resistant to TRAIL–induced apoptosis (22–24). Studies in the literature have investigated the combination of a wide range of chemotherapeutic drugs and radiation with TRAIL agonists to potentiate cell death and/or overcome resistance in breast cancer cells (24, 25).

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Inhibitors

WEE1 inhibitor II [6-butyl-4-(2-chlorophenyl)-9-hydroxyprrolo[3,4-c]carbazole-1,3-(2H,6H)-dione; Calbiochem, 681641]; the pan-caspase inhibitor, Z-VAD-FMK (Biomol International, P416) and caspase-3 inhibitor, Ac-DEVD-CHO (Biomol International, P410) were dissolved in dimethyl sulfoxide (DMSO) and used at 10 or 100 µmol/L concentrations, as described in the text. The glutathione S-transferase (GST)-TRAIL construct and the isolation of recombinant GST-TRAIL fusion protein have been described previously (23). The GST-TRAIL protein (at 0.5 mg/mL in culture media) was stored at −70°C in aliquots until used.

Cell viability assays

Cellular cytotoxicity was assessed by the MTS assay using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega Corporation, G3582) as previously described (23). All MTS measurements were done in replicates of 6 wells, and each experiment was carried out at least 3 times. Results are given as the mean ± SEM of at least 3 independent experiments.

Trypan blue staining

Cells were incubated with Trypan blue stain (Invitrogen, 15250–061) after transfection with siRNA or treatment with inhibitors and/or GST-TRAIL. Cells that excluded the dye (viable) and cells that retained the dye (dead) were counted.

siRNA transfection

The WEE1 ON-TARGET plus SMARTpool siRNA (siWEE1, L-005050–00) and a nontargeting siRNA control pool (siNEG, D-001810–10) were purchased from Dharmacon. HCC38 cells were transfected in solution with the siRNA pool at a final concentration of 50 nmol/L using Oligofectamine (Invitrogen, 12252011; 7 µL/mL). Transfected cells were plated in 6-well plates (2 × 10⁵ cells per well). Eight hours later, one well with the siNEG-transfected cells and one well with the siWEE1-transfected cells were treated with 2.5 ng/mL TRAIL. The percentage of dead cells was determined after 15 hours using Trypan blue stain. Results shown represent mean ± SEM of 3 experiments. Other cells in the 6-well plate were used for protein analysis.

Sub-G₁ analysis

Cells (1 × 10⁶) were plated overnight, treated with 10 µmol/L WEE1 inhibitor for 4 hours followed with 100 ng/mL TRAIL for 17 hours. Then, the cells were incubated with 10 µmol/L bromodeoxyuridine (BrdU; Sigma-Aldrich, 5002) for 15 minutes, trypsinized, washed with PBS, and fixed in cold 70% ethanol. Staining was conducted with anti-BrdU-FITC antibody (BD Biosciences, 347583) and 5 µg/mL propidium iodide (Invitrogen, P3566). Flow cytometry was conducted on a BD FACS Calibur (BD Biosciences) and analyzed using FlowJo software.

Annexin V staining

After treatment with 10 µmol/L WEE1 inhibitor for 4 hours or TRAIL (100 ng/mL) for 17 hours or their combination, cells were stained using Annexin V-FITC Apoptosis Detection kit II (BD Pharmingen, 51-6710AK) as per the manufacturer’s protocol. Flow cytometry was conducted on a BD FACS Calibur and analyzed using FlowJo software.

Caspase-Glo 3/7 assay

Cells incubated with Z-VAD-FMK or DEVD-CHO (100 µmol/L) or DMSO for 1 hour were treated with 10 µmol/L WEE1 inhibitor or DMSO for 4 hours, and then with 100 ng/mL TRAIL for 2 hours at 37°C. The assay was
done as described previously (12). Results are shown as mean ± SEM of 3 independent experiments normalized to DMSO-treated cells.

Flow cytometry for TR1 and TR2
MB231 cells treated with DMSO or WEE1 inhibitor (10 μmol/L) for 24 hours were trypsinized into sorter medium (PBS with 1% fetal calf serum and 0.5 mmol/L EDTA and 0.02 mmol/L sodium azide). All antibodies used for fluorescence-activated cell-sorting (FACS) analysis were purchased from R&D Systems. One million cells in 50 μL were incubated with 10 μL of mouse IgG1-PE (IC002P) or mouse IgG2B (IC0041P) or anti-TR1-PE (FAB347P) or anti-TR2-FITC (FAB6311P) for 45 minutes on ice. TR1 and TR2 surface expression was measured by flow cytometry conducted on a BD FACSCalibur and analyzed using FlowJo software.

Lysate preparation and immunoblotting
Cell lysates were made, and immunoblotting was conducted as described earlier (23). The following antibodies were used: caspase-8 (Clone 1C12, 9746), pY15CDC2 (4539), CDC2 (9112), caspase-9 (9502), caspase-3 (9662), BCL-2 (2872), BCL-XL (2762), and GAPDH (2118) from Cell Signaling Technology; caspase-10 (M059–3) from MBL; WEE1 (5285), MCL-1 (819), caspase-2 (625), ERK2 (154), HSC70 (7298), and PARP (7150) from Santa Cruz Biotechnology; TR1 (Genetex, 28414); TR2 (Prosci Inc., 2019); FLIP (Imgenex, 104); and survivin (R&D Labs, AF886).

Statistics
Statistical comparison of mean values was conducted using the paired Student t tests. All P values are 2 tailed.

Results
WEE1 inhibition sensitizes breast cancer cells to TRAIL treatment
Previously, we have shown that WEE1 loss or inhibition induces cell-cycle arrest and caspase-mediated cell death in breast cancer cell lines whereas nontransformed cell lines are not affected (12). This cell death was observed only after more than 2 days of WEE1 loss or inhibition. TNBC cells with a mesenchymal phenotype (so called basal B) are more sensitive to TRAIL than the other subsets of breast cancer cell lines, and this cell death can be observed within less than 24 hours of TRAIL treatment (13, 20, 21, 23, 24). We tested the effect of the combination of WEE1 inhibition on TRAIL-mediated apoptosis using a panel of breast cancer cell lines, including basal B/TNBC (MB231, HCC38, MB157, BT549, Hs578T), basal A/TNBC (BT20, HCC1937), HER-2–amplified (BT474, SKBR3) and ER+ (MCF7, T47D) breast cancer cell lines. In these experiments, cells were preincubated with or without WEE1 inhibitor II (26) for 4 hours, sub-IC50 doses of TRAIL were then added, and the viability was assessed 17 hours later by MTS assay. Basal B/TNBC cells were the most sensitive to the combined treatment. In the basal B/TNBC cells, WEE1 inhibition alone caused 20% to 30% decrease in viability, whereas treatment of cells with sub-IC50 doses of TRAIL alone caused approximately 5% to 30% loss in viability. However, the pretreatment of cells with the WEE1 inhibitor followed by TRAIL treatment resulted in enhanced cell death (60%–90%) compared with the untreated controls (Fig. 1A). Also, the basal A/TNBC HCC1937 cells and the HER-2–amplified cells were found to be sensitive to the WEE1 inhibitor and TRAIL combination but to a lesser extent than the basal B/TNBC cells. The ER+ cell lines, MCF7 and T47D, were relatively resistant to each agent alone and did not show any effect to the combination therapy at these sub-IC50 doses of TRAIL. To assess whether higher doses of TRAIL would enhance cell death in the MCF7 cell line, we treated MB231 or MCF7 cells with 10 μmol/L WEE1 inhibitor for 4 hours and then with different doses of TRAIL for 17 hours (Fig. 1B). In MB231 cells, pretreatment with WEE1 inhibitor significantly reduced the IC50 of TRAIL (from >1,000 to ~8 ng/mL), whereas in MCF7 cells, there was no change in viability in the presence or absence of WEE1 inhibitor over the wide range of TRAIL concentrations tested. The WEE1 inhibitor blocked WEE1 function as measured by the decrease in the phosphorylation on Tyr15 of CDC2 in both of these cell lines (Fig. 1C). These data indicate that the WEE1 inhibitor significantly increases the sensitivity of the basal B/TNBC cells to TRAIL-mediated inhibition, modestly increases the sensitivity to TRAIL-mediated inhibition in basal A/TNBC cell lines and HER-2–amplified cell lines but does not increase TRAIL sensitivity in resistant ER+ cell lines.

Whereas WEE1 inhibitor II is relatively specific for WEE1, it is possible that the effects seen above were due to off-target inhibition of other kinases (26). To confirm that the effects of the WEE1 inhibitor were acting through inhibition of WEE1, we measured the effects of loss of WEE1 by RNAi on TRAIL-mediated cell death. WEE1 was knocked down by gene-specific siRNA for 8 hours followed by treatment of cells with TRAIL for 17 hours, and the cell death was determined by counting dead cells as measured by the uptake of Trypan blue. WEE1 silencing resulted in decreased WEE1 protein levels and decreased Tyr15 phosphorylation of CDC2 (Fig. 2A). Silencing of WEE1 alone for 24 hours caused cell death in 10% of the cells, only slightly more than cells treated with the control siRNA (siNEG; Fig. 2B). Treatment of the siNEG-transfected cells with a sub-therapeutic concentration of TRAIL did not significantly enhance cell death compared with the untreated cells. However, cells in which WEE1 protein has been reduced by siRNA exhibited a 4-fold increase in the percentage of dead cells when these cells were treated with TRAIL, confirming that loss of WEE1 function sensitizes cells to TRAIL-induced cell death.

Cell death caused by WEE1 inhibition and TRAIL is caspase-dependent apoptosis
TRAIL acts through the extrinsic death pathway inducing caspase-mediated apoptosis (16). Previously, we showed that
WEE1 inhibition causes apoptotic cell death in breast cancer cells (12). To determine whether the treatment of WEE1-inhibited cells with TRAIL would cause an increase in apoptosis compared with either of the 2 independent drugs, we measured the fraction of cells with sub-G\(_1\) DNA content using propidium iodide (Fig. 3A) and the Annexin V–positive cells (Fig. 3B) by FACS analysis. The combination of WEE1 inhibitor and TRAIL resulted in a significantly increased percentage of cells with sub-G\(_1\) DNA content compared with each of the other treatments or untreated cells (\(P < 0.01\); Fig. 3A). WEE1 inhibition alone significantly increased the percentage of cells with sub-G\(_1\) DNA content (\(P < 0.005\)), suggesting that there is increased DNA damage in cells treated with WEE1 inhibitor as we have previously shown (ref. 12; Fig. 3A). TRAIL treatment alone resulted in a borderline increase in the percentage of cells with sub-G\(_1\) DNA content (\(P = 0.07\)). Also, the combination treatment increased the percentage of Annexin V–positive cells significantly compared with any of the single treatments or untreated cells (\(P < 0.0005\); Fig. 3B). Under these conditions, neither WEE1 inhibitor nor TRAIL alone resulted in a significant increase in Annexin V–positive cells.

Caspase activation is a key step in apoptosis induced by TRAIL receptors and WEE1 inhibition (12, 13). Cells treated with WEE1 inhibitor, TRAIL, or with both in the presence or absence of the pan-caspase inhibitor Z-VAD-FMK were stained with Trypan blue and counted for the number of dead cells. In the TNBC cell lines, MB231 (Fig. 4A) and HCC38 (Fig. 4B), WEE1 inhibition or TRAIL treatment alone resulted in approximately 10% to 15% dead cells whereas the combined treatment resulted in 40% to 50% dead cells. Pretreatment with Z-VAD-FMK completely abrogated the induction of cell death by WEE1 inhibition, TRAIL treatment, and the combination.
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Figure 2. Silencing of WEE1 by siRNA in the breast cancer cell line HCC38 sensitizes the cells to TRAIL-induced cell death. A, Western blot analyses show the knockdown of WEE1 expression and decrease in pY15CDC2 by synthetic siRNA (50 nmol/L) at 24 hours. Total CDC2 and ERK2 proteins are shown as loading controls. B, control siRNA (siNEG) or siWEE1-transfected cells were treated with or without TRAIL (2.5 ng/mL) 8 hours after transfection. Cell counts with Trypan blue were conducted in the siNEG or siWEE1-transfected cells in the absence (white bars) or the presence (black bars) of TRAIL for 17 hours. The values represent mean values ± SEM of 3 independent experiments. IB, immunoblotting.

Together, the results shown in Figs. 3 and 4 are consistent with the induction of caspase-dependent apoptosis by the combination of WEE1 inhibitor and TRAIL.

Caspase-8 is the initiator caspase

Caspases are activated by proteolytic cleavage from a precursor form (pro-caspases) to active cleaved products (27). To investigate the differential activation of caspases by WEE1 inhibitor, TRAIL, or the combination, we measured the disappearance of pro-caspase-8, -9, and -3 (Fig. 5A). WEE1 inhibition alone did not result in detectable activation of any of the caspases as measured by the loss of the precursors (indicated by the arrows). Treatment with TRAIL alone resulted in activation of caspase-8 and -3 but no appreciable activation of caspase-9 (as measured by the loss of the precursor). Also, there was appearance of the cleaved form of caspase-8 with TRAIL treatment (arrowhead in top panel of Fig. 5A). The combination of WEE1 inhibitor and TRAIL resulted in greater activation of caspase-8 and -3 and also activation of caspase-9 as measured by pro-caspase loss. Thus, there is more caspase-8 cleavage product in the cells treated with the combination. PARP-1 is cleaved by activated caspase-3 (27). We used loss of full-length PARP-1 (arrow) and the appearance of cleaved PARP-1 (arrowhead) as a measure of caspase activity (Fig. 5A, panel 4). WEE1 inhibition alone did not lead to measurable PARP-1 cleavage whereas TRAIL alone resulted in appearance of cleaved PARP-1 (Fig. 5A, panel 4, lanes 2 and 3). The combination of WEE1 inhibitor and TRAIL treatment resulted in significantly increased PARP-1 cleavage as measured by loss of the full-length PARP-1 and the appearance of more cleaved PARP-1 (Fig. 5A, panel 4, lane 4). Thus, the combination of WEE1 inhibitor and TRAIL results in greater activation of caspases.

We measured upstream (caspase-8) and downstream (caspase-3/7) caspase activity using Caspase-Glo assays to quantify the increase in caspase activity in MB231 cells (Fig. 5B). TRAIL treatment alone resulted in a 2- to 3-fold increase in both caspase-8 and caspase-3/7 activity whereas WEE1 inhibition alone had no significant effect (Fig. 5Bi and ii, respectively, white bars). The combination of WEE1 inhibitor and TRAIL resulted in an approximately 30% to 40% increase in both caspase-8 and caspase-3/7 activity compared with TRAIL alone and in an approximately 4-fold increase compared with either untreated cells or cells treated with WEE1 inhibitor alone [Fig. 5B (i) and (ii) respectively, white bars]. Activation of caspase-8 and caspase-3/7 was completely blocked by addition of the pan-caspase inhibitor Z-VAD-FMK [Fig. 5B (i) and (ii) respectively, striped bars]. Caspase-8 can be activated in a retrograde manner by caspase-3 (28).

To test whether the increased activity of caspase-8 seen upon treatment of cells with WEE1 inhibitor and TRAIL was downstream of caspase-3, we inhibited caspase-3 with DEVD-CHO (Fig. 5B, black bars). Caspase-3 activity was completely blocked by DEVD-CHO for all treatments [Fig. 5B (ii)]. In contrast, caspase-8 activity was only slightly decreased by the caspase-3 inhibitor [Fig. 5B (i), black bars]. Importantly, the increase in caspase-8 activity induced by the combination of WEE1 inhibition with TRAIL compared with TRAIL alone was maintained in the presence of DEVD-CHO [Fig. 5B (i)]. This suggests that the WEE1 inhibitor increases TRAIL-induced caspase-8 activation.

Caspases are activated by distinct stimuli and the first caspases activated are known as initiator caspases (24, 27). TRAIL binds to its agonistic receptors (TR1 and TR2) and activates caspase-8 and/or caspase-10. The caspase assays above (Fig. 5B) suggested that the combined treatment of WEE1 inhibitor and TRAIL initiates caspase activation via caspase-8. However, it is also possible that WEE1 inhibition activates a distinct initiator in addition to caspase-8 or caspase-10 and that this accounts for the increase in caspase-3/7 activity seen upon treatment with WEE1 inhibitor and TRAIL. To identify the initiator caspase involved in the dual treatment, each of the known initiator caspases (caspase-2, -8, -9, and -10) were silenced using specific siRNAs in MB231 cells and then the cells were treated with DMSO, WEE1 inhibitor, or TRAIL or the combination (Fig. 6). Cells were stained with Trypan blue, and the dead cells were counted for each treatment and compared with those of the nontargeting siRNA (siNEG). In siNEG-transfected cells, the combination of WEE1 inhibitor and TRAIL resulted in a significantly
higher percentage of dead cells than either treatment alone or untreated cells [Fig. 6A (i) and (ii), white bars], similar to those previously shown in Fig. 4A (black bars). WEE1 inhibitor and TRAIL alone resulted in a small increase in the percentage of dead cells compared with untreated controls [Fig. 6A (i) and (ii), black bars]. Knockdown of caspase-8 resulted in a significant decrease in the percentage of dead cells when cells were treated with TRAIL or with the combination of WEE1 inhibitor and TRAIL [Fig. 6A (i) and (ii), black bars]. However, knockdown of caspase-2, -9, or -10 did not result in the inhibition of cell death in the presence of WEE1 inhibitor, TRAIL, or the combination [Fig. 6A (i) and (ii)]. The knockdown of each caspase was confirmed by immunoblotting (Fig. 6B). These studies suggest that caspase-8 is the key initiator caspase in cell death caused by TRAIL in cells sensitized by WEE1 inhibition.

The decrease in pro-caspase-9 (shown above in Fig. 5A) in cells treated with WEE1 inhibitor and TRAIL suggests the involvement of the intrinsic apoptosis pathway. The intrinsic pathway could be activated by caspase-8 or the WEE1 inhibitor could be independently activating the intrinsic pathway. To determine whether the activation of the intrinsic pathway is dependent on initiator caspase-8 or is independent of it, loss of precursor caspase-9 was monitored in cells in which caspase-8 was knocked down (Fig. 6C). The combined treatment with WEE1 inhibitor and TRAIL resulted in the decrease of both pro-caspase-8 and -9 in the siNEG-transfected cells (Fig. 6C, lane 4). Strikingly, in the absence of caspase-8, there was no evidence for a decrease in pro-caspase-9 (Fig. 6C, lane 8). This indicates that in cells treated with WEE1 inhibitor and TRAIL, the intrinsic pathway of apoptosis is triggered by the activation of the initiator caspase-8.

Altogether, these results indicate that WEE1 treatment enhances TRAIL-mediated apoptosis through increased activation of the initiator caspase, caspase-8.
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The expression of caspase-8, -9, or -3 did not change upon WEE1 inhibitor treatment (Fig. 5A). Similarly, expression of FLIP, BCL-xL, survivin, BCL-2, and MCL-1 proteins was measured, but no changes in levels of any of these proteins were observed (Fig. 7A). We measured the transcripts of the death receptors after WEE1 inhibitor treatment by real-time PCR. A significant increase in the mRNA levels of TR1 (4-fold) and TR2 (8-fold) at 24 hours was observed, compared with the DMSO controls (Fig. 7B). No significant change in the transcript levels was seen at 8 hours of WEE1 inhibition. In addition to the transcripts, surface expression of the death receptors was assessed by flow cytometry. A 2-fold increase in the TR2 surface expression ($P < 0.05$) and a 1.5-fold change in TR1 ($P = 0.7$) surface expression was observed at 24 hours of WEE1 inhibition (Fig. 7C). Thus, WEE1 inhibition increases TRAIL receptor surface expression, and this increase is likely to account for the increased activation of caspase-8 upon the treatment of cells with the combination of WEE1 inhibitor and TRAIL.

**Discussion**

TNBC is an aggressive subtype of breast cancer which does not benefit from hormone receptor or anti-HER-2–targeted therapies. Patients with TNBC have a poor prognosis relative to those patients with other breast cancer subtypes (1). TRAIL and its agonists have been shown to rapidly induce apoptosis in TNBC cell lines with a mesenchymal phenotype (designated basal B breast cancer cells; refs. 21, 23). We have reported that the inhibition of WEE1, a tyrosine kinase involved in cell-cycle regulation, resulted in caspase-dependent apoptotic cell death in breast cancer cell lines of all subtypes but not in the nontransformed immortalized MCF10A (12). In contrast to the rapid death induced by TRAIL, the loss of WEE1 function induces cell death after 72 hours (12). In this study, we show that pretreatment of breast cancer cells with a WEE1 inhibitor for 4 hours enhanced TRAIL-mediated cell death by 24 hours and that the effects were most striking in basal B breast cancer cells (Fig. 1). WEE1 inhibitor alone caused little observable death at this early time point. WEE1 inhibitor increased cell death caused by TRAIL in sensitive cell lines but did not affect the TRAIL-resistant cells unlike other drugs such as histone deacetylase (HDAC) inhibitors that sensitized even resistant cells to TRAIL (29). In other studies of WEE1 inhibition, it has been reported that MK-1775, a WEE1 inhibitor, enhances the antitumor efficacy of various DNA-damaging agents such as 5-fluorouracil, doxorubicin, camptothecin, and pemtrexed in colon carcinoma cells and gemcitabine in pancreatic cancer (30–32). To our knowledge, our study is the first to show the effects of using the WEE1 inhibitor along with TRAIL in cancer cells.

In this study, treatment with the combination of WEE1 inhibitor and TRAIL resulted in a significant increase in the cleavage of multiple caspases involved in the death receptor pathway. The combination augmented the activation of 2 caspases considered initiators: caspase-8 (of the extrinsic death pathway) and caspase-9 (of the intrinsic death pathway).
Addition of a pan-caspase inhibitor abrogated the cell death caused by the WEE1 inhibitor and TRAIL, showing that the synergism is due to an increase in caspase activation (Fig. 5B). RNAi-mediated silencing of initiator caspases (caspase-2, -8, -9, and -10) showed that only caspase-8 was required for the cell death seen by the combination of WEE1 inhibition and TRAIL (Fig. 6). Our data suggest that the mechanism by which the WEE1 inhibitor sensitizes cells to TRAIL-induced apoptosis is by increasing caspase-8 activation upon the combined treatment. Whether there are other mechanisms that contribute to the enhanced activation of caspase-8 by TRAIL in the presence of WEE1 inhibitor is not known.

Sensitization to TRAIL by chemotherapeutic drugs has been attributed to multiple molecular mechanisms including the upregulation of TRAIL receptors (33), activation of the mitochondrial pathway (34), or enhanced caspase-8 recruitment to the DISC (35). DNA-damaging agents such as doxorubicin, etoposide, and radiation induce TR2 gene expression via a p53-dependent mechanism (22, 36, 37). Also, HDAC inhibitors were shown to increase the mRNA and protein levels of TR2 and proapoptotic BCL-2 family members. This increase correlated with an increase in caspase activity and in apoptosis thereby sensitizing TRAIL-resistant and TRAIL-sensitive breast cancer cells to TRAIL (29). In this study, we report an increase in transcripts and cell surface expression of TR1 and TR2 levels upon WEE1 inhibitor (Fig. 7). We have shown previously that TR2 activation is important in inducing TRAIL-mediated apoptosis in TNBC cell lines such as the MB231 cells (23). Also, Zhang and Zhang reported that loss of cellular surface expression of TR1 and TR2 accounts for resistance to TRAIL and its agonists (38). The increase in TRAIL receptors in cells treated with WEE1 inhibitor is p53-independent as MB231 cells are p53-deficient (6). Sheikh and colleagues have reported p53-independent upregulation of TR2 by genotoxic stress induced by methyl methanesulfonate (39). A number of studies have found that treatments altering the level or activity of antiapoptotic proteins such as BCL-2, BCL-xL, FLIP, NF-kB, or survivin can alter the sensitivity of cells to TRAIL (40–45). However, we did not observe any change in their levels upon WEE1 inhibitor treatment alone or with TRAIL.
WEE1 Inhibition and TRAIL Toxicity in Breast Cancer Cells

In our previous work, we have shown that the WEE1 inhibitor induces a DNA damage signal as measured by increased amounts of the phosphorylated form of histone H2AX (γH2AX) that is observed as early as 4 hours after treatment with WEE1 inhibitor (12). The γH2AX induced by WEE1 inhibition is not blocked by caspase inhibition (12). Also, TRAIL treatment induces the formation of γH2AX. However, this is blocked by caspase inhibitors (12). Thus, the mechanism leading to the DNA damage signal by WEE1 inhibition is independent of caspase activation, whereas the induction of γH2AX by TRAIL is downstream of and dependent on caspase activation (12). Earlier studies have linked DNA damage to TRAIL-induced apoptosis. Silencing of CHK1 or CHK2 in human prostate cancer cells enhances sensitivity to the combination treatment of CPT-11 or aphidicolin and Apo2L/TRAIL (46). This occurs by abrogating the proteolysis of CDC25A (a phosphatase) and thereby enhancing the dephosphorylation of pTyr15 on CDC2 which induces an S-phase arrest. This is similar to our model wherein silencing or inhibition of the tyrosine kinase WEE1 results in decreased pTyr15 on CDC2 (Fig. 2) and in the arrest of cells in S-phase (shown earlier in ref. 12). Hence, the enhancement of CDC25A phosphatase activity or the inhibition of WEE1 would be expected to phenocopy one another. Both result in S-phase arrest and both sensitize cells to TRAIL-mediated apoptosis.

Another mode of sensitization of cells to TRAIL by DNA-damaging agents is attributed to both the activation and inhibition of ATM kinase. Upon DNA damage, activated ATM/ATR trigger the activation of their downstream targets CHK1/CHK2 which, in turn, phosphorylate and activate WEE1 kinase. This leads to G2-M arrest. ATM kinase activation in response to DNA damage triggers a series of events that leads to the proteolytic degradation of cFLIP, an antiapoptotic protein, and sensitization of hepatic carcinoma cells to TRAIL (47). In another model, inhibition of ATM by pharmacologic inhibitors or RNAi followed by radiation and TRAIL treatment increased the sensitivity of melanoma cells to TRAIL because of the upregulation of surface TR2 levels and downregulation of FLIP levels (48). Inhibition of WEE1 in our study would be expected to work in a mechanism similar to ATM inhibition. Also, in our analysis we found upregulation of TRAIL receptors, but we did not observe a downregulation of FLIP.

Our study and other published data suggest that WEE1 inhibition has selective toxicity in cancer cells compared with normal cells (12, 49). This is due, likely in part, to the fact that cancer cells are deficient in the G1 checkpoint and therefore rely more on the G2-M checkpoint regulated by WEE1. Our study and other published data suggest that WEE1 inhibition has selective toxicity in cancer cells compared with normal cells (12, 49). This is due, likely in part, to the fact that cancer cells are deficient in the G1 checkpoint and therefore rely more on the G2-M checkpoint regulated by WEE1. This is an added advantage in combining TRAIL with the targeted inhibition of WEE1 as both the drugs show a relative specificity for killing cancer cells. WEE1 inhibitors and the TRAIL agonists are presently being tested in phase I and II clinical trials (25, 50). Therefore, our data suggest that the combination of WEE1 inhibition with TRAIL may be a promising therapy for...
TNBC/basal breast cancer in the clinic and well worth further exploration.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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WEE1 Inhibition Sensitizes Basal Breast Cancer Cells to TRAIL-Induced Apoptosis

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