

Full Paper

S1, a Novel Pan-BH3 Mimetic, Induces Apoptosis in Mcl-1-Overexpressing Cells Through BakTing Song^{1,2,3}, Xilong Chang³, Zhichao Zhang^{1,*}, Yubo Liu³, and Xiaoyun Shen³¹State Key Laboratory of Fine Chemicals, School of Chemistry, ³School of Life Science and Technology, Dalian University of Technology, Dalian 116024, People's Republic of China²State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing 100191, People's Republic of China

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Abstract. Mcl-1, an anti-apoptotic Bcl-2 homolog that has a structurally divergent BH3-binding pocket, non-redundant action model, and unique characteristic of short life confers complete resistance to the BH3 mimetic ABT-737. Herein, we used S1, previously identified as a Mcl-1/Bcl-2 dual inhibitor and a pure BH3 mimetic, to explore the mechanism of Mcl-1's action and supply a strategy to challenge Mcl-1's protection. Apoptosis assay in SMMC-7721, HCT116, and K562 cells demonstrated that S1 can effectively challenge Mcl-1's anti-apoptotic effect. Notably, we discovered an unexpected dynamic change of Mcl-1 that directly correlates with resistance or commitment to apoptosis induced by both ABT-737 and S1. Co-immunoprecipitation assays demonstrated that Mcl-1 increase results from Bim trafficking from Bcl-2 to Mcl-1, while subsequent Bak released by S1 determines Mcl-1 decrease and full-blown apoptosis. Further experiments using Bak shRNA testified that Bak accounts for S1-induced apoptosis and Mcl-1 decrease. Consistently, Bax-deficient DU145 cells are sensitive to S1, whereas Bak-mutant MKN-28 cells are significantly more resistant. The in vitro model could be extended to an in vivo mouse xenograft model in which Mcl-1 confers resistance by increased protein level, and the release of Bak could serve as a biomarker of apoptosis.

[Supplementary materials: available only at <http://dx.doi.org/10.1254/jphs.12103FP>]**Keywords:** BH3 mimetic, Bcl-2/Mcl-1 dual inhibitor, Mcl-1 dynamics, Bax, Bak**Introduction**

The Bcl-2 protein family includes anti-apoptotic as well as pro-apoptotic members that show homology in up to four conserved regions, termed the Bcl-2 homology (BH) domains BH1, BH2, BH3, and BH4 (1). The anti-apoptotic members, including mammalian Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1, each contain three or four BH domains. They function until engaged by their distant relatives, the pro-apoptotic BH3-only proteins. The latter, which includes mammalian Bim, Puma, Bmf, Bad, Bik, Hrk, Bid, and Noxa, and the former are related to each other and the wider family only by the short BH3 domain (2). Multi-domain pro-apoptotic proteins such as Bax and Bak induce mitochondrial dysfunction upon receipt

of death signals (3). In viable cells, these proteins exist as monomers or are restrained by Bcl-2-like proteins. In response to a variety of death stimuli, however, homo-oligomers and/or hetero-oligomers are assembled and proceed to promote apoptosis (4 – 6).

Apoptosis is controlled by complex interactions between members of the Bcl-2 family. Although Bcl-2 is the first identified anti-apoptotic member, attention has recently been drawn to a relatively neglected member, Mcl-1, since it was found that Mcl-1 confers complete resistance to the clinically ongoing BH3 mimetic ABT-263 (7). Mcl-1 has a divergent BH3-binding groove (8). Additionally, Mcl-1 has a critical role separate from that of Bcl-2 in the regulation of apoptosis (9). Whereas Bcl-2 restrains Bax, Mcl-1 mainly guards Bak (10, 11). Importantly, Mcl-1 is a short-lived protein that undergoes dynamic changes at both the transcriptional and protein levels upon certain death stimuli (12, 13). Unlike Bcl-2, which protects cancer cells only by over expression, a

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more complicated mechanism may be involved in Mcl-1's protection. For example, previous work has demonstrated that Mcl-1 could serve as a buffer for pro-apoptotic Bcl-2 family members (14). Additionally, down-regulation of Mcl-1 results in a limited apoptotic effect due to cross-talk with Bcl-2 (15). As such, there is an urgent need, as well as a challenge for both chemists and biologists, to redesign the Bcl-2 inhibitor for Mcl-1 binding and to redefine the molecular mechanism for how a pan-BH3 mimetic kills cancer cells by antagonizing the anti-apoptotic Bcl-2 family, especially Mcl-1.

We previously identified the small-molecule BH3 mimetic S1 that exhibits high affinity toward Bcl-2 and Mcl-1 ($K_i = 310$ and 58 nM, respectively) (16). Herein, we assessed the effect of S1 in three Mcl-1-overexpressing cells. Meanwhile, we investigated the molecular mechanism underlying S1-induced apoptosis, with emphasis on protein interactions between Bcl-2 family members. Results revealed a mechanism of apoptosis regulated by dynamic change of Mcl-1. To our knowledge, this is the first study of a mechanism of drug-induced, dynamic changes in Mcl-1 linked to the Bcl-2 interaction network. We found that Mcl-1 had a cross-talk with Bcl-2, binding Bim after it is released from Bcl-2, increasing itself and keeping Bak in check. We further found that releasing Bak from Mcl-1 by S1 is the lethal strike on Mcl-1. These findings may provide a theoretical framework for drugs that target a broad spectrum of Bcl-2 interactions.

Materials and Methods

Cell culture

The human liver cancer cell line SMMC-7721, colon cancer cell line HCT116, human myeloid leukemia cell line K562, lung cancer cell line NCI-H345, prostate cancer cell line DU145, gastric cancer cell line MKN-28, and mouse liver carcinoma H22 cells were purchased from the China Center for Type Culture Collection (Wuhan, China) in October 2010. Before receipt, all the cell lines were well characterized through STR (short tandem repeat) fingerprinting by the center. Cells were cultured in 75-cm² Falcon flasks (Becton Dickinson, Franklin Lakes, NJ, USA) in phenol-red-free RPMI medium 1640, which was purchased from Life Technologies Corporation (A10491-01; Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 10 mM HEPES, 24 mM sodium bicarbonate, 1 mM sodium pyruvate, 0.05 μ M 2-mercaptoethanol, 60 μ g/mL penicillin, and 100 μ g/mL streptomycin. Cells were cultured in a humidified incubator with 5% CO₂ at 37°C.

Compounds

S1 was synthesized as previously described (16) and ABT-737 was obtained from Selleck Chemicals (S1002; Houston, TX, USA). These two compounds were dissolved in DMSO (10 mM) and subsequently stored as a stock solution in dark-colored bottles at 4°C until use.

Quantitative reverse transcription (RT)-PCR

Quantitative RT-PCR was performed as previously described (17). Quantitative PCR was performed using the SYBR PrimeScript RT-PCR Kit from Takara (DRR083A, Otsu) and the Thermal Cycler Dice instrument (Takara) according to the manufacturer's instructions. Amplification of DMSO-treated cells was carried out for each sample as a control. Primer sequences for Mcl-1 were forward: 5'-GTGCTTTGACAAATTCCCA TCTGA-3' and reverse: 5'-CTTACTGTCCCGGATCT TGTCCA-3'. The relative expression ratio of the Mcl-1 gene was calculated according to the Pfaffl equation using control cells as in Ref. 18.

Assessment of apoptosis

Apoptosis was determined by flow cytometric measurement of phosphatidylserine exposure using Annexin V-FITC purchased from Beyotime Institute of Biotechnology (C1063; Suzhou, China). Cells were washed twice with PBS and incubated with a 1:40 solution of FITC-conjugated Annexin V for 10 min at room temperature. Stained cells were analyzed by flow cytometry.

Western blotting and co-immunoprecipitation

For analysis of protein levels and protein-protein interactions, standard western blotting and co-immunoprecipitation experiments were applied. For details of experiments, see Supplementary Materials and Methods (available in the online version only).

RNA interference

Bak shRNA and nonspecific control shRNA were obtained as described in a previous study (14). Transfection of shRNA was performed using LipofectamineTM 2000 from Invitrogen (11668-019; Carlsbad, CA, USA) according to the manufacturer's protocol.

Xenograft studies in mice

Mice bearing the ascitic-type hepatocarcinoma H22 were purchased from the Institute of Materia Medica, Chinese Academy of Medical Sciences (Beijing, China). Healthy BALB/c mice (CLA grade, 10 weeks, weight 34–36 g) were obtained from Dalian Laboratory Animal Center (Dalian, China). All experiments were performed according to institutional ethical guidelines on animal care approved by the Dalian University of Medical Sci-

ence. For details of experiments, see Supplementary Materials and Methods.

Statistical analysis

The data are presented as means \pm S.E.M. with $P < 0.01$ considered significant. Statistical significance was determined using Student's unpaired *t*-test with equal variance.

Results

S1 as a single agent kills Mcl-1-overexpressing cancer cells

We recently showed that S1 can compete with BH3 peptide binding to Mcl-1 in vitro (16, 19). It has been determined that Mcl-1 confers resistance to a promising BH3 mimetic, ABT-737. Therefore, we tested S1 lethality in three selected ABT-737-resistant cell lines, SMMC-7721, HCT116, and K562 cells, as well as an ABT-737-sensitive cell line, NCI-H345. Mcl-1 and Bcl-2 protein levels in the four cell lines were examined firstly. We found that Mcl-1 protein levels varied between the cell lines, whereas Bcl-2 expression was nearly equivalent. Cells were then exposed to increasing doses of S1 (0, 2.5, 5, or 10 μ M) and ABT-737 (5 μ M), respectively, for 24 h, and apoptosis was identified by the flow cytometric measurement of phosphatidylserine exposure using Annexin V staining. Consistent with previous reports, the level of Mcl-1 and/or the ratio between Mcl-1 and Bcl-2 expression determined ABT-737 resistance (20). In contrast, S1 induced an efficient, dose-dependent apoptotic response in all the four cell lines, regardless of the Mcl-1 level or the ratio between Mcl-1 and Bcl-2 (Fig. 1A). So far, it suggests that S1 can antagonize Mcl-1 as a single agent.

To further test the kinetics of S1-induced apoptosis, cells were examined at different time points after 10 μ M S1 treatment. As shown in Fig. 1B, S1 induced rapid apoptosis in NCI-H345 cells whose Mcl-1 level is the lowest among the four cell lines; 37% of apoptotic cells were found within 12 h and 72% cells underwent apoptosis at 24 h. However, only 11% of the cell population was apoptotic in the three Mcl-1-overexpressing cell lines upon 12-h S1 treatment, and substantial apoptosis (52% apoptotic cells) did not occur until 18 h. Between 12 and 18 h of treatment, the proportion of apoptotic cells remained constant. These data indicate that different from the rapid death in cells with low-level Mcl-1, S1 induced apoptosis was delayed until 18 h in cells with high-level Mcl-1.

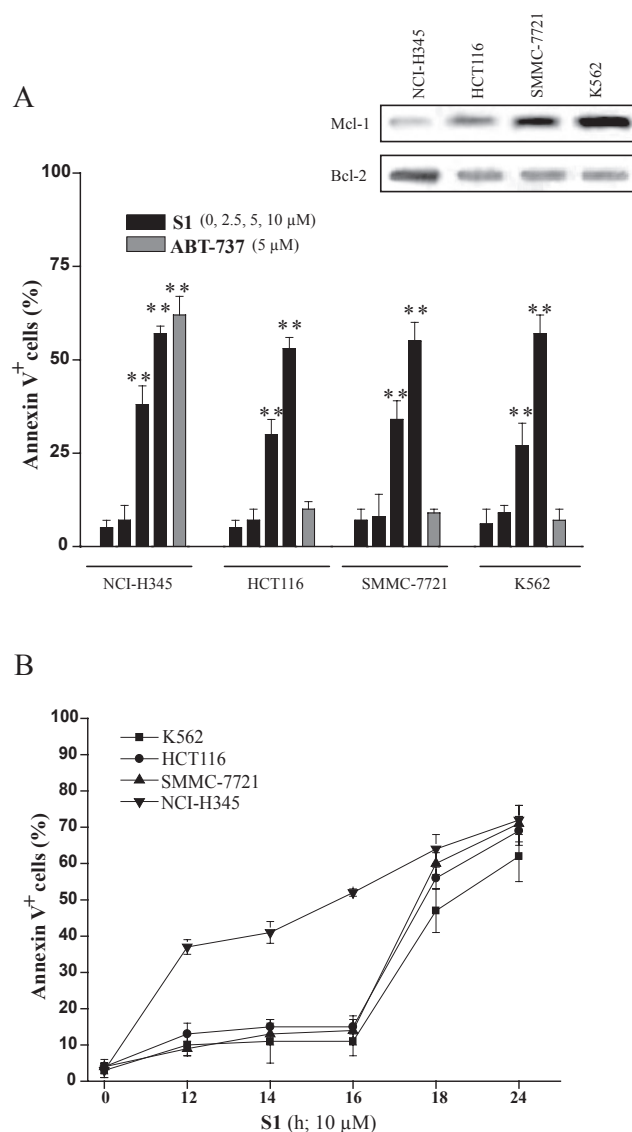


Fig. 1. S1 induces apoptosis in Mcl-1-overexpressing cells. A: S1 induces dose-dependent apoptosis in HCT116, SMMC-7721, K562, and NCI-H345 cells and ABT-737 causes apoptosis only in NCI-H345 cells. The levels of Mcl-1 and Bcl-2 in these cancer cells were first examined by western blotting. Cells were treated with S1 (0, 2.5, 5, or 10 μ M) or 5 μ M ABT-737 for 24 h, and the percentage of apoptotic cells was determined via Annexin V staining and flow cytometry. B: Kinetics of S1-induced apoptosis. Cells were treated with 10 μ M S1 for the indicated time and then apoptotic cells were determined via Annexin V staining. ** $P < 0.01$, highly significant.

Dynamic change of Mcl-1 confers a temporary protection against S1-induced apoptosis

Mcl-1 is a short-lived protein, and dynamic change in protein levels has been found to occur under certain stressful stimuli (12, 13). To investigate whether and how the dynamic change of Mcl-1 regulates S1-induced apoptosis, we treated NCI-H345 cells and HCT116 cells,

respectively, with ABT-737 or S1, and then performed a real-time examination of protein levels of Mcl-1 and Bcl-2 by means of western blotting. In NCI-H345 cells, the same progressive degradation of Mcl-1 accompanied with cleavage of caspase 3 was found between ABT-737 and S1 (Fig. 2: A and C). However, a different dynamic change of Mcl-1 between ABT-737 and S1 was found in HCT116 cells. ABT-737 induced a progressive increase of Mcl-1 levels, which is consistent with the resistance of this cell line to it (Fig. 2D). In contrast, S1 induced Mcl-1 increase from the initial treatment to 16 h, followed by a quick decline to a very low level from 18 to 24 h (Fig. 2B). The time frame of Mcl-1 level increase was just the same as delayed apoptosis. Since 18 h, Mcl-1 started to decline. The Mcl-1 dynamic change and caspase 3 cleavage was also found in K562 and SMMC-7721 cells (Supplementary Fig. 1: available in the online version only). Bcl-2 levels were remained unchanged throughout the treatment. These results indicate that increased Mcl-1 delays but can not block S1-induced apoptosis.

S1 induced dynamic change of Mcl-1 through a combination of the proteasome system and the caspase-mediated pathway

We next investigated the mechanism by which Mcl-1 level is manipulated. First, we evaluated it at the transcriptional level. SMMC-7721 cells were treated with 10 μ M S1 for 12 or 18 h. *Mcl-1* mRNA levels were then assessed by real-time PCR. Cells treated with distamycin, a DNA-groove binder that can inhibit transcription, were examined in parallel as a positive control (21). β -Actin, a housekeeping gene, was measured as an internal control

gene. No changes in *Mcl-1* mRNA levels were observed in S1-treated cells relative to DMSO-treated cells under any treatment conditions. In contrast, a 2-fold decrease of *Mcl-1* mRNA levels in cells treated with distamycin was found (Fig. 3A). These results suggest that S1 regulates the expression of Mcl-1 at the post-transcriptional level.

To test whether S1 affects the stability of Mcl-1, we used cycloheximide that blocks protein translation to measure the half-life of Mcl-1. SMMC-7721 cells were treated with 100 μ g/mL cycloheximide in the absence or presence of 10 μ M S1, and the corresponding protein extracts were analyzed for their content of Mcl-1 and other Bcl-2 family proteins. In control cells, Mcl-1 level rapidly decrease, with an estimated half-life of 2 – 3 h. The turn-over of Mcl-1 was nearly abolished upon cell exposure to S1 (Fig. 3B). However, the turn-over of the other proteins of the Bcl-2 family in S1-treated cells was nearly the same as the control (Supplementary Fig. 2: available in the online version only). We therefore concluded that S1 specifically prevents the rapid turn-over of Mcl-1 at the post-translational level.

Post-translational regulation of Mcl-1 involves the ubiquitin–proteasome degradation system and caspase pathway (22). To explore the pathways through which S1 induced change of Mcl-1 protein levels, we immunoprecipitated Mcl-1 from cell lysates and performed a western blot using an anti-ubiquitin antibody. In contrast with untreated cells, S1 induced a decrease of high molecular weight forms of Mcl-1 polyubiquitinated proteins at 12 h, which is consistent with the Mcl-1 accumulation that occurs at this time point (Fig. 3C). However, an increase

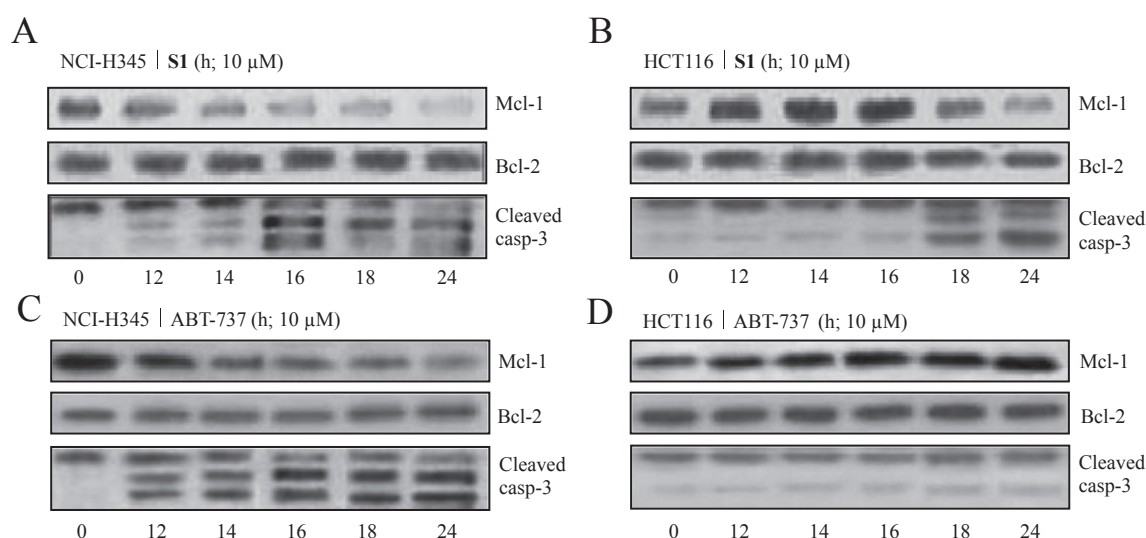


Fig. 2. Dynamic change of Mcl-1 correlates with S1-induced apoptosis. A – D: NCI-H345 and HCT116 cells were treated with 10 μ M S1 and ABT-737, respectively for the indicated time. Cells were lysed, and lysates containing 100 μ g of protein were analyzed by western blotting for Mcl-1 and Bcl-2, respectively. Cleaved caspase 3 (casp-3) was detected in parallel.

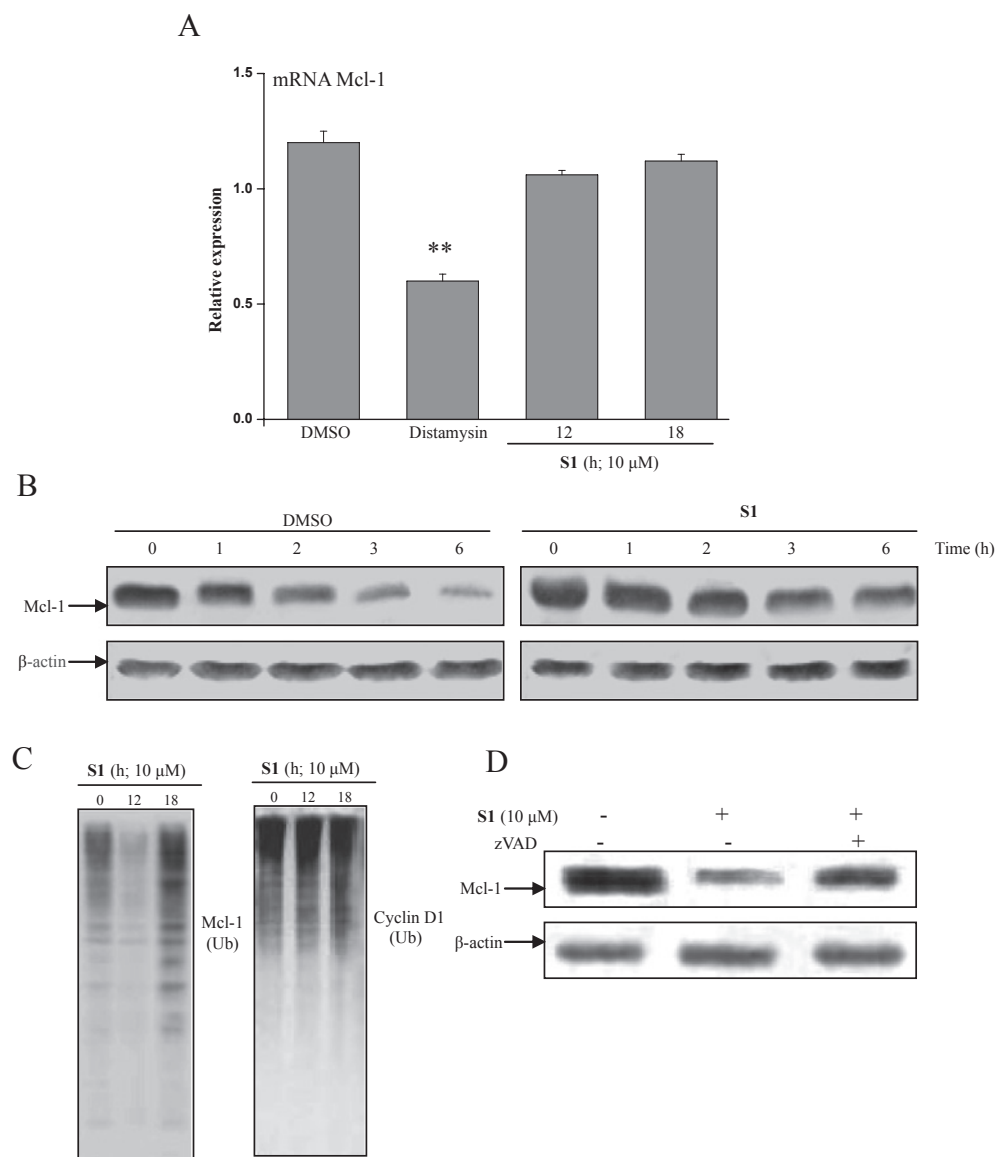


Fig. 3. S1 induced dynamic change of Mcl-1 levels through a combination of the proteasome and caspase pathway. **A:** SMMC-7721 cells were treated with 10 μ M S1 for 12 or 18 h. Distamycin was used as a positive control. *Mcl-1* mRNA levels were measured by quantitative RT-PCR and assayed by the CT method. The level of *Mcl-1* mRNA normalized to β -actin mRNA level was expressed relative to the DMSO-treated control set at 1. Data are shown as the mean \pm S.E.M. of three independent experiments. **B:** SMMC-7721 cells were pre-treated for 30 min with 50 μ M cycloheximide and treated with 10 μ M S1. Total protein extracts containing 100 μ g of protein were analyzed by western blotting for Mcl-1. **C:** Cells treated with 10 μ M S1 for 12 or 18 h were lysed in CHAPS buffer, and lysates containing 500 μ g of protein were prepared. Mcl-1 and cyclin D1, respectively, was immunoprecipitated, and the immunoprecipitates were subjected to western blot analysis with an anti-ubiquitin antibody. **D:** SMMC-7721 cells were treated with 10 μ M S1 alone or in the presence of zVAD-fmk for 24 h. Mcl-1 levels were then analyzed by western blotting. β -Actin was used as a loading control. ** $P < 0.01$, highly significant.

of ubiquitinated Mcl-1 was found after an 18-h treatment with 10 μ M S1, which may at least partly account for the loss of Mcl-1 during this period (Fig. 3C). In order to test the possibility that S1 might generally inhibit ubiquitinylation, we examined the effect of S1 on the ubiquitinylation of cyclin D1 protein, which is also a well-known short-life protein (23). Different with Mcl-1, the ubiquitinylation of cyclin D1 was stable during S1 treatment. Therefore, we concluded that S1 specifically induces Mcl-1 increase through the ubiquitinylation-proteasome pathway.

Because loss of Mcl-1 occurred at approximately the same time as caspase 3 activation, we next examined whether caspase 3 acted to cleave Mcl-1 proteins. S1 at 10 μ M was applied in combination with the caspase inhibitor zVAD-fmk. As shown in Fig. 3D, the decrease in

Mcl-1 levels was partially but not fully prevented by the addition of zVAD-fmk, indicating that caspase-mediated Mcl-1 degradation is indeed occurring. As such, we concluded that S1 induced dynamic change of Mcl-1 through a combination of the proteasome system and the caspase-mediated pathway.

Released Bim from Bcl-2 transfers to Mcl-1 and stabilizes it, while released Bak induces Mcl-1 down-regulation

As dynamic change of the post-translational level of Mcl-1 has been shown to be closely correlated with a delay to S1-induced apoptosis in the Mcl-1-overexpressing cell lines, we wanted to evaluate the contribution of Bcl-2 family members to this dynamic change. After SMMC-7721 cells were treated with ABT-737 or S1, Bcl-2 or Mcl-1 was immunoprecipitated, and the samples

were probed by western blotting for Noxa, Bim, Bax, and Bak. Because the level of Mcl-1 was changing while that of Bcl-2 was constant, we normalized all of the protein levels to compare their content both alone and in complexes. As shown in Fig. 4A, after 12 h of drug treatment, both ABT-737 and S1 had released Bax and Bim from Bcl-2, respectively. Interestingly, increased levels of Bim and Bak were found in association with Mcl-1 as the level of Mcl-1 itself was increased. As the total Bim content remained constant, we determined that the Bim released from Bcl-2 transferred to Mcl-1. The above events of Mcl-1 up-regulation, Bim and Bak shift, were similar in cells treated with either ABT-737 or S1, but a turning point at 18 h indicated a difference. At that time, we observed that S1, but not ABT-737, induced a 20% decrease in total Mcl-1 content accompanied by a 70% reduction of Bak in complex with Mcl-1. However, Bim was steadily bound with Mcl-1 with a constant ratio. We suspected that specific Bak release from Mcl-1 by S1 is the determining event for full-blown apoptosis. To test

this, we used the caspase inhibitor zVAD-fmk to prevent Mcl-1 decrease and analyzed cells after 18 h of S1 treatment. As expected, the same amount of Bak was released from Mcl-1 independent of Mcl-1 levels. Once Bak was released, cytochrome *c* was detected in the cytosolic fraction (Fig. 4B). When Bak was released from Mcl-1, Bim was found still largely bound.

Bak shRNA cells exhibit constant Mcl-1 increase and resistance to S1-induced apoptosis

To figure out the contribution of Bak as a specific mediator of disarming Mcl-1's protection during S1 treatment, we applied Bak shRNA to delete Bak expression in SMMC-7721 cells (Supplementary Fig. 3: available in the online version only). Nonspecific (NS) shRNA was used as a control. After cells were treated with a concentration gradient of S1, Mcl-1 protein levels and apoptotic cells were examined in parallel. As shown in Fig. 5, A and B, in wild-type cells, S1 at a concentration lower than 5 μ M failed to trigger apoptosis and up-regu-

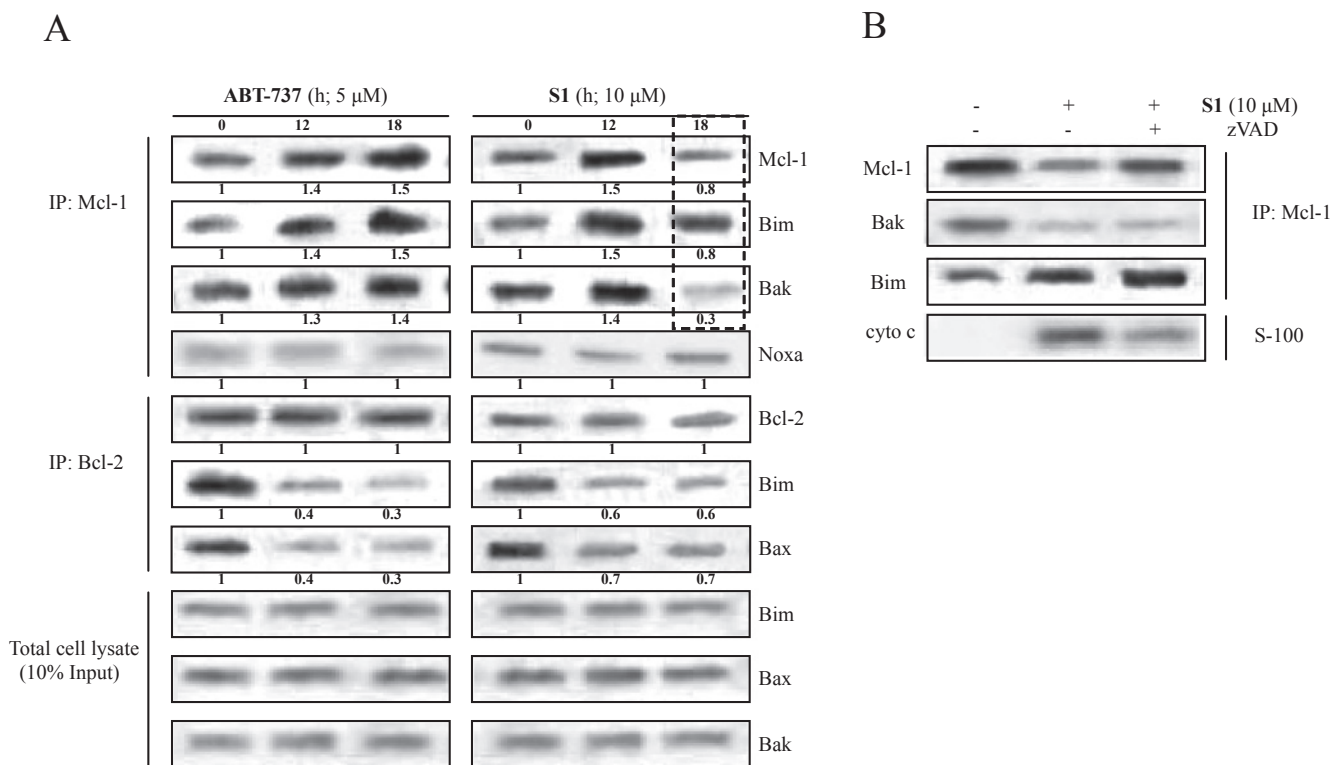


Fig. 4. Trafficking of Bcl-2 family members determines Mcl-1 dynamics. A: SMMC-7721 cells were treated with 5 μ M ABT-737 or 10 μ M S1 for 12 and 18 h. Harvested cells were subjected to immunoprecipitation of Bcl-2 followed by western blot analysis for Bax and Bim. Another group of treated cells was subjected to immunoprecipitation of Mcl-1 followed by western blot analysis for Bak, Bim, and Noxa. Each protein level normalized to β -actin level was expressed relative to the DMSO treated control set at 1. The expression of Bim, Bax, and Bak in each sample prior to immunoprecipitation was determined by western blotting. B: SMMC-7721 cells were treated with 10 μ M S1 alone or in the presence of zVAD-fmk for 18 h. Then cells were subjected to immunoprecipitation of Mcl-1 followed by western blot analysis for Bak and Bim. Cells were also lysed in digitonin lysis buffer, and the supernatants (S-100, cytosolic fraction) were collected and subjected to western blot analysis for cytochrome *c*.

lated Mcl-1 levels. When the concentration of S1 increased up to 5 μM , a dose-dependent apoptosis corresponding to Mcl-1 decrease occurred. However, in the Bak shRNA cells, apoptosis was still minimal under treatment of 10 μM S1, while Mcl-1 level increased to the highest level to protect cancer cells. Therefore, all the data suggest that Bak plays a dominant role during S1-induced apoptosis. In agreement with the Bak-led apoptotic response, a great amount of cytochrome *c* release and cleaved caspase 3 was detected in wild-type cells, but not in Bak shRNA cells (Fig. 5C). To determine the contribution of Bax to S1-induced apoptosis, we compared the apoptotic response of cells deficient in Bax and Bak, respectively, upon S1 treatment. Consistently, cells deficient in Bak showed obvious resistance to S1, while cells deficient in Bax showed comparable apoptosis with

wild-type cells (Fig. 5D). These data indicate that S1-induced apoptosis was totally through Bak.

S1 induces apoptosis in DU145 cells, but not in MKN-28 cells

We suspected that Bak could be used as a biomarker to predict sensitivity to S1 in human malignancies. To test this, we examined the effects of S1 on Bax-deficient DU145 and Bak-mutated MKN-28 cell lines. The two cell lines were treated with 10 μM S1 for 24 or 48 h. Live/Death/Apoptosis staining was applied to detect the response of cells. As shown in Fig. 6A, after 24 h of exposure, a large proportion of the DU145 cells exhibited signs of early apoptosis (cells in which nuclei present pyknosis); after 48 h, many DU145 cells were clearly in the late phase of apoptosis (cells with shrinkage and less

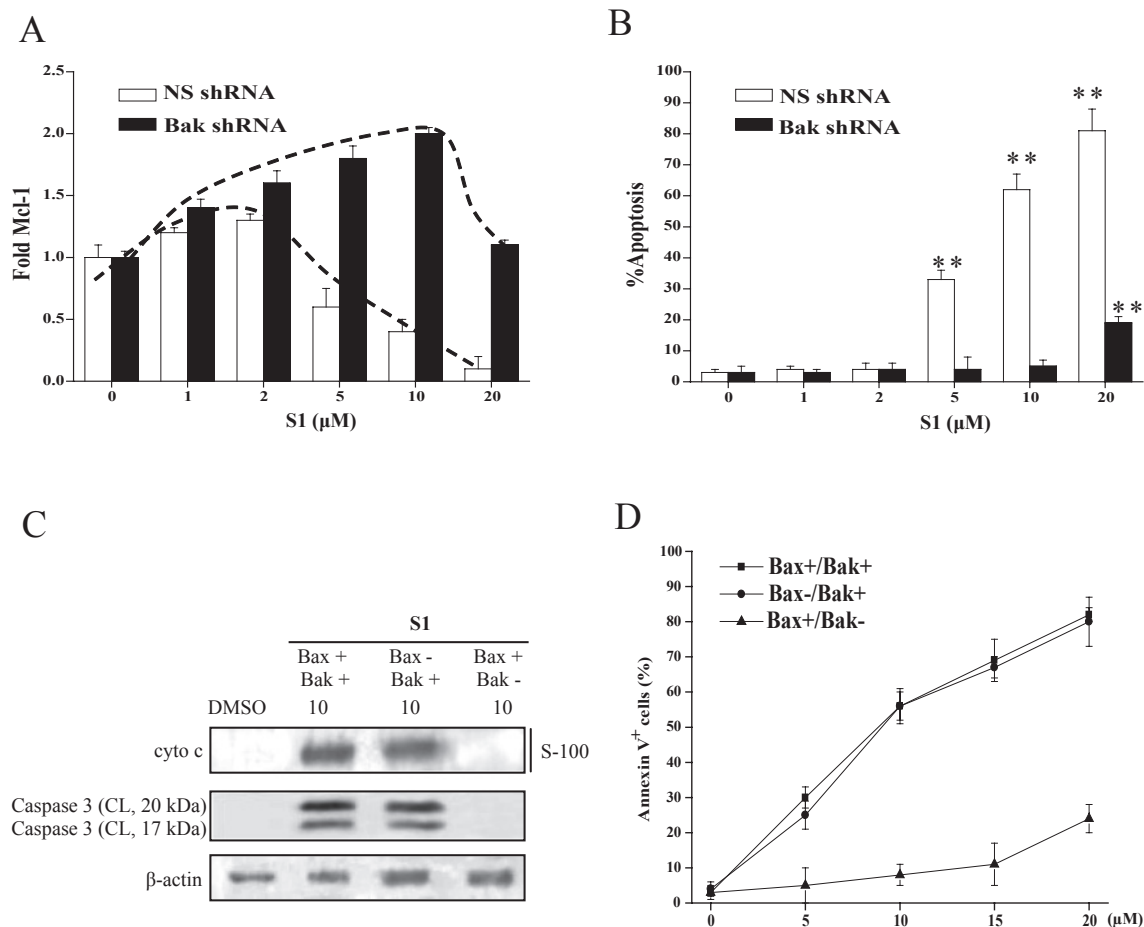


Fig. 5. S1 induced apoptosis and Mcl-1 decrease are dependent on Bak more than Bax. A, B: SMMC-7721 cells transfected with NS shRNA and Bak shRNA, respectively, were treated with S1 (0, 1, 2, 5, 10, 20 μM) for 24 h, after which the level of Mcl-1 and apoptosis were analyzed by western blotting and Annexin V staining in parallel. C: Cells transfected with Bax shRNA and Bak shRNA, respectively, were treated in parallel with 10 μM S1 for 24 h. Cells were lysed in digitonin lysis buffer, and the supernatants (S-100, cytosolic fraction) were collected and subjected to western blotting for cytochrome *c* (cyto c). Cleaved caspase 3 was analyzed by western blotting. D: Cells transfected with corresponding shRNA were treated with a gradient dose of S1 for 24 h and apoptosis was analyzed by Annexin V staining. ** $P < 0.01$, highly significant.

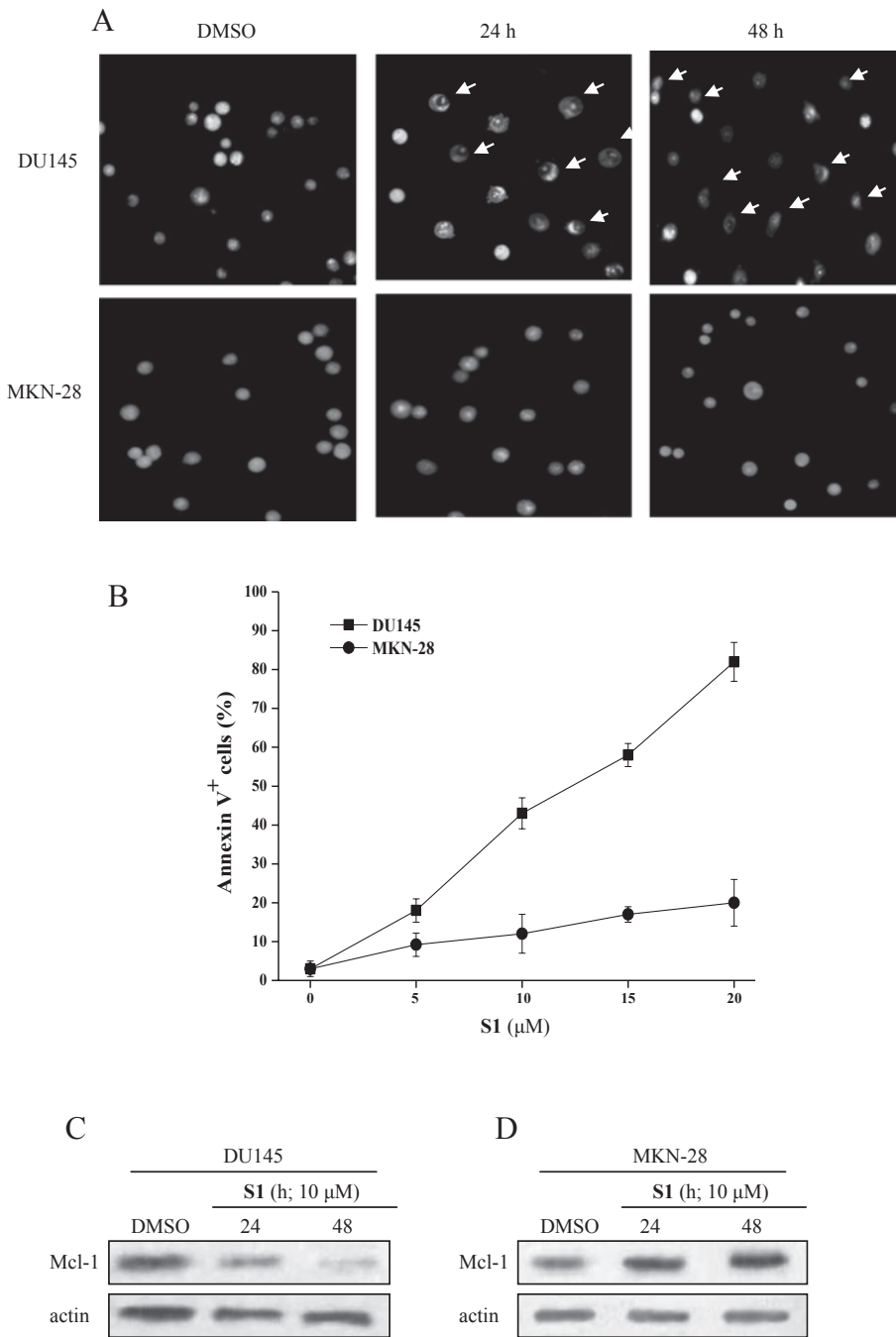


Fig. 6. S1 induced apoptosis in Bak-deficient DU145 cells, but not in Bak-mutated MKN-28 cells. **A:** S1 induces apoptosis-related change in DU145 cells but not in MKN-28 cells. Live/Dead/Apoptosis cell staining was used to measure cell apoptosis. The left panel indicates live cells treated with DMSO as a control. The middle panel indicates cells exposed to 10 μ M S1 for 24 h (arrows pointing to early apoptotic cells) and the right panel indicates cells exposed for 48 h (arrows pointed to late apoptotic cells). **B:** DU145 cells and MKN-28 cells, respectively, were treated with increased concentration of S1 for 24 h. Percentage of apoptotic cells was determined via Annexin V staining assessed by flow cytometry. **C, D:** Mcl-1 is down-regulated in DU145 cells, but up-regulated in MKN-28 cells. Each cell line was treated with 10 μ M S1 for 24 h or 48 h, after which the level of Mcl-1 was analyzed by western blotting.

visibility). In contrast, almost no apoptotic staining could be detected in the Bak-mutated MKN-28 cells, even after 48 h. Furthermore, apoptotic DU145 cells assayed by Annexin V-FITC showed S1-induced concentration-dependent induction of apoptosis. In contrast, MKN-28 cells exhibited significant resistance to S1. Only 17% of the cells were apoptotic after a 48-h treatment with 20 μ M S1 ($P < 0.01$, compared with 82% in the DU145 cells) (Fig. 6B).

Western blot analyses showed that after treatment with

S1, Mcl-1 levels were significantly diminished in DU145 cells and increased in MKN-28 cells (Fig. 6: C and D). These results further support the idea that S1-induced apoptosis requires the presence of Bak.

S1 promotes apoptosis in vivo via Bak displacement and Mcl-1 down-regulation

Next, we extended the mechanism-based apoptosis induced by S1 to H22 xenograft models. For these experiments, an ineffective dose of 0.05 mg/kg and an ef-

fective dose of 0.3 mg S1/kg body weight were applied. Thirty mice bearing palpable H22 tumors (average tumor volume = $63 \pm 18 \text{ mm}^3$) were randomly placed into three separate groups and given intraperitoneal injections of DMSO (control) or S1 at one of the two concentrations (0.05 and 0.3 mg/kg body weight) every other day for 10 days. Meanwhile, tumor volumes were measured in two dimensions with calipers. As shown in Fig. 7A, S1 substantially suppressed the growth of H22 solid tumors at the 0.3 mg/kg dose, while it had no effect at the 0.05 mg/kg dose. On day 11, the mice were euthanized, and the solid tumors were harvested. Co-immunoprecipitation from the tumor tissues showed that the relatively high dose of S1 (0.3 mg/kg) did release Bak from Mcl-1 and

down-regulate Mcl-1 levels. In contrast, the 0.05 mg/kg S1 treatment resulted in Mcl-1 accumulation and persistent Bak binding to Mcl-1. One representative sample from each group is shown with its co-immunoprecipitation results in Fig. 7, B and C, and statistical analyses for each sample are shown in Fig. 7D. Together, these data suggest that tumor regression is induced by S1 through Bak released from Mcl-1, and degradation of Mcl-1 best predicted whether S1 reached the efficient dose to kill.

Discussion

Overexpression of Mcl-1 is known to confer resistance to diverse chemotherapy as well as clinically ongoing

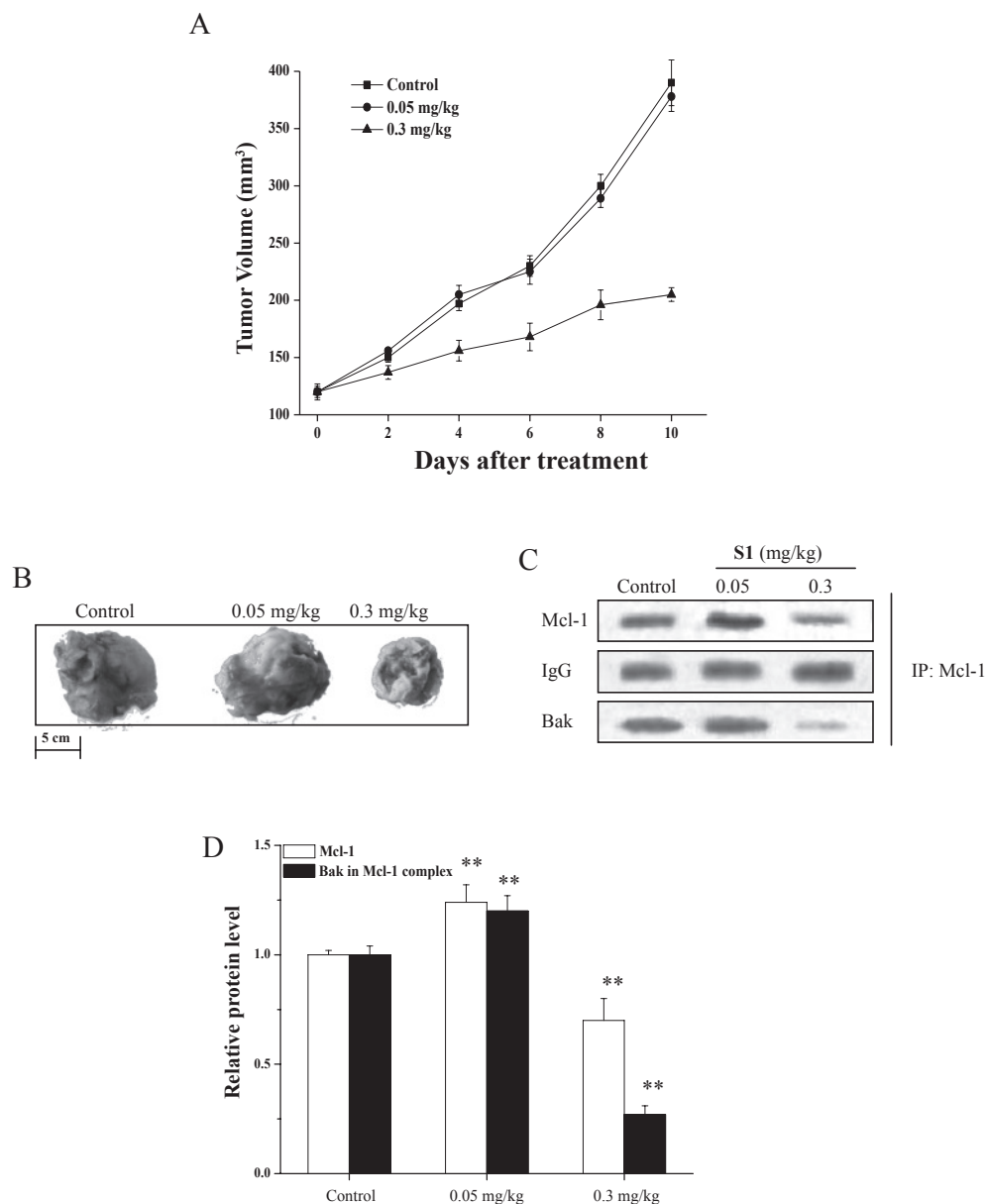


Fig. 7. S1 kills by Bak displacement and Mcl-1 down-regulation in vivo. The H22-bearing mice model was set up and administered with S1 (0, 0.05, 0.3 mg/kg body weight; $n = 10$, respectively) every other day for 10 days. A: S1 is effective at 0.3 mg/kg and ineffective at 0.05 mg/kg. On the same day as S1 treatment, the tumor volume of each mouse was determined by measuring the tumor in two dimensions with calipers. The results are the average of 10 mice in each group and expressed as the mean \pm S.E.M. On day 11, mice were killed. Tumor tissue from each mouse was stripped. B: Tumor masses of one selected mouse from each group are shown. C: Immunoprecipitation of the selected mouse is shown. The stripped tumors were ground in CHAPS buffer. Lysates equivalent to 500 μg of protein were prepared. Mcl-1 was immunoprecipitated, and the immunoprecipitates were subjected to western blot analysis for Bak. D: The average amount of Bak in the Mcl-1/Bak complex and Mcl-1 level among 10 mice of each group was displayed as relative protein level after normalizing to the DMSO-treated group. Elevated Bak in the Mcl-1/Bak complex and Mcl-1 level in the control group vs. 0.05 mg/kg group was notable. Decreased Bak in the Mcl-1/Bak complex and Mcl-1 level in the control group vs. 0.3 mg/kg group was notable (** $P < 0.01$).

BH3 mimetic ABT-263. Therefore, there is an urgent need to understand how Mcl-1 supplies such a broad protection to various apoptotic stimuli. In view of Mcl-1's binding affinity with multiple pro-apoptotic proteins and its unique feature of short half-life, drug-induced stress related to one pro-apoptotic protein is not sufficient to explain cell death in the absence of a functional link to the network of the Bcl-2 family including BH3 members and Bax/Bak as well as other Bcl-2-like relatives. S1, a direct Bcl-2/Mcl-1 antagonist that acts completely via Bax/Bak, will be a valuable tool to explore the exact role of Mcl-1 in the prevention of apoptosis. In this study, we revealed the mechanism by which Mcl-1 protects cancer cells as a buffer for freed Bim and Bak. The transferred Bim results in Mcl-1 level increase, which in turn boosts Mcl-1's function. In this way, Mcl-1 successfully rescues cancer cells from ABT-737 and confers a temporary protection to S1-induced apoptosis. Apoptosis finally occurs when S1 releases Bak from Mcl-1, which results in Mcl-1 down-regulation.

We emphasize three interesting aspects in the chain of events. First, S1 monotherapy can induce efficient apoptosis in Mcl-1-overexpressing cancer cells through Mcl-1 down-regulation. Herein, we observed intrinsic Mcl-1 level was not associated with S1-induced apoptosis. For example, S1 exhibited comparable killing ability in ABT-737-resistant and -sensitive cells whose Mcl-1 levels are quite different. Regardless of Mcl-1 base levels, down-regulation of Mcl-1 was universally observed be occurring with both S1-induced apoptosis *in vitro* and tumor regression *in vivo*. In the three Mcl-1-overexpressing cancer cells, apoptosis did not occur until 18 h, where massive cells began to undergo apoptosis (52% apoptotic cells) as well as cleavage of caspase 3. In NCI-H345 cells, apoptosis was induced at 12 h. Mcl-1 was continuously decreased, accompanied with caspase 3 activation from 12 to 24 h upon S1 treatment (Fig. 2A). No accumulation was found in this cell line. By means of caspase inhibitors, we identified that the caspase-mediated pathway was mainly responsible for Mcl-1 decrease upon S1 treatment. When apoptosis occurred, activated caspase 3 acted to cleave Mcl-1, leading to further significant decreases in protein levels. The loss of Mcl-1 released more Bak to form pores in the mitochondrial membrane. This feedback loop could enforce the apoptotic signal. This loop may explain the efficient apoptosis induced by S1 as a single agent. Conversely, when the dose of S1 was too low to induce apoptosis (0.05 mg/kg for animals), Mcl-1 was found to be persistently up-regulated. The same phenomenon was found in Bak-mutated MKN-28 cells. Consistently, ABT-737 could also induce Mcl-1 increase in resistant cancer cells reported by others (24, 25).

Second, while it is not necessarily surprising that S1 can overwhelm Mcl-1's protection supported by its down-regulation, the finding that a transient Mcl-1 up-regulation related to a delay for S1-triggered apoptosis was indeed unexpected. We identified a role of Bim on Mcl-1 up-regulation, which were in agreement with the previous findings. Willeme-Toumi et al. have reported that Bim induces Mcl-1 increase by inhibiting its ubiquitination (26). In our studies, the turning point for Mcl-1 level change was 18 h upon S1 treatment. Before 18 h, Mcl-1 protein levels were increased in the three Mcl-1-overexpressing cancer cell lines, accompanied with Bim shift and decrease of Mcl-1 ubiquitination (Fig. 3C). It is consistent with the previous discovery that Bim could stabilize Mcl-1 by reducing ubiquitination. At 18 h, Bak was released by S1. At this point, although the recaptured Bim could still stabilize Mcl-1, it can not antagonize caspase 3 cleavage due to Bak release. As another aspect, Bak release also resulted in increase of ubiquitination as we found in Fig. 3C. As such, Mcl-1 decreased. These results further illustrated our previous finding that S1-induced apoptosis is Bim-independent (16). When the accumulation was less sufficient to release Bak, as 0.05 mg/kg S1 did *in vivo*, no apoptosis occurred to antagonize Mcl-1 stabilization and its level was continuously increased.

Third, unique roles for Bax and Bak were found in S1-induced apoptosis. Bak, but not Bax, plays the predominant role in Mcl-1 down-regulation. Although minor Bax released from Bcl-2 was found, it might be too weak to trigger substantial apoptosis. Previous studies have shown that Bax activation requires Bim. Bim was found in our system buffered by Mcl-1, which may account for the limitation of Bax oligomerization. Different from Bax, Bak has been reported to be able to induce apoptosis only when freed. In agreement with this, when Bak was released by S1, apoptosis immediately occurred and Mcl-1 decreased. Our previous structure-activity relationship studies strongly supported the ability of S1 to release Bak from Mcl-1. S1 can form hydrogen bonds with the conserved R263 of Mcl-1 (19). A previous study identified a mutation of R263 in Mcl-1 that abolished its binding to Bak (14). Most probably, S1 sets free Bak through interaction with R263. The specific role of Bak was further confirmed in shRNA experiments showing that Bak itself is responsible for Mcl-1 downregulation and apoptosis.

These observations not only provide an explanation for the ability of Mcl-1 to modulate BH3-mimetic-induced apoptosis, but also have potentially important implications for the preclinical and clinical development of a pan-BH3 mimetic.

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