SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
TARGETED THERAPIES
DRUG DEVELOPMENT

Received
9 July 2013

Accepted
4 December 2013

Published
20 December 2013

Correspondence and requests for materials should be addressed to Z.-C.H. (zchua@nju.edu.cn)

Annexin V-TRAIL fusion protein is a more sensitive and potent apoptotic inducer for cancer therapy

Fan Qiu[1], Minjin Hu[2], Bo Tang[2], Xiufeng Liu[1], Hongqin Zhuang[1], Jie Yang[1] & Zi-Chun Hua[1,2]

[1]The State Key Laboratory of Pharmaceutical Biotechnology, College of Life Sciences and School of Stomatology, Affiliated Stomatological Hospital, Nanjing University, Nanjing, 210093, P.R. China, [2]Changzhou High-Tech Research Institute of Nanjing University and Jiangsu TargetPharma Laboratories Inc., Changzhou, 213164, P.R. China.

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising cancer therapeutic agent, which kills cancer cells selectively, while leaving normal cells unharmed. However, the emerging resistance of tumor cells and patients to TRAIL-induced apoptosis limits its further application. In this study, we developed a chimeric protein Annexin V-TRAIL (designated as TP8) with higher efficacy than TRAIL both *in vitro* and *in vivo*. *In vitro*, the EC_{50} of TP8 on a series of tumor cells was much lower than wild-type TRAIL. Annexin V provided this recombinant protein with higher efficacy, while leaving tumor specificity of TRAIL unchanged since TP8 had no effects on normal cells. *In vivo*, TP8 effectively suppressed tumor growth and prolonged tumor doubling time and tumor growth delay time in mouse xenografts involving multiple cancer cell types including A549, Colo205 and Bel7402. This study provides a new rational strategy to treat TRAIL-resistant cancers.

Cancer is a widespread disease and considered as one of the leading causes of death worldwide. Chemotherapy and radiotherapy are the main approaches for cancer therapy in addition to surgery. However, due to the lack of tumor specificity, they kill both cancer cells and normal cells, eliciting severely toxic side effects[1]. Hence, ideal agents for cancer treatment are those can selectively kill cancer cells without harming normal cells. So TRAIL is a promising candidate for cancer therapy, as it can selectively kill tumor cells but with no or little harm toward normal cells. And clinical trials involving recombinant rhTRAIL also showed good outcomes without obvious side effects.

TRAIL or Apo2L, a type II transmembrane protein, with an extracellular region forming a soluble molecular when cleaved by protease, is a member of the tumor necrosis factor (TNF) family. Compared with other members of this family, TRAIL can induce apoptosis of various transformed cells and xenografts, leaving normal cells unharmed *in vitro*[2–4]. *In vivo*, TRAIL differs from TNF-α and FasL. Besides inducing apoptosis of cancer cells, TNF causes severe inflammatory responses and FasL causes liver damage, when administered systemically[5]. However, TRAIL activates nuclear factor κB (NF-κB) very weakly and it is unlikely to initiate inflammatory cascades when administered systemically. *P*53, which is considered as a critical factor of cancer therapies involved in chemotherapeutic agents or radiation[6], is mutated in at least 50% of all human cancers[7]. However, TRAIL-induced apoptosis does not depend on *p*53 status, for this reason, TRAIL can be used to overcome chemotherapeautic or radiotherapeautic resistance caused by *p*53 dysfunction[8]. Therefore, TRAIL is a promising candidate of cancer therapy. Since its discovery in 1995, TRAIL has been intensely investigated. However, the applications of TRAIL were impeded because certain TRAIL preparations were toxic against primary human hepatocytes via a caspase-dependent mechanism that involved the activation of the extrinsic death pathway[7,9]. Further studies have suggested that the use of native TRAIL, not tagged TRAIL, can prevent such toxicities[10].

Two membrane-associated death receptors, DR4 or/and DR5, are required in TRAIL induced-apoptosis. The interaction of TRAIL with DR4 or DR5 is an initial step of TRAIL-induced apoptosis[11,12]. The binding of trimeric TRAIL to death receptors results in receptors homotrimerization[13,14]. Activated death receptors recruit Fas-associated DD (FADD), which is an adaptor protein that can directly bind to the intracellular death domain of death receptors. Through the interactions of DED of FADD and pro-caspase-8/10, the pro-caspase-8/10 is recruited, thereby forming the death-inducing signaling complex (DISC)[15–17]. In DISC, pro-caspase-8/10 is auto-cleaved to generate an active form. According to the need of the mitochondrial pathway to trigger TRAIL-induced apoptosis completely, cells are divided in two groups, namely, type I cells and type II cells[15]. In type I cells,

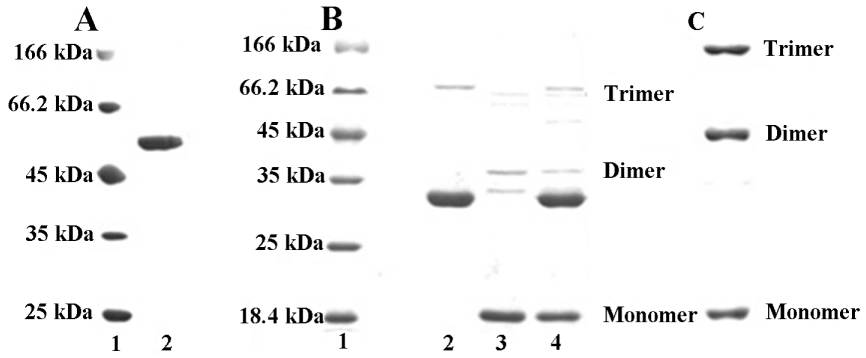

Figure 1 | SDS-PAGE analysis for purified TP8, TRAIL or Annexin V + TRAIL. (A) TP8 analyzed under reducing condition. Lane 1: molecular weight markers; Lane 2: purified TP8. Full-length gel is presented in Supplementary Figure 1. (B) TRAIL or Annexin V + TRAIL analyzed under non-reducing condition. Lane 1: molecular weight markers; Lane 2: Annexin V; Lane 3: TRAIL; Lane 4: Annexin V + TRAIL. Full-length gel is presented in Supplementary Figure 2. (C) TP8 analyzed under non-reducing condition. Full-length gel is presented in Supplementary Figure 3.

activated caspase-8 is sufficient to activate downstream effector caspases such as caspase-3 and caspase-7 to execute apoptosis[18–20]. In type II cells, activated caspase-8 is not sufficient to trigger apoptosis and mitochondria pathway is needed to amplify apoptotic signal[20–22].

Sensitivity to TRAIL-induced apoptosis is a key factor influencing efficacy of cancer treatment. However, it has been reported that not all the cancer cells are sensitive to TRAIL-induced apoptosis due to the acquired or inherent resistance[23]. The resistance of cancer cells has been attributed to dysfunction of different steps in TRAIL-induced apoptosis pathway or elevation of survival signals[24]. Studies have indicated that the resistance appeared to be mediated by the following factors: mutation, modification and location of death receptors; increased expression of caspase inhibitors such as c-FLIP, X-linked inhibitor of apoptosis protein (XIAP), cellular inhibitor of apoptosis protein (cIAP) and surviving; or increased expression of Bcl-2 protein family, including Bcl-2 and Bcl-X_L[25–27]. The activation of other survival pathways, including AKT and NF-κB pathways, also leads to cell survival and chemoresistance[28]. However, the resistant mechanisms are not fully understood now. Studies on intracellular mechanisms that mediate the resistance of TRAIL-induced apoptosis may help develop TRAIL-based approaches for cancer therapy. The combinations of TRAIL with chemotherapy or radiotherapy are used to overcome resistance of some cancers, in which monotherapy with TRAIL, chemical agents or radiation is ineffective.

Annexin V is a calcium phospholipid-binding protein that belongs to the Annexin superfamily. It is widely expressed in skeletal, cardiac, smooth musle, Leyding cells, endothelia, chondrocytes and some neurons[29–33]. Previous studies have revealed that Annexin V is involved in various extracellular and intracellular processes, including blood coagulation, signal transduction, anti-inflammatory processes, membrane trafficking and ion channel activity[34–36]. Besides, a recent study has revealed that Annexin V competes with tumor-derived microvesicles containing activated EGFR to bind to PS of tumor-derived vascular endothelial cells and inhibits tumor angiogenesis[37].

In this study, a chimeric protein Annexin V-TRAIL (designated as TP8) with higher efficacy than TRAIL was developed, which selectively induced various kinds of tumor cell apoptosis *in vitro* and *in vivo*. *In vitro*, the EC_{50} of TP8 was lower. TP8 exhibited long-term effects in addition to rapid induction of apoptosis because colony growth was suppressed by TP8. *In vivo*, TP8 effectively suppressed tumor growth in mouse xenografts involving multiple cancer cell types including A549, Colo205 and Bel7402. Besides, tumor doubling time and tumor growth delay time were also evidently prolonged by TP8's treatment, but not by PBS, Annexin V, TRAIL or Annexin V + TRAIL. All the results suggested TP8 might be a potential candidate to treat TRAIL-resistant cancer. This study provides a new rational strategy to treat cancer.

Results

Annexin V endowed the TRAIL fused-protein TP8 with higher polymerization ability. After purification, the purity of TP8 final product was over 98% (Figure 1A). The purified recombinant protein TP8 was comprised of 31.69% monomer, 38.70% dimer and 29.61% trimer. However, 70.14% monomer, 27.25% dimer and 2.62% trimer were present in TRAIL alone or TRAIL + Annexin V (Figure 1B and 1C). The different trimer and dimer proportions between TRAIL and TP8 indicated that these molecules might exhibit different bioactivities, as one previous study has reported that the apoptosis-inducing activity of TRAIL is related to its ability to form homotrimers[38]. The presence of Annexin V endowed the fused TRAIL protein with higher trimer and dimer formation ability, indicating that TP8 might have enhanced apoptosis-inducing ability.

TP8 enhanced the antitumor activity of TRAIL but maintained tumor specificity of TRAIL *in vitro*. Human MDA-MB-231, Colo-205, A549, Bel7402, MCF-7, HeLa, HepG2, U937, H446, H1229 and PLC cells, treated with varying concentrations of TRAIL or TP8 for the indicated time, were assessed by MTT assay to determine the cytotoxicity of TRAIL and TP8. For TRAIL-sensitive MDA-MB-231 and Colo-205 cells, the EC_{50} of TRAIL was 5.19 and 2.74-fold that of TP8, respectively. In TRAIL moderate-sensitive or TRAIL-resistant cancer cells, TP8 also exhibited severe cytotoxicity even at low concentrations (Table 1). In contrast to wild-type TRAIL, TP8 induced significant and extensive apoptosis (>50%) rapidly, when they were applied at the same concentration. For example, the robust apoptosis of Colo-205 and MDA-MB-231 cells was induced at

Table 1 | The EC50 (nM) of TRAIL and TP8 on different tumor cell lines

	Drug	
Cell line	TRAIL	TP8
Colo-205[a]	1.67	0.61
MDA-MB-231[a]	2.96	0.57
HeLa[b]	33.75	0.84
H446[b]	48.63	0.85
PLC[b]	78.97	0.46
H1229[b]	67.71	1.01
Bel7402[b]	389.5	1.58
A549[b]	> 1000	5.82
MCF-7[b]	> 1000	0.57
U937[b]	> 1000	2.96
HepG2[b]	> 1000	3.82

[a]EC50 was analyzed after 4 hours' treatment with TRAIL or TP8;
[b]EC50 was analyzed after 24 hours' treatment with TRAIL or TP8.

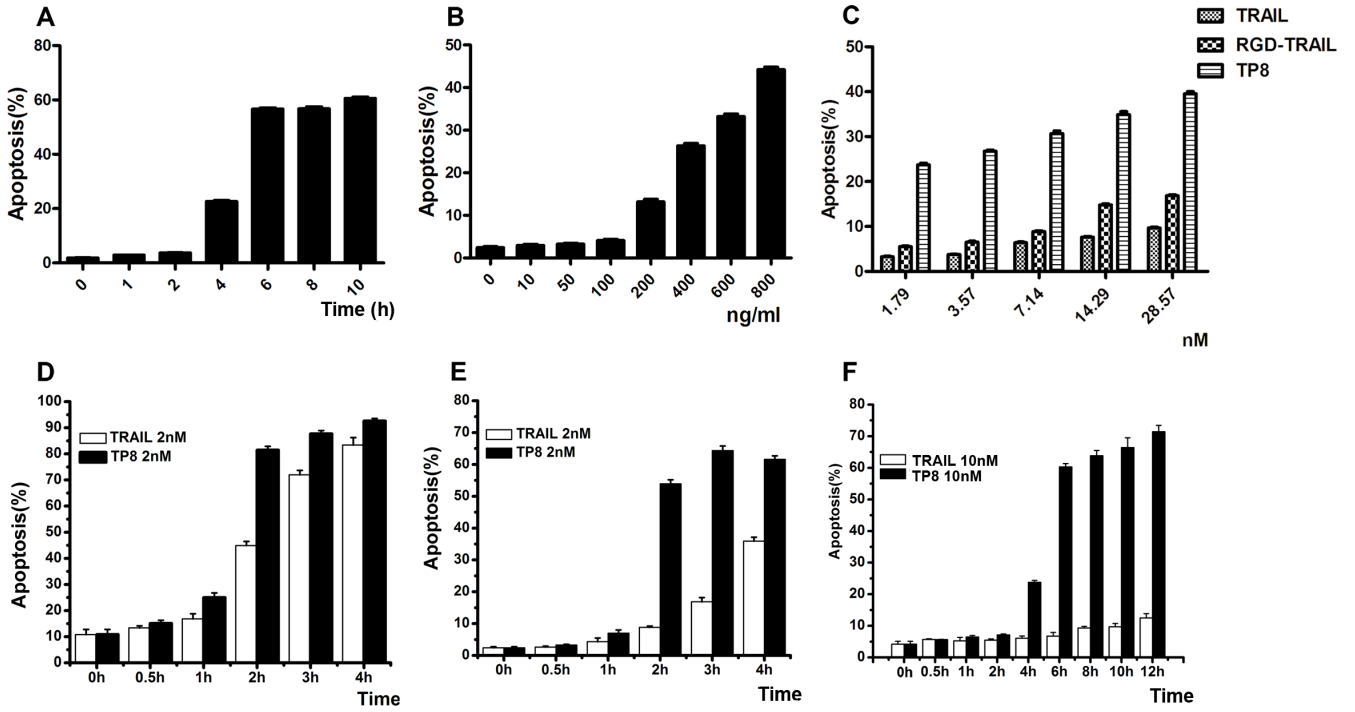

Figure 2 | TP8 induced apoptosis more rapidly than TRAIL and with better effects than TRAIL and RGD-TRAIL. (A) Kinetics of TP8-induced apoptosis in A549 cells. Cells were incubated with TP8 (600 ng/ml) for indicated time. (B) Dose dependency of TP8 in A549 cells. Cells were incubated with TP8 for 6 hours at the concentrations indicated. (C) Antitumor effect comparisons of TRAIL, RGD-TRAIL and TP8 toward A549 cells. TP8 induced intensive apoptosis of (D) Colo-205, (E) MDA-MB-231 and (F) A549 cells more rapidly than TRAIL. Columns, means of three independent experiments; bars ± SD.

2 hours, whereas the apoptosis of A549 cells was induced at 6 hours (Figure 2D, 2E and 2F). Moreover, TP8 was more effective than RGD-TRAIL, a TRAIL derivative with the ability to target TRAIL to tumor vasculature, which was reported to exhibit 3–5 fold higher apoptosis-inducting activity than TRAIL *in vivo*[39] (Figure 2C). Although TP8 greatly enhanced the apoptosis-inducing activity of TRAIL, tumor specificity remained unchanged. 293 T cells were used to evaluate the cytotoxicity of TP8 toward normal cells. The results of Fig. 3D showed that TP8 had no effects on 293 T cells, as the apoptosis did not increase with the increase of TP8 concentration, even when the cells were treated with 800 ng/ml TP8 for 72 hours, no significant apoptosis occurred. That's to say, TP8 exerted no cytotoxicity on normal 293 T cells (Figure 3D).

TP8 induced apoptosis and reduced colony growth of A549 cells. To investigate the apoptosis-inducing activities of Anneixn V, TRAIL, Annexin V + TRAIL and TP8, A549 cells were incubated with PBS or the aforementioned proteins for 6 hours. Consistent with cell viability results, only TP8 induced robust apoptosis of A549 cells, whereas Annexin V, TRAIL or Annexin V + TRAIL were ineffective (Figure 3A). To confirm these results, we investigated the changes of cellular morphology. A549 cells, treated with PBS, Annexin V, TRAIL, Annexin V + TRAIL or TP8, were examined by TEM. Only the cells treated with TP8 exhibited apoptotic morphology (Figure 3B).

To assess whether or not TP8 affects the long-term growth of A549 cells, we examined the colony formation of cells treated with PBS, Annexin V, TRAIL, Annexin V + TRAIL or TP8. The results showed that the colony growth of A549 cells was obviously reduced only when TP8 was applied (Figure 3C). All the results above suggested that TP8 exhibited both short and long-term effects.

Time- and dose-dependence of TP8-induced A549 cell apoptosis. To investigate the effects of treatment time and TP8 dose on the apoptosis of A549 cells, we treated the cells with various concentrations of TP8 or for various treatment periods. Apoptosis increased as the treatment period was extended. The apoptotic rate reached the maximum when the cells were treated for 6 hours (Figure 2A). After 6 hours, the apoptotic rate increased further as treatment period was prolonged, but this increase was not significant. During treatment with different concentrations of TP8 for the same duration, apoptosis rose with the increase of TP8 dosage (Figure 2B).

TP8 induced apoptosis in a caspase-dependent manner. The binding ability of TP8 to death receptors was not enhanced, compared with that of TRAIL or Annexin V + TRAIL (Figure 4A). The expression levels of DR4 and DR5 were not altered by TP8, compared with PBS, Annexin V, TRAIL or Annexin V + TRAIL (Figure 4B, 4C and 4D). However, western blot results showed that PARP, the substrate of caspase-3, was significantly activated. Besides, both the results of western blot and enzymatic activity assay suggested that caspase-8 and caspase-3 were evidently activated only after the A549 cells were treated with TP8 (Figure 4B, 4E and 4F). The pretreatment with Z-IETD-FMK, a caspase-8 inhibitor, inhibited TP8-induced apoptosis, but the pretreatment with caspase-9 inhibitor Z-LEHK-FMK did not reduce apoptosis induced by TP8 significantly (Figure 4G). When Cys230 of TRAIL domain of TP8 was mutated to Gly, the protein TP8-V could not induce apoptosis anymore, indicating that the activity of TP8 was dependent on the TRAIL domain (Figure 4H). Hence, all the results above demonstrated that TP8 induced apoptosis in the same way as TRAIL, indicating that TP8 would not cause additional cytotoxicity than TRAIL.

Administration of TP8 significantly suppressed tumor growth and prolonged tumor doubling time and tumor growth delay time. We confirmed the aforementioned results *in vivo*. A549, Colo205 and Bel7402 xenografts, which represent non-small cell lung cancer, colon cancer and liver cancer, respectively, were used

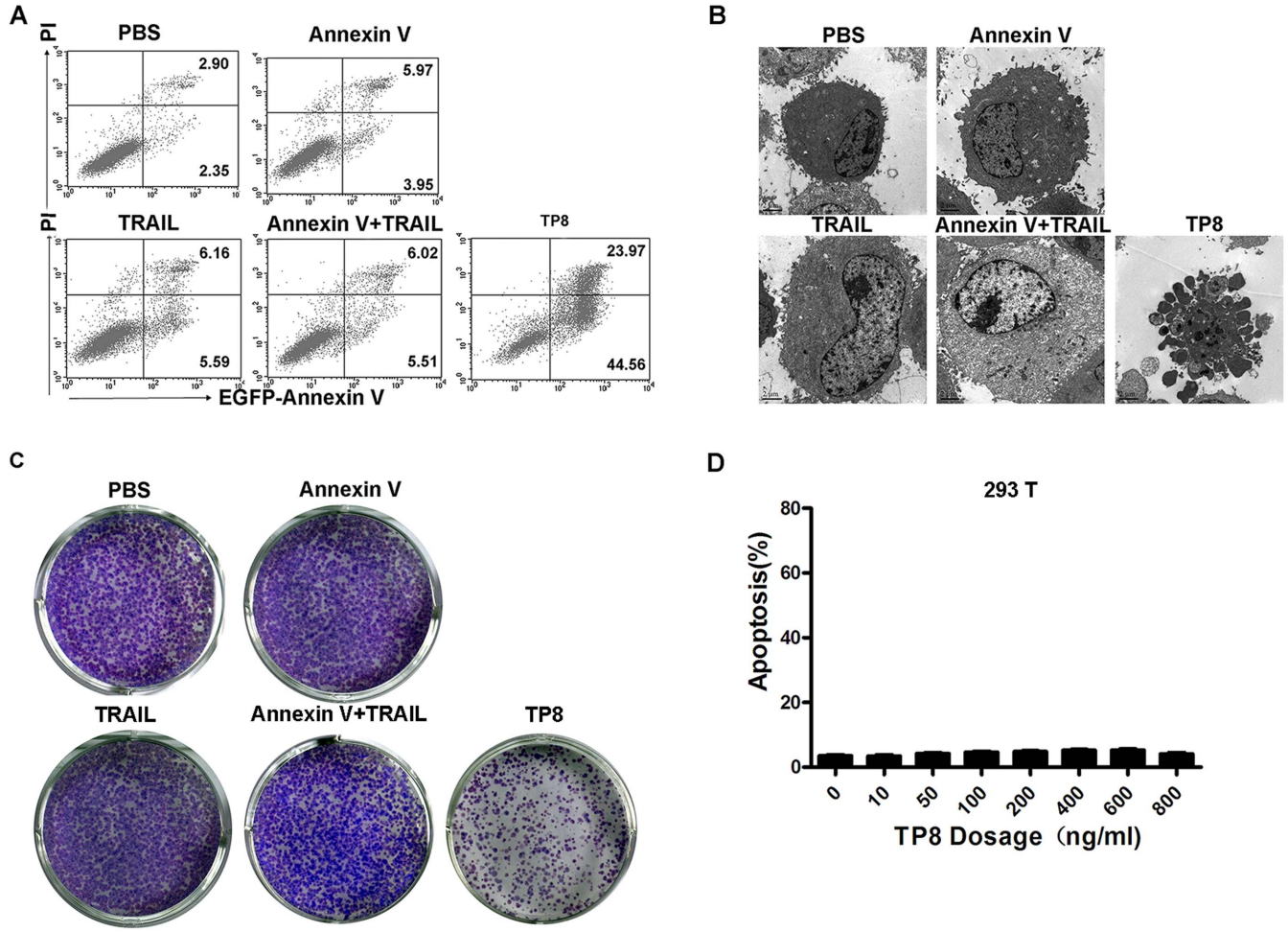

Figure 3 | The *in vitro* cytotoxicity of TP8 towards A549 cells and 293 T cells. (A) A549 cells were treated with 10.74 nM Annexin V, TRAIL, Annexin V + TRAIL or TP8 for 6 hours and cell death was analyzed using flow cytometer. (B) TEM images for A549 cells morphology after treatment with 10.74 nM Annexin V, TRAIL, Annexin V + TRAIL or TP8 for 6 hours. (C) Colony-forming assay for A549 cells after treatment with 1.79 nM Annexin V, TRAIL, Annexin V + TRAIL or TP8 for indicated time. (D) 293 T cells were treated with 10, 50, 100, 200, 400, 600 and 800 ng/ml TP8 TP8 for 72 hours. No significant apoptosis was observed. Columns, means of three independent experiments; bars ± SD.

to construct tumor models in athymic nude mice. In these three tumor models, tumor growth was effectively suppressed by TP8, but not by equal dosage of Annexin V, TRAIL or Annexin V + TRAIL. TP8 also retarded tumor growth in a dose-dependent manner, because the inhibitory efficacy was enhanced as dosage increased (Figure 5A, Figure 6A and 6C). Additionally, the tumor volume in mice treated with TP8 was significantly smaller than that of the controls (Figure 5A, Figure 6A and 6C). Furthermore, tumor growth of the TP8 group was slower than that of the controls. In mice bearing A549 xenograft, tumor doubling time was prolonged from 5.3 days (CI, 4.9–5.9 days) in mice receiving PBS, 6.1 day (CI, 5.6–6.8 days) in mice receiving Annexin V, 6.1 day (CI, 5.6–6.7 days) in mice receiving TRAIL, or 6.0 days (CI, 5.5–6.6 days) in mice receiving Annexin V + TRAIL, to 7.4 days (CI, 6.6–8.4 days) in mice receiving TP8 (12.5 mg/kg) (Figure 5B and Table 2). In mice bearing Bel7402 xenograft, tumor doubling time was prolonged from 7.3 days (CI, 7.0–7.7 days) in mice receiving PBS, 7.8 day (CI, 7.4–8.2 days) in mice receiving Annexin V, 8.0 days (CI, 7.7–8.4 days) in mice receiving TRAIL, or 7.6 days (CI, 7.3–8.0 days) in mice receiving Annexin V + TRAIL, to 9.3 days (CI, 8.9–9.6 days) in mice receiving TP8 (12.5 mg/kg) (Figure 6B and Table 2). In mice bearing Colo-205 xenograft, tumor doubling time was prolonged from 10.0 days (CI, 9.5–10.6 days) in mice receiving PBS, 10.2 days (CI, 9.8–10.7 days) in mice receiving Annexin V, 12.5 days (CI, 11.9–13.1 days) in mice receiving TRAIL, or 10.4 days (CI, 10.0–10.9 days) in mice receiving Annexin V + TRAIL, to 14.5 days (CI, 13.9–15.2 days) in mice receiving TP8 (12.5 mg/kg) (Figure 6D and Table 2). TP8 also caused a markedly delay of 6.9 days (CI, 5.5–8.4 days) in mice bearing A549 xenograft, 18.3 days (CI,15.7–21.0 days) in mice bearing Colo-205 xenograft and 10.0 days (CI, 7.5–12.5 days) in mice bearing Bel7402 xenograft for tumor growth to 1000 mm^3 compared with PBS control (Figure 5C, Figure 6B, 6D and Table 2).

TP8 induced tumor cell apoptosis *in vivo*. The tumor slices obtained from the mice in each group were subjected to TUNEL staining after the treatments were completed. The results showed an increase in the number of TUNEL-positive cells from the mice treated with TP8 compared with those from the mice treated with PBS, Annexin V, TRAIL or Annexin V + TRAIL (Figure 5D).

Discussion

The American Cancer Society reported that cancer is a widespread disease. In the US, approximately 1,660,290 new cases and 580,350 deaths are expected in 2013. Cancer is the second leading cause of death (next to heart disease) and accounts for 1 out of 4 deaths. Chemotherapy and radiotherapy are the two main approaches for cancer treatment besides surgery. *P*53 dysfunction, resistance caused by repeatedly administration of chemical agents or the severe side effects often result in the failure of tumor treatments.

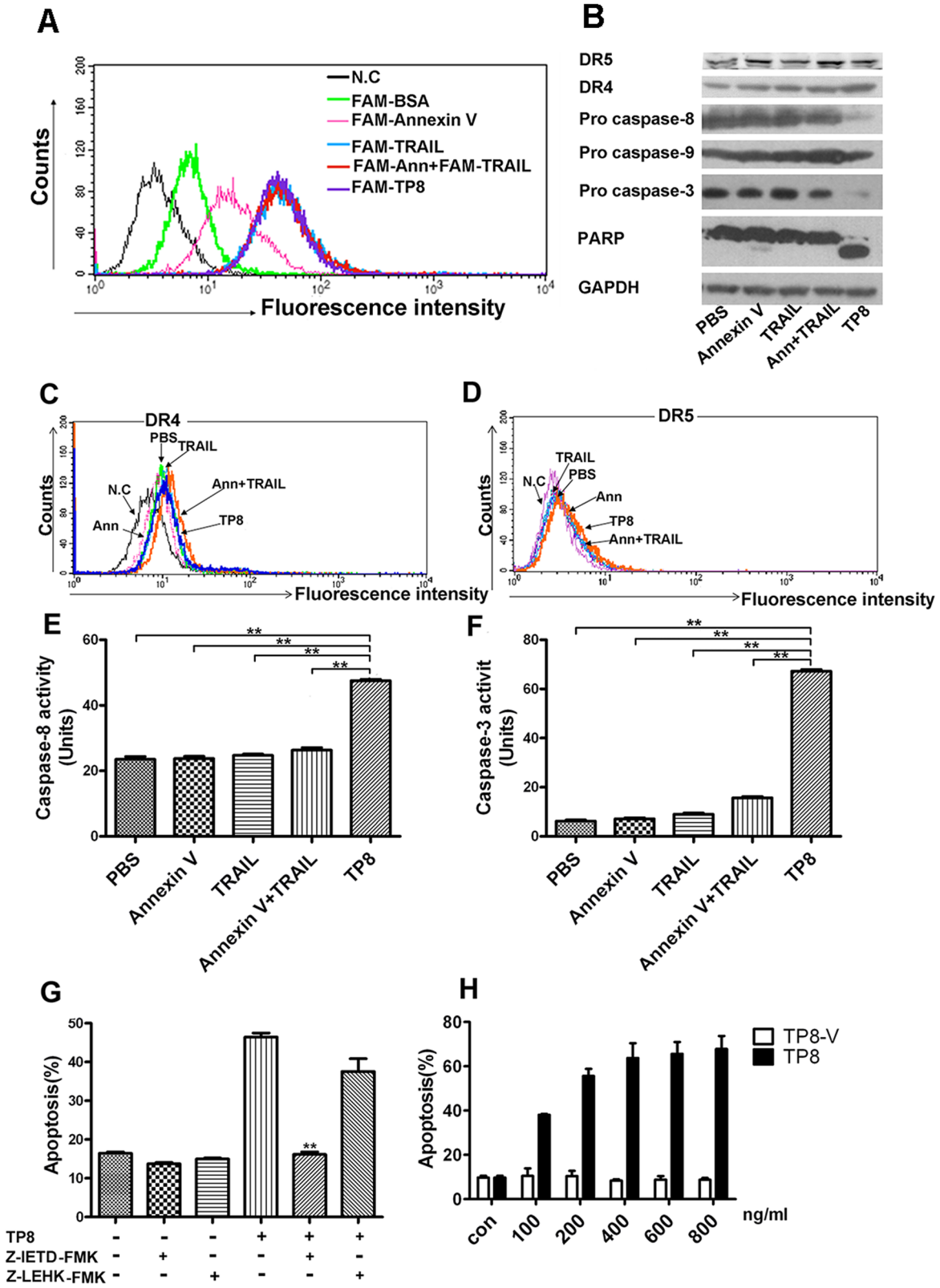

Figure 4 | TP8 induced cancer cells death in the same manner as TRAIL. (A) Flow cytometer assay for the binding abilities of Annexin V, TRAIL, Annexin V + TRAIL and TP8 with A549 cells. (B) Western blotting detection for DR4, DR5, pro-caspase-8, pro-caspase-9, pro-caspase-3 and PARP. Full-length blots are presented in Supplementary Figure 4. (C) DR4 and (D) DR5 expression levels were analyzed using flow cytometer. (E) Caspase-8 activation determined with caspase-8 activity assay kits and (F) caspase-9 activation determined with caspse-9 activity assay kits. (*Columns*, means of three independent experiments; bars ± SD, $^{**}p < 0.01$ compared with each corresponding treatment). (G) Caspase inhibitors study for the roles of caspases in TP8 induced apoptosis (*Columns*, means of three independent experiments; bars ± SD, $^{**}p < 0.01$ compared with TP8 treatment). (H) Mutation of Cys230 of TRAIL in TP8 attenuated TP8 induced apoptosis. (*Columns*, means of three independent experiments; bars ± SD).

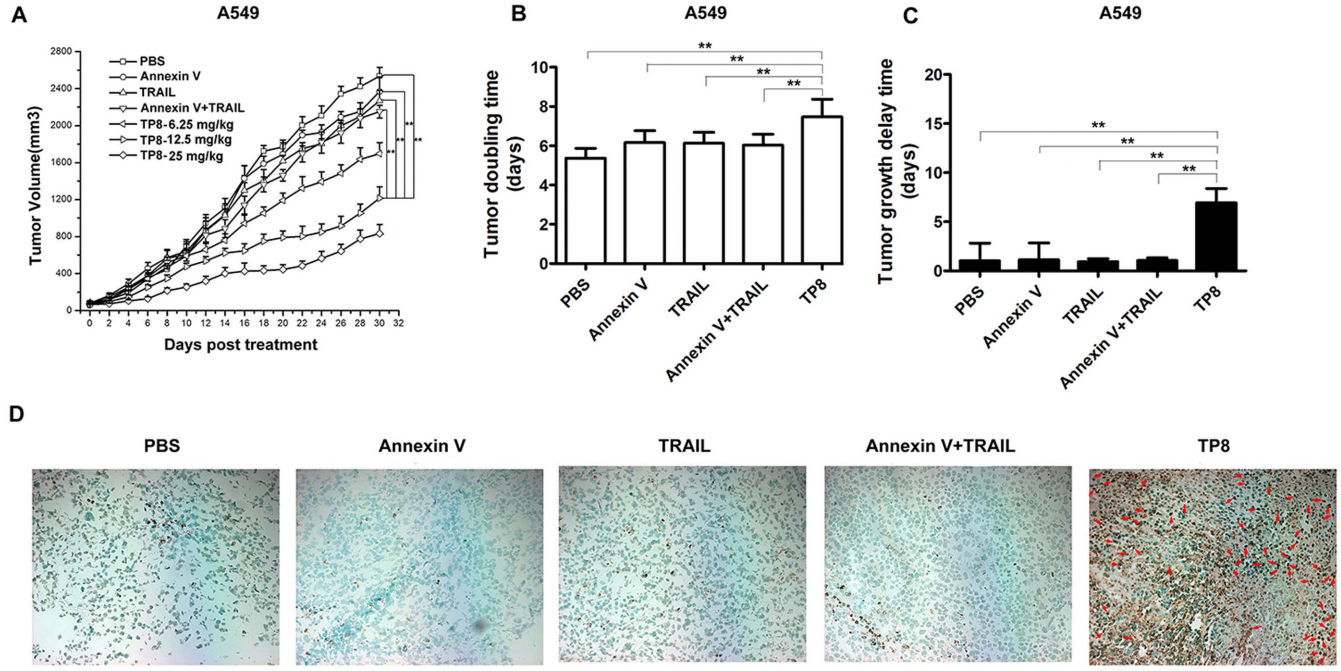

Figure 5 | Evaluation of antitumor effects of TP8 in nude mice bearing A549 carcinomas. (A) Tumor volumes comparison among different groups (Mean ± SEM, n = 8, **p < 0.01 compared with each corresponding treatment). (B) Tumor doubling time comparison among different groups (Mean ± SD, n = 8, **p < 0.01 compared with each corresponding treatment). (C) Tumor growth delay time comparison among different groups (Mean ± SD, n = 8, **p < 0.01 compared with each corresponding treatment). (D) TUNEL analysis for tumor cells undergoing apoptosis within tumor tissues (original magnification × 400). The TUNEL-positive cells are indicated with red arrows.

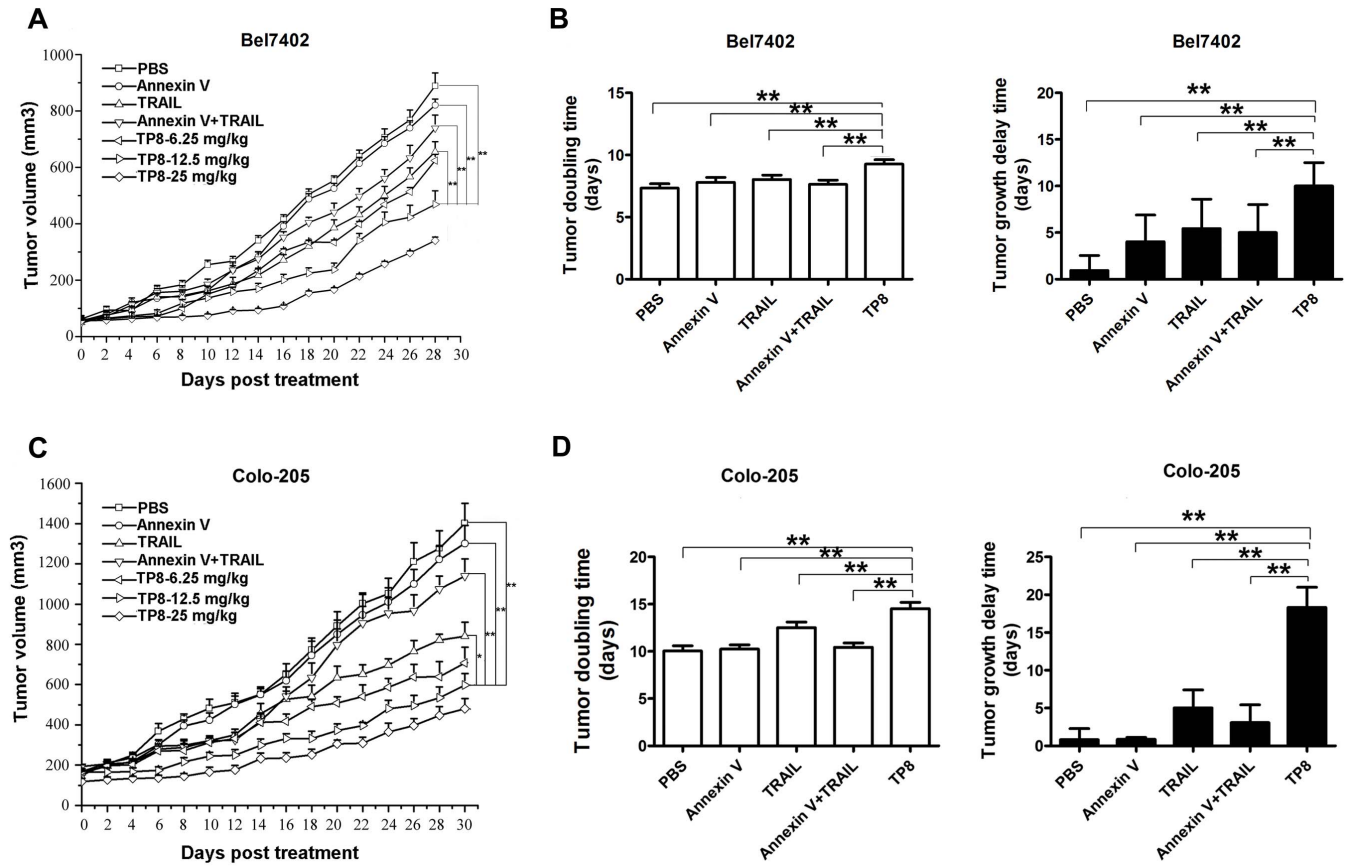

Figure 6 | Evaluation of antitumor effects of TP8 on Bel7402 carcinoma and Colo-205 carcinoma *in vivo*. Tumor volumes comparison among different groups in nude mice bearing (A) Bel7402 carcinomas and (C) Colo-205 carcinomas (Mean ± SEM, n = 8, *p < 0.05, **p < 0.01 compared with each corresponding treatment). Tumor doubling time and tumor growth delay time comparison among different groups in nude mice bearing (B) Bel7402 carcinomas and (D) Colo-205 carcinomas (Mean ± SD, n = 8, **p < 0.01 compared with each corresponding treatment).

Table 2 | Regression analysis for treatment effects on tumor growth

	Treatment	n	Growth curve[a] *v(d)*	r	Tumor doubling time *(d)*[b]	Tumor growth delay *(d)*[c]
A549	PBS	8	$\ln(V) = 0.1303 \times d + 4.87$	0.95	5.3 (4.9–5.9)	0 (0–3.1)
	Annexin V	8	$\ln(V) = 0.1133 \times d + 5.14$	0.95	6.1 (5.6–6.8)	0 (0–3.1)
	TRAIL	8	$\ln(V) = 0.1137 \times d + 5.07$	0.95	6.1 (5.6–6.7)	0.6 (0.4–0.8)
	Annexin V + TRAIL	8	$\ln(V) = 0.1157 \times d + 4.98$	0.95	6.0 (5.5–6.6)	1.1 (0.8–1.3)
	TP8-6.25 mg/kg	8	$\ln(V) = 0.1072 \times d + 4.93$	0.94	6.5 (5.9–7.2)	2.8 (2.1–3.4)
	TP8-12.5 mg/kg	8	$\ln(V) = 0.0942 \times d + 4.78$	0.92	7.4 (6.6–8.4)**	6.9 (5.5–8.4)**
	TP8-25 mg/kg	8	$\ln(V) = 0.0876 \times d + 4.42$	0.95	7.9 (7.3–8.7)$^{++}$	12.8 (8.7–16.9)$^{++}$
Colo205	PBS	8	$\ln(V) = 0.0694 \times d + 5.32$	0.98	10.0 (9.5–10.6)	0 (0–2.5)
	Annexin V	8	$\ln(V) = 0.0678 \times d + 5.30$	0.99	10.2 (9.8–10.7)	0.9 (0.6–1.1)
	TRAIL	8	$\ln(V) = 0.0554 \times d + 5.23$	0.98	12.5 (11.9–13.1)	4.6 (2.9–7.6)
	Annexin V + TRAIL	8	$\ln(V) = 0.0666 \times d + 5.18$	0.99	10.4 (10.0–10.9)	3.1 (0.7–5.4)
	TP8-6.25 mg/kg	8	$\ln(V) = 0.0497 \times d + 4.68$	0.99	14.0 (13.5–15.5)	12.2 (9.4–15.0)
	TP8-12.5 mg/kg	8	$\ln(V) = 0.0479 \times d + 5.23$	0.99	14.5 (13.9–15.2)**	18.3 (15.7–21.0)**
	TP8-25 mg/kg	8	$\ln(V) = 0.0463 \times d + 5.00$	0.99	15.0 (14.5–15.5)$^{++}$	22.0 (19.1–25.0)$^{++}$
Bel7402	PBS	8	$\ln(V) = 0.0945 \times d + 4.36$	0.96	7.3 (7.0–7.7)	0 (0–2.8)
	Annexin V	8	$\ln(V) = 0.0889 \times d + 4.03$	0.96	7.8 (7.4–8.2)	4.0 (1.2–6.9)
	TRAIL	8	$\ln(V) = 0.0865 \times d + 4.23$	0.98	8.0 (7.7–8.4)	5.4 (2.3–8.6)
	Annexin V + TRAIL	8	$\ln(V) = 0.0907 \times d + 4.01$	0.98	7.6 (7.3–8.0)	5.0 (2.0–8.0)
	TP8-6.25 mg/kg	8	$\ln(V) = 0.0773 \times d + 4.05$	0.99	9.0 (8.7–9.2)	9.3 (6.5–11.9)
	TP8-12.5 mg/kg	8	$\ln(V) = 0.0748 \times d + 4.20$	0.97	9.3 (8.9–9.6)**	10.0 (7.5–12.5)**
	TP8-25 mg/kg	8	$\ln(V) = 0.0684 \times d + 3.80$	0.95	10.1 (9.6–10.8)$^{++}$	18.5 (14.4–21.7)$^{++}$

[a]Regression growth curves summarize volume (*V*, mm^3) dependence on time (*d*, days) from initial treatment, with correlation coefficients indicated by *r*.
[b]Tumor doubling time was derived from exponential growth curves.
[c]Growth delay was determined by assessing the time interval to 1000 mm^3 compared with the PBS control. (Mean ± SD, **$p < 0.01$ compared with PBS. $^{++}p < 0.01$ compared with TP8-6.25 mg/kg).

TRAIL is a tumor-specific agent for cancer therapy, which is relatively safe when administered systemically compared with TNFα and FasL. Besides induction apoptosis of a variety of tumor cells, TRAIL elicits antitumor effects in xenograft models, including breast cancer, colon cancer, multiple myeloma, glioma and prostate cancer[40,41]. Nevertheless, the resistance to TRAIL-mediated apoptosis is a great challenge when it is applied in cancer treatment, because substantial numbers of cancer cells are resistant to TRAIL-induced apoptosis, particularly some highly malignant tumors. Combinations TRAIL with chemotherapy or radiotherapy are the main strategies used in clinic to overcome TRAIL-resistance. The modifications of TRAIL mainly focused on enhancement of tumor targeting of TRAIL. These approaches enhanced antitumor effects of TRAIL through increased concentration of TRAIL in tumor and did not increase TRAIL-sensitivity fundamentally. In this study, a completely new protein TP8 was developed to overcome TRAIL-resistance. The antitumor efficacy was greatly enhanced compared with TRAIL both *in vitro* and *in vivo*. *In vitro*, the EC_{50} of TP8 on a range of tumor cells, including TRAIL-sensitive cells, moderate-sensitive cells and TRAIL-resistant cells, was no more than 6 nM. To assess the *in vivo* antitumor activity of TP8, Colo-205, Bel7402 and A549 cells were selected to construct xenograft models, which also represent TRAIL-sensitive, TRAIL moderate-sensitive and TRAIL-resistant tumor cells, respectively. And the results revealed that TP8 effectively inhibited tumor growth and prolonged tumor doubling time and tumor growth delay time. These results above further indicated that TP8 greatly broadened antitumor spectrum of TRAIL both *in vitro* and *in vivo*.

The half-life of rhTRAIL is only 23 to 31 min in non-human primates and mouse tumor models, so high dose of rhTRAIL is needed to achieve antitumor effects due to its fast clearance[42]. And repeatedly administration of rhTRAIL at high dose may cause unexpected side effects. The recombinant replication-deficient adenoviral vector encoding human TRAIL (Ad5-TRAIL) was constructed to achieve better antitumor activity[43]. Contrary to rhTRAIL, TRAIL is expressed continuously after intratumor injection of Ad5-TRAIL and it's not necessary to be administered repeatedly. However, intratumor injection is not a convenient and conventional tumor therapy approach, and intratumor-injected Ad5-TRAIL is unlikely to travel to other parts of the body, so it's less effective to eliminate metastasis. Agonistic anti-TRAIL-receptor antibodies, with the half-life of approximately 14–21 days, were developed to overcome the resistance caused by expression of TRAIL-R3/TRAIL-R4[40]. Nevertheless, each antibody is specific to one death receptor and the expression of death receptors varies among tumor cells. Therefore, individualized detection of expressed death receptor impedes effective treatment with agonistic anti-TRAIL receptor antibodies. Compared with TRAIL, TP8 induced substantial apoptosis rapidly after administration, indicating that less TP8 protein was needed to achieve the same antitumor effects. Compared with Ad5-TRAIL, TP8 could travel to other parts of the body and eliminate metastasis effectively. Compared with agonistic anti-TRAIL receptor antibodies, TP8 could also induce apoptosis of tumor cells those expressed either DR4 or DR5. Additionally, TP8 could effectively prevent cytotoxicity caused by antibody crosslinks.

TP8 induced cell death in the same manner as TRAIL and both pathways were caspase-dependent. Through the increased activation of caspase-8, TP8 induced apoptosis of TRAIL-resistant A549 cells. Cys230 was critical to maintain the antitumor activity of both TRAIL and TP8. That's to say, Annexin V endowed the fusion protein TP8 with enhanced antitumor activity, without causing additional cytotoxicity.

In summary, a novel TRAIL chimeric protein TP8 was developed and both the *in vitro* and *in vivo* investigations showed promising results. All of our findings suggested that this novel approach could be used to overcome TRAIL-resistance and may have profound implications in cancer therapy.

Methods

Cell culture and reagents. A549, Bel7402, MDA-MB-231, MCF-7, HeLa, HepG2, U937, H446, H1229, PLC, Colo-205 and 293 T cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). U937 and Colo-205 were cultured in RPMI 1640 (HyClone, Logan, UT). Others were cultured in DMEM (HyClone, Logan, UT). All the medium were supplemented with 10% FBS (HyClone, Logan, UT), 100 IU/ml penicillin-streptomycin (Invitrogen, Burlington, Canada). All the cells were grown at 37°C in 5% CO_2.

EGFP-Annexin V, TRAIL, RGD-TRAIL and Annexin V were prepared in our lab.

Preparation of TP8 and TP8-V. The human TRAIL cDNA fragment (amino acid residues 114–281) was amplified by PCR from the pET32a-TRAIL plasmid and Annexin V cDNA fragment was amplified from pET28a-Annexin V plasmid,

respectively. And the fragments were linked by overlapping PCR and cloned into pET28a expression plasmid (Novagen). The plasmids pET32a-TRAIL and pET28a-Annexin V were stored in our lab. Recombinant TP8 was expressed in *Escherichia coli* BL21 (DE3) cells (Novagen) and purified by metal affinity chromatography on Zn-sepharose and anion exchange chromatography on DEAE-Sepharose. All of the solutions were prepared with sterile, endotoxin-free water. Protein concentrations were determined using BCA Protein Assay Reagent (Galen Biopharm). After purification, TP8 was analyzed by SDS-PAGE under reducing (12%) and non-reducing (8%) conditions using Coomassie blue staining, while TRAIL was analyzed by non-reducing SDS-PAGE (12%). Cys230 is the activity center of TRAIL. In order to study the pathway TP8 induced apoptosis of tumor cells, we mutated the Cys230 of TRAIL domain of TP8 to Gly and this mutated protein was called TP8-V. TP8-V cDNA fragment was obtained from pET28a-TP8 plasmid by overlapping PCR and then cloned into pET28a expression plasmid. And TP8-V was purified in the same manner as TP8.

Cell viability assay. 10^3–10^4 cells were seeded triplicate in 96-well plates. After 12 hours incubation, the Colo-205 and MDA-MB-231 cells, which are sensitive to TRAIL-induced apoptosis, were treated with various concentrations of TRAIL or TP8 for 4 hours. The A549, MCF-7, PLC, HepG2, Hela, U937, H446 and H1229 cells, which are moderate-sensitive or resistant to TRAIL-induced apoptosis, were treated for 24 hours. Cell viability was determined by MTT assay as described previously[44]. In brief, after drug treatment, MTT (5 mg/ml, 100 μl/well) was added to each well and after additional 4 hours incubation, the supernatant was removed and 100 μl/well DMSO was added. After shaking the plates for 10 min to dissolve the formazan, absorbance of each well was recorded with a microplate reader (Safire, TECAN, Switzerland) at 570 nm.

Clonogenic survival assay. A549 cells were seeded as single cells (300 cells/well) in 6-well plates, incubated 24 hours to allow adhesion and treated with same concentration (1.79 nM) of Annexin V, TRAIL, Annexin V + TRAIL or TP8 for 24 hours. Cells were then washed twice and cultured in drug-free regular medium for additional 14 days. The medium was changed every five days. After the treatments were completed, the colonies formed were fixed with methanol and stained with Gimsa reagent. Cells treated with PBS were used as negative control.

Transmission electron microscopy analysis of cell morphology. Images of cell morphology were obtained using transmission electron microscope (H-60, HITACHI, Japan) as described before[45]. Briefly, the A549 cells were harvested and washed twice with PBS after these cells were treated with indicated concentrations (10.74 nM) of Annexin V, TRAIL, Annexin V + TRAIL or TP8 for 6 hours. Afterward, the cells were incubated with 2.5% glutaraldehyde **at** 4°C overnight, dehydrated gradually with 70%, 80%, 90% and 100% ice-cold alcohol and embedded in EPON 812. The blocks were cut into ultrathin sections by ultramicrotome and stained with uranyl acetate/lead citrate. The cells treated with PBS were used as the negative control.

Apoptosis assay. Cells, treated with indicated concentrations of different proteins for indicated time, were stained with EGFP-Annexin V and propidium iodide (Sunshine Biotechnology, Nanjing, China) to measure the rate of apoptosis. Then the cells were analyzed using flow cytometer and the rate of apoptosis was analyzed with CELLQuest software (Becton Dickinson, Mountain View, CA). All of the experiments were three independent experiments and each experiment was repeated thrice. 293 T cells were used to assess cytotoxicity of TP8 against normal cells.

DRs binding ability of Annexin V, TRAIL, Annexin V + TRAIL and TP8 assay *in vitro*. Firstly, the proteins were labeled with FAM (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Then the A549 cells were incubated with equal molar concentrations of FAM-conjugated proteins at 37°C for 30 min, harvested, washed thrice with PBS and analyzed using flow cytometer. The fluorescence intensity was analyzed with CELLQuest software.

Western blot assay of signaling pathway. In order to study the changes of important proteins of apoptosis pathway, the A549 cells were treated with 10.74 nM Annexin V, TRAIL, Annexin V + TRAIL or TP8 for 6 hours. After drug treatment, the cells were harvested and the whole cell lysates were prepared as described before[46] with protease inhibitor cocktail (Roche, Milan, Italy). A total of 50 μg of protein was electrophoresed on 12% SDS-PAGE gels using a Tris-glycine system and transferred to polyvinylidene difluoride membranes (Millipore, Bedord, MA). The membranes were blocked with 5% non-fat dry milk in PBST for 1 hour and probed with antibodies against DR4 (IMGENEX, San Diego, CA), DR5 (IMGENEX, San Diego, CA), caspase-3 (Cell Signaling Technology, Beverly, MA), caspase-8 (Cell Signaling Technology, Beverly, MA), caspase-9 (Cell Signaling Technology, Beverly, MA), PARP (Oncogene) and GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. The blots were labeled with a secondary antibody (HRP-conjugated anti-rabbit or anti-mouse IgG) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and visualized with enhanced chemiluminescence detection reagents (Cell Signaling Technology, Beverly, MA).

Flow cytometer analysis of DR4 and DR5 expression levels. After treatment with PBS, Annexin V, TRAIL, Annexin V + TRAIL or TP8 for 6 hours, the A549 cells were stained with fluorescence probe-conjugated antibodies: PE-anti-DR4 (E14958-101, eBioscience) or FITC-anti-DR5 (E11072-102, eBioscience). The expression levels of DR4 and DR5 were analyzed using flow cytometer. The fluorescence intensity was assessed with CELLQuest software. The cell staining experiments were repeated at least thrice.

Enzymatic activity assay of caspase-8 and caspase-3. A549 cells, treated with indicated concentrations (10.74 nM) of Annexin V, TRAIL, Annexin V + TRAIL or TP8 for 6 hours, were lysed with lysis buffer (Beyotime, Nantong, China). Caspase-8 activity was determined with caspase-8 assay kit (Beyotime, Nantong, China), in which Ac-IETD-*p*NA was used as substrate. And caspase-3 activity was determined with caspase-3 assay kit (Beyotime, Nantong, China), in which Ac-DEVD-*p*NA was used as substrate. For these caspase activity assays, the cell lysates were incubated with reaction buffer and substrates at 37°C for 1–2 hours and the absorbance was read at the wavelength of 405 nm. Caspase activity was evaluated by enzyme unit.

Xenograft experiments. Female BALB/c nude mice (5–6 weeks old), which were obtained from Beijing Animal Centre, were housed in environmentally controlled conditions (22°C; a 12 hours/12 hours light/dark cycle with the light cycle from 6:00 to 18:00 and the dark cycle from 18:00 to 6:00) and provided with pathogen-free food and water. Cancer cells were grown in medium as described before[47] and 1×10^6 cells in 100 μl PBS were injected into the mid-right flank of mice, which developed tumors in 7–10 days with the size of approximately 50–100 mm^3. For every experiment, the mice were randomized into 7 groups (8 mice per group) and intraperitoneally injected with PBS, 7.5 mg/kg Annexin V, 5 mg/kg TRAIL, 7.5 mg/kg Annexin V+5 mg/kg TRAIL, 6.25 mg/kg TP8, 12.5 mg/kg TP8 or 25 mg/kg TP8 daily for continuous 14 days. Tumor volume was measured every two days after the initial injection with calipers and determined as mm^3 using the equation: $A \times B^2 \times 0.52$[47], where A was the length (mm) and B was the width (mm). Tumor doubling time refers to the time for a tumor to double in volume and tumor growth delay time is the time interval to reach 1000 mm^3 compared with the PBS control group[48].

Animal care and use were performed strictly in accordance with the ethical guidelines by the Nanjing University Animal Care and Use Committee, and the study protocol was approved by the local institution review board.

TUNEL staining for tumor tissue sections. Tumor tissue sections (5 μm in thickness) were prepared according to standard protocols and then assessed for apoptosis. DNA fragmentation [terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) staining] was determined according to the manufacturer's instructions (Calbiochem, San Diego, CA).

Statistical analysis. Data are reported as mean with their 95% confidence intervals in parentheses. Statistical analysis was carried out using the Student's *t*-test. Data of apoptosis and caspase activity were expressed as mean ± SD and data of tumor sizes were expressed as the mean ± SEM. All the data were analyzed by use of SPSS software. The significance was shown as follows: *, $P < 0.05$; **, $P < 0.01$.

1. Wang, S. The promise of cancer therapeutics targeting the TNF-related apoptosis-inducing ligand and TRAIL receptor pathway. *Oncogene* **27**, 6207–6215 (2008).
2. Wiley, S. R. *et al.* Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* **3**, 673–682 (1995).
3. Pitti, R. M. *et al.* Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* **271**, 12687–12690 (1996).
4. Ashkenazi, A. *et al.* Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* **104**, 155–162 (1999).
5. Ashkenazi, A. & Dixit, V. M. Death receptors: signaling and modulation. *Science* **281**, 1305–1308 (1998).
6. Hymowitz, S. G. *et al.* A unique zinc-binding site revealed by a high-resolution X-ray structure of homotrimeric Apo2L/TRAIL. *Biochemistry* **39**, 633–640 (2000).
7. Seol, D. W. & Billiar, T. R. Cysteine 230 modulates tumor necrosis factor-related apoptosis-inducing ligand activity. *Cancer Res* **60**, 3152–3154 (2000).
8. Menendez, D., Inga, A. & Resnick, M. A. The expanding universe of p53 targets. *Nature reviews. Cancer* **9**, 724–737 (2009).
9. Jo, M. *et al.* Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. *Nat Med* **6**, 564–567 (2000).
10. Lawrence, D. *et al.* Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. *Nat Med* **7**, 383–385 (2001).
11. Pan, G. *et al.* The receptor for the cytotoxic ligand TRAIL. *Science* **276**, 111–113 (1997).
12. Walczak, H. *et al.* TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *EMBO J* **16**, 5386–5397 (1997).
13. Zheng, S. J., Wang, P., Tsabary, G. & Chen, Y. H. Critical roles of TRAIL in hepatic cell death and hepatic inflammation. *J Clin Invest* **113**, 58–64 (2004).
14. Bodmer, J. L. *et al.* TRAIL receptor-2 signals apoptosis through FADD and caspase-8. *Nat Cell Biol* **2**, 241–243 (2000).
15. Kischkel, F. C. *et al.* Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J* **14**, 5579–5588 (1995).
16. Sprick, M. R. *et al.* FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. *Immunity* **12**, 599–609 (2000).

17. Kischkel, F. C. *et al.* Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. *Immunity* **12**, 611–620 (2000).
18. Srivastava, R. K. TRAIL/Apo-2L: mechanisms and clinical applications in cancer. *Neoplasia* **3**, 535–546 (2001).
19. Eggert, A. *et al.* Resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in neuroblastoma cells correlates with a loss of caspase-8 expression. *Cancer Res* **61**, 1314–1319 (2001).
20. Daniel, P. T., Wieder, T., Sturm, I. & Schulze-Osthoff, K. The kiss of death: promises and failures of death receptors and ligands in cancer therapy. *Leukemia* **15**, 1022–1032 (2001).
21. Liu, X., Kim, C. N., Yang, J., Jemmerson, R. & Wang, X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86**, 147–157 (1996).
22. Kluck, R. M., Bossy-Wetzel, E., Green, D. R. & Newmeyer, D. D. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275**, 1132–1136 (1997).
23. Muzio, M. Signalling by proteolysis: death receptors induce apoptosis. *Int J Clin Lab Res* **28**, 141–147 (1998).
24. Kim, M. R. *et al.* Ionizing radiation can overcome resistance to TRAIL in TRAIL-resistant cancer cells. *FEBS Lett* **505**, 179–184 (2001).
25. Zhang, L. & Fang, B. Mechanisms of resistance to TRAIL-induced apoptosis in cancer. *Cancer gene Ther* **12**, 228–237 (2005).
26. Wagner, K. W. *et al.* Death-receptor O-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL. *Nat Med* **13**, 1070–1077 (2007).
27. Muppidi, J. R. & Siegel, R. M. Ligand-independent redistribution of Fas (CD95) into lipid rafts mediates clonotypic T cell death. *Nat Immunol* **5**, 182–189 (2004).
28. Krueger, A., Baumann, S., Krammer, P. H. & Kirchhoff, S. FLICE-inhibitory proteins: regulators of death receptor-mediated apoptosis. *Mol Cellular Biol* **21**, 8247–8254 (2001).
29. Doubell, A. F., Lazure, C., Charbonneau, C. & Thibault, G. Identification and immunolocalisation of annexins V and VI, the major cardiac annexins, in rat heart. *Cardiovasc Res* **27**, 1359–1367 (1993).
30. Giambanco, I., Pula, G., Ceccarelli, P., Bianchi, R. & Donato, R. Immunohistochemical localization of annexin V (CaBP33) in rat organs. *J Histochem Cytochem* **39**, 1189–1198 (1991).
31. Hamre, K. M., Chepenik, K. P. & Goldowitz, D. The annexins: specific markers of midline structures and sensory neurons in the developing murine central nervous system. *J Comp Neurol* **352**, 421–435 (1995).
32. Mayran, N., Traverso, V., Maroux, S. & Massey-Harroche, D. Cellular and subcellular localizations of annexins I, IV, and VI in lung epithelia. *Am J Physiol* **270**, L863–871 (1996).
33. Rahman, M. M., Iida, H. & Shibata, Y. Expression and localization of annexin V and annexin VI during limb bud formation in the rat fetus. *Anat Embryol* **195**, 31–39 (1997).
34. Woolgar, J. A., Boustead, C. M. & Walker, J. H. Characterization of annexins in mammalian brain. *J Neurochem* **54**, 62–71 (1990).
35. Raynal, P. & Pollard, H. B. Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. *Biochim Biophys Acta* **1197**, 63–93 (1994).
36. Liemann, S. & Lewit-Bentley, A. Annexins: a novel family of calcium- and membrane-binding proteins in search of a function. *Structure* **3**, 233–237 (1995).
37. Al-Nedawi, K., Meehan, B., Kerbel, R. S., Allison, A. C. & Rak, J. Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. *Proc Natl Acad Sci U S A* **106**, 3794–3799 (2009).
38. Trabzuni, D., Famulski, K. S. & Ahmad, M. Functional analysis of tumour necrosis factor-alpha-related apoptosis-inducing ligand (TRAIL): cysteine-230 plays a critical role in the homotrimerization and biological activity of this novel tumoricidal cytokine. *Biochem J* **350 Pt 2**, 505–510 (2000).
39. Cao, L. *et al.* Enhancement of antitumor properties of TRAIL by targeted delivery to the tumor neovasculature. *Mol Cancer Ther* **7**, 851–861 (2008).
40. Walczak, H. *et al.* Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* **5**, 157–163 (1999).
41. Gazitt, Y. TRAIL is a potent inducer of apoptosis in myeloma cells derived from multiple myeloma patients and is not cytotoxic to hematopoietic stem cells. *Leukemia* **13**, 1817–1824 (1999).
42. Kelley, S. K. *et al.* Preclinical studies to predict the disposition of Apo2L/tumor necrosis factor-related apoptosis-inducing ligand in humans: characterization of in vivo efficacy, pharmacokinetics, and safety. *J Pharmacol Exp Ther* **299**, 31–38 (2001).
43. Griffith, T. S. & Broghammer, E. L. Suppression of tumor growth following intralesional therapy with TRAIL recombinant adenovirus. *Mol Ther* **4**, 257–266 (2001).
44. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**, 55–63 (1983).
45. Mo, Z. T., Fang, Y. Q., He, Y. P. & Zhang, S. beta-Asarone protects PC12 cells against OGD/R-induced injury via attenuating Beclin-1-dependent autophagy. *Acta Pharmacol Sin* **33**, 737–742 (2012).
46. Seol, D. W. *et al.* Signaling events triggered by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL): caspase-8 is required for TRAIL-induced apoptosis. *Cancer Res* **61**, 1138–1143 (2001).
47. Jia, L. J. *et al.* Tumor-targeting Salmonella typhimurium improves cyclophosphamide chemotherapy at maximum tolerated dose and low-dose metronomic regimens in a murine melanoma model. *Int J Cancer* **121**, 666–674 (2007).
48. Ganai, S., Arenas, R. B. & Forbes, N. S. Tumour-targeted delivery of TRAIL using Salmonella typhimurium enhances breast cancer survival in mice. *Br J Cancer* **101**, 1683–1691 (2009).

Acknowledgments

This study was supported, in part, by grants from the Chinese Ministry of Science and Technology (2012CB967004, 2014CB744501, 2011CB933502, 2012AA020304, 2012AA020809), the Chinese National Nature Sciences Foundation (81121062, J1103512, 31071196, 31070706), the Ministry of Education of China (20130091130003), the Jiangsu Provincial Nature Science Foundation (BZ2012050, BE2013630) and Bureau of Science and Technology of Changzhou (WF201207, CM20122003, CZ20120004, CZ20130011, CE20135013).

Author contributions

F.Q. designed and performed most experiments and data analysis. M.J.H. purified Annexin V, TP8 and analyzed data. B.T. purified TRAIL. X.F.L., H.Q.Z. and J.Y. contributed cell apoptosis analysis and EC_{50} analysis. Z.C.H. designed and directed the overall project, and wrote the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors applied patent for this project.

How to cite this article: Qiu, F. *et al.* Annexin V-TRAIL fusion protein is a more sensitive and potent apoptotic inducer for cancer therapy. *Sci. Rep.* **3**, 3565; DOI:10.1038/srep03565 (2013).