Perifosine Synergistically Enhances TRAIL-Induced Myeloma Cell Apoptosis via Up-Regulation of Death Receptors

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Abstract

Purpose: The aim of this study is to investigate the efficacy of a novel Akt inhibitor, perifosine, in combination with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in human myeloma cells and primary patient samples.

Experimental Design: The activity of perifosine in combination with TRAIL was evaluated with experiments testing the effect of perifosine on DR4/DR5 expression by the use of chimeric blocking antibodies, as well as siRNA.

Results: DR4 and DR5 expression was induced by exposure to single-agent perifosine. After exposure of human myeloma cell lines or primary patient samples to increasing doses of perifosine with exogenous TRAIL, we identified synergistically enhanced apoptosis when compared with the perifosine alone, which was achieved with levels well below clinically achievable concentrations for both agents. Transfection with siRNA against DR4, and DR5 reduced the level of apoptosis induced by the combination but did not result in total abrogation of the combination effect. Overexpression of activated Akt, the proposed target for perifosine, did not inhibit the combination effect. Anti-DR4 and DR5 chimeric proteins blocked the cytotoxicity induced by the combination, and the use of c-FLICE-like interleukin protein (FLIP) siRNA enhanced the efficacy at the combination, further supporting the importance of the DR4/DR5 axis in the effect of perifosine.

Conclusion: Our observation seems to be independent of the effects of perifosine on Akt signaling, and may represent an additional mechanism of action for this agent, and supports future clinical trials combining these two agents.

The phosphatidylinositol-3-OH (P13K) kinase/Akt axis represents an attractive target for cancer therapy as signaling through the phosphatidylinositol-3-OH kinase/Akt axis has profound effects on tumor cell proliferation, survival, differentiation, and apoptosis (1–8). There are several agents that directly inhibit phosphatidylinositol-3-OH kinase signaling (LY294002; refs. 9–11), or whose combination inhibits Akt activation (tipifarnib/bortezomib; refs. 12, 13) with subsequent suppression of this critical pathway, but few have been clinically tested. The alkylphospholipid perifosine is an oral Akt inhibitor that has been studied in a number of different tumor models and is well-known to down-regulate p-Akt expression and function (14), as well as the upstream regulator of Akt, PDK-1 (15, 16). Perifosine induces synergistic apoptosis with etoposide (17), temozolomide (18), UCN-1 (19), and histone deacetylase inhibitors (20) in cancer cells. The effect of perifosine on myeloma survival and signaling was initially reported by Hideshima et al. (14) where they confirmed the effect on Akt, and also showed an apparent compensatory increase in extracellular signal-regulated kinase (ERK) activation after in vitro exposure to perifosine. Additionally, constitutive activation of Akt via overexpression did not abrogate the proapoptotic effect of perifosine (14), leading others to explore additional mechanisms of action. Gajate and Mollinedo (21) recently showed that perifosine induces death receptors migration into lipid rafts, yet the consequence of this surface accumulation has not been well-defined. Signaling through death receptors of the tumor necrosis factor receptor superfamily have been well-elucidated, and death receptors are now being targeted via small molecules and monoclonal antibodies in cancer (22–26). We show that the up-regulation of DR4/DR5 receptors on the surface of malignant plasma cells (cell lines and primary human cells) in response to perifosine exposure sensitizes tumor cells to tumor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis, and that this effect is independent of the effects of perifosine on Akt inhibition, and represents an additional mechanism of action for this novel agent.

Materials and Methods

Reagents. Perifosine (NSC639966), a synthetic substitute heterocyclic alkyl phospholipid, was provided by Keryx Biopharmaceuticals.

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doi:10.1158/1078-0432.CCR-08-0016
This agent was dissolved in PBS and stored at -70°C. Generally, 100 mmol/L stock solution was diluted to the appropriate concentrations with 5% fetal bovine serum (Cellgro) containing growth medium immediately before use. Human TRAIL was purchased from PeproTech, Inc. and also from R&D systems. The DR4 and DR5 chimera blocking proteins were purchased from R&D systems.

**Cells.** MM.1S (Dexamethasone sensitive) and MM.1R (dexamethasone resistant) human multiple myeloma cell lines were kindly provided by Dr. Steven Rosen (Northwestern University Chicago, Illinois). RPMI8226 and U266 cells were purchased from American Type Tissue Culture. Cells were grown in RPMI 1640 containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL Ampicillin, B, and Penn/strep (Cellgro), and Plasminogen at 2.5 μg/mL (a Mycoplasma preventive from In Vivogen) at 37°C in a humidified CO2 chamber containing 95% air and 5% CO2.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay. Cytotoxicity assays were done as previously published (12). Typically, multiple myeloma cells (50,000) were seeded in the RPMI 1640 complete medium (96-well plates; Costar) containing 5% fetal bovine serum. To establish a dose response to perifosine or TRAIL or combination, cells were incubated for a minimum of 24 h up to 72 h in 200-μL volume. After continuous exposure, 20 μL 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT proliferation kit from American Type Tissue Culture) dye was added 4 h before the end of incubation. The insoluble formazan complex was solubilized with 100 μL of the detergent, followed by incubation for a minimum of 4 h, and the absorbance was measured at 570 nm (Molecular Devices).

**Translational Relevance**

The dramatic change in therapeutic options and clinical outcomes for patients with multiple myeloma has occurred as a direct result of well-defined preclinical studies. Perifosine, an AKT/PKB inhibitor, is an agent that is currently in several clinical trials in patients with relapsed myeloma. Our article identifies and highlights additional mechanisms of action of this agent, including the up-regulation of key death receptors. Preclinical identification of additional mechanisms allows clinicians to more accurately identify combination drug strategies for subsequent clinical trials. Given the improvement in preclinical understanding and, more importantly, clinical data with drug combinations, we propose that the combination of perifosine and TRAIL will induce synergistic plasma cell apoptosis in vitro and thereby provide a rationale to test this combination in humans with relapsed myeloma.

**Fig. 1.** A, MM.1S (■), MM.1R (▲), U266 (●), and RPMI8226 (▲), myeloma cell lines (1.5 × 10⁶ cells/mL) were treated with 2 to 10 μmol/L concentrations of perifosine for 48 h, and MTT assays were done. Points, mean from triplicate wells; bars, SD. B, DR4/DR5 expression by Western blot in a time-dependent manner after perifosine treatment in MM.1S. C, autoradiography films were scanned from B, and the area of DR4 and DR5 bands were quantified by the imager. The readings were plotted as intensity (area) versus duration of treatment. D, MM.1S cells were treated with 5 μmol/L perifosine for 24 and 48 h. Surface detection of DR4/DR5 receptors was done using flow-cytometry as described in Materials and Methods. Samples were triplicates and Student’s t test was done to determine the significance of the mean fluorescence intensity (P) between the untreated and treated samples for 24 and 48 h. Columns, mean of triplicate values; bars, SD.
Fig. 2. A, MM.1S cells were treated with escalating doses of TRAIL for 18 h. Cells were analyzed by flow cytometry after Annexin V staining to determine apoptosis. B, MM.1S cells treated with escalating doses of perifosine with or without TRAIL for 24 h were analyzed for growth inhibition by MTT assay. Open bars, cell death with increasing concentrations of perifosine by itself; solid bars, cell death in the presence of TRAIL (50 ng/mL) or TRAIL by itself. Columns, mean; bars, SD. C, MM.1S cells were treated with 5 μmol/L perifosine or TRAIL (50 ng/mL) or in combination for 18 h, and apoptotic cells were analyzed by flow cytometry. Error bars, SD at each time point. D, MM.1R cells were similarly treated as in B. E, the MTT data obtained by combining perifosine from 2 to 7 μmol/L with 50 ng/mL of TRAIL were used to generate a synergy graph. The X-axis represents the absorbance values obtained from the cell death assays (MTT assay), and the Y-axis represents the combination index. The numbers in the graph refers to the drug concentration. The combination index values lower than 1 indicates synergy. F, MM.1S cells were treated with 5 μmol/L perifosine or TRAIL (50 ng/mL) or in combination for 15 h, and the cells were analyzed by Western blotting using antibodies for caspases 3, 8, 9, and poly-ADP ribose polymerase. The cleaved bands indicate the active forms generated from the pro-caspases.
A. RPMI8226 myeloma cell lines were similarly treated as in Fig. 2B in the presence or absence of DR4 or DR5 blocking chimeras (100 ng/mL) containing perifosine, TRAIL, or the combination, and MTT assays were done after 24 h. Columns, mean; bars, SD. Similar results were noted with MM.1S and MM.1R cell lines (data not shown).

B. siRNA for DR4, DR5 (100 nm), or control siRNA were transfected; in MM.1S cells overnight, the cells were collected and treated with perifosine or TRAIL or in combination for 18 h. The cells were collected and stained with CD138-phycoerythrin and Annexin V-FITC – conjugated antibodies to assess apoptosis by flow cytometry. An aliquot was lysed and the efficiency of siRNA inhibition of DR4 and DR5 was analyzed by Western blotting using DR4 and DR5 specific antibodies.

C. FLIP siRNA (25 nm) and also with control nontarget siRNA (100 nmol/L) were transfected in U266 cells overnight. After transfection, the cells were collected and treated with perifosine or TRAIL or in combination or 18 h. The cells were collected and stained with CD138-phycoerythrin and annexing-FITC – conjugated antibodies for analysis by flow cytometry. The Annexin V – positive cells from the control sample was subtracted from each treatment group and the differences between perifosine alone, TRAIL alone, and in combination are presented (I); *, P value of 0.05 compared with control. An aliquot was lysed and the efficiency of FLIP siRNA inhibition was analyzed by Western blotting using FLIP-specific antibodies (II), and an aliquot was lysed after the treatment by Western blotting for caspase cleavage (III).
Flow cytometry for the surface detection of DR4 and DR5 death receptors. Cell surface expression of death receptors was analyzed by flow cytometry as described previously (21), using specific monoclonal antibodies conjugated to phycoerythrin, against the extracellular domains of human DR4 or DR5 receptors. Phycoerythrin-conjugated mouse IgG1 was used as an isotype control (eBioscience). Cells (4 × 10^6/mL) were treated for 24 or 48 h, and the cells were collected and stained in Annexin V binding buffer (BD Bioscience), with DR4 or DR5 or isotype controls (eBioscience) on ice for 30 min. The cells were washed twice with Annexin V binding buffer and resuspended in the same fluorescence intensity was calculated using the FlowJo software (Treestar).

Densitometry. Autoradiography films were scanned by Typhoon 9210 scanner (Amersham Biosciences) to quantitative DR4 and DR5 expression, and the images were quantified using Image Quant Densitometry. Western blot analysis. Multiple myeloma cells were harvested and lysed in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 5 mmol/L NaF, 2 mmol/L NaN3, 1 mmol/L phenylmethysulfonyl fluoride, 5 μg/mL leupeptine, and 5 μg/mL aprotinin. Whole-cell lysates were subjected to SDS-PAGE using precast gels (Bio-RAD Laboratories) and transferred to polyvinylidene difluoride membrane (Bio-RAD Laboratories). The antibodies used for immunoblotting included the following: Anti-DR4 and DR5 are from ProSci Incorporated; anti-(phos)-Akt, anti–caspase-8, anti–caspase-9, anti–caspase-3, anti-poly-ADP-ribose polymerase, and anti-FLIP from Cell Signaling Technology; and α-actin was from Sigma.

Table 1. Combination index

<table>
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<tr>
<th>Dose of Perifosine (μg/mL)</th>
<th>Dose of TRAIL (ng/mL)</th>
<th>Combination index</th>
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<tbody>
<tr>
<td>5.0</td>
<td>50.0</td>
<td>0.453</td>
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<tr>
<td>10.0</td>
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Results

DR4 and DR5 death receptors are up-regulated after perifosine exposure. Perifosine induces plasma cell growth inhibition across a number of different myeloma cell lines as reported by Hideshima et al. (14) with an IC50 of 5 to 10 μmol/L in these human cell lines (Fig. 1A). After exposure of MM.1S cells to perifosine, the expression of DR4 and DR5 death receptors was increased in a time-dependent (0-24 hours) fashion (5 μmol/L perifosine) as observed by Western blot analysis (Fig. 1B).
revealed that DR4 was steadily increasing from 2 hours, whereas DR5 was significantly increasing from 6 to 24 hours (Fig. 1C). These results were consistent between experiments. Also, treatment of MM.1S cells with 10 μmol/L perifosine showed similar levels of DR4 and DR5 expression (data not shown). Next, we determined if the increased protein levels of DR4 and DR5 as identified by Western blotting after perifosine exposure translated into greater surface expression of DR4 and DR5. Flow cytometry confirmed that surface expression of DR4 and DR5 increased after perifosine treatment (Fig. 1D). Kinetics of both protein and surface expression of DR4 and DR5 suggest that the effect on DR4 is more rapid and pronounced than what was observed for DR5. Perifosine induced a 2.1- and 2.7-fold increase in DR4 and DR5 expression, respectively, based on mean fluorescence intensity when compared with baseline. The observation of perifosine-induced DR4/DR5 on the surface as well as at the protein levels in MM.1S cells has been consistent between experiments. However, the functional consequence of this increased expression was unknown. This led us to combine perifosine with TRAIL and to further define the functional significance of this combination.

**TRAIL combined with perifosine induces synergistic apoptosis.**

We next evaluated escalating doses of TRAIL as a single agent or the combination of TRAIL with perifosine to test the consequences of perifosine induced DR4/DR5 expression. Although single-agent TRAIL induced only modest growth inhibition (Fig. 2A), the addition of low dose of TRAIL (50 ng/mL) to escalating doses of perifosine induced a significantly more growth inhibition than perifosine alone (Fig. 2B). However, sequential administration of TRAIL to perifosine did not effect the pro-apoptotic effect of the combination. The pro-apoptotic effect of the combination was confirmed using flow cytometry, and showed significant enhancement in apoptosis compared with either single agent alone (Fig. 2C). An additional multiple myeloma cell line, MM.1R showed similar levels of increased cytotoxicity when both agents were combined (Fig. 2D).

The interaction between the perifosine and TRAIL was analyzed using the Compusyn software program (2B) to determine whether this combination induces additive, synergistic, or antagonistic cytotoxicity. MM.1S cells were treated with increasing concentrations of perifosine from 1 to 7 μmol/L with 50 ng/mL of TRAIL. Growth inhibition was measured by MTT assay, and the absorbance values were used to determine the combination index as shown in Fig. 2E. The perifosine (2-7 μmol/L) concentrations in combination with 50 ng/mL of TRAIL showed a synergistic effect with combination index values of <1. These doses of perifosine (1-5 μmol/L) are clinically achievable as shown by phase I/II trials (29, 30).

To further evaluate the proapoptotic effects of combination therapy, we measured poly-ADP ribose polymerase and caspase cleavage with perifosine, TRAIL, or the combination of both agents by Western blot analysis (Fig. 2F). These data show that the combination induced more caspase 8, 9, and 3 cleavage as well as poly-ADP ribose polymerase activation than when either agent was used alone. Given the enhanced cleavage of both caspases 8 and 9, these data suggest that both the extrinsic and intrinsic apoptotic pathways are effectively initiated with the combination.

**Synergy of perifosine + TRAIL can be blocked at the cell surface.**

We than evaluated the contribution of the addition of TRAIL and its effect on apoptosis induction by using anti-DR4 or DR5 chimeric blocking soluble proteins. Administration of the blocking DR4 or DR5 proteins significantly inhibited the combination effect as measured by MTT assay (Fig. 3A). These data suggests, while perifosine has single agent activity, the addition of TRAIL significantly enhances apoptosis, which can be blocked by the addition of DR4 or DR5 soluble blocking proteins. To further explore our observation implicating the importance of DR4/DR5 death receptor signaling in perifosine-induced cell death, we investigated whether either DR4 or DR5 plays a more central role in the combination effect. MM.1S cells were transiently transfected with either DR4, DR5, or control siRNA, and treated with or without perifosine and TRAIL. In the presence of control siRNA, 80% apoptosis was observed when exposed to perifosine and TRAIL. When either DR4 or DR5 siRNA was added, there was only a modest reduction in apoptosis (73% and 61%, respectively), but when DR4 and DR5 siRNAs were combined, apoptosis was reduced to 48% similar to perifosine alone (Fig. 3B). This result suggests that both DR4 and DR5 signaling are equally important for the combination effect, and that inhibition of only one receptor likely allows the remaining unblocked receptor to compensate for the blocked pathway.

**FLIP siRNA enhanced TRAIL- and perifosine-induced apoptosis in U266 cells.**

Given the importance of FLIP as a negative regulator of DR4/DR5 intracellular signaling and Fas-associated death domain-mediated apoptosis, we tested the effect of FLIP siRNA on apoptosis induction. FLIP siRNA was transfected in the U266 cell line, and apoptosis was evaluated after treatment with perifosine and TRAIL (Fig. 3C). Although the level of apoptosis seen with perifosine and TRAIL was less than we have seen in other cell lines, the addition of FLIP siRNA significantly enhanced sensitivity either to perifosine alone or to the combination, suggesting that in this relatively insensitive myeloma cell line, the augmentation of extrinsic pathway-induced apoptosis had a major effect on cell survival. The enhanced sensitivity of FLIP siRNA treatment has been shown by caspase cleavage (Fig. 3C, II and III).

**Perifosine induces apoptosis in primary myeloma cells.**

We then tested the combination of these agents in primary human myeloma cells. When bone marrow cells obtained from a myeloma patient were exposed to 5 μmol/L perifosine, we showed the up-regulation of DR4 and DR5 receptors up to 6 h, similar to what was seen in cell lines (Fig. 4A). We then tested the proapoptotic effect of either single agent or combination therapy in primary human multiple myeloma cells. Data from the primary cells for two patients (Fig. 4B and C) also showed that the combination of perifosine (5 μmol/L) with TRAIL (50 ng/mL) induced more apoptosis of CD38+ and CD138+ cells than when either agent was used alone representing combination is at least beneficial. Combination index values (0.453) indicate that the perifosine combination is synergistic with TRAIL in primary cells (Table 1).

**siRNA inhibition of death receptors reduced the synergy more than siRNA inhibition of Akt.**

The combination of perifosine and TRAIL inhibited phos-Akt expression in 6 hours as shown
in Fig. 5A. Given the known effect of perifosine on Akt signaling, we evaluated if these two potential mechanisms of action (Akt inhibition and death receptor up-regulation) were independent of each other. Akt siRNA or DR4/DR5 siRNA were transfected separately in myeloma cell lines. Maximal apoptosis (75%) was achieved with the combination of perifosine+TRAIL in control siRNA–treated cells as described in the previous figures. However, there was a minimal effect of Akt siRNA on perifosine+TRAIL (64% Akt siRNA treated versus 75% control siRNA treated) as the combination effect is able to overcome the effects of Akt siRNA. However, perifosine alone (59% control siRNA treated versus 44% Akt siRNA treated) was significantly affected by Akt siRNA as that is the main mechanism by which single agent perifosine is effective. When the combination is exposed to DR4/DR5 siRNA the effects on apoptosis was more marked with a significant inhibition of perifosine+TRAIL-induced apoptosis (46% DR4/DR5 siRNA treated versus 46% Akt siRNA treated) as shown in Fig. 5B. This result indicates that death receptor signaling enhanced by the perifosine and TRAIL combination plays a more crucial role in the induction of apoptosis than Akt-mediated signaling.

Discussion

The use of novel agents directed at specific signaling pathways is an approach that is clearly advancing the care of patients with cancer. Agents such as imatinib, rituximab, trastuzumab, and bortezomib are all examples of targeted agents that are relatively specific in their mechanism of action, and have significant single agent activity. However, few agents have a single mechanism of action, and in diseases where there multiple aberrant signaling pathways, a truly “targeted” agent is unlikely to have significant efficacy. Even the prototypic “targeted agent,” Imatinib, has shown activity in diseases other than chronic myelogenous leukemia as a result of other non–BCR-ABL mechanisms (31–33).

Targeting the TRAIL/Death receptor complex has been a clinically challenging endeavor. The TRAIL receptor family in humans consists of two active receptors (DR4 and DR5) as well as two decoy receptors (DcR1 and DcR2) that do not result in triggering intracellular effects. Interaction of TRAIL either with DR4 or DR5 results in conformational changes that create the death-inducing signal complex, which then interacts with the Fas-associated death domain subsequently cleaving caspase 8 and activating the extrinsic pathway of apoptosis. Although most cytotoxic and novel agents induce their proapoptotic effect via activation of the intrinsic pathway, the extrinsic pathway has been largely unexplored in clinical practice. The initiation of both the intrinsic and extrinsic cascades as a net result of a rationally designed combination trials has the potential to overcome drug resistance, result in a higher response rate, and should be the goal of therapies that seek to circumvent known and potential mechanisms of resistance.

The activity of perifosine in myeloma was first described by Hideshima et al. (14, 34). Myeloma cell lines and primary human myeloma cells showed sensitivity with IC_{50} concentrations around 5 μmol/L. Although the in vitro activity of perifosine was shown to be associated with reduction in Akt activation, transfection with constitutively activated Akt did not seem to abrogate the cytotoxic effect of perifosine. This could be interpreted in two different ways. First, the effect of perifosine could be so potent that it is able to overcome even the effect of constitutively activated Akt, or alternatively, this inhibition of Akt may be one of many mechanisms that are at work when cells are exposed to perifosine. Our observation that the exposure of perifosine induces up-regulation of DR4/DR5 death receptors expression has been shown by our group, as well as others (21, 29). However, the functional effect of this up-regulation is unknown. Our data shows that this increased DR4/DR5 expression does have significant functional consequences in myeloma. Cotreatment with TRAIL, the natural ligand for the DR4/DR5 receptors, significantly enhances the in vitro efficacy of perifosine, and does so with lower doses of both agents. This functional effect of the combination was confirmed by extracellular blocking studies (Fig. 3A), siRNA directed at DR4 and DR5 (Fig. 3B), and was able to induce cell death in a TRAIL-resistant cell line with the use of FLIP siRNA (Fig. 3C). The siRNA experiments directed at DR4/DR5 inhibition (Fig. 5B) were effective despite submaximal reduction in the of DR4/DR5 message, further supporting the critical role of the extrinsic pathway in the combination effect.

Clinical experience with perifosine to date has shown predominately stable disease or minor responses (35). Two phase I/II trials combining both lenalidomide and bortezomib with perifosine based on preclinical data suggested additive benefits (30, 36). Interestingly, the combination with bortezomib has two potential consequences. First, for a short period of time after exposure to bortezomib, multiple myeloma cell lines seem to up-regulate Akt activity, likely as a survival
mechanism. This transient dependence on Akt target may induce sensitive cells to the proapoptotic effect of perifosine. Additionally, our group has also shown that bortezomib enhances DR4/DR5 expression on the cell surface (29), providing preclinical rationale for a perifosine/bortezomib/TRAIL triplet.

Given the broad nature of activity for most novel agents, exploration of additional mechanisms of action is an important part of modern drug discovery. If we are to maximally capitalize on the utility of these agents, a mechanistic understanding and translational application of this knowledge is critically important. The observation that up-regulation of DR4/DR5 after exposure of multiple myeloma cells to perifosine has real clinical implications as outlined by our data, and combination phase I studies are planned to further test this laboratory observation to improve the outcome for patients with myeloma.

Disclosure of Potential Conflicts of Interest

J.L. Kaufman has received honoraria from Millenium, Celgene, and Genentech. R. Sinha has received honoraria from Genentech.
References


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*Clin Cancer Res* 2008;14:5090-5098.

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