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RAD001 (everolimus) enhances TRAIL cytotoxicity in human leukemic Jurkat T cells by upregulating DR5

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), either alone or in combination with other anti-cancer agents, is a promising new strategy for the treatment of cancer. However, aberrant PI3K/Akt/mTOR survival signaling may confer TRAIL resistance by altering the balance between pro- and anti-apoptotic proteins. In the present study, we showed that the Akt/mTOR inhibitor RAD001 (everolimus) induced cell death in a dose-dependent manner and enhanced TRAIL-induced apoptosis in human leukemic Jurkat T cells, which show PI3K/Akt/mTOR pathway activation and basal expression levels of death receptor (DR) 5 (TRAIL-R2). Investigation of the effect of RAD001 treatment on the expression of TRAIL receptors (TRAIL-Rs) in Jurkat T cells showed that RAD001 significantly upregulated DR5 by up to 51.22%, but not other TRAIL-Rs such as DR4 (TRAIL-R1), decoy receptor (DcR) 1 (TRAIL-R3), and DcR2 (TRAIL-R4). Pretreatment with DR5:Fc chimera abrogated the RAD001-induced increase of TRAIL cytotoxicity, indicating that the upregulation of DR5 by RAD001 plays a role in enhancing the susceptibility of Jurkat T cells to TRAIL. Our results indicate that combination treatment with RAD001 and TRAIL may be a novel therapeutic strategy in leukemia.

1. Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), also known as Apo-2 ligand, is a member of the TNF family and selectively induces apoptosis in tumor cells [1,2]. TRAIL interacts with two types of receptors, apoptosis-inducing death receptors (DRs) 4 (TRAIL-R1) and 5 (TRAIL-R2), and the non-apoptosis-inducing decoy receptors (DcRs) 1 (TRAIL-R3) and 2 (TRAIL-R4). DR4 and DR5 share highly homologous cysteine-rich extracellular domains and intracellular domains that include a death domain (DD). The extracellular domains of DcRs are similar to those of DRs, but DcR1 lacks a cytoplasmic DD, and DcR2 has a truncated DD [3,4]. Binding of TRAIL to DRs induces receptor trimerization and a conformational change in the intracellular DD resulting in the recruitment of the Fas-associated death domain (FADD) and pro-caspase-8 and -10 to the death-inducing signaling complex. The recruited caspases are self-activated and, in turn, activate downstream effector caspases, such as caspase-3 and caspase-9, which transmit signals leading to apoptosis. By contrast, when TRAIL binds to DcRs, FADD cannot be recruited and apoptosis is not triggered [5,6]. TRAIL has been considered as a potential therapeutic agent in cancer treatment because of its proapoptotic activity in cancer cells and minimal cytotoxicity to normal cells.

RAD001 (everolimus) is an orally active rapamycin derivative that is clinically approved for immunosuppression following organ transplantation [7]. RAD001 is under investigation as an anti-cancer agent because it inhibits growth-promoting signaling downstream of mammalian target of rapamycin (mTOR) [8]. Previous reports showed that RAD001 induces senescence and apoptosis in T-cell lymphomas [9] and caspase-independent cell death in childhood acute lymphoblastic leukemia (ALL) [10]. Moreover, RAD001 was shown to sensitize childhood ALL to vincristine therapy [11] and to act synergistically with chemotherapeutic agents, ionizing radiation, and proteasome inhibitors to induce cell death in pre-B ALL [12]. Collectively, these findings

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suggest that the combination of RAD001 with TRAIL, which is a promising protein because of its tumor-specific induction of apoptosis in a DR-dependent manner, is a potential novel strategy for the treatment of human leukemia.

In the present study, we evaluated the effects of combination treatment with RAD001 and TRAIL in lymphoblastic leukemia cells. Our results show that RAD001 enhances TRAIL susceptibility in Jurkat T cells by upregulating DR5 expression, suggesting that the combination of RAD001 and TRAIL could be a novel therapeutic strategy in leukemia.

2. Materials and methods

2.1. Reagents

RAD001 (everolimus) was purchased from InvivoGen (San Diego, CA). Recombinant human TRAIL was purchased from Life Technologies (Frederick, MD). Recombinant human DR5 (TRAIL-R2)/Fc chimera (DR5:Fc) protein and antibodies specific for DR4 (TRAIL-R1), DR5, DcR1 (TRAIL-R3), and DcR2 (TRAIL-R4) were purchased from R&D Systems (Minneapolis, MN). Hoechst 33258 was purchased from Thermo Fisher Scientific (Rockford, IL). Alamar Blue® was purchased from Gibco-Invitrogen (Rockville, MD). Antibodies specific for poly ADP/ribose-polymerase (PARP), caspase-8, caspase-9, caspase-3, phospho-Akt (Thr 308), phospho-Akt (Ser 473), and total Akt were obtained from Cell Signaling Technology (Danvers, MA). Antibodies specific for c-FLICE-like inhibitory protein $(FLIP)_L$, c-$FLIP_S$, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

The human lymphoblastic leukemia cell lines Reh, Daudi, Jurkat, Molt-4, and CCRF-CEM were grown in non-coated T75 culture flasks (Nalge Nunc, Naperville, IL) in RPMI 1640 (Gibco-Invitrogen). All cell lines were grown in media supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-Invitrogen), 100 U/ml penicillin (Gibco-Invitrogen), and 100 mg/ml of streptomycin (Gibco-Invitrogen) in a humidified 5% CO_2 atmosphere at 37 °C. The media were changed every 3 days.

2.3. Cell viability: Alamar Blue assay

Cells (5×10^4 cells/well) were seeded on 96-well plates (Nalge Nunc) in 90 μl of RPMI 1640 containing 1% FBS in the absence of phenol red. Cells were treated for 72 h with either DR5:Fc, RAD001, or TRAIL separately and in different combinations, as described in the individual experiments. Eleven microliters of 1 × Alamar Blue® was added at 3 h before the end of the incubation. Absorbance at 570 nm and 600 nm was measured with an ELISA Reader (Molecular Devices, Sunnyvale, CA).

2.4. Immunoblotting

Cells were washed with cold Dulbecco's phosphate-buffered saline (DPBS; Gibco-Invitrogen), and lysed in 300 μl of cold RIPA buffer (50 mM Tris–HCl, pH 7.5, containing 1% Triton X-100, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), and 1% sodium deoxycholate) with a protease inhibitor cocktail (Thermo Fisher Scientific). Cell lysates were centrifuged at 3000 × g for 15 min at 4 °C. The supernatant was harvested, and protein concentration was analyzed using a BCA protein assay kit (Thermo Fisher Scientific). For electrophoresis, 50 μg of protein was dissolved in sample buffer (60 mM Tris–HCl, pH 6.8, containing 14.4 mM β-mercaptoethanol, 25% glycerol, 2% SDS, and 0.1% bromophenol blue), boiled for 5 min, and separated on a 10% SDS reducing gel. Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Buckinghamshire, UK) using a trans-blot system (Gibco-Invitrogen). Blots were blocked for 1 h in Tris-buffered saline (TBS) (10 mM Tris–HCl, pH 7.5, with 150 mM NaCl) containing 5% non-fat dry milk (BD Biosciences, San Jose, CA) at room temperature, washed three times with TBS, and incubated at 4 °C overnight with primary antibodies (all antibodies, 1:1000 dilution) in TBST (10 mM Tris, pH 7.5, containing 150 mM NaCl and 0.01% Tween 20) containing 3% non-fat dry milk. The next day, blots were washed three times with TBST, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, 1:2000 or 1:5000 dilution) in TBST containing 3% non-fat dry milk at room temperature. After washing three times with TBST, protein was visualized with an ECL detection system (GE Healthcare).

2.5. Phase contrast and nuclear staining images

Cells were treated for 24 h with either DR5:Fc, RAD001, or TRAIL separately and in different combinations, as described in the individual experiments. For phase contrast imaging, cells were examined with a Nikon Eclipse TE2000-U inverted fluorescence microscope equipped with a Nikon LH-M100C-1 camera (Nikon Corporation Instruments Company, Japan). Images of each plate were captured with the digital camera. For imaging of nuclear staining, the cells were incubated with Hoechst 33258 at a final concentration of 5 μg/ml in HEPES buffer (Gibco-Invitrogen) for 20 min at 37 °C in a 5% CO_2 incubator. Cells were examined with a Nikon Eclipse TE2000-U inverted fluorescence microscope equipped with a Nikon LH-M100C-1 camera. Images of each plate were captured with the digital camera.

2.6. Flow cytometry

For measurement of TRAIL receptor expression, a total of 10^6 cells were resuspended in 0.2 ml of DPBS and incubated with phycoerythrin (PE)-conjugated antibodies for 30 min at room temperature. PE-conjugated human IgGs (R&D systems) were used as isotype controls at the same concentration as the specific antibodies. The fluorescence intensity of the cells was evaluated by flow cytometry (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ), and data were analyzed using CELLQUEST software (Becton Dickinson).

2.7. Statistical analysis

Data were presented as the mean ± standard deviation (SD). Statistical significance was determined using Student's *t*-test. Results were considered significant when $P < 0.05$.

3. Results

3.1. RAD001 induces cell death in a dose-dependent manner in Jurkat and CCRF-CEM T cells

The Akt pathway was strongly activated in human leukemic Jurkat and CCRF-CEM cells, whereas its activity in the other cells (Reh, Daudi, and Molt-4) was relatively low (Fig. 1A). Cells were treated with RAD0001 (everolimus), an inhibitor of Akt/mTOR, at various doses for 72 h, which induced cell death in a dose-dependent manner in Jurkat and CCRF-CEM T cells (Fig. 1B and Fig. S1A). RAD0001 did not have a significant effect on inducing cell death in Reh, Daudi, and Molt-4 cells (Fig. S2).

3.2. Pretreatment with RAD001 enhances TRAIL-induced apoptosis in Jurkat T cells

Treatment with TRAIL induced cell death in a dose-dependent manner in Jurkat cells, which express DR5 at basal levels (Fig. 1C), but not in CCRF-CEM, Daudi, and Molt-4 cells, which do not express TRAIL-Rs (Figs. S1 and S2). To explore a possible increase in TRAIL susceptibility in lymphoblastic leukemia cells, the effect of pretreatment with RAD001 on TRAIL-induced cell death was examined. As shown in Fig. 2A, treatment with 750 or 1000 nM RAD001 increased TRAIL-induced apoptosis by almost 100%. Consistent with these data, combination treatment with RAD001 and TRAIL significantly induced the activation of caspase-8, -9, and -3 (Fig. 2B) and apoptotic cell death, as evidenced by nuclear condensation and fragmentation (Fig. 2C). In CCRF-CEM cells, which are unresponsive to TRAIL, no additive effect was observed by pretreatment with RAD001 (Fig. S1). In Reh, Daudi, and Molt-4 cells, which are unresponsive to RAD001, concurrent treatment with RAD001 and TRAIL did not induce significant cell death (Fig. S2).

3.3. RAD001 enhances TRAIL susceptibility in Jurkat T cells by upregulating DR5 expression

To elucidate the mechanism underlying the enhancement of TRAIL-induced apoptosis by RAD001, the expression levels of TRAIL-Rs were examined in RAD001-treated Jurkat cells by flow cytometry. As shown in Fig. 3, RAD001 significantly upregulated DR5 expression by up to 51.22% in Jurkat T cells, whereas it did not affect the expression of the other TRAIL receptors. TRAIL-induced cell death was significantly blocked upon treatment with the DR5:Fc protein, a TRAIL-specific antagonist (Fig. 4A). The recovery of TRAIL-treated cells from apoptotic cell death, accompanied by nuclear condensation and fragmentation, by pretreatment with DR5:Fc protein (Fig. 4) indicated that RAD001 enhances TRAIL susceptibility in Jurkat T cells by upregulating DR5 expression.

4. Discussion

Rapamycin is a bacterial product that inhibits mTOR by associating with its intracellular receptor FK506-binding protein (FKBP). The FKBP12-rapamycin complex binds directly to the FKBP12-rapamycin binding domain of mTOR and inhibits its activity [13]. mTOR is a member of the phosphatidylinositol 3-kinase (PI3K)-like family of serine/threonine kinases that integrates signals derived from growth factor stimulation and nutrient sensing to modulate a number of biological processes, including cell growth, proliferation, protein translation, differentiation, and autophagy [14]. The use of mTOR inhibitors as single agents was shown to improve treatment outcomes in several types of cancer. The combination of mTOR inhibitors with different treatment modalities is under active clinical investigation, and the combination of mTOR inhibitors with other anti-cancer agents has been reported in the literature [13,14]. The rapamycin analog RAD001 is currently being tested in clinical trials for the treatment of several hematologic and solid tumors [15,16]. However, RAD001 alone shows a modest antitumor effect. To improve the clinical efficacy of RAD001, several combination therapies with cytotoxic or molecular targeted agents were investigated, and RAD001 showed definitive synergy or additive effects in combination therapy [17,18].

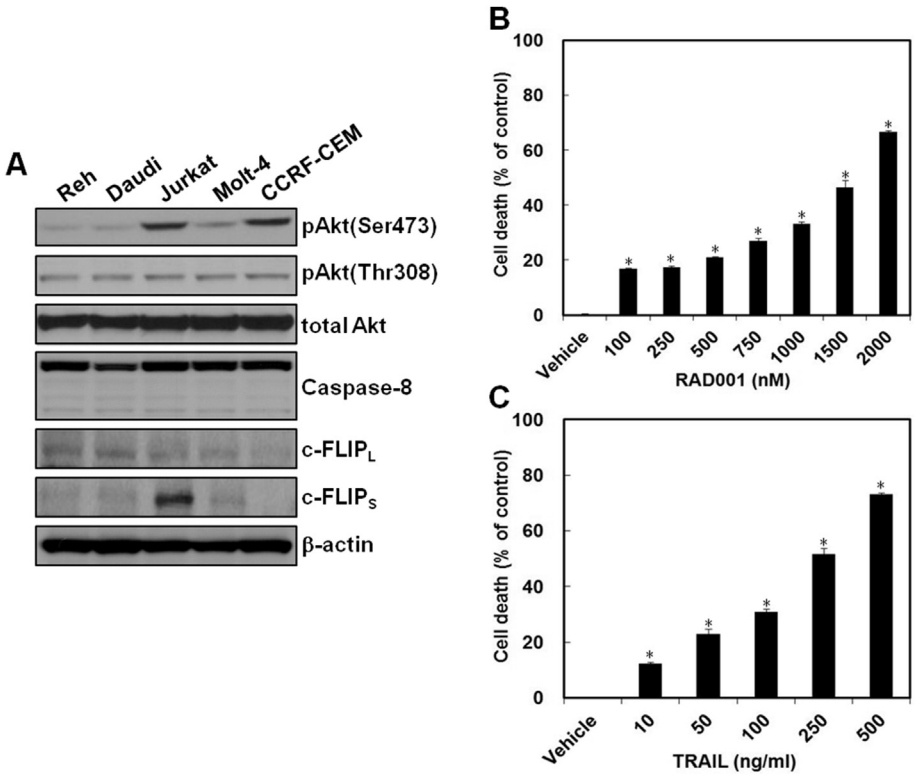

Fig. 1. RAD001 or TRAIL respectively induces cell death in human Jurkat T cells. (A) Immunoblot analysis of phospho-Akt (Thr308), phospho-Akt (Ser473), total Akt, caspase-8, c-FLIP$_L$, c-FLIP$_S$, and β-actin in human leukemic Reh, Daudi, Jurkat, Molt-4, and CCRF-CEM cells. (B, C) Cell death in response to treatment with either RAD001 or TRAIL for 72 h in human leukemic Jurkat T cells was determined by the Alamar Blue assay. Data are expressed as a percentage of vehicle-treated control cells and represent the mean ± SD of three independent experiments. $^*P < 0.05$ versus untreated control cells.

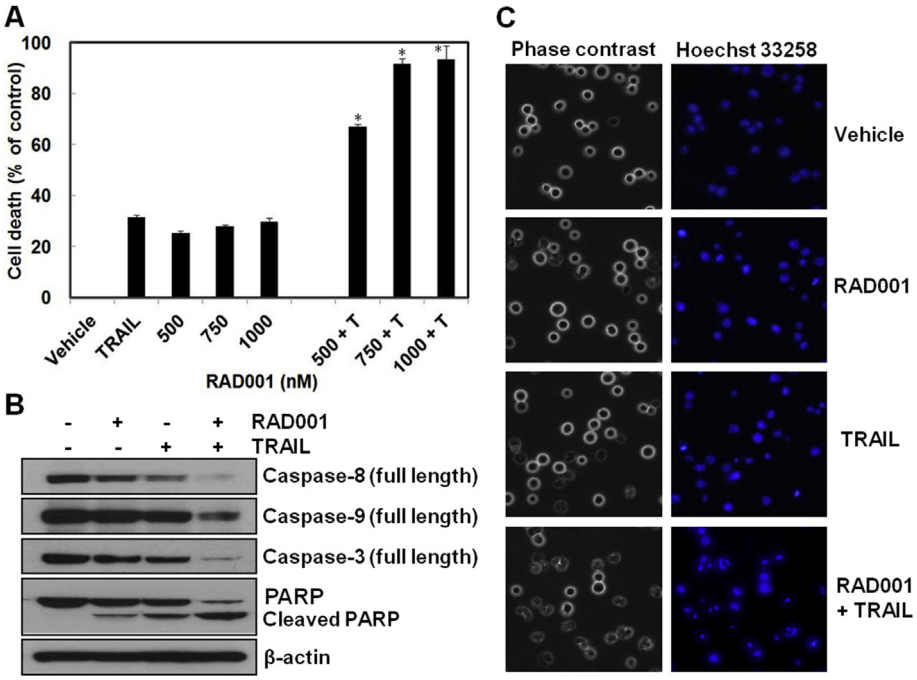

Fig. 2. Pretreatment with RAD001 enhances TRAIL-induced apoptosis in Jurkat T cells. (A) Cell death in response to treatment with 500, 750, and 1000 nM RAD001 alone and in combination with 100 ng/ml of TRAIL for 72 h in Jurkat T cells was determined by the Alamar Blue assay. Data are expressed as a percentage of vehicle-treated control cells and represent the mean ± SD from three independent experiments. *$P < 0.05$ versus TRAIL-treated cells. (B) Jurkat T cells were treated with 750 nM RAD001 and 100 ng/ml of TRAIL alone and in combination for 24 h. Immunoblot analysis of caspase-8, -9, -3, PARP, and β-actin. (C) Jurkat T cells were treated with 750 nM RAD001 and 100 ng/ml of TRAIL alone and in combination for 36 h. Cells were then treated with 5 μg/ml of Hoechst 33258 fluorescent dye for 20 min. Images were acquired using an inverted fluorescence microscope.

The selectivity of TRAIL towards certain cancer cells combined with its relatively low toxicity makes it a very attractive potential therapeutic agent in different cancers [19,20]. However, sensitivity to TRAIL may vary between individual tumors, as demonstrated here in ALL cells and elsewhere [21,22]. The mechanism underlying the resistance to TRAIL is not completely understood. Reports in the literature [23–25] have suggested various mechanisms to explain the reduction of TRAIL sensitivity, including differential expression of receptors or decoy receptors, increased expression of anti-apoptotic factors such as FLIP or inhibitor of apoptosis proteins (IAPs), or increased activation/expression of oncoproteins involved in cell survival. On the other hand, a number of studies explored combination therapies with TRAIL or TRAIL receptor antibodies currently in clinical trials include cytotoxic regimens such as cisplatin or radiotherapy, which enhance the intrinsic apoptotic pathway leading to the amplification of TRAIL-induced apoptosis [26]. Combining TRAIL or TRAIL receptor agonists with selective molecular targeted agents is being evaluated as a strategy to amplify TRAIL-induced apoptosis [27,28]. The use of targeted therapies in combination with TRAIL has at least two potential advantages, namely, the amplification of TRAIL-induced apoptosis and the reduction of side-effects compared to combinations involving cytotoxic agents. Therefore, we investigated the effects of combination treatment with RAD001 and TRAIL in ALL cells.

In the present study, our results showed that RAD001 enhances TRAIL susceptibility in Jurkat T cells by upregulating DR5 expression. To the best of our knowledge, this is the first study demonstrating that RAD001 could function as a sensitizer of TRAIL-induced apoptosis by upregulating DR expression. Clinically approved doses of RAD001 in oncology are 5–10 mg per day, which is significantly higher than the doses used in organ transplantation. RAD001 administered to patients at 5 mg/day was reported to produce a C_{max} of 33.4 nM [29]. Therefore, we included concentrations of 100–2000 nM in this study to assess the inhibitory effect of RAD001 over a wide range of concentrations. RAD001 had a dose-dependent effect on the proliferation of Jurkat and CCRF-CEM T cells, which have a highly activated PI3K/Akt/mTOR pathway, over this range of concentrations, whereas it did not significantly affect the proliferation of Reh, Daudi, and Molt-4 cells, which lack PI3K/Akt/mTOR pathway activation. Moreover, an additive effect of RAD001 was not observed in CCRF-CEM cells, which are unresponsive to TRAIL. In Reh, Daudi, and Molt-4 cells, which show a limited response to RAD001 and TRAIL, concurrent treatment with RAD001 and TRAIL did not significantly increase the rate of cell death. By contrast, RAD001 increased TRAIL-induced apoptosis by almost 100% by upregulating DR5 expression in TRAIL-sensitive Jurkat T cells, which are responsive to RAD001. Previous reports showed that inhibition of the PI3K/Akt/mTOR pathway and downregulation of FLIP, Mcl-1, IAPs, and survivin sensitize various cancer cells to TRAIL cytotoxicity [30–32]; however, further studies are necessary to elucidate the precise molecular and cellular mechanisms underlying the effects of the upregulation of DR5 expression by RAD001.

In summary, we evaluated the effects of combined treatment with RAD001 and TRAIL in lymphoblastic leukemia cells. Our results suggest that RAD001 enhances TRAIL susceptibility in Jurkat T cells by upregulating DR5 expression. Given that RAD001 plays an important role as an anti-cancer agent by inducing cell death in leukemia and TRAIL-based therapeutics have entered clinical trials, our findings provide a potential novel strategy based on the combination of RAD001 with TRAIL for the treatment of leukemia. The current findings are of clinical significance and warrant further evaluation of the efficacy of RAD001 combined with TRAIL in preclinical and clinical studies.

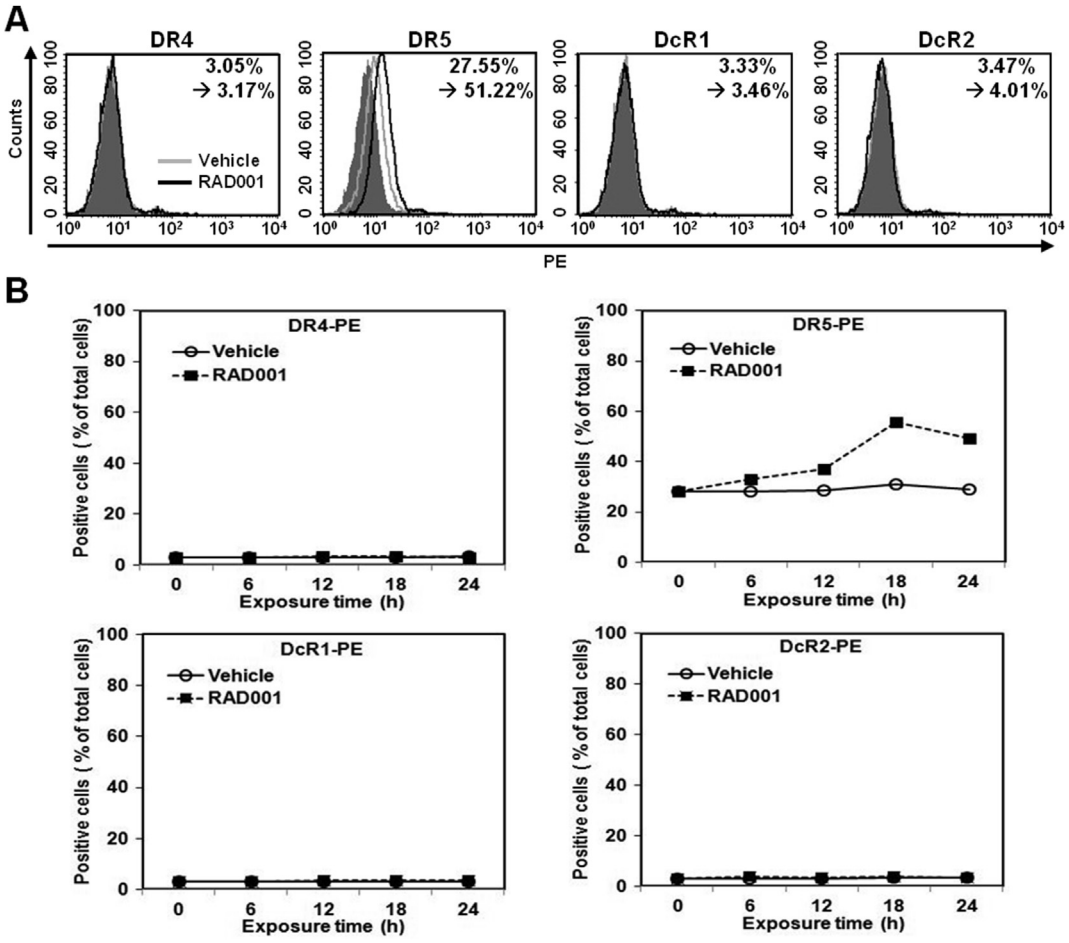

Fig. 3. RAD001 upregulates the expression of DR5 in Jurkat T cells but not that of other TRAIL receptors. Human leukemic Jurkat T cells were treated with 750 nM RAD001 alone for 24 h. The expression of TRAIL receptors including DR4, DR5, DcR1, and DcR2 was determined by flow cytometry using PE-conjugated specific antibodies for each receptor. (A) The expression of surface antigens was plotted against the appropriate IgG isotype controls (gray histogram). Data obtained at 18 h after treatment with vehicle (gray line) or RAD001 (black line) are presented. (B) Changes in the expression of TRAIL receptors in response to RAD001 treatment for the indicated times are presented as line graphs.

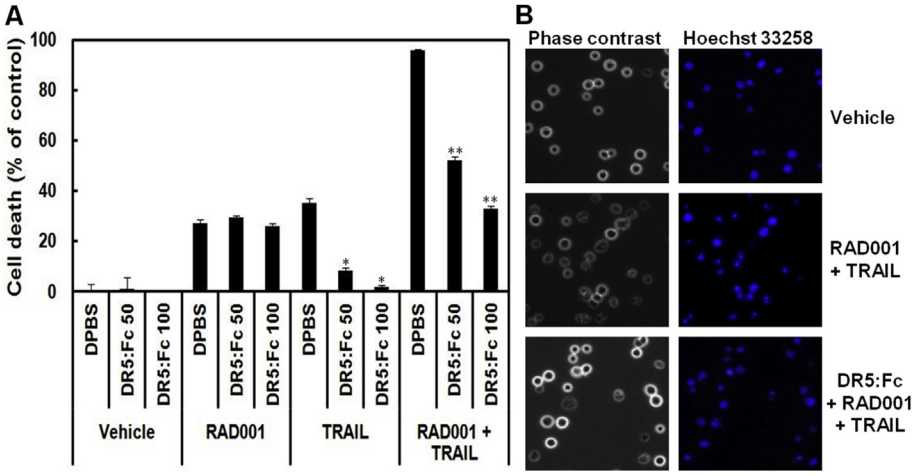

Fig. 4. Pretreatment with DR5:Fc abrogates the enhancement of TRAIL-induced apoptosis by RAD001 in Jurkat T cells. (A) Cell death in response to treatment with 750 nM RAD001 and 100 ng/ml of TRAIL alone and in combination in the presence of 50 or 100 μM DR5:Fc for 72 h was determined by the Alamar Blue assay. Data are expressed as a percentage of vehicle-treated control cells and represent the mean ± SD from three separate experiments. $^{*}P < 0.05$ versus TRAIL-treated cells; $^{**}P < 0.05$ versus RAD001 and TRAIL concurrently-treated cells. (B) Jurkat T cells were treated with 100 ng/ml of TRAIL after pretreatment with 750 nM RAD001 in the absence or presence of 100 μM DR5:Fc for 36 h. Cells were then treated with 5 μg/ml of Hoechst 33258 fluorescent dye for 20 min. Images were acquired using an inverted fluorescence microscope.

Conflict of interest

The authors report no potential conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.133.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.133.

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