WEE1 inhibition sensitizes basal breast cancer cells to TRAIL-induced apoptosis

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Abstract

Tumor Necrosis Factor (TNF)-Related Apoptosis Inducing Ligand (TRAIL) is a member of the TNF super family 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 based on 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 while 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. Pre-treatment with WEE1 inhibitor or knockdown of WEE1 increased the toxicity of TRAIL in the basal/triple-negative breast cancer cell lines compared to 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 since 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.
Introduction

Breast cancer is a heterogeneous group of diseases. Approximately 60-70% of breast cancers express estrogen receptors (ER) and/or progesterone receptors (PR), and approximately 20-30% of breast cancers have amplified HER-2 and thus express high levels of the HER-2 protein (1, 2). In approximately 15-20% 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 demonstrated 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 RNAi screen of the human tyrosine kinome 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 a.k.a. DR4) or TRAIL-R2 (TR2 a.k.a. 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) (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 xenografts without affecting normal tissues (14). Human Phase I studies have demonstrated 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 demonstrated that most TNBC/basal cell lines and a few of the HER2 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 (ATCC); BT20 and HCC1937 were obtained from Reinhard Ebner (Avalon Pharmaceuticals). ATCC cell lines are authenticated by STR analysis. On receiving the cell lines, they were immediately cultured and expanded to prepare frozen ampule stocks. Cells were passaged for no more than 2 to 3 months before establishing new cultures from the early passage frozen ampules. MB231 cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), (R5); all other cells were grown in RPMI 1640 medium supplemented with 10% FBS (R10). All growth media contained 100 units/ml of penicillin and 100 units/ml of streptomycin.

Inhibitors

WEE1 inhibitor II (6-Butyl-4-(2-chlorophenyl)-9-hydroxypyrrolo[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 DMSO, and used at 10 μM or 100 μM concentrations, as described in the text. The 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 Cell Titer 96AQueous One Solution Cell Proliferation Assay (Promega Corporation, G3582) as previously described (23). All MTS measurements were done in replicates of six wells, and each experiment was carried out at least three times. Results are given as the mean ± the standard error of the mean (SE) of at least three 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-TARGETplus SMARTpool siRNA (siWEE1, L-005050-00) and a non-targeting siRNA control pool (siNEG, D-001810-10) were purchased from Dhharmacon. HCC38 cells were transfected in solution with the siRNA pool at a final concentration of 50 nM using Oligofectamine (Invitrogen, 12252011) (7 µl/ml). Transfected cells were plated in 6-well plates (2x10^5 cells/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 h using Trypan blue stain. Results shown represent mean +/- SE of three experiments. Other cells in the 6-well plate were used for protein analysis.
Sub-G1 analysis

Cells (1x10^6) were plated overnight, treated with 10 μM WEE1 inhibitor for 4 h followed with 100 ng/ml TRAIL for 17 h. Then the cells were incubated with 10 μM BrdU (Sigma-Aldrich, 5002) for 15 min, trypsinized, washed with PBS, and fixed in cold 70% ethanol. Staining was performed with anti-BrdU-FITC antibody (BD Biosciences, 347583) and 5 μg/ml propidium iodide (PI) (Invitrogen, P3566). Flow cytometry was performed on a BD FACSCalibur (BD Biosciences) and analyzed using FLOWJO software.

Annexin V staining

After treatment with 10 μM WEE1 inhibitor for 4 h or TRAIL (100ng/ml) for 17 h 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 performed on a BD FACSCalibur and analyzed using FLOWJO software.

Caspase-Glo 3/7 assay

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

Flow cytometry for TR1 and TR2
MB231 cells treated with DMSO or WEE1 inhibitor (10 μM) for 24 h were trypsinized into sorter medium (PBS with 1% FCS and 0.5 mM EDTA and 0.02 mM sodium azide). All antibodies used for 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 min on ice. TR1 and TR2 surface expression was measured by flow cytometry performed on a BD FACSCalibur and analyzed using FLOWJO software.

Lysate preparation and immunoblotting

Cell lysates were made, and immunoblotting was performed as described earlier (23). The following antibodies were used: caspase-8 (Clone 1C12, 9746), pY15CDC2 (4539), CDC2 (9112), Caspase-9 (9502), Caspase-3 (9662), BCL2 (2872), BCL-xL (2762) and GAPDH (2118) from Cell Signaling Technology; Caspase-10 (M059-3) from MBL; WEE1 (5285), MCL1 (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 performed using the paired Student’s 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 while non-transformed cell lines are not affected (12). This cell death was observed only after more than two days of WEE1 loss or inhibition. TNBC cells with a mesenchymal phenotype (so called basal B) are more sensitive to TRAIL than the other sub-sets of breast cancer cells and this cell death can be observed within less than 24 h 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 pre-incubated with or without WEE1 inhibitor II (26) for 4 h, sub-IC50 doses of TRAIL were then added, and the viability was assessed 17 h 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 20-30% decrease in viability while treatment of cells with sub-IC50 doses of TRAIL alone caused approximately 5-30% loss in viability. However, the pre-treatment of cells with the WEE1 inhibitor followed by TRAIL treatment resulted in enhanced cell death (60-90%) compared to the untreated controls (Fig 1A). Also the basal A/TNBC HCC1937 cells and the HER2 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. In order to assess if higher doses of TRAIL would enhance cell death in the MCF7 cell line, we treated MB231 or MCF7 cells with 10 μM WEE1 inhibitor for 4 h and then with different doses of TRAIL for 17 h (Fig 1B). In MB231 cells, pre-treatment with WEE1 inhibitor significantly reduced the IC50 of TRAIL (from >1000 ng/ml to ~8 ng/ml) while 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.

While 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 h followed by treatment of cells with TRAIL for 17 h 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 h 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 to 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 demonstrated that WEE1 inhibition causes apoptotic cell death in breast cancer cells (12). In order to determine whether the treatment of WEE1 inhibited cells with TRAIL would cause an increase in apoptosis compared to either of the two independent drugs, we measured the fraction of cells with sub-G1 DNA content using propidium iodide (PI) (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-G1 DNA content compared to each of the other treatments or untreated cells (p<0.01) (Fig 3A). WEE1 inhibition alone significantly increased the percentage of cells with sub-G1 DNA content (p<0.005) suggesting that there is increased DNA damage in cells treated with WEE1 inhibitor as we have previously shown (12) (Fig 3A). TRAIL treatment alone resulted in a borderline increase in the percentage of cells with sub-G1 DNA content (p=0.07). Also, the combination treatment increased the percentage of Annexin V-positive cells significantly compared to 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 either 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 ~10-15% dead cells whereas the combined treatment resulted in 40-50% dead cells. Pre-treatment with Z-VAD-FMK completely abrogated the induction of cell death by WEE1 inhibition, TRAIL treatment, and the combination.

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. Also there is more caspase-8 cleavage product in the cells treated with
the combination. Poly (ADP-ribose) polymerase (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 four). WEE1 inhibition alone did not lead to measurable PARP-1 cleavage while 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 utilizing 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 while 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 30-40% increase in both caspase-8 and caspase-3/7 activity compared to TRAIL alone and in an approximately four-fold increase compared to either untreated cells or cells treated with WEE1 inhibitor alone (Fig 5Bi and 5Bii 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 5Bi and 5Bii 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 5Bii). In contrast, caspase-8 activity was only slightly decreased by the caspase-3 inhibitor (Fig 5Bi black bars). Importantly, the increase in caspase-8 activity induced by the combination of WEE1 inhibition with TRAIL compared to TRAIL alone was maintained in the presence of DEVD-CHO (Fig 5Bi). 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. In order 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 to those of the non-targeting siRNA (siNEG). In siNEG-transfected cells the combination of WEE1 inhibitor and TRAIL resulted in a significantly higher percentage of dead cells compared to either treatment alone or untreated cells (Fig 6Ai and 6Aii, 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 to untreated controls (Fig 6Ai and Aii white 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 6Ai and Aii 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 6Ai and Aii). 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. In order to determine if 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.

*WEE1 inhibition results in increased levels of the agonistic TRAIL receptors*
The expression of pro-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 (four-fold) and TR2 (eight-fold) at 24 h was observed, compared to the DMSO controls (Fig 7B). No significant change in the transcript levels was seen at 8 h of WEE1 inhibition. In addition to the transcripts, surface expression of the death receptors was assessed by flow cytometry. A two-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 h 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 (so called basal B breast cancer cells) (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 non-transformed 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 pre-treatment of breast cancer cells with a WEE1 inhibitor for 4 h enhanced TRAIL-mediated cell death by 24 h 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 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 anti-tumor efficacy of various DNA damaging agents such as 5-FU, doxorubicin, camptothecin and pemetrexed 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 two caspases considered initiators: caspase-8 (of the extrinsic death pathway) and caspase-9 (of the intrinsic death pathway) (Fig 5A). Addition of a pan-caspase inhibitor abrogated the cell death caused by the WEE1 inhibitor and TRAIL, demonstrating 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) demonstrated 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 like doxorubicin, etopside, and radiation induce TR2 gene expression via a p53-depandent mechanism (22, 36, 37). Also, HDAC inhibitors were shown to increase the mRNA and protein levels of TR2 and pro-apoptotic 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 inhibition (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 et al. 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 anti-apoptotic proteins such as BCL-2, BCL-XL, FLIP, NFκB, 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.

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 h 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 while 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 their 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 (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 anti-apoptotic protein, and sensitization of hepatic carcinoma cells to TRAIL (47). In another model, inhibition of ATM by pharmacological inhibitors or RNAi followed by radiation and TRAIL treatment increased the sensitivity of melanoma cells to TRAIL due to 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 also, 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 to 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 (10). This is an added advantage in combining TRAIL with the targeted inhibition of WEE1 as both the drugs demonstrate 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.

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Figure Legends

Figure 1: WEE1 inhibition sensitizes basal breast cancer cells to TRAIL. (A) A panel of breast cancer cell lines were treated with WEE1 inhibitor (10 μM) for 21 h, with TRAIL for 17 h, or pre-treated with WEE1 inhibitor for 4 h and then treated with TRAIL for 17 h. HCC38 cells were treated with 0.5 ng/ml TRAIL and MB157 cells were treated with 50 ng/ml TRAIL. All other cell lines were treated with 125 ng/ml of TRAIL. Cell viability was measured by MTS assay and is plotted as a percent change relative to the DMSO control. The combined treatment was compared to either treatment alone by a two tailed Student’s t-test (*: viability of combined treatment less than either treatment alone p<0.05; #: viability of combined treatment not significantly different from either treatment alone). (B) Sensitization of the triple-negative cell line MB231 and ER-positive cell line MCF7 by WEE1 inhibitor was measured at different doses of TRAIL by MTS assay. (C) WEE1 inhibition was assessed by immunoblotting for pY15CDC2. Total CDC2 and HSC70 are shown as loading controls. All MTS values represent the mean of three experiments ± SE.

Figure 2: Silencing of WEE1 by siRNA in the breast cancer cell line HCC38 sensitizes the cells to TRAIL-induced cell death. (A) Western blots demonstrate the knockdown of WEE1 expression and decrease in pY15CDC2 by synthetic siRNA (50 nM) at 24 h. 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) eight hours after transfection. Cell counts with Trypan blue were performed in the siNEG or siWEE1-transfected cells in the absence (white bars) or the presence (black bars) of
TRAIL for 17 h. The values represent mean values ± SE of three independent experiments.

Figure 3: WEE1 inhibition followed by TRAIL treatment results in apoptotic cell death in MB231 cells. (A) The percentage of cells with sub-G1 DNA content was measured by PI staining after 21 h of WEE1 (10 μM) inhibition or 17 h of treatment with TRAIL (100 ng/ml) or pre-treatment with WEE1 inhibitor (10 μM) for 4 h followed by TRAIL treatment (100 ng/ml) for 17 h. The graph represents the mean values ± SE of three independent experiments. (B) The percentage of Annexin V-FITC positive cells seen following the treatment with either WEE1 inhibitor or TRAIL or the combination of both as described above. The graph represents the mean values ± SE of three independent experiments.

Figure 4: Cell death caused by WEE1 inhibitor and TRAIL treatment is caspase-dependent. (A) MB231 or (B) HCC38 cells were pre-treated with DMSO (black bars) or 100 μM Z-VAD-FMK (white bars) for 1 h followed by WEE1 inhibitor (10 μM) for 4 h and 100 ng/ml TRAIL for 17 h or both. Cells were then stained with Trypan blue and the number of dead cells was counted in each of the treatments. Combined treatment with WEE1 inhibitor and TRAIL resulted in a significant increase in the percentage of dead cells in MB231 and HCC38 compared to either treatment alone or to the DMSO treated control cells (p<0.0005). Pre-treatment with Z-VAD-FMK significantly blocked cell
death caused by the dual treatment in MB231 and HCC38 (comparison of combined treatment with or without Z-VAD-FMK, p<0.0005).

Figure 5: Activation of caspases by treatment with WEE1 inhibitor and TRAIL in MB231 cells. (A) Activation of caspases was monitored by Western Blot analysis. Lysates of MB231 cells obtained from the treatments (described above) were analyzed for the decrease in the precursor form (→) as seen in caspase-8, -9, -3 and PARP-1 or the appearance of the cleaved form (▼) as seen in caspase-8 and PARP-1. (B) Caspase-8, -3 and -7 activities were measured by Caspase-Glo Assay. The cells were pre-treated with either DMSO (□) or 0.3 μM of DEVD-CHO (■) or 100 μM of Z-VAD-FMK (□) for 1 h, followed by WEE1 inhibitor (10 μM) for 4 h and then with TRAIL (100ng/ml) for 2 h after which the caspase activity was measured. Data represents mean values ± SE of three independent experiments.

Figure 6: Caspase-8 is the initiator caspase of apoptotic cell death. (A) The initiator caspases-2, -8, -9, -10 were knocked down independently by synthetic siRNA (50 nM) along with the control siRNA (siNEG) in MB231 cells for 48 h. The cells were then treated with either 10 μM WEE1 inhibitor for 21 h or with 100 ng/ml TRAIL for 17 h or pre-treated with WEE1 inhibitor for 4 h followed by TRAIL for 17 h. Treated cells were stained with Trypan blue and the dead cells were counted. (B) The decrease in the expression of the four caspase protein levels by siRNA was measured by immunoblotting. GAPDH levels are shown as loading controls. (C) Activation of caspase-9 is dependent on caspase-8 activation. Cells transfected with either control
(siNEG) or caspase-8 (siCASP8) siRNA were treated with WEE1 inhibitor, TRAIL or the combination as described above. Activation of caspase-8 and caspase-9 were measured by disappearance of the precursor forms of the caspases. The pro-caspases are indicated by the arrow and the cleaved forms of caspase-8 are indicated by the arrow head.

Figure 7: Expression of death receptors and apoptotic proteins with the combined treatment of WEE1 inhibitor and TRAIL. (A) The levels of pro-/anti-apoptotic proteins were assayed by immunoblotting in MB231 cell lysates. The cells were either treated with WEE1 inhibitor (10 μM) for 21 h or with 100 ng/ml TRAIL for 17 h or pre-treated with of WEE1 inhibitor (10 μM) followed by 100 ng/ml of TRAIL for 17 h. (B) Transcript levels of TR1 and TR2 were measured by real-time PCR after 8 h (white bars) or 24 h (black bars) of WEE1 inhibitor (10 μM) treatment. The results represent the average of three experiments ± SE. The mRNA expression of WEE1 inhibitor treated cells was compared to the other samples as indicated. (C) Surface expression of TR1 and TR2 was measured after 24 h of WEE1 inhibition by flow cytometry. Surface levels of the receptors after WEE1 inhibition (black bars) were compared to the DMSO controls (white bars).
Figure 1

A

% Viability

\[ \text{MB231, HCC38, MB157, BT549, HS578T, BT20, HCC1937, BT474, SKBR3, MCF7, T47D} \]

Basal B | Basal A

Triple-Negative/Basal-like | HER2 amplified | ER+

DMSO | TRAIL | WEE1 inhibitor | WEE1 inhibitor and TRAIL

B

MB231 | MCF7

% Viability

\[ \text{TRAIL (ng/ml)} \]

DMSO | WEE1 inhibitor

C

WEE1 inhibitor | TRAIL

IB: pCDC2 | CDC2 | HSC70

MB231 | MCF7
Figure 2

A

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<th>siRNA</th>
<th>NEG</th>
<th>WEE1</th>
<th>NEG</th>
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<td>2.5ng/ml TRAIL</td>
<td>-</td>
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</tbody>
</table>

IB: WEE1
IB: pCDC2
IB: CDC2
IB: ERK2

B

![Bar chart showing % Dead cells for different conditions](chart.png)

- **Control**
- **2.5ng/ml TRAIL**

<table>
<thead>
<tr>
<th>siRNA (50nM)</th>
<th>si NEG</th>
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Figure 3

A

![Bar graph showing % Sub-G1 cells for DMSO, WEE1 inhibitor, TRAIL, and WEE1 inhibitor and TRAIL.]

B

![Bar graph showing % Annexin V-positive cells for DMSO, WEE1 inhibitor, TRAIL, and WEE1 inhibitor and TRAIL.]

Figure 4

A

MB231

% Dead cells

DMSO  WEE1 inhibitor  TRAIL  WEE1 inhibitor and TRAIL

no ZVAD  with ZVAD

B

HCC38

% Dead cells

DMSO  WEE1 inhibitor  TRAIL  WEE1 inhibitor and TRAIL

no ZVAD  with ZVAD
Figure 5

A

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<td></td>
<td></td>
<td></td>
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<tr>
<td>TRAIL</td>
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IB: CASP8
IB: CASP9
IB: CASP3
IB: PARP-1
IB: GAPDH

B (i)

Caspase 8

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<th>WEE1 inhibitor and TRAIL</th>
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B (ii)

Caspase 3/7

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<th>TRAIL</th>
<th>WEE1 inhibitor and TRAIL</th>
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</table>
Figure 6

A (i)

B

C

siNEG siCASP2 siCASP8 siNEG siCASP9 siCASP10

IB: CASP

IB: GAPDH

siNEG siCASP8

WEE1 inhibitor TRAIL

IB: CASP8

IB: CASP9

IB: GAPDH
Figure 7

A

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<th>MCL1</th>
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<tr>
<td>TRAIL</td>
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<td>-</td>
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</tbody>
</table>

B

**TRAIL-R1 (DR4) mRNA levels**

- Fold change in transcript levels at 8h:
  - DMSO: 1
  - WEE1 inhibitor: 5
- Fold change in transcript levels at 24h:
  - DMSO: 2
  - WEE1 inhibitor: 6

- p < 0.005

**TRAIL-R2 (DR5) mRNA levels**

- Fold change in transcript levels at 8h:
  - DMSO: 1
  - WEE1 inhibitor: 2
- Fold change in transcript levels at 24h:
  - DMSO: 2
  - WEE1 inhibitor: 12

- p < 0.05

Molecular Cancer Research

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