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An MMP-2/MMP-9 inhibitor, 5a, enhances apoptosis induced by ligands of the TNF receptor superfamily in cancer cells

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Abstract

Several studies have shown that matrix metalloproteases (MMPs) promote tumor growth, invasion, and metastasis. Consequently, MMP inhibitors have been developed as a new class of anticancer drugs, many of which are in clinical trials. The exact mechanism of the antineoplastic activity of MMP antagonists is unknown. To investigate the mechanism, we hypothesized that MMP inhibitors enhance the actions of apoptosis-inducing agents. To test this hypothesis, we treated breast, melanoma, leukemia, osteosarcoma, and normal breast epithelial cells with (2R)-2-[(4-biphenylsulfonyl)amino]-3-phenylproprionic acid (compound 5a), an organic inhibitor of MMP-2/MMP-9, alone or in combination with TNFα or other apoptotic agents. FACS analysis showed that 5a interacted synergistically with ligands of the TNF receptor superfamily, including TNFα and TNF receptor-like apoptosis-inducing ligand (TRAIL), and with a Fas-cross-linking antibody (CH11), UV, paclitaxel, thapsigargin, and staurosporin, to induce apoptosis in a cell-type-specific manner. Other MMP inhibitors did not synergize with TNFα. Compound 5a did not act directly on the mitochondrion or via changes in protein synthesis. Instead, the mechanism requires ligand–receptor interaction and caspase 8 activation. Investigation of the effect of 5a on tumor growth *in vivo* revealed that continuous treatment of subcutaneous melanoma with a combination of 5a plus TRAIL reduced tumor growth and angiogenesis in nude mice. Our data demonstrate that 5a possesses a novel proapoptotic function, thus providing an alternative mechanism for its antineoplastic action. These observations have important implications for combination cancer therapy.

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Keywords: apoptosis; matrix metalloproteinase inhibitors; synergy; receptor; tumor growth

Abbreviations: MMP, Matrix metalloproteinase; MMPI, matrix metalloproteinase inhibitor; FACS, flow-activated cell sorter; MTP, mitochondrial transmembrane potential; SODD, silencer of death domain; FADD, Fas-associated death domain; TNFα, tumor necrosis factor alpha; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; RIPA, radioimmune precipitation; TRAIL, TNF receptor-like apoptosis-inducing ligand; ECL, enhanced chemiluminescence; $DiOC_6$, 3,3-dihexyloxacarbocynine iodide; IPA, inhibitor of apoptosis protein; FLIP, Flice-inhibiting protein.

Introduction

One of the critical issues in cancer treatment today is that many tumors become resistant to single-agent therapy. [1,2] Combination therapies have been developed to address this problem,[3–7] but some tumors still respond poorly to combination therapies, suggesting the existence of some yet unknown molecular variations that are not targeted by such therapies.[8] Consequently, there is a need to identify critical molecular characteristics of cancer cells that would guide the development of new cancer therapies. In this regard, the propensity of tumor cells to invade normal tissues and metastasize to distant sites[9] is promoted by matrix metalloproteinases (MMPs), which are zinc-dependent endopeptidases that degrade extracellular matrices.[10–13] Consequently, in recent years much attention has been focused on developing MMP inhibitors (MMPIs) as a new class of anticancer drugs. Several of the MMPIs have been reported to possess antineoplastic activities.[14,15] Some of them are presently at various stages of clinical trials.[16–21]

While the antineoplastic activity of MMPIs is presumed to be because of the inhibition of MMP enzymes, the exact mechanism is not yet completely understood. Some studies reported that MMPs promote tumor growth by releasing growth factors from their inactive membrane-bound form,[22–24] suggesting that MMP antagonists would inhibit cell growth, while other studies have reported that MMPIs inhibit tumor proliferation by inducing apoptosis via the release of ligands such as TRAIL and TNFα from their membrane-bound inactive forms.[25] Furthermore, MMPIs have been implicated in the inhibition of shedding of stress-induced membrane-bound receptors.[26–29] However, these studies have not yet clearly elucidated the exact mechanisms by which MMPIs antagonize tumor growth, invasion, and metastasis.

In the present study, we hypothesized that MMPIs modulate the expression or function of proteins involved in the induction of apoptosis. We tested this hypothesis by treating breast, melanoma, leukemia, and normal transformed cells with (2R)-2-[(4-biphenylsulfonyl) amino]-3-phenylproprionic acid (5a),[30,31] an MMP-2/MMP-9 inhibitor derived from *N*-sulfonylamino acid, to determine whether it would enhance apoptosis

induced by ligands of the TNFR receptor superfamily. We report here that 5a interacted synergistically with ligands of the TNF receptor superfamily to enhance apoptosis in cancer cells in a cell-type-specific manner and that combination treatment reduced tumor growth and angiogenesis *in vivo*.

Results

Effect of compound 5a on TNFα-induced apoptosis

To investigate the mechanism of antineoplastic activity of MMPIs, we treated 9D3S breast cancer cells with 0. 2% DMSO alone, 200 nM 5a alone, 20 ng/ml TNFα alone, and 200 nM 5a followed by 20 ng/ml TNFα. FACS analyses of DNA fragmentation, a measure of cell death, showed that neither 0.2% DMSO, nor 200 nM 5a, nor 20 ng/ml TNFα alone induced significant cell death after 12–20 h (Figure 1a, panels 1–2, 3–4, and 5–6). In contrast, 5a enhanced the apoptotic effect of TNFα by 3–10-fold (Figure 1a, panels 7–8). In the presence of 5a, TNFα-induced cell death was accelerated and reached a maximum level within 12–20 h instead of 48 h, as we previously demonstrated.[32] It should be noted that adding TNFα before 5a had the same effect as adding TNFα alone.

We next determined the optimum preincubation time necessary to achieve synergy. As shown in Figure 1b, adding TNFα simultaneously with 5a (0 h) or 5 and 6 h after preincubation with 5a was less effective in enhancing apoptosis than adding TNFα 1–3 h after the cells were preincubated with 5a. Subsequently, we tested whether the failure of 5a to synergize with TNFα following 5–6 h of preincubation might be because of a depletion of 5a. To that end, at the 5 and 6 h time points, we treated cells with additional DMSO, 5a, TNFα alone as before, or 5a followed by TNFα 1 h later. We found that additional DMSO, 5a, and TNFα alone had no effect on cell death, whereas the addition of 5a followed by TNFα restored synergy between 5a and TNFα (Figure 1b, columns 5+ and 6+).

Furthermore, we investigated the effect of 5a concentration needed to synergize with TNFα to induce apoptosis. As shown in Figure 1c, 200–400 nM 5a alone did not induce cell death (columns 200, 300, and 400). We next determined whether simultaneous treatment of cells with higher concentration of 5a (300 and 400 nM) and 20 ng/ml of TNFα would enhance apoptosis. Figure 1c shows that 300 nM 5a added simultaneously with 20 ng/ml of TNFα did not have any effect (300+T), whereas 400 nM 5a and 20 ng/ml of TNFα had a remarkable effect on cell death (400+T). From these observations, we conclude that 5a induces a transient change that requires an optimum concentration that can be achieved in 2–3 h of treatment with 200 nM before adding TNFα or by adding 400 nM 5a, and 20 ng TNFα simultaneously for sensitizing cells to TNFα-induced apoptosis.

Compound 5a, a unique MMP-2/MMP-9 inhibitor that enhances cytokine-induced apoptosis

We screened several other MMPIs (Calbiochem-Novabiochem Corp.,. San Diego, CA, USA) for synergistic interaction with TNFα at 100 nM, which was far above their IC_{50} or their K_i values, to test whether they could also sensitize breast cancer cells to TNFα-induced apoptosis. They included MMP-2/MMP-9 inhibitor II (IC_{50} 17 and 30 nM, respectively), MMP-8 inhibitor I (IC_{50}=4 nM), and MMP-9/MMP-13 inhibitor I (IC_{50}=900 pM) and GM8914, a broad-based MMP inhibitor (MMP-1: K_i=0.2 nM; MMP-2: K_i=500 nM; MMP-3: K_i=20 nM, MMP-8 and MMP-9: K_i=100 nM). They were all tested at 100 nM. FACS analyses presented in Figure 2a showed that none of the other MMP inhibitors synergized with TNFα to enhance apoptosis.

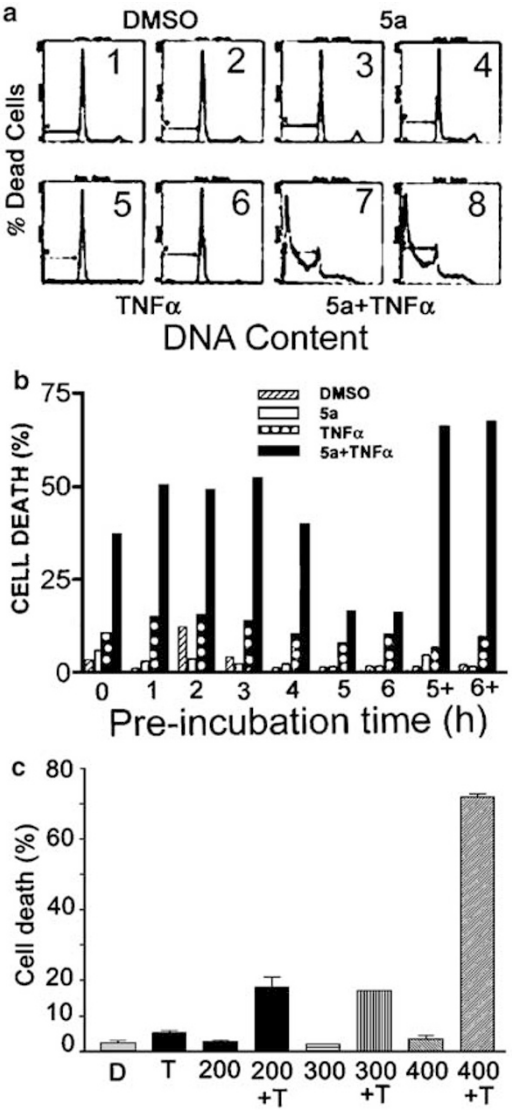

Figure 1 Compound 5a interacts synergistically with TNFα to enhance apoptosis in cancer cells. (**a**) FACS analyses of DNA fragmentation of 9D3S breast cancer cells treated with 0.2% DMSO (panels 1and 2), 200 nM 5a (panels 3 and 4), 20 ng/ml TNFα (panels 5 and 6), and 200 nM 5a for 3 h followed by 20 ng/ml of TNFα (panels 7 and 8). (**b**). FACS analyses of 9D3S cells treated with 200 nM 5a for various times followed by 20 ng/ml of TNFα (columns 0–6). Some cells were treated for 5 or 6 h with 200 nM 5a followed by an additional 200 nM 5a and then 20 ng/l TNFα 1 h later (columns 5+ and 6+). This is a representative experiment of three performed. (**c**) FACS analysis of 9D3S cells treated with 0.2% DMSO, 20 ng/ml TNFα, and various concentrations of 5a (200, 300 and 400 nM) alone (D, T, 200, 300 and 400), or with 5a plus 20 ng/ml TNFα (200+T, 300+T, and 400+T)

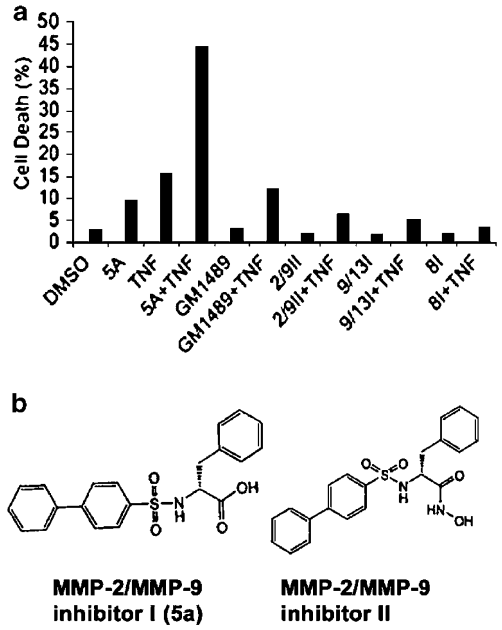

Figure 2 Compound 5a is the only tested MMP inhibitor that synergized with TNFα to enhance apoptosis. All the inhibitors used here were obtained from Calbiochem-Novabiochem Corp., San Diego, CA, USA (**a**) FACS analysis of 9D3S cells treated with DMSO, 5a, TNFα, the general MMP inhibitor (GM1489), inhibitors of MMP-2/MMP-9 II (2/9II), MMP-9/MMP-13 (9/13I), and MMP-8 (8I) alone or in combination with TNFα. This is a representative experiment of three performed. (**b**) Chemical structures of two closely related MMP inhibitors: MMP-2/MMP-9 I (5a) and MMP-2/MMP-9 inhibitor II

The uniqueness and specificity of compound 5a was further demonstrated by studying another structurally related MMP-2/MMP-9 antagonist in more detail. Although MMP-2/MMP-9 inhibitor II is structurally very similar to 5a (Figure 2b), it did not synergize with TNFα (Figure 2a, eighth column from the left) to enhance apoptosis compared to 5a plus TNFα (Figure 2a, fourth column from the left). This was validated for different concentrations ranging from 100 to 800 nM (data not shown). The highest concentration was about 30–50-fold higher than its IC_{50} of 17 and 30 nM, respectively. These results show that the proapoptotic function of 5a is not a general property of MMP inhibitors and it is not because of their ability to chelate zinc.

Compound 5a synergizes only with ligands of the TNF receptor superfamily

Since many agents induce apoptosis, it was necessary to determine whether other apoptotic factors synergize with compound 5a to enhance apoptosis. This question was relevant to the investigation of the mechanism by which 5a enhances apoptosis. To that end, various apoptotic agents [UV light,[33] thapsigargin,[34] staurosporin,[35] paclitaxel,[36] a Fas-crosslinking antibody (CH11),[37] and TRAIL[38]] were examined. We found that in addition to TNFα, anti-Fas (data not shown), and TRAIL interacted synergistically with compound 5a to induce apoptosis. As shown in Figure 3, DMSO, 5a, and TNFα alone did not significantly induce cell death. However, in

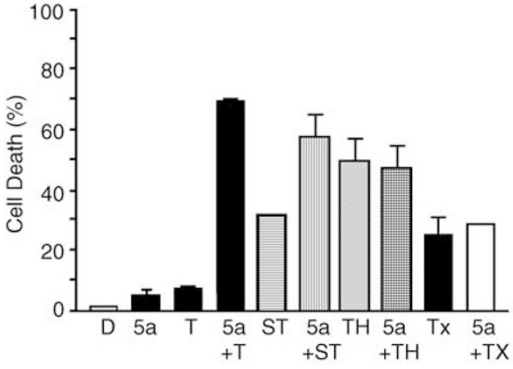

Figure 3 Compound 5a interacts synergistically only with ligands of the TNF receptor superfamily. FACS analysis of 9D3S cells treated with DMSO (D), 5a and TNFα (T); staurosporin (ST), thapsigargin (TH), or paclitaxel (TX) alone or with 5a in combination with TNFα, staurosporin, thapsigargin, or paclitaxel (columns 5a+T, 5a+ST, 5a+TH, and 5a+TX).

combination, 5a+TNFα enhanced apoptosis by about seven-fold (Figure 3, fourth column from the left). In contrast, 200 J/cm^2 of UV light (data not shown), 20 nM staurosporin (ST), 100 nM paclitaxel (TX), and 20 nM thapsigargin (TH) all induced cell death. However, while 5a had an additive effect with staurosporin, it did not synergize with any of them to enhance apoptosis (Figure 3, 5a+ST, 5a+TH, and 5a+TX). From these observations, we conclude that only TNFα, TRAIL, and the Fas-cross-linking antibody, all ligands of the TNF receptor superfamily, synergized with 5a to enhance apoptosis.

Synergy is cell-type specific

We next determined whether the synergy between 5a and TNFα was unique to the 9D3S breast cancer cell line. To that end, we tested several other cell lines, including a nontumorigenic transformed breast epithelial cell line (HBL100)[39] and the aggressively metastatic MDA-MB-231[40] breast cancer cell line. We also tested the poorly metastatic melanoma cell line A375P and its metastatic variant melanoma cell line A375SM,[41] and three osteosarcoma cell lines (MG63, SOAS-2, and TA29), a lymphoblastic leukemia (Z119), and four acute mylogenous leukemia cell lines (AML-2, AML-3, KBM5, and KBM7). FACS analyses revealed that the nontumorigenic transformed normal breast epithelial cell line HBL100 was not sensitive to DMSO, 5a, or TNFα alone or to the combination treatment of 5a plus TNFα (Figure 4a, panels 1–4). 9D3S cells were sensitive to all three ligands (anti-Fas, TNFα, and TRAIL). MDA-MB-231 and the melanoma cell lines (A375P, A375SM) were resistant to TNFα (data not shown). Analysis of cell viability determined by the Trypan blue dye-exclusion method showed that MDA-MB-231 cells were not sensitive to DMSO, 5a, or TRAIL alone (Figure 4b, columns D, 5a, and TR), but they were sensitive to combination treatment with 5a and TRAIL (Figure 4b, column 5a+TR). FACS analysis of the melanoma cell lines also showed that they were sensitive to combination treatment of 5a and TRAIL (data not shown). All the leukemia cells were resistant to TNFα (data not shown). However, two of the acute myelogenous leukemia

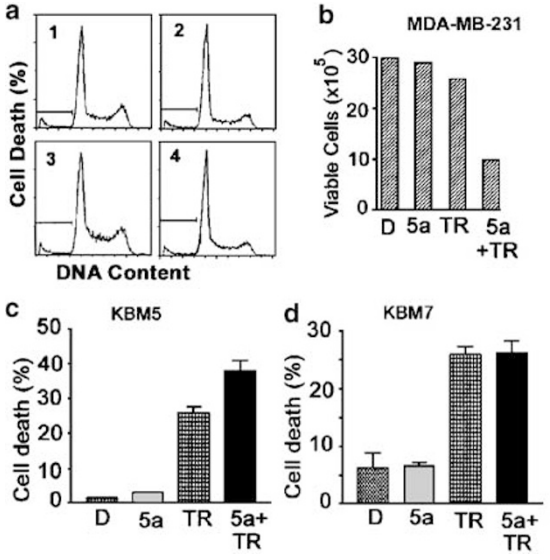

Figure 4 Synergy between 5a and TRAIL to enhance apoptosis is cell-type specific**.** These are representative experiments done in duplicate several times. (**a**) Representative FACS analyses of HLB100-transformed normal breast epithelial cells treated with 0.2% DMSO, 5a or TRAIL alone (panels 1–3, respectively) or 5a in combination with TRAIL (panel 4). (**b**) Viable cells were determined by the Trypan blue dye exclusion method after MDA-MB-231 breast cancer cells were treated with 0.2% DMSO (D), 5a, or TRAIL (TR) alone, or 5a in combination with TRAIL (5a+TR). (**c**) FACS analysis of KBM5 leukemia cells treated with 0.2% DMSO (D), 5a and TRAIL (TR) alone, or 5a in combination with TRAIL (5a+TR). (**d**) Similar analyses were performed with the KBM7 leukemia cells

cell lines (KBM5 and KBM7) were sensitive to TRAIL and the killing was somewhat enhanced by 5a in KBM5 but not in KBM7 cells (Figure 4c and d). All three osteosarcoma cell lines were resistant to TNFα and TRAIL alone or in combination with 5a (data not shown). Thus, 5a enhanced apoptosis in a cell-type specific manner. Therefore, while cell sensitivity to apoptosis induced by certain apoptotic agents was required, it was not sufficient for such agents to interact synergistically with 5a to enhance apoptosis. Table 1 shows a list of cells used and the results of various treatments.

The effect of compound 5a on protein synthesis

In an effort to determine the mechanism by which compound 5a potentiates apoptosis, we next examined the possibility that 5a might, like cyclohexamide,[42] enhance apoptosis by inhibiting protein synthesis. We measured the total protein yield in untreated and treated cells by the Bradford method. As shown in Figure 5a, cells treated with DMSO (column 2), 5a (column 3) and TNFα (column 4) alone, or 5a plus TNFα (column 5) all had about a 15–20% reduction in total proteins as compared to the untreated control (column 1). We next measured TCA-precipitable L-[^{35}S]methionine incorporation following different treatments to determine if 5a inhibited global *de novo* protein synthesis. As shown in Figure 5b, *de novo* synthesized proteins calculated per unit of protein were reduced by about 40% cells treated with DMSO, 5a, or TNFα alone (columns 2–4, respectively) compared to that in untreated control cells (column 1) and there was no significant difference among the different treatments. Lastly, we analyzed the pattern of newly synthesized proteins by autoradiography following sodium dodecyl sulfate polyacrylamide gel (10%) electrophoresis (SDS-PAGE) to determine whether expression of some proteins were down- or upregulated. There was no detectable differential effect on *de novo* protein synthesis during the 2-h labeling period (Figure 5c). Collectively, these data indicate that 5a does not act through the inhibition of *de novo* protein synthesis.

Table 1 Synergistic effect of 5a on ligand-induced apoptosis in different cell lines

Cell Lines	5a+TNFα	5a+TRAIL	5a+Anti-fas (CH11)
1. HLB100	No	No	ND
2. 9D3S	Yes	Yes	Ye
3. MDA-MB-231	No	Yes	ND
4. A375P	No	Yes	ND
5. A375SM	No	Yes	ND
6. AML2	No	No	ND
7. KBM5	No	Additive	ND
8. KBM7	No	No	ND
9. MG63	No	No	ND
10. SAOS-2	No	No	ND
11. TA29	No	No	ND

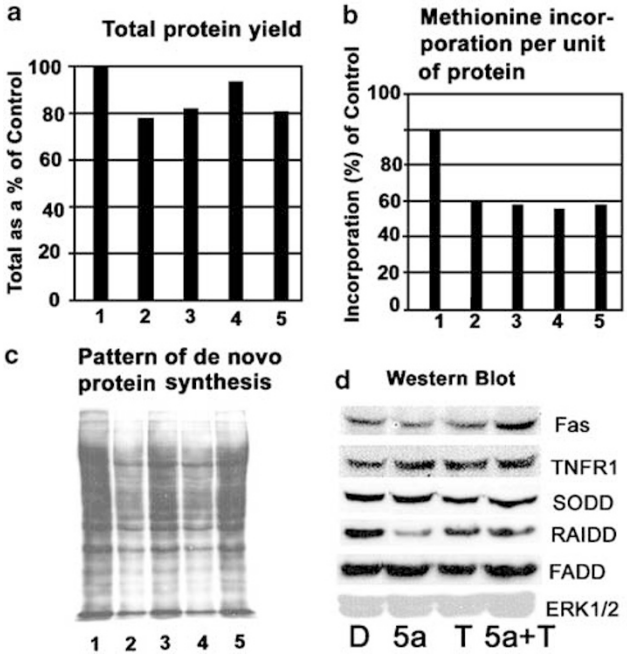

Figure 5 Compound 5a does not affect protein synthesis. These experiments were done twice and shown here are representative results. (**a**) Total protein yield (measured by the Bradford method) of extracts of cells that were untreated (1), treated with DMSO, 5a, or TNFα alone or a combination of 5a and TNFα (columns 1–5, respectively). (**b**) TCA-precipitable L-[^{35}S]methionine incorporation per unit protein in the same samples described in (**a**). (**c**) Autoradiographic analyses of the pattern of *de novo* synthesized proteins of the same samples described in (**a**). (**d**) Western blot analysis of proteins that constitute the death-inducing signaling complex (DISC), including Fas, TNFR1, SODD RAIDD, and FADD, after treatment with DMSO (lane D), 5a (lane 5a), or TNFα (lane T) alone or 5a in combination with TNFα (lane 5a+T). ERK1/2 was included to show equal loading

We next analyzed proteins extracted from cells treated with DMSO, 5a or TNFα alone, or with 5a in combination with TNFα for the expression of pro- and antiapoptotic proteins by Western blotting. The same membrane was stripped several times and reprobed with different antibodies. We did not detect any significant difference in the expression levels of the antiapoptotic factors including the silencer of the death domain (SODD)[43,44] (Figure 5d) and Flice-inhibiting protein (FLIP)[45] (data not shown) or the pro-apoptotic factors Fas,[46] TNFR1,[47] FADD,[48] RAIDD[49] (Figure 5a) and caspase 8[50] (data not shown). ERK1/2 was included to assure equal loading (Figure 5d). These observations indicate that 5a does not target the expression of these proteins.

We next performed detailed time course experiments in which we analyzed TNFR1 expression at 0, 0.5, 1, 2, 3, and 6 h after initiating treatment with DMSO, 5a, or TNFα alone, or 5a followed by TNFα. Cytosolic and membrane-associated proteins were analyzed by Western blotting for TNFR1. There was no significant difference in the level of TNFR1 expression during this period (Figure 6) despite the fact that the highest level of synergy between 5a and TNFα occurred when TNFα was added 2–3 h after pre-incubation with 5a (see Figure 1b).

To distinguish between membrane-bound and cytosolic TNFR1, we used the indirect biotinylated-avidin immunofluorescent method as described in Materials and Methods. Results of FACS analysis (data not shown) show that 5a did not affect the level of membrane-bound TNFR1. Additionally, we searched for receptor shedding by Western blotting, for truncated forms of TNFR1 in cell culture medium that had been concentrated by a factor of 100. Although we detected intact TNFR1 in the medium, we did not find any evidence that TNFR1 was shed from the cell membrane into the medium, which would be characterized by truncated receptors (data not shown).

Collectively, these observations show that the mechanism by which 5a enhances ligand-induced apoptosis is not mediated by changes in protein synthesis or inhibition of shedding of membrane-bound members of the TNF receptor superfamily.

TRAIL decoy receptor 1 (DcR1) abrogates synergy between 5a and TRAIL

To investigate the role of the receptor–ligand interaction in synergy between 5a and TNFα, we pretreated cells with the

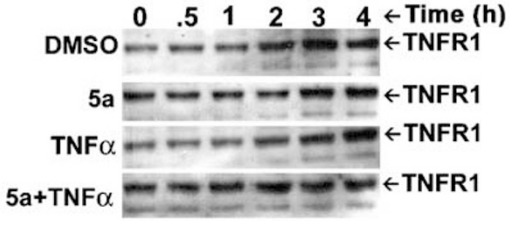

Figure 6 Time course of TNFR1 expression. Results of Western blot analysis of TNFR1 expression in 9D3S breast cancer cells. Cells from different treatment groups were prepared at different time points as indicated in the figure. No changes were observed in TNFR1 expression between the different treatment groups. This experiment was done twice and a representative result is shown here

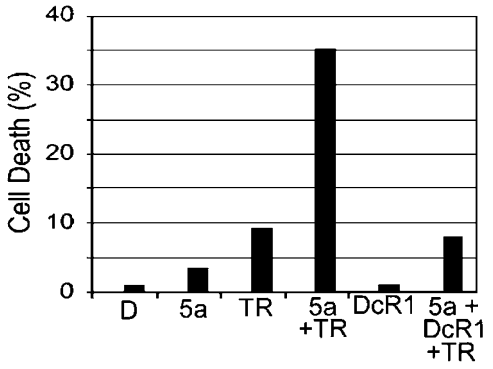

Figure 7 TRAIL decoy receptor 1 abrogates synergy between 5a and TNFα to enhance apoptosis. FACS analyses of cells treated with DMSO (D), 5a, TRAIL (TR) and TRAIL decoy receptor 1 (DcR1) alone, 5a in combination with TRAIL (5a+TR), or 5a followed by DcR1 and TRAIL (5a+DcR1+TR). This is a representative experiment of three performed

TRAIL DcR1[51,52] and then treated them with TRAIL. This receptor binds the ligand, but because it lacks the intracellular domain, it cannot transduce the death signal into the cell. As shown in Figure 7, DMSO (D), 5a, TRAIL (TR), and DcR1 alone had no significant effect on cell death. However, treatment with a combination of 5a and TRAIL (5a+TR) enhanced apoptosis. The DcR1 effectively blocked the synergistic interaction between TRAIL and 5a, indicating that ligand–receptor interaction is essential for initiating the death-signaling cascade that leads to the enhancement of apoptosis.

Caspase activation

We next determined whether 5a affects death signaling downstream of the receptor–ligand interaction. To that end, we examined cell extracts for the activity of caspase 8, which initiates the caspase death-signaling cascade. Firstly, we observed that the general caspase anagonist z-Val-Ala-Asp(OMe)-fluoromethyl ketone (zVADfmk) inhibited 5a-enhanced apoptosis (data not shown), as did the caspase 8 inhibitor, z-Ile-Glu(OMe)-Thr-Asp(Ome)-fluoromethyl ketone (zIETDfmk) (Figure 8a, column I+5a+T). Secondly, using a colorimetric assay for activated caspase 8, we showed that DMSO, 5a, and TNFα alone had no significant effect on caspase 8 activity at 12 or 20 h (Figure 8b), but combination treatment elicited a substantially high level of caspase 8 activity (Figure 8b). These findings constitute the first evidence that the most-upstream change in the caspase cascade that occurs in 5a-enhanced apoptotic cells is the activation of caspase 8.

Mitochondrial transmembrane potential

Some apoptotic agents alter the mitochondrial transmembrane potential (MTP), which leads to the release of apoptosis-related factors, including cytochrome *c*[53] and Smac.[54,55] We investigated whether 5a alone or in combination with TNFα alters the MTP. Figure 9 shows that DMSO (D), 5a, and TNFα (T) alone did not alter MTP, but TNFα-5a combination reduced the MTP by about 35% (Figure 9,

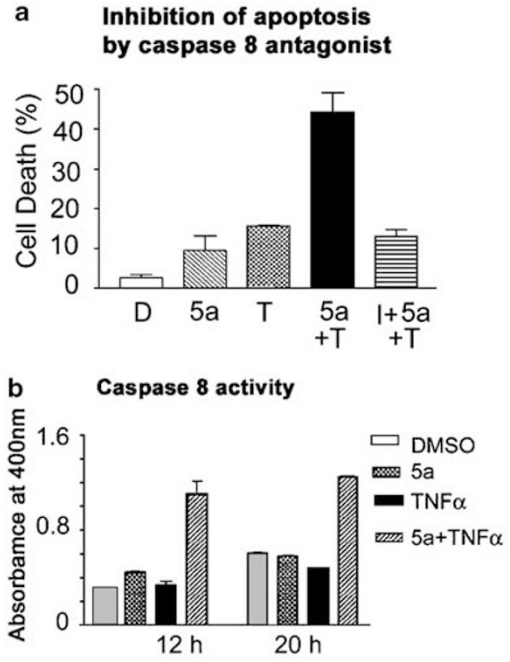

Figure 8 Compound 5a interacts synergistically with TNFα to activate caspase 8. (**a**) FACS analyses of 9D3S cells treated with DMSO (D), 5a and TNFα (T) alone, 5a in combination with TNFα (5a+T), or caspase 8-preferred inhibitor followed by 5a and TNFα (I+5a+T). (**b**). Caspase 8 activity measured by the colorimetric method in cells treated for 12 or 20 h as described in (**A**)

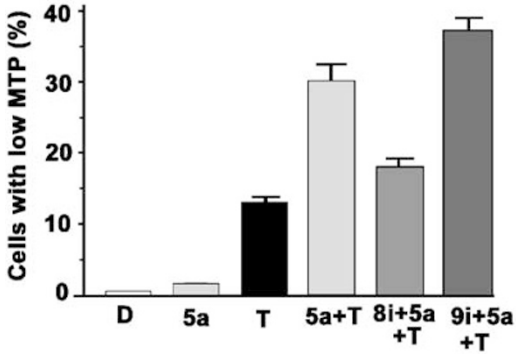

Figure 9 Compound 5a interacts synergistically to reduce MTP. Changes in the MTP were measured by the reduction in the amount of $DiCO_6$ loaded into cells following various treatments. MTP is shown in cells treated with DMSO (D), 5a and TNFα (T) alone, and those treated with a combination of 5a and TNFα in the absence (5a+T) or in the presence of caspase 8 inhibitor (8i+5a+T) and caspase 9 inhibitor (9i+5a+T)

column 5a+T). This reduction in the MTP was inhibited if the cells were preincubated with an antagonist of caspase 8 (zIETDfmk) (8i+5a+T) but not with an inhibitor of caspase 9, z-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethyl ketone (zLEHDfmk) (9i+5a+T). These results suggest that the signal that altered the MTP came from activated caspase 8 and not from within the mitochondrion. The failure of the caspase 9 inhibitor to abrogate the reduction in MTP induced by combination treatment was consistent with the conclusion that the signal for synergy between 5a and TNFα to enhance apoptosis was initiated upstream of the mitochondrion.

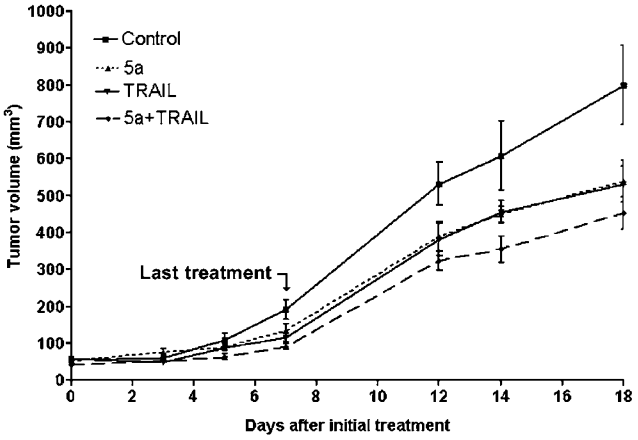

Figure 10 Effect of 5a and TRAIL treatment on tumor growth of human melanoma cells. Athymic nude mice (6–8 weeks old) were injected subcutaneously with 5×10^5 A375SM melanoma cells in 200 μl PBS. When tumors became palpable after a week, the mice were divided into four treatment groups containing 10 animals each. The treatment groups were: untreated control, 5a alone, TRAIL alone, and 5a+TRAIL (i.p. for 7 days). Tumor volumes were measured twice a week for 18 days. Each plot is the average of tumor volumes of 10 animals per group. Note that treatment with 5a+TRAIL inhibited the growth of A375SM melanoma cells by two-fold as compared to control groups ($P<0.05$)

In vivo study

To determine if the proapoptotic effect of 5a observed *in vitro* also occurs *in vivo,* we next monitored the effect of individual and combination treatment on the growth of A375SM melanoma cells implanted subcutaneously into nude mice. Tumor volume in each animal was measured twice a week. Figure 10 shows that by the end of 18 days, the tumors in the control group reached the size of 800 mm^3. While treatment with 5a or TRAIL alone reduced the growth of A375SM cells by about the same amount, combination treatment with 5a+TRAIL was the most effective and resulted in tumor size of only $\sim$450 mm^3. The reduction in tumor growth by combination treatment compared to 5a or TRAIL alone was significantly different ($P<0.05$). The data also show that once the treatment was stopped, the tumor resumed growth, suggesting that it may require continuous or a more extended course of combination treatment (>7 days) to completely arrest tumor growth.

Tumor sections were also examined for apoptosis by the TUNEL method and for microvessel formation by immunohistochemical method using a CD31-specific antibody. Shown in Figure 11 are representative pictures of TUNEL staining (second column) and CD31 staining (third column) and the composite picture of TUNEL staining superimposed on CD31 staining (first column). Untreated tumors had no apoptotic (TUNEL positive) cells, but they did have abundant microvessels (Figure 11a). Cells treated with 5a alone had some apoptotic cells but a reduced number of blood vessels (Figure 11b). In contrast, tumors treated with 5a plus TRAIL had a higher number of apoptotic cells, concomitant with a reduced number of blood vessels (Figure 11c). These results suggest that 5a in combination with TRAIL inhibits tumor growth by enhancing apoptosis and by inhibiting angiogenesis *in vivo*.

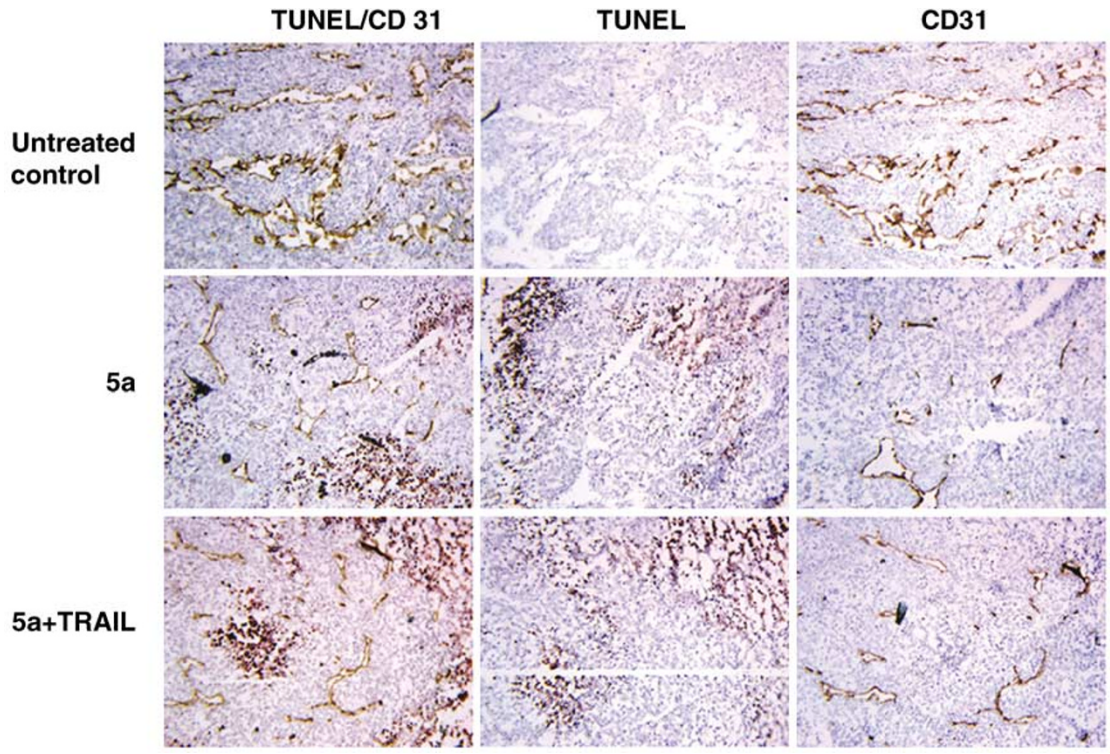

Figure 11 Apoptosis and tumor microvessels in the same subcutaneous melanoma described above (Figure 10). After 7 days of daily treatment, mice were kept for another 11 days for further observations. Tumor sections were stained for apoptosis by TUNEL and for microvessels by CD31-specific antibody. Shown are pictures of TUNEL staining alone (second column), CD31 staining alone (third column), and superimposed pictures of TUNEL and CD31 (first column). Untreated control tumor (top row), 5a-treated tumor (middle row) and 5a plus TRAIL-treated tumor (bottom row) A375SM melanoma cells. These are representative fields

Discussion

In this study, we have demonstrated that an MMP-2/MMP-9 inhibitor, 5a, selectively augments apoptosis induced by ligands of the TNF receptor superfamily, specifically TNFα, TRAIL, and the crosslinking anti-Fas antibody, in a cell-type specific manner. Several factors could account for the unique characteristics of 5a. For example, the finding that 5a is the only MMPI that interacted synergistically with ligands of the TNF receptor superfamily to induce apoptosis might be because of its chemical structure. As shown in Figure 2b, the most structurally similar inhibitors (5a and MMP-2/MMP-9 inhibitor II) differ at least in three moieties, which could account for the differences in their behavior. The inhibitors may also differ in their ability to be absorbed onto or into cells, and once absorbed, the inhibitors may differ in their stability. Some of them may be rapidly inactivated by secondary modifications such as phosphorylation, methylation, or sulfonation. Furthermore, the inhibitors could be confined to an inaccessible cellular compartment or pumped out such that the intracellular concentration never reaches the critical level required for synergy. Since the mechanism of MMP inhibition involves chelation of zinc, yet synergy was selective, it is conceivable that the uniqueness of 5a to synergize with ligands of the TNF receptor superfamily to enhance apoptosis derives from its ability to utilize a yet unknown additional factor.

Cell-type specificity of the observed synergy could be explained in several ways. Different cell types express different amounts of the TNF receptor superfamily,[56] pro-apoptotic factors, including caspases[57,58] and Smac,[54,55] or antiapoptotic factors such as SODD,[43,44] FLIP,[45] and IAP.[59] Cells that express more receptors and proapoptotic factors or less antiapoptotic factors would be predicted to facilitate synergy between 5a and ligands of the TNF receptor superfamily.

At present, there is no information on the targets of 5a apart from MMP-2/MMP-9 described in the original studies[30,31]. It is not yet known whether 5a targets exclusively MMP-2/MMP-9 or other proteases such as TACE and other members of the ADAM family. Studies are underway using radiolabeled 5a to determine whether 5a binds to a specific molecule or a class of molecules, including proteins and nucleic acids.

Our observations are significant because of the growing interest in the potential for developing MMPIs as a new class of cancer drugs.[60] Many MMPIs have been reported to be active against tumor growth, invasion, and metastasis.[61,62] However, since these MMPIs are structurally different from 5a, and since some of them are effective against cells that are refractive to 5a-enhanced apoptosis,[62] it is likely that their mechanisms of action are different. Although it is assumed that the antineoplastic activity of MMPIs is because of their inhibition of MMPs, through their zinc-chelating activity, our findings do not support this notion. For example, not all MMPIs, including those that are more efficient than 5a in chelating zinc, do not all enhance TNFα-induced apotosis. Additionally, the cells in which synergy between 5a and ligands of the TNF receptor superfamily have been observed

express variable levels of MMP-2/MMP-9 enzymes. Furthermore, despite the fact that some studies have reported receptor shedding induced by MMPs,[26] suggesting that MMPIs would act to stabilize receptors, we did not find any evidence to support such a notion.

It should be pointed out that other studies have also shown that MMPIs such as AG3340 work against tumors by inhibiting angiogenesis[63] whereas batimistat (BB-94)[64] was reported to act by perturbing the cell cycle. However, in both cases, the exact mechanism is not yet known. The induction of cell cycle arrest and apoptosis by MMPIs was also reported in kidney inflammatory cells.[65] Whether or not the same mechanism works in tumor cells is yet to be determined.

Based on our findings, the most likely mechanism of 5a augmentation of TNFα-induced apoptosis is the enhancement of caspase 8 activation. However, the exact mechanism by which 5a augments the activation of caspase 8 is not yet known. It should be pointed out that in a study of a prostatic carcinoma cell line, LNCaP, it was demonstrated that caspase 8 activation was necessary but not sufficient for TRAIL-induced apoptosis.[66] Collectively, these findings suggest that the proapoptotic activity of 5a might be unrelated to the inhibition of MMP-2/MMP-9 enzymes.

The synergistic interaction between 5a and TRAIL in particular has important implications for cancer treatment. Compound 5a was effective in synergizing with TRAIL in nanomolar concentrations, whereas, 5a by itself at such low concentrations, had no adverse effect on cells. Since 5a appeared to be active only against TRAIL-sensitive cells, its action should be selective for tumor cells because TRAIL does not kill normal cells.[67–70] This was also evident from our observation that the nontumorigenic transformed normal breast epithelial cell line (HLB100) was not sensitive to combination treatment with 5a and TRAIL. Indeed, preliminary *in vivo* study showed that combination treatment of subcutaneously planted melanoma cells with 5a and TRAIL reduced tumor volumes by at least 50% compared to that of the untreated control tumors during a week of daily treatment (Figure 10).

In summary, we provide evidence that compound 5a is an MMP-2/MMP-9 inhibitor that enhances apoptosis induced by ligands of the TNF receptor superfamily, specifically TNFα, TRAIL, and the Fas-crosslinking antibody. The synergy was observed only in breast and melanoma cell lines and not in transformed normal breast epithelial cell line, leukemia, or osteosarcoma cell lines, indicating that it is cell-type specific. Our data also indicate that the 5a-enhanced TNFα-induced apoptosis is not mediated by changes in protein synthesis, nor does it involve 5a acting directly on the mitochondrion. Instead, the mechanism requires a ligand–receptor interaction and activation of caspase 8. Furthermore, we showed that combination treatment of subcutaneous melanoma with 5a and TRAIL inhibited tumor growth and angiogenesis in nude mice. However, the inhibition of tumor growth appears to require continuous treatment. These observations show that 5a can be a useful tool for investigating the role of ligand–receptor interaction in modulating tumor growth, invasion, and metastasis, and it also offers opportunities for developing novel cancer therapies.

Materials and Methods

Cell culture

Cell line 9D3S was derived from the ZR-75-1 as described previously.[32] HBL100 was obtained from Dr. Michael Tainsky (Wayne State University, Detroit, MI, USA, who obtained it initially from the American Type Culture Collection). The following cell lines were obtained from colleagues at the University of Texas MD Anderson Cancer Center: MDA-MB-231 breast cancer cell line (Dr. Janet E Price); MG63, TA29, and SAOS2 osteosarcoma cell lines (Dr. Eugenie Kleinerman); and AML2, AML3, KBM5, KBM7, and Z119 leukemia cell lines (Dr. Srdan Verstovsek).

Breast cancer cells were maintained in 100-mm plastic dishes in RPMI 1640 medium, supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 units of penicillin, 100 μg/ml of streptomycin, and a 1 $\times$ solution of sodium pyruvate (GIBCO-BRL Life Technologies, Grand Island, NY, USA) in a humidified incubator with 5% CO_2 atmosphere. Cultures were replenished with fresh medium every 3–4 days and were split 1 : 4 whenever they reached about 90% confluence. Adherent cells were harvested by trypsinization in calcium-free phosphate-buffered saline (PBS). Unattached 9D3S cells were harvested by repeatedly pipetting the medium.

Treatment with MMP inhibitors

Cells were seeded at 2.5×10^6 per 100 $\times$ mm dish in 6 ml of normal medium overnight. They were then treated with 200 nM 5a for 3 h followed by 20 ng/ml TNFα and incubated for another 17 h. Samples were analyzed for the effect of treatment by specific assays as described in specific subsections below. For FACS analysis, 2.5×10^5 cells in 3 ml of medium in six-well dishes were used instead. The concentration of other MMPIs were adjusted to at least three-fold their IC_{50} or K_i values. All MMPIs were purchased from Calbiochem-Novabiochem Corp., San Diego, CA, USA.

Protein extraction

Subcellular fractions were prepared according to a modified method of Dignam[71] in which 2 μM instead of 0.2 mM. phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemicals, St. Louis, MO, USA) together with other protease inhibitors were used. All extraction buffers contained a cocktail of protease inhibitors including 1 μg/ml of aprotinin, 1 μg/ml of leupeptin, 10 μM iodoacetamide, 1 μM benzamidine, and 2 μM PMSF as previously described.[72] Briefly, cells were grown to 70–80% confluence, scraped off the plates, washed once with PBS and once with hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 2 μM PMSF, and 0.5 mM DTT), and chilled in hypotonic buffer in ice for 10 min. Cells were lysed in a 2-ml glass homogenizer with a loose-fitting pestle (type B). Lysates were separated into nuclei (pellet) and other components (supernatant) by centrifugation at 230 $\times$ *g* in a Tommy desktop centrifuge. The nuclear pellet was resuspended in 0.2 $\times$ volume of the original cell pellet of 0.02 M KCl followed by an equal volume of 1.2 M KCl and extracted by gentle agitation for 30 min at 4°C. The supernatant (containing cytoplasmic proteins and membrane fragments) and the nuclear extract were clarified at 20 000 $\times$ *g* at 4°C for 45 min. The protein concentration of each extract was determined by the Bradford method according to the manufacturer's instructions (BioRad, Hercules, CA, USA). Extracts were divided into 50-μl aliquots and stored at -80°C until analyzed.

Western blot analysis

Total, cytoplasmic, and nuclear cell extracts were analyzed by Western blot as described previously.[73] Briefly, 10–25 μg of each subcellular protein or 75–100 μg/ml of total cellular proteins were resolved by SDS-PAGE in a BioRad Mini Protein III gel apparatus, transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA, USA), and blocked with 3% nonfat dry milk in Tris-buffered saline consisting of 20 mM Tris base, 137 mM sodium chloride, and 0.05% Tween 20, pH 7.6 (TBST) for 1 h at room temperature or overnight at 4°C. This was followed by incubation with the desired dilution of the primary antibodies in TBST containing 1% nonfat dry milk for 1 h at room temperature or overnight at 4°C. Excess antibody was washed off twice for 15 min with 20 ml of TBST, and the membrane was incubated with a 1 : 3000 dilution of horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Membranes were washed as above. The enhanced chemiluminescence (ECL) reagents that detect bands of reactive proteins were used as described by the manufacturer (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). Chemiluminescent signals were captured on Kodak Bio-MAX MR X-ray films, which were obtained from Sigma (St. Louis, MO, USA).

Flow cytometry

To detect TNFα-induced DNA fragmentation, cells were prepared as described previously.[32] Briefly, cells were treated with the appropriate agents as described elsewhere in this paper. At least 5×10^5 cells were removed from the plate, pelleted at $900 \times g$ in a swing bucket centrifuge, washed once with PBS, and resuspended in 0.75 ml of a 50 μg/ml solution of propidium iodide, comprising 3 mM sodium citrate and 0.1% Triton X-100. Cell suspensions were incubated at 4°C for 12 h. They were then analyzed for DNA fragmentation and cell cycle stage by an Epics Profile flow cytometer (Coulter Corp., Miami, FL, USA). The percentage of apoptotic cells was measured by the percentage of subdiploid cells.

Biosynthetic protein labeling

The question of whether compound 5a nonspecifically inhibits protein synthesis was addressed by labeling newly synthesized proteins with radioactive methionine.. Briefly, 9D3S cells were seeded in complete RPM1640 medium at 2.5×10^5 per well of a 12-well plastic plate overnight. The medium was removed by centrifugation, and cells were washed once in 5 ml of PBS and resuspended in labeling medium consisting of DMEM without methionine, supplemented with 5% dialyzed fetal bovine serum. At time zero, cells were divided into five groups. The first group was left untreated, the second and fourth groups were treated with DMSO, and the third and fifth groups were treated with 200 nM (2R)-2-[(4-biphenylsulfonyl)amino]-3-phenylproprionic acid (compound 5a) (Calbiochem-Novabiochem Corp., San Diego, CA, USA). After 3 h, the fourth and fifth groups were treated with 20 ng/ml of TNFα. An hour later, the medium was supplemented with 10 μCi of L-[^{35}S]methionine per well. Incubation was continued for 2 h. At 6 h, cells were harvested, and the unincorporated radiolabel was removed by centrifugation. Cell pellets were washed once with ice-cold PBS and lysed in 200 μl of radioimmune precipitation assay (RIPA) buffer (PBS containing 0.03 M sodium citrate and 0.5% NP-40). The protein concentration was determined by the Bradford method. Aliquots of 5 μg of protein were precipitated in 500 μl of ice-cold 20% trichloroacetic acid (TCA) on ice for 30 min. The precipitate was collected on glass microfiber filters (Whatman International Ltd, Maidstone, England) and washed with 3 ml of 10% ice-cold TCA, and the radioactivity was determined in a scintillation counter.

Colorimetric assay for activated caspases

To detect activated caspase 8, cells were treated with 0.2%. DMSO, 200 nM 5a, and 20 ng/ml TNFα for the desired time and then processed using activated caspase detection kits according to the manufacturer's instructions (Biosourse International, Camarillo, CA, USA). Briefly, 2.5×10^6 cells were seeded per 100-mm plastic dish overnight. Control cells were pretreated with 0.2% DMSO, while others were pretreated with 200 nM 5a for the desired time and washed once with 5 ml of PBS, and the pellet was resuspended in 50 μl of cold lysis buffer. Aliquots of 100 μl were assayed for caspase 8 using the synthetic substrate Ile-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin (IETD-AFC), according to the manufacturer's instruction. Caspase activity was measured by determining the absorbance at 400 nm using Ultrospec 3000pro spectrophotometer (Amershampharmacia Biotech, Cambridge, England).

Time course experiment

Breast cancer cells (9D3S) were seeded at 3×10^5 per well of a six-well plate in 3 ml of medium and incubated overnight. Cells in 12 wells (26-well plats) were treated with 0.2% DMSO and an equal number of wells were treated with 200 nM 5a. Cells were removed at 0, 0.5, 1, 2, and 4 h following treatment, washed once with PBS, and blocked with 3% BSA (Pierce, Rockford IL, USA) for 1 h at 4°C. This was followed by incubation with a 1 : 250 dilution of rabbit anti-human TNFR1 for 1 h at 4°C, and 2 washes in 1 ml of PBS. Cells were then treated with a 1 : 2500 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 1 h at 4°C, washed twice as before, resuspended in 500 μl of PBS, and analyzed by FACS.

A similar experiment was set up with 3×10^6 cells per 100×20 mm^2 dish for analyzing protein expression. Cytosolic and membrane-associated proteins were extracted according to the Dignam method.[71] Cells were removed at the same time points as described above.

MTP

To determine whether the mechanism by which compound 5a enhances TNFα-induced apoptosis involves primarily the mitochondrion, we examined cells for changes in the MTP according to the method of Rottenberg and Wu.[74] Briefly, 9D3S cells seeded at 2.5×10^5 per well in six-well plates in 3 ml of normal medium in duplicate were incubated overnight. The wells were numbered 1–10. At time zero, cells in wells 3–4 and 7–8 were treated with 0.2% DMSO, while those in wells 5–6 and 9–10 were treated with 200 nM 5a. At time 2 h, cells in wells 7–10 were treated with 20 ng of TNFα. At time 4 h, cells were harvested, pelleted at 1000 rpm for 5 min, and resuspended in 200 μl of PBS in 5 ml polystyrene tubes. We then treated cells in tubes 3–10 (i.e. all except the untreated cells) with 25 nM 3,3-dihexyloxacarbocyanine iodide ($DiOC_6$) solution for 20 min at 37°C in the dark. The reaction mixture was diluted with 400 μl of PBS and analyzed for a decrease in the mitochondrion transmembrane potential by FACS using excitation wavelength at 484 nm and maximum emission wavelength at 501 nm.

Animal study

Athymic BALB/c nude mice (6–8 weeks old) were purchased from the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD, USA). They were housed under pathogen free conditions. The mice were allowed to acclimate for 2 weeks. Exponentially growing A375SM melanoma cells were harvested by trypsinization, washed once with 10 ml of PBS, and injected into 30 athymic nude mice subcutaneously at 5×10^5 cells in $200 \times \mu$l of PBS per animal as described previously.[41] After 1 week, when mice had palpable tumors, three treatment groups consisting of 10 mice each were selected. The treatment groups consisted of nontreated control, 1 mg 5a, 10 μg TRAIL and 1 mg 5a plus 10 μg of TRAIL. One week after tumor injection, mice were injected intraperitoneally with 200 μl of 5a suspended in 25% DMSO in PBS or with 200 μl of TRAIL dissolved in PBS, in the tail vein 1 h after the injection of 5a according to the treatment grouping. Daily injection was performed for 7 days. The length and width of tumors were measured with calipers every 2 days, and the volume was estimated by the formula: the square of the width x length (w^2l). As a result of the limited available reagents, we could not sustain the treatment beyond 7 days.

After 7 days of treatment, the mice were kept for 11 more days for further observations. Tumor volumes continued to be measured as before. On day 18, all mice were sacrificed, and nonnecrotic tumors were removed from five mice from each treatment group. Representative sections were processed and stained for apoptotic cells using the TUNEL method, and for microvessel formation using anti-CD31 IgG as described previously.[75]

The data on tumor growth following different treatments were analyzed for statistical significance by the *t*-test (GraphPad Software Inc). The confidence limit was set at 0.95.

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