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Small-molecule activation of the TRAIL receptor DR5 in human cancer cells

Gelin Wang[1]*, Xiaoming Wang[2], Hong Yu[1], Shuguang Wei[1], Noelle Williams[1], Daniel L Holmes[1], Randal Halfmann[1], Jacinth Naidoo[1], Lai Wang[3], Lin Li[3], She Chen[4], Patrick Harran[5], Xiaoguang Lei[2,4]* & Xiaodong Wang[4]*

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) activates apoptosis through the death receptors DR4 and DR5. Because of its superior safety profile and high tumor specificity compared to other TNF family members, recombinant soluble TRAIL and agonistic antibodies against its receptors are actively being developed for clinical cancer therapy. Here, we describe the identification and characterization of the small molecules that directly target DR5 to initiate apoptosis in human cancer cells. The activity was initially discovered through a high-throughput chemical screen for compounds that promote cell death in synergy with a small-molecule mimetic of Smac, the antagonist for inhibitor of apoptosis protein. Structure-activity relationship studies yielded a more potent analog called bioymifi, which can act as a single agent to induce DR5 clustering and aggregation, leading to apoptosis. Thus, this study identified potential lead compounds for the development of small-molecule TRAIL mimics targeting DR5 for cancer therapy.

Apoptosis in mammalian cells is executed by the proteolytic activities of caspases that are activated in response to apoptotic stimuli via two distinct signaling pathways[1]. These pathways have different initiator caspases that cleave and activate a common set of executioner caspases, including caspases 3, 6 and 7. The intrinsic (or mitochondrial) pathway of apoptosis is activated in response to DNA damage and other stress signals within the cell. Cytochrome *c* is consequently released from mitochondria into the cytosol and binds its adaptor protein, Apaf-1, to form the large 'apoptosome' complex, which recruits and activates caspase-9 (ref. 2). The extrinsic pathway is triggered from the exterior of the cell by members of the TNF superfamily, which bind and activate their corresponding death receptors[3–5]. For example, binding of TRAIL to the extracellular domain (ECD) of the death receptors DR4 (TRAIL-Receptor 1 (TRAIL-R1)) and DR5 (TRAIL-R2) promotes clustering of these receptors. The ligand–receptor complex in turn engages the adaptor protein Fas-associated death domain (FADD) via its cytoplasmic death domains. FADD then recruits the initiator caspase-8 through its N-terminal death-effector domain to form a death-inducing signaling complex (DISC)[6–9].

Caspase activation is tightly regulated by members of the inhibitor of apoptosis protein (IAP) family, such as XIAP, cIAP1 and cIAP2 (refs. 10–12). In response to death signals, a second mitochondria-derived activator of apoptosis (Smac; also called Diablo) is released from the mitochondria into the cytosol, where it binds the Bir domains of XIAP to relieve its inhibition of caspases and the Bir domains of cIAPs to induce their degradation. The four N-terminal residues (alanine, valine, proline and isoleucine) of Smac are sufficient for this interaction and have been mimicked using a peptidomimetic approach to generate cell-permeable small molecules that function similarly to the Smac protein[13–15]. Previously, we demonstrated that Smac mimetics not only effectively kill cancer cell lines with an autocrine TNFα signal but also substantially increase the cell-killing efficiency of TRAIL[13–15].

Selective activation of the apoptotic pathway provides tremendous therapeutic potential for cancer treatment[16]. Various strategies are currently under active clinical investigation, including the use of Bcl-2 inhibitors, Smac mimetics or IAP antagonists and death-receptor agonists[17]. Among the death receptor–targeted agents, the therapeutic use of TNFα and agonistic CD95-specific antibodies has been hampered by their toxic side effects, particularly a severe inflammatory response through NF-κB activation[3,18]. However, mounting evidence from clinical trials has indicated that targeting DR4 and DR5 with agonist antibodies or recombinant TRAIL selectively eliminates tumor cells while sparing normal cells[19,20]. Although targeting TRAIL pathways may be promising as a safe anticancer therapy, the clinical use of TRAIL itself is limited by its fast turnover rate in the blood. The efficacy of the antibody may also be limited by the dimeric nature of the antibody, whereas death receptors are activated as a trimer[19]. In the current study, we uncovered small molecules that specifically bind and activate DR5.

RESULTS

Identification of a Smac mimetic synergist

We screened a collection of ~200,000 drug-like compounds to identify agents capable of promoting cell death in combination with a Smac mimetic in T98G human glioblastoma cells (**Supplementary Results**, **Supplementary Fig. 1**). The initial screen identified 2,068 compounds that scored below 0.70 of normalized data, which indicated more than 30% dead cells. We chose these active compounds to generate new master plates and retested whether Smac mimetic sensitized the cells to the compounds. T98G cells were treated with three concentrations (15 μM, 5 μM and 1.7 μM) of compounds in the absence or presence of 1 μM Smac mimetic. In combination with Smac mimetic, 22 compounds at the 15-μM concentration and 2 compounds at the 5-μM concentration reduced cell viability to less than 50% of the viability resulting from treatment with the respective compound alone. Of the 24 identified hits, the majority

[1]Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas, USA. [2]School of Pharmaceutical Science and Technology, Tianjin University, Tianjin, China. [3]Joyant Pharmaceuticals, Dallas, Texas, USA. [4]National Institute of Biological Sciences, Beijing, China. [5]Department of Chemistry and Biochemistry, University of California–Los Angeles, Los Angeles, California, USA. *e-mail: gelin.wang@utsouthwestern.edu, leixiaoguang@nibs.ac.cn or wangxiaodong@nibs.ac.cn

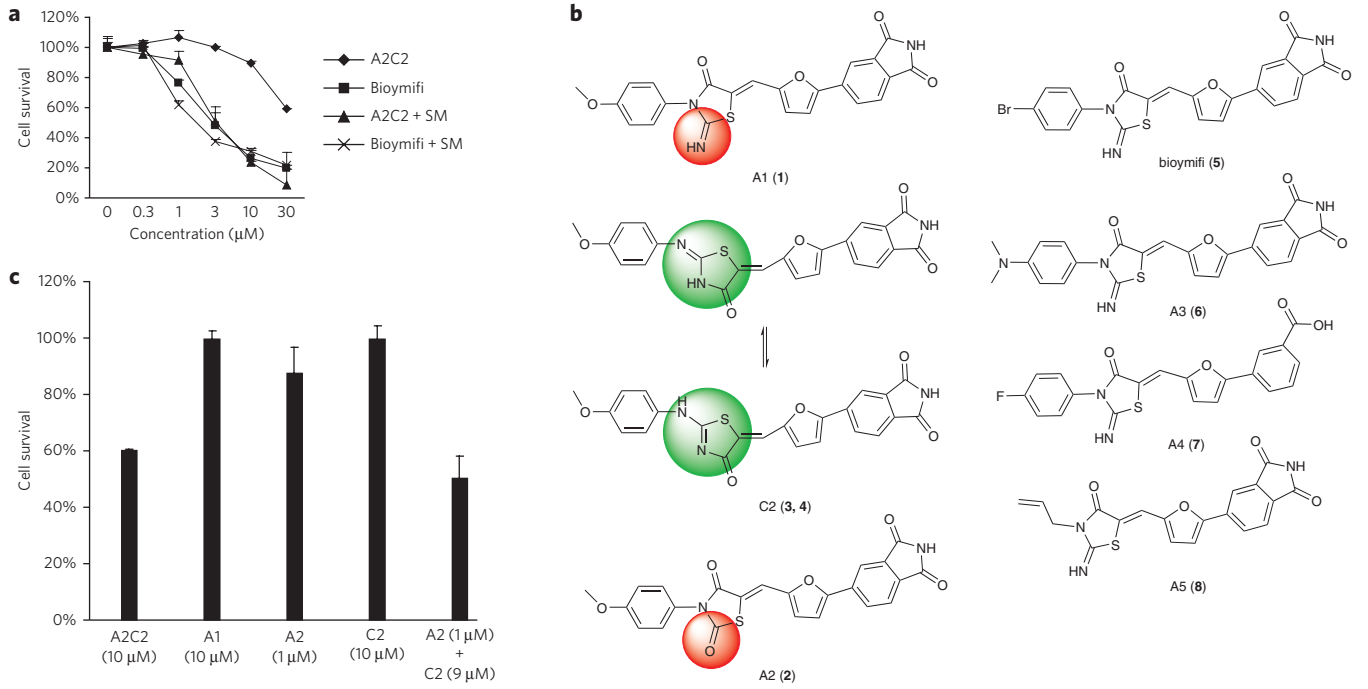

Figure 1 | Identification and chemical elucidation of the Smac synergists from the chemical library screen. (**a**) Dose-response curves of bioymifi and A2C2 in T98G cells were plotted as a function of A2C2 or bioymifi concentration. Human glioblastoma (T98G) cells were treated with various concentrations of A2C2 or bioymifi alone or in combination with 1 μM Smac mimetic (SM) for 48 h. The corresponding cell survival is normalized to the treatment without A2C2 or bioymifi. The data shown here are representative of four independent experiments. (**b**) The chemical structures of the compounds used in this study. A1 represents the initially assigned structure of A2C2. A2 and C2 are components found in A2C2. (**c**) A cell-survival assay was performed to assess the cell-killing activity of A2C2 and the individual components of A2C2. Error bars in **a** and **c** represent the s.d. of experimental duplicates.

were eliminated because of cytotoxicity. We chose two 5-μM hits and two 15-μM hits that showed relatively low cytotoxicity for retesting in a dose-response analysis. Among them, compound A2C2 showed the most robust synergy with the Smac mimetic in inducing cell death (**Fig. 1a**). Notably, A2C2 was also the only hit in an additional screen using 5 μM of various compounds in triplicate plates (**Supplementary Fig. 1**).

To validate the chemical identity of A2C2, we synthesized compound A1 (**1**) according to the originally assigned structure of A2C2 (**Fig. 1b**). To our surprise, the resynthesized A1 had little or no effect on cell survival (**Fig. 1c**), suggesting that A2C2 has a chemical identity that is different from the previous structural assignment. Indeed, through extensive NMR and LC/MS analyses, we confirmed that A2C2 was a mixture of three compounds (**Supplementary Note 1**). To elucidate the chemical components of A2C2, we performed the chemical synthesis and purification of A2C2 and its derivatives. A2 (**2**) and C2, with a molar ratio of 1:9, represented the true identity of A2C2. The chemical structures of A2 and C2 were finally unambiguously confirmed by the independent chemical synthesis of A2 and C2 and the corresponding spectroscopic analysis. Notably, C2 (**3** and **4**) was determined to be a mixture of two tautomers on the basis of the results of NMR studies showing the coalescence of signals at higher temperatures (**Fig. 1b** and **Supplementary Note 1**). In a cell-based assay, either 10 μM C2 or 1 μM A2 alone barely affected cell survival, whereas a mixture of 1 μM A2 and 9 μM C2 showed potent activity comparable to 10 μM of the original A2C2 stock (**Fig. 1c**). Taking these findings together, we concluded that A2C2-induced cell death is attributable to the combined activity of compounds A2 and C2.

Bioymifi induces caspase-8–dependent apoptosis

We further applied the structure-activity relationship (SAR) approach to search for a functional analog or derivative of A2C2 with a single and well-defined chemical entity (**Supplementary Table 1**). Among two dozen synthesized analogs, the majority had little effect on the viability of T98G cells. However, one related compound with a bromo substituent, (*Z*)-5-(5-[(3-[4-bromophenyl]-2-imino-4-oxothiazolidin-5-ylidene)methyl]furan-2-yl)isoindoline-1,3-dione, which we named bioymifi, showed enhanced toxicity toward T98G cells. In comparison with A2C2, bioymifi (**5**) was able to promote cell death without the need for the Smac mimetic in T98G cells (**Fig. 1a**). In addition to testing bioymifi's effects on T98G cells, we tested its effects in other human cancer cell lines, including the lung cancer cell lines H460 and H1155, the cervical cancer cell line HeLa, the osteosarcoma cell line U2OS, the pancreatic carcinoma cell line Miapaca and the colon cancer cell line HT29. All of these cell lines showed single-agent sensitivity to bioymifi (**Supplementary Fig. 2**). We therefore focused our further investigations on bioymifi-induced cell death.

The characteristic caspase requirements distinguish apoptosis from other forms of cell death. The pan-caspase inhibitor Z-VAD effectively blocked the cell death induced by cotreatment with bioymifi and the Smac mimetic (**Fig. 2a**), which indicates that bioymifi promotes apoptotic cell death. A2C2 plus Smac mimetic, TRAIL plus Smac mimetic or bioymifi plus Smac mimetic activated both caspase-3 and caspase-8, as evidenced by the proteolytic processing of procaspase-3, procaspase-8 and a caspase-3 substrate, poly (ADP-ribose) polymerase (PARP). Notably, at a 10-μM concentration, bioymifi induced processing of caspase-3 into smaller fragments. Z-VAD inhibited these caspase-mediated cleavages (**Fig. 2b** and **Supplementary Fig. 3**). Caspase-3 activation in the bioymifi-treated cells was also measured by monitoring the cleavage of a fluorogenic substrate of caspase-3 in a time-course experiment. We showed that caspase-3 was rapidly activated as early as 2 h after bioymifi treatment of T98G cells. The caspase-3 activity was markedly increased after 8 h of treatment (**Fig. 2c**).

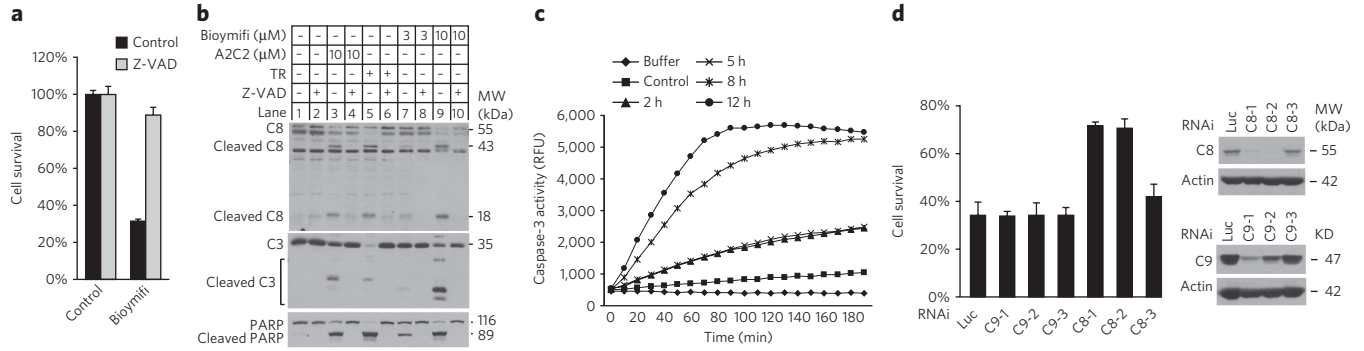

Figure 2 | A functional analog of A2C2, bioymifi induces apoptosis. (**a**) Bioymifi-induced cell death is blocked by caspase inhibition. T98G cells were treated as indicated for 48 h; 20 μM Z-VAD was added 2 h before treatment with 5 μM bioymifi plus Smac mimetic. (**b**) Caspase-3 (C3) and caspase-8 (C8) activation after bioymifi treatment as indicated by western blotting with antibodies specific for caspase-8, caspase-3 and PARP. MW, molecular weight. (**c**) Kinetic caspase-3 activation in T98G cells by bioymifi was measured in the fluorogenic assay. RFU, relative fluorescence units. (**d**) Left: bioymifi-induced cell death is blocked by knockdown of caspase-8 (C8), but not caspase-9 (C9). Three siRNA oligos targeting different regions of the mRNA of C8 or C9 are numbered 1, 2 and 3. Right: western blot analysis was performed to assess the knockdown efficiency of the siRNAs. Actin was used as a loading control throughout this study. In **a** and **d**, error bars represent the s.d. of experimental duplicates.

To determine which apoptotic pathway is activated by bioymifi, we searched for the apical caspase that initiates cell death. We knocked down caspase-8 or caspase-9 in T98G cells, each with three independent siRNA oligos targeting the coding region (**Fig. 2d** and **Supplementary Fig. 3**). Knockdown of caspase-8, but not caspase-9, rescued cells from bioymifi- and Smac mimetic–induced apoptosis. Furthermore, the three oligos decreased the amount of caspase-8 to different degrees, and knockdown efficiency correlated with the degree of apoptosis inhibition, indicating that caspase-8 and the related extrinsic apoptotic pathway are essential for bioymifi-induced cell death.

Bioymifi induces apoptosis via death receptor DR5

We then performed a candidate RNAi screen to search for the responsible death receptor from a small siRNA library including the known members of the death domain–containing TNF superfamily receptors, including TNFR1, DR3, DR4, DR5, DR6 and Fas. Notably, depletion of TRAIL-R2 (also known as DR5) rescued the T98G cells from bioymifi- or A2C2-induced cell death, whereas knockdown of other receptors had little effect on bioymifi- or A2C2-mediated apoptosis (**Fig. 3a**). Knockdown of DR5 but not DR4 blocked TRAIL- plus Smac mimetic–induced death (**Fig. 3b**), suggesting that DR5 is the predominant receptor for TRAIL in T98G cells. As expected, knockdown of TNFR1 or Fas blocked cell death induced by TNFα or by FasL with cotreatment with Smac mimetic, respectively (**Fig. 3b**), indicating that good knockdown efficiency was achieved toward those death receptors.

To validate the requirement of DR5 in bioymifi-induced apoptosis, we knocked down DR5 with three different siRNA oligos in T98G cells. DR5 but not DR4 expression was selectively silenced by these three siRNA oligos, as revealed by quantitative RT-PCR, and the knockdown efficiency correlated with the extent of apoptosis inhibition (**Fig. 3c** and **Supplementary Fig. 4**), confirming that bioymifi-induced apoptosis is mediated primarily by DR5.

Both bioymifi and TRAIL induce DR5-dependent death pathways. To dissect the signaling events leading to apoptosis by bioymifi, we first examined whether bioymifi induces a DR5-dependent proximal DISC as TRAIL does. The lack of a validated DR5-specific antibody prompted us to engineer a cell line stably expressing C-terminal Flag-tagged DR5. This was carried out in the human lung cancer cell line HCC15, which was sensitive to TRAIL alone. The engineered stable cells showed a responsiveness to bioymifi similar to that of the parental cells (**Supplementary Fig. 5**). We pulled down the DR5 immunocomplex from these cells by using a Flag-specific antibody. As shown in **Figure 3d** and **Supplementary Figure 6**, when treated with bioymifi, FADD was rapidly recruited to the DR5 complex within 1.5 h. Caspase-8 was detected in the complex slightly later.

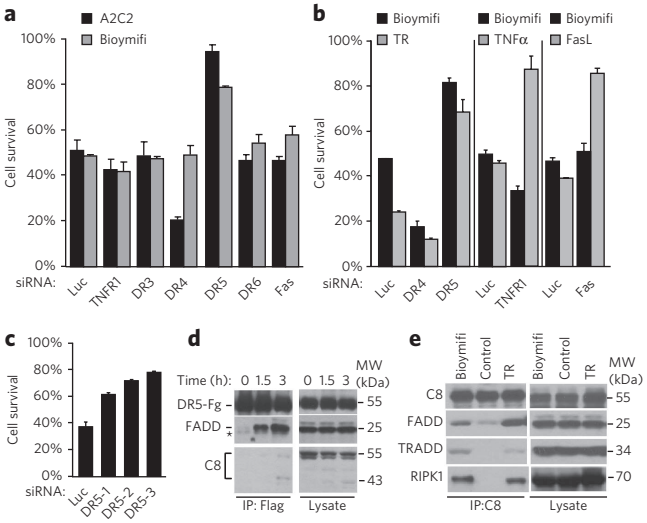

Figure 3 | Bioymifi-induced apoptosis occurs through a DR5-dependent extrinsic pathway. (**a**) A candidate siRNA library screen for a mediator of the bioymifi-activated extrinsic apoptosis pathway. T98G cells were transfected with siRNA for the indicated death receptor (DR) or luciferase (Luc). (**b**) T98G cells were transfected with siRNA for DR4 or DR5, TNFR1, or Fas. After 48 h of transfection, the cells were treated with 5 μM bioymifi or 100 ng ml^{-1} TRAIL (TR) plus Smac mimetic (SM), 5 μM bioymifi or 20 ng ml^{-1} TNFα plus SM, or 5 μM bioymifi or 50 ng ml^{-1} FasL plus SM for 24 h. (**c**) T98G cells were transiently transfected with multiple individual siRNAs for DR5. Three siRNA oligos targeting different regions of DR5 mRNA are numbered 1, 2 and 3. (**d**) Bioymifi induces formation of proximal DISC containing DR5, FADD and caspase-8. Stable cells expressing Flag-tagged DR5 (DR5-Fg) were treated with 10 μM bioymifi plus Smac mimetic for the indicated time. Cell lysates were immunoprecipitated (IP). The immune complexes and the cell lysates were analyzed by western blotting using the indicated antibodies. Asterisk indicates the band for the IgG light chain. (**e**) Bioymifi induces the formation of a complex containing caspase-8, FADD, TRADD and RIPK1. The protein amounts of caspase-8, FADD, TRADD and RIPK1 in the immunocomplex or the cell lysates were measured by western blot analysis. In **a**–**c**, error bars represent the s.d. of experimental duplicates.

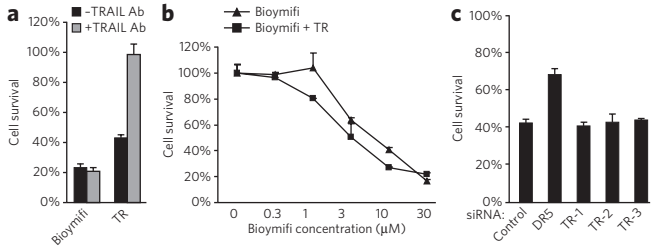

Figure 4 | Bioymifi promotes cell death independent of TRAIL. (**a**) The neutralizing antibody (Ab) for TRAIL blocks cell death induced by TRAIL (TR) but not by bioymifi. T98G cells were preincubated with 10 μg ml^{-1} TRAIL-specific antibody for 1 h and treated with 5 μM bioymifi or with 10 nM recombinant TRAIL plus Smac mimetic (SM) for 48 h. (**b**) T98G cells were cotreated with SM and various concentrations of bioymifi or in combination with 100 ng ml^{-1} TRAIL for 48 h. The corresponding percentage cell survival is normalized to that measured in treatment without bioymifi. (**c**) Depletion of TRAIL does not inhibit bioymifi-induced cell death. Three siRNA oligos targeting different regions of TRAIL mRNA are numbered 1, 2 and 3. In **a**–**c**, error bars represent the s.d. of experimental duplicates.

We next used a caspase-8 antibody to immunoprecipitate the caspase-8–activating complex from bioymifi- and Smac mimetic–treated cells. Z-VAD was added to enhance the complex formation[14]. FADD, TRADD and RIPK1 were present in the caspase-8 immunocomplex. The basal amounts of FADD in the control sample probably represent the IgG light chain, which runs the same size as FADD in western blot analysis. TRAIL also promoted DISC recruitment of FADD, TRADD, RIPK1 and caspase-8 (**Fig. 3e** and **Supplementary Fig. 6**). Because TRADD is part of a TRAIL-stimulated DISC, we examined the importance of TRADD in TRAIL-induced apoptosis in mouse embryonic fibroblasts (MEFs) prepared from embryos of TRADD knockout mice. As shown in **Supplementary Figure 7a**, when the cells were treated with mouse TRAIL, MEFs from the wild-type animal committed to death, whereas death was attenuated in cells from TRADD knockout embryos, suggesting that TRADD has a positive role in TRAIL-induced apoptosis. Consistent with the role of FADD as the primary adaptor protein for TRAIL signaling, TRAIL-induced cell death was abolished in cells from FADD knockout embryos. Notably, transient knockdown of RIPK1 led to cell death (**Supplementary Fig. 7b**,**c**), suggesting that RIPK1 has a pro-survival role and is not required for DISC formation in T98G cells. Recruitment of RIPK1 may represent the formation of the so-called ripoptosome, a similar complex induced by the Smac mimetic independently of TNF death ligands[21].

Bioymifi-induced apoptosis is independent of TRAIL

DR5 activation is triggered upon binding to its cognate ligand TRAIL. To examine whether TRAIL mediates bioymifi-induced cell death, we pretreated cells with TRAIL-neutralizing antibody before applying bioymifi or soluble recombinant TRAIL. TRAIL antibody inhibited the death-inducing effect of TRAIL but not that of bioymifi (**Fig. 4a**), suggesting that bioymifi does not induce TRAIL secretion to initiate cell death. We then treated cells with bioymifi plus TRAIL. TRAIL did not have any competitive effect on bioymifi-induced cell death (**Fig. 4b**). In addition, knockdown of TRAIL by siRNA could not block the killing activity of bioymifi (**Fig. 4c** and **Supplementary Fig. 8**). Taken together, these data indicate that bioymifi acts on DR5 independently of TRAIL.

Bioymifi directly binds DR5

To identify the cellular target of bioymifi in triggering DR5-dependent apoptosis, we first tested whether bioymifi directly acts on and activates DR5. We generated recombinant DR5 and DR4 proteins containing only the ECD (**Supplementary Fig. 9**). Isothermal titration calorimetry (ITC) was used to determine the direct molecular interaction between bioymifi and ECDs. As shown in **Figure 5a**,**b**, bioymifi bound the ECD of DR5 with a K_d of 1.2 μM but showed little binding affinity to the DR4 ECD. We observed that bioymifi has poor solubility in buffer solutions. Cloudy yellow precipitates were obvious at concentrations higher than 10 μM. The absorption spectra of the compound in the solution showed maximal absorbance at 420 nm. Measured $D_{420\,nm}$ values as a function of compound concentration yielded a nonlinear curve (**Supplementary Fig. 10**). Therefore the calculated molar ratio (N = 0.1–0.2) of the DR5 ECD versus bioymifi from the ITC assay might not be accurate. The dimethylamino derivative A3 (**6**) retained its binding capability, which was indistinguishable from that of bioymifi or A2C2, but showed substantially lower cytotoxic activity (**Supplementary Table 2**). Compounds with other structural modifications (A4 (**7**) and A5 (**8**)) had lower binding affinities for the DR5 ECD (that is, a K_d of approximately 5 μM; **Supplementary Table 2**). In fact, A4 was chosen from the chemical library owing to its structural similarity to A1, but it did not have any effect on cell survival in either screen or upon retesting. In agreement with this result, caspase-8 cleavage was not detectable in A3-, A4- or A5-treated cells (**Fig. 5c** and **Supplementary Fig. 11**). These results indicate that the binding of the compound to the receptor was not sufficient for DR5 and caspase-8 activation.

Bioymifi promotes DR5 aggregation and activation

To investigate the mechanism for DR5 activation by A2C2 and/or bioymifi, we tested whether treatment with the compound altered the biochemical property of DR5 *in vitro*. We purified full-length Flag-tagged DR5 from cells with anti-Flag M2 Affinity Gel. We used semidenaturing detergent agarose gel electrophoresis (SDD-AGE), which was previously used in the detection of prion-like structures[22] and the formation of the large MAVS complex resulting from viral infection[23]. In 1.5% semidenaturing detergent agarose gel, a small fraction of DR5-Flag formed the SDS- and β-mercaptoethanol (β-ME)-resistant high-molecular-weight aggregates after treatment with bioymifi or A2C2 for 1 h, whereas treatment with DMSO or inactive analog A3 did not have such an effect. DR5 aggregates that appeared in the bioymifi-treated sample were larger than those in the A2C2-treated sample (**Fig. 6a** and **Supplementary Fig. 11**). To determine whether the ECD domain mediates DR5 aggregation induced by bioymifi, we incubated recombinant DR5 ECD and DR4 ECD with increasing concentrations of bioymifi. Bioymifi stimulated the formation of high-molecular-weight DR5 homo-oligomeric

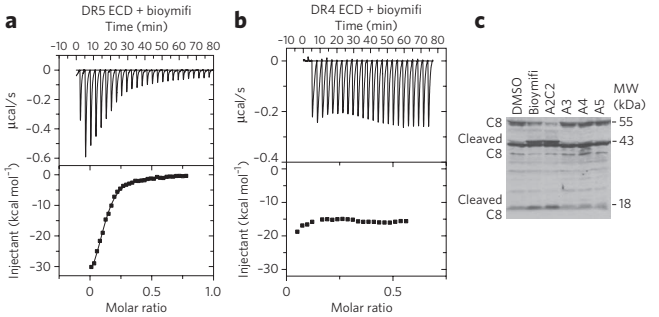

Figure 5 | Bioymifi selectively binds the ECD of DR5. (**a**,**b**) The binding assays were performed in an ITC assay. A total of 120 μM recombinant DR5 ECD (**a**) or DR4 ECD (**b**) was titrated into the sample cell containing 30 μM bioymifi (model: one site; χ^2/degree of freedom = 1.283 × 10^5; N = 0.118; K = 8.82 × 10^5; DH = −4.087 × 10^4; DS = −112). The data shown here are representative of three independent experiments. (**c**) T98G cells were treated with DMSO, 10 μM bioymifi and A2C2, A3, A4, or A5 plus Smac mimetic for 16 h. Cell lysates were subjected for immunoblot with caspase-8 (C8)-specific antibody.

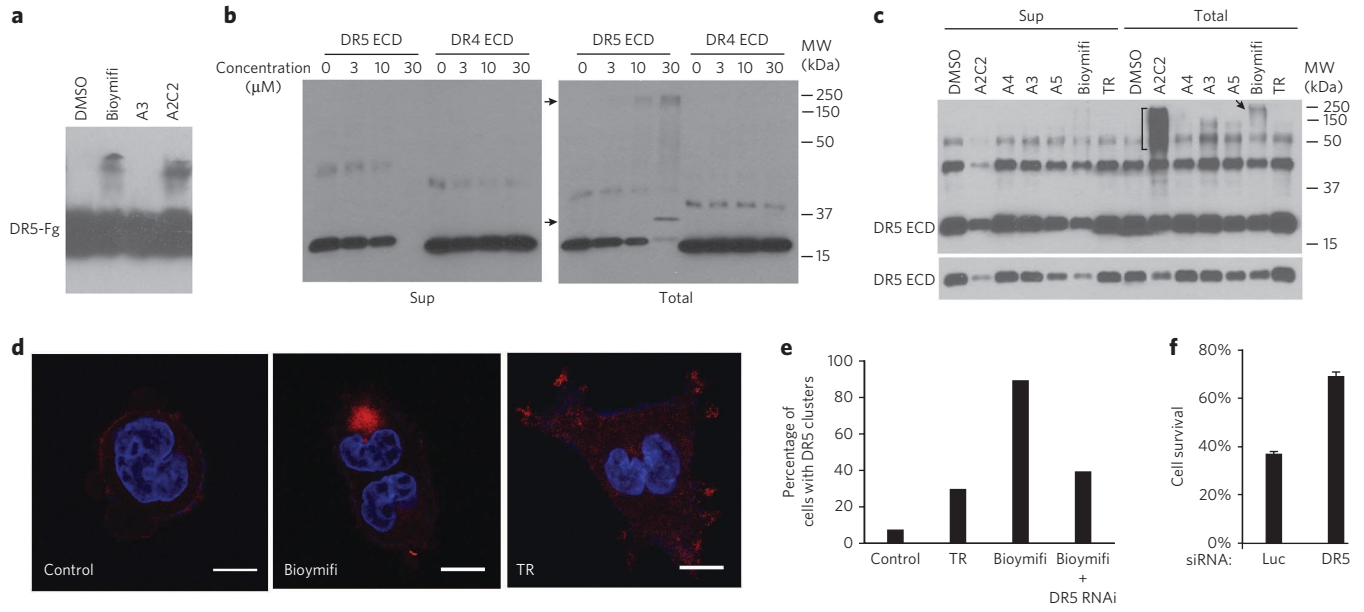

Figure 6 | Bioymifi and A2C2 promote DR5 aggregation and activation. (**a**) Purified full-length DR5-Flag (DR5-Fg) from cells was incubated with DMSO, 10 μM bioymifi, A2C2 or A3 for 1 h. The reaction mixtures were analyzed by immunoblotting following SDD-AGE. (**b**) One micromolar DR5 ECD or DR4 ECD was incubated with bioymifi as indicated. The total lysate or supernatant (sup) of the reaction mixtures was analyzed by western blotting with His-specific antibody. The slow-migrating oligomeric forms of DR5 are indicated by arrows. (**c**) A total of 0.5 μM DR5 ECD was incubated with DMSO, 10 μM A2C2, A4, A3, A5, bioymifi or 2.5 μM TRAIL (TR). The total lysate or supernatant of the reaction mixtures was analyzed using western blotting with His-specific antibody. The oligomeric forms of DR5 are indicated by a square bracket and an arrow in the A2C2- and bioymifi-treated samples, respectively. The lower panel shows only the monomeric form of DR5 ECD on the same membrane upon a shorter exposure. (**d**) Bioymifi causes DR5 aggregation in cells. DR5-Flag–expressing cells were incubated with DMSO (control), 10 μM bioymifi or 1 μg ml^{-1} TR. The cells were immunostained with Flag-specific antibody (red) and mounted in a DAPI-containing (blue) medium. Scale bars, 10 μm. The results are representative of three independent experiments. (**e**) Depletion of DR5 reduces the receptor clustering induced by bioymifi. The percentage of the cells showing clustering DR5 are quantified (n = 200). (**f**) Depletion of DR5 decreases bioymifi sensitivity in DR5-overexpressing stable cells. Luc, luciferase. In **e** and **f**, error bars represent the s.d. of experimental duplicates.

complexes in a dose-dependent manner but had little effect on the DR4 ECD (**Fig. 6b** and **Supplementary Fig. 11**). These high-molecular-weight aggregates were resistant to SDS solubilization and β-ME breakage and could be collected by centrifugation. We applied this aggregation assay to the compounds listed in **Supplementary Table 2**. As detected by SDS-PAGE (**Fig. 6c** and **Supplementary Fig. 11**), the active compounds (A2C2 and bioymifi) induced the formation of DR5 ECD aggregates. These results suggest that the ECD domain alone is sufficient to form DR5 aggregates in response to bioymifi or A2C2. In contrast, the inactive compounds A3, A4 and A5 had little effect in promoting DR5 ECD aggregates (**Fig. 6c**). Thus, DR5 ECD aggregate formation correlated well with caspase-8 activation and cell-killing activity of the tested compounds (**Figs. 5c** and **6c** and **Supplementary Table 2**).

To examine DR5 receptor clustering upon bioymifi binding in cells, we used size-exclusion chromatography to analyze the high-molecular-weight aggregates in DR5-expressing stable cells. DR5 can undergo preligand association and elute in an oligomer form (**Supplementary Fig. 12**). Bioymifi stimulated further clustering of DR5 as it was shifted to higher-molecular-weight fractions. A small amount of FADD and caspase-8 eluted together with DR5 in the higher-molecular-weight fractions. Notably, caspase-8 in the stimulated cells was cleaved to smaller activator fragments in the absence and presence of Smac mimetic. Consistent with these observations, imaging studies of stable cells expressing DR5-Flag showed that treatment with bioymifi led to the formation of visible DR5 aggregates. Prominent foci structures were formed in the cytosol in approximately 90% of the cells in 1 h but not in control cells. The size of foci varied from 1 μm to 4 μm. TRAIL induced smaller DR5 clusters (0.3–1 μm) at the membrane in around 30% of cells (**Fig. 6d**). We performed transient knockdown of DR5 using siRNA oligos in these cells. The number and intensity of the foci induced by bioymifi was decreased in DR5 knockdown cells (**Fig. 6e** and **Supplementary Fig. 13a**), and bioymifi-induced cell death was attenuated by about a factor of two (**Fig. 6f**). The partial apoptosis rescue may be a result of incomplete DR5 depletion (**Supplementary Fig. 13b**). Finally, to determine whether bioymifi specifically aggregates DR5, we also stained the cells with antibodies detecting other death receptors such as DR4, TNFR1 and Fas (**Supplementary Fig. 14**). Bioymifi had little effect on TNFR1 or Fas and induced small DR4 clusters in a very low percentage of cells (~10%). As bioymifi does not interact with DR4 directly and DR4 can form heterodimers with DR5 (ref. 24), it is possible that the formation of DR4-containing foci is mediated by DR5 aggregation. Because the ligand-induced clustering of receptors such as Fas, DR4 and DR5 is important for the recruitment of the DISC and the consequent activation of caspase-8 (refs. 24–26), these data strongly suggest that bioymifi and A2C2 promote apoptosis by directly binding to and facilitating aggregation of DR5.

DISCUSSION

The initial screen used in this study was carried out in a human glioblastoma cell line in which the Smac mimetic induces apoptosis synergistically with death receptor ligands such as TRAIL and TNFα[13]. Although Smac synergizes with other cytotoxic therapies even in resistant tumors[27], the screen was designed to exclude those compounds from the list of hits because of the cytotoxicity associated with these therapies. On the basis of this design, we successfully identified what is to our knowledge the first synthetic chemical compound, A2C2, that induces apoptosis by directly targeting the TRAIL receptor DR5.

A2C2 was ultimately determined to be a mixture of three constituent compounds. Our subsequent SAR study based on A2C2 identified a more potent single agent, bioymifi. Bioymifi is capable of inducing apoptosis without the need for a Smac mimetic in a variety of cancer cell lines, even in U2OS and HT29 cell lines, which are unresponsive to TRAIL up to concentrations of 1 μg ml^{-1} but undergo apoptosis upon treatment together with Smac mimetic. It seems that the critical determinant of cell sensitivity is bioymifi concentration, not DR5 expression. When bioymifi reaches micromolar concentration, its capability to aggregate DR5 is strong enough to induce apoptosis in various cancer cells.

Both bioymifi and A2C2 harbor agonistic activity toward DR5. They specifically bind the ECD of DR5 and induce the formation of DR5 aggregates. The DR5 ECD alone is sufficient to aggregate upon compounds stimulation. These DR5 polymers are resistant to detergent solubilization and reducing agent disruption. Binding of cognate homotrimeric ligand TRAIL promotes DR5 clustering and subsequent binding of DR5 to FADD[5]. TRAIL also stimulates the formation of SDS-stable, high-molecular-weight forms of DR5 (ref. 26). However, DR5 oligomers induced by TRAIL are sensitive to treatment with reducing agents, suggesting disulfide bond formation within the active complexes.

Bioymifi shares the same DR5-dependent extrinsic apoptotic pathway with TRAIL. Binding of bioymifi to DR5 recruits FADD, which in turn engages with the apical caspase-8 to form a DISC. The DISC mediates autocatalytic processing of caspase-8 and releases the active caspase-8 in the cytoplasm. To our surprise, we found that bioymifi and TRAIL also stimulate the recruitment of RIPK1 and TRADD, which were previously found in the TNFα-induced proximal complex[28], indicating that cross-talk may occur between different death receptor signaling.

In our *in vitro* aggregation assays, a small fraction of DR5 is converted to large SDS- and β-ME–resistant polymers, as detected by SDS-PAGE or SDD-AGE. The aggregates seem to be very heterogeneous. It is unclear how bioymifi binds and aggregates DR5. Further investigation should focus on bioymifi-based SAR studies and structural studies of the compound-DR5 complex, which may lead to the identification of compounds with higher efficacy. The findings presented here establish a foundation for the future development of TRAIL mimics and DR5-targeted anticancer therapies.

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METHODS

Methods and any associated references are available in the online version of the paper.

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Author contributions

Xiaodong Wang, X.L. and G.W. designed the study; G.W., H.Y., N.W., D.L.H., R.H., L.L. and L.W. performed and analyzed the biological experiments; Xiaoming Wang, J.N., P.H. and X.L. performed all of the chemical syntheses; S.C. performed the MS analysis; S.W. implemented the chemical library screen; and Xiaodong Wang, X.L. and G.W. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Supplementary information and chemical compound information is available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to G.W., X.L. or Xiaodong Wang.

ONLINE METHODS

Compound library screen for small molecules sensitizing Smac mimetic. The synthetic chemical library in the HTS core of UT Southwestern comprises a total of 198,080 compounds. They were purchased from ChemDiv (100,000), ChemBridge (75,500), ComGenex (22,000), Prestwick (1,100) and TimTek (500). Compounds were selected for being able to pass 48 structure-based filters that identified undesirable characteristics as well as for satisfying a relaxed set of Lipinski's rules for good bioavailability. In the primary screen, all ~200,000 compounds were screened in a single copy using a cell-based survival assay, before which T98G cells were treated with 5 μM of each compound in the presence of 1 μM Smac mimetic for 48 h. Next we cherry-picked 2,068 active compounds to generate new master plates and tested whether Smac mimetic sensitizes the cell response to these compounds. Briefly, T98G cells were treated with 3 concentrations (15 μM, 5 μM and 1.7 μM) of compounds in the absence or presence of 1 μM Smac mimetic. In a parallel screen with triplicate plates, T98G cells were treated with 5 μM compounds in the absence or presence of 1 μM Smac mimetic.

Cell survival assay. In the chemical library screen and follow-up studies on the compound A2C2, the cell survival assay was performed using the CellTiter-Glo Luminescent Cell Viability Assay kit (Promega). The CellTiter-Glo reagent was diluted by adding two volumes of PBS buffer containing 1% Triton X-100. Fifty microliters of diluted reagent was added to 100 μl of cell culture medium. The plates were incubated at room temperature for 10 min. Luminescence was recorded by Tecan SPCTRAFluor Plus reader (Tecan) except that the compound screen was carried out in the HTS Core (University of Texas Southwestern Medical Center) using an EnVison multimode plate reader (PerkinElmer).

***In vitro* binding assay.** The binding affinity of the DR5 ECD with A2C2 or its analogs was determined by ITC. All titrations were performed at 15 °C in VP-ITC (MicroCal Inc, Northampton, MA). The concentration of compound in the calorimeter cell was 30 μM, and the concentration of DR5 ECD in the injection syringe was 120 μM. Data analyses and curve fittings were carried out using one-site binding algorithm in Origin 7 software (Origin Lab).

SDD-AGE. The reaction mixtures of compounds with DR5-Flag were resuspended in 4× sample buffer (2× TAE; 20% (v/v) glycerol; 4% (w/v) SDS; 1% (v/v) β–ME; bromophenol blue), allowed to incubate for 5 min at room temperature and loaded onto a 1.5% agarose gel containing 1× TAE and 0.1% SDS. Electrophoresis was carried out at 100 V, followed by capillary transfer to nitrocellulose.

Caspase-3 activity assay. Cells were treated with compounds and harvested. Twenty micrograms of cell lysates were incubated with 10 μM fluorogenic substrate (Ac-DEVD-AMC, Calbiochem) in a 20-μl total reaction. Fluorescence was measured on the Tecan SPCTRAFluor Plus reader (Tecan) every 10 min for 19 cycles after incubation with the fluorogenic substrate (Ac-DEVD-AMC).

General reagents. Z-VAD was purchased from Calbiochem. Compound A4 was purchased from ChemBridge. The antibodies for western blotting were specific for the following: caspase-3 (Cell Signaling, 9662, used at 1:1,000), caspase-8 (Cell Signaling, 9746, used at 1:1,000), caspase-9 (Cell Signaling, 9508, used at 1:1,000), FADD (Santa Cruz Biotech, SC-56093, used at 1:2,000), TRADD (Cell Signaling, 3684, used at 1:1,000), RIPK1 (BD Biosciences, 551041, used at 1:2,000), actin (Sigma, A2066, used at 1:5,000), Flag (Sigma, F-3165, used at 1:10,000), tubulin (Sigma, T7816, used at 1:10,000), PARP (Cell Signaling, 9542, used at 1:5,000). The antibodies for immunostaining were specific for DR4 (ProSci, 1139), TNFR1 (Santa Cruz Biotech, SC-7895) and Fas (Upstate, 05-351), and all of these antibodies were used at 1:200. Caspase-8 antibody for immunoprecipitation was purchased from Santa Cruz Biotech (SC-6136). Blocking antibodies used were specific for TRAIL (BioLegend, 308208) and recombinant FasL (R&D, 126-FL/CF).

Plasmids and siRNA transfection. In general, plasmid transfections were done using Lipofectamine 2000 reagent, whereas siRNA transfections were done using Lipofectamine RNAiMAX reagent according to the manufacture's instruction (Invitrogen). For reverse siRNA transfections in 96-well plate, 5 pmol siRNA and 0.25 μl RNAiMAX reagent were used to transfect 5,000 cells in each well. All siRNAs were obtained from Dharmacon. The pooled siRNA from siGENOME SMARTpool were for TNFR1, DR3, DR4, DR5, DR6, caspase-8, caspase-9 and RIPK1. The individual oligos were for:

Caspase-8-1 target sequence: 5′-UGAAGAUAAUCAACGACUAUU-3′
Caspase-8-2 target sequence: 5′-UGGAUUUGCUGAUUACCUAUU-3′
Caspase-8-3, obtained from Dharmacon (J-003466-14)
Caspase-9-1 target sequence: 5′-GAUGCCUGGUUGCUUUAAUUU-3′
Caspase-9-2 obtained from Dharmacon (J-003309-05)
Caspase-9-3 obtained from Dharmacon (J-003309-06)
DR5-1 target sequence: 5′-GCAAGUCUUUACUGUGGAA-3′
DR5-2 target sequence: 5′-CAAGGUCGGUGAUUGUACA-3′
DR5-3 target sequence: 5′-UCAUGUAUCUAGAAGGUAA-3′
TRAIL-1 target sequence: 5′-CCAAACAUACUUUCGAUUU-3′
TRAIL-2 target sequence: 5′-GCAACUCCGUCAGCUCGUU-3′
TRAIL-3 target sequence: 5′-CAAGUUAUCCUGACCCUAU-3′

Quantitative RT-PCR analysis. Total RNA was extracted with TRIzol reagent (Invitrogen). cDNAs were synthesized with oligo(dT) primer using SuperScript II Reverse Transcriptase (Invitrogen). PCR was performed on an AB 7900HT fast real-time PCR system (Applied Bioscience) using DR5-, DR4- or TRAIL-specific primers. The primer pair for DR5 is 5′-GGCCACAGGGACACCTTGTA-3′ (forward) and 5′-TCGCCCGGTTTTGTTGA-3′ (reverse). The primer pair for DR4 is 5′-GCAGCTGGACCTCACGAAAA-3′ (forward) and 5′-CCTG GGCCTGCTGTACCA-3′ (reverse). The primer pair for TRAIL is 5′-GCTCTG GGCCGCAAAAT-3′ (forward) and 5′-AGGAATGAATGCCCACTCCTT-3′ (reverse).

Generation of stable cell lines. Full-length DR5 cDNA with 3× Flag at the C terminus was cloned into retroviral vector pMXs-IRES-Blasticidin (Cell Biolabs). The HCC15 lung cancer cell line was infected with the viral suspension. Cells were grown in DMEM containing 1 μg/ml blasticidine. After 2 to 3 weeks, clones were picked up and checked for exogenous DR5 expression using western blotting with Flag-specific antibody.

Preparation of recombinant proteins. cDNA corresponding to the ECD of DR5 (DR5 ECD, amino acids 54–180) or DR4 (DR4 ECD, amino acids 109–230) was cloned into pET21a to produce fusion proteins with a His_6 tag at the C terminus. These recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) strain and purified using Ni-NTA agarose beads (Qiagen).

Caspase-8 antibody immunoprecipitation. Cells were grown on 100-mm plates and preincubated with 10 μM z-VAD for 2 h before TRAIL or bioymifi treatment. Cells were harvested in 5× volume lysis buffer (50 mM Tris-Cl (pH 8.0), 130 mN NaCl, 1 mM EDTA, 10% glycerol, 0.1 mM PMSF, 10 mM NaF, 1% Triton X-100 and complete protease inhibitor (Roche)). Two micrograms of caspase-8 antibody were coupled to 20 μl protein A agarose beads (Pierce) in 250 μl PBS (supplemented with 5 mg/ml BSA). The beads were then washed and incubated with 2 mg cell lysates overnight at 4 °C. The bound proteins were eluted off the beads using low-pH elution buffer (Pierce). Elution buffer was neutralized by adding 1:20 1 M Tris-HCl (pH 9.4).

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