Published in final edited form as:

Cancer Lett. 2008 February 8; 259(2): 198–208. doi:10.1016/j.canlet.2007.10.012.

SHA 14-1, a stable and ROS-free antagonist against anti-apoptotic Bcl-2 proteins, bypasses drug resistances and synergizes cancer therapies in human leukemia cell

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Abstract

HA 14-1, a small-molecule antagonist against anti-apoptotic Bcl-2 proteins, was demonstrated to induce selective cytotoxicity toward malignant cells and to overcome drug resistance. Due to its poor stability and the reactive oxygen species (ROS) generated by its decomposition, chemical modification of HA 14-1 is needed for its future development. We have synthesized a stabilized analog of HA 14-1 – sHA 14-1, which did not induce the formation of ROS. As expected for a putative antagonist against anti-apoptotic Bcl-2 proteins like HA 14-1, sHA 14-1 disrupted the binding interaction of a Bak BH3 peptide with Bcl-2 or Bcl-X_L protein, inhibited the growth of tumor cells through the induction of apoptosis, and circumvented the drug resistance induced by the over-expression of anti-apoptotic Bcl-2 and Bcl-X_L proteins. Interestingly, the impairment of extrinsic apoptotic pathway induced moderate resistance to sHA 14-1. The moderate resistance suggested that sHA 14-1 generated part of its apoptotic stress through the intrinsic pathway, possibly through its antagonism against anti-apoptotic Bcl-2 proteins. The resistance indicated that sHA 14-1 generated apoptotic stress through the extrinsic apoptotic pathway as well. The ability of sHA 14-1 to induce apoptotic stress through both pathways was further supported by the synergism of sHA 14-1 towards the cytotoxicities of Fas ligand and dexamethasone in Jurkat cells. Taken together, these findings suggest that sHA 14-1 may represent a promising candidate for the treatment of drug-resistant cancers either as a monotherapy or in combination with current cancer therapies.

Keywords

sHA 14-1; stability; Bcl-2; apoptosis; drug resistance; synergism

1. Introduction

Over-expressing anti-apoptotic Bcl-2 (B-cell leukemia/lymphoma 2) family proteins is one mechanism for tumors to acquire resistance to cancer therapies.[1,2] Intense efforts, therefore, have been devoted to identify small-molecule antagonists against these proteins with the hope

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that such antagonists would resensitize drug-resistant malignancies to standard anticancer treatment.[3–12] HA 14-1, one of the earliest small-molecule antagonists against Bcl-2 protein, has been established to synergize the anticancer activities of cancer therapies with diverse mechanism of action, including dexamethasone, TRAIL ligand, bortezomib, doxorubicin, to name a few.[13–25] These results demonstrated the potential of HA 14-1 in combination therapy for cancer treatment. Mechanistically, several studies suggested that reactive oxygen species (ROS) may be generated *in vitro* upon HA 14-1 antagonism against anti-apoptotic Bcl-2 proteins and such ROS may induce the synergism of HA 14-1 with other anticancer drugs.[13,14,26] Furthermore, HA 14-1 selectively killed malignant cells that are drug-resistant through over-expression of Bcl-2 protein, indicating that HA 14-1 may be a selective therapy against drug-resistant malignancies.[16,17,27]

We recently observed, however, that HA 14-1 was not stable – it rapidly decomposed to inactive species with a half-life of 15 min in cell culture medium.[28] More importantly, the decomposition of HA 14-1 directly generated ROS that were the major species responsible for the observed *in vitro* cytotoxicity. The stability of HA 14-1, therefore, needs to be improved for its development into anticancer agents. The concomitant generation of ROS also raised the question whether the observed synergism from HA 14-1 to various cancer therapies were derived from its antagonism of anti-apoptotic Bcl-2 proteins or from its decomposition-generated ROS.

Based on the decomposition study about HA 14-1, we developed a stable analog of HA 14-1 – sHA 14-1. This study describes our biochemical and biological evaluation of sHA 14-1. We showed that sHA 14-1 was stable in cell culture medium with a half-life of over 24 hours, and had improved the binding interaction with the anti-apoptotic Bcl-2 proteins compared to HA 14-1. We further demonstrated that sHA 14-1 displayed a similar *in vitro* cytotoxicity as HA 14-1. Notably, sHA 14-1 did not generate ROS in cell culture medium and *in vitro*, which eliminates the potential contribution to cytotoxicity from ROS. Multiple assays established that sHA 14-1 inhibited tumor cell growth through the induction of apoptosis. Like HA 14-1, sHA 14-1 nullified drug resistance developed through the over-expression of anti-apoptotic Bcl-2 proteins. Moreover, sHA 14-1 induced apoptotic stress through both the intrinsic and the extrinsic apoptotic pathways and synergized the *in vitro* cytotoxicity of various anticancer agents in Jurkat cells, including Fas ligand and dexamethasone. Since HA 14-1 has attractive properties in cancer therapy and sHA 14-1 is a significantly more stable and more potent antagonist against anti-apoptotic Bcl-2 proteins, and intrinsically ROS free, sHA 14-1 is a promising candidate for future anticancer agent development.

2. Materials and methods

2.1. Chemicals

HA 14-1 [Ethyl-2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate][29] and sHA 14-1 [Ethyl-2-amino-6-phenyl-4-(2-ethoxy-2-oxoethyl)-4*H*-chromene-3-carboxylate] (manuscript about the synthesis and SAR is in preparation and information can be provided upon reviewer's request) were synthesized in house. Both compounds were characterized by NMR and mass spectrometry for identity and purity. CellTiter Blue Assay Kit and Apo-ONE® Homogeneous Caspase-3/-7 Assay Kit were purchased from Promega (Madison, WI). H₂DCFDA (2',7'-Dichlorodihydrofluorescein diacetate, a cell-permeable indicator for reactive oxygen species) was purchased from Molecular Probes (Eugene, OR). Cell Lysis Buffer, 2 × Reaction Buffer, dithiothreitol (DTT), and Apoptotic DNA Ladder Extraction Kit were purchased from Biovision (Mountain View, CA). Anti-cytochrome C and anti-FADD were purchased from BD Biosciences (San Jose, CA). Anti-beta-Actin, anti-rabbit IgG peroxidase conjugate, anti-mouse-peroxidase conjugate, and anti-goat IgG peroxidase conjugate were purchased from Sigma Aldrich (Saint Louis,

MO). Anti-caspase-2, anti-caspase-8, and anti-caspase-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell cultures

Normal, FADD dominant-negative, and caspase-8 dominant-negative Jurkat cells were purchased from ATCC (Manassas, VA). Bcl-2 over-expressing and Bcl-X_L over-expressing Jurkat cells were provided by Dr. Claus Belka at University of Tuebingen and Dr. Daniel Johnson at the University of Pittsburgh respectively and characterized as previously described. [29] All these Jurkat cells were cultured in RPMI medium supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 10 % fetal bovine serum at 37 °C with 5 % CO₂ in air atmosphere.

2.3. Binding interaction assay

The binding interactions of sHA 14-1 toward recombinant Bcl-2 and Bcl-X_L proteins were evaluated according to our established procedures.[29] Briefly, recombinant Bcl-2 protein (1 μM) or Bcl-X_L protein (130 nM) was incubated with a Flu-Bak peptide (10 nM) for 1 hour at room temperature to form the protein-peptide complex. Such a complex was then mixed with varying concentrations of sHA 14-1 or HA 14-1. Fluorescence polarization (FP) of the solution was then determined by using a Tecan GENios Pro multi-well plate reader. The binding of sHA 14-1 or HA 14-1 to the recombinant proteins would release the Flu-Bak peptide from the protein-peptide complex, resulting in the decrease of FP. A more potent small-molecule binder would result in greater decrease of FP.

2.4. Cell viability measurement

The *in vitro* cytotoxicity of various candidates was assayed by determining their effect to inhibit the growth of the tumor cells. In brief, the tumor cells were plated in a 96 - well plate (the density of 1×10^4 cells/well). A series of the test compounds of varied concentrations with 1 % DMSO in the final cell medium was used for the cell treatment (cells treated with medium containing 1 % DMSO served as a control). For stability studies, an identical set of the test compounds were incubated at 37 °C for the specified time period and then were used along with freshly prepared samples to treat the cells. With a 24-hour treatment, the relative cell viability in each well was determined by using CellTiter-Blue Cell Viability Assay kit or through viable cell count with trypan blue staining. The IC₅₀ of each candidate was determined by fitting the relative viability of the cells to the drug concentration by using a dose-response model in Prism program from GraphPad Software, Inc. (San Diego, CA).

Synergistic interactions between sHA 14-1 and test agents (Fas ligand or Dexamethasone) were examined by using median dose-effect analysis as described by Chou and Talalay.[30] Briefly, tumor cells were treated with serial dilutions of each agent individually and in combination with sHA 14-1 simultaneously at a fixed dose ratio for 24 hours. The cytotoxic effect of the treatment was measured by evaluating the cell viability using the cell viability assay and the long term colony. Fractional effect was calculated as fraction of cells killed by the individual agent or the combination, in treated versus untreated cells. Median dose effect analysis was performed using CompuSyn program from ComboSyn, Inc. (Paramus, NJ). The software computes combination index (CI) values based on the following equation: $CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2 + (D)_1(D)_2/\{(D_x)_1(D_x)_2\}$, where (D)₁ and (D)₂ are the doses of drug 1 and drug 2 that have x effect when used in combination and (D_x)₁ and (D_x)₂ are the doses of drugs 1 and 2 that have the same x effect when used alone. The CI values indicate synergism (< 1), additivity (1), or antagonism (> 1). The CIs of 0.1–0.3, 0.3–0.7 and 0.7–0.85 are considered to indicate strong synergism, synergism, and moderate synergism, respectively.

2.5. Assay of ROS generation

In cell-free system, dichlorodihydro-fluorescein, which can be oxidized by ROS to dichlorofluorescein, was prepared from H₂DCFDA by alkaline hydrolysis, according to the methods of Cathcart et al.[31] Briefly, 6.4 μ l of 25 mM H₂DCFDA in DMSO was added to 32 μ l of aqueous NaOH (100 mM), which was allowed to stand at room temperature for 30 min. The hydrolyte was then neutralized with 4 ml PBS and kept on ice before use. Within 3 hours of its preparation, 50 μ l of the de-acetylated probe was transferred to each well in a 96-well plate, 50 μ l of the compound of interest in PBS with 2% DMSO was then added to each well. The formation of ROS was measured kinetically with excitation at 485 nm and emission at 530 nm. ROS intensity in each well was normalized to the vehicle-treated control. HA 14-1 was used as a positive control that generates ROS.

In a systems with cells, Jurkat cells were adjusted to a cell density of 8×10^5 /ml in PBS and 5 ml of the cell suspension (5 ml) was transferred to a 5-cm round Petri dish. To one cell sample, 4 μ l 25 mM H₂DCFDA dye in DMSO was added. To another cell sample, 4 μ l DMSO was added, which served as control. These cell samples were incubated at 37 °C for 30 min to allow intracellular de-esterification of H₂DCFDA to dichlorodihydro-fluorescein. Such cell samples were collected by centrifugation, washed twice with PBS, re-suspended in PBS (3 ml). These cell samples (0.25 ml) were mixed with the compound of interest in PBS with 2 % DMSO (0.25 ml). ROS generation was followed kinetically with excitation at 485 nm and emission at 530 nm in a 96-well plate with 100 μ l sample in each well. ROS intensity in each well was normalized to the vehicle-treated control. HA 14-1 also was used as a positive control that generates ROS.

2.6. Apoptosis analyses using flow cytometry

Apoptotic cells were stained by annexin V using Annexin V-FITC Apoptosis Detection Kit and identified by flow cytometry. Briefly, Jurkat cells were treated with various concentrations of sHA 14-1 for 24 hours, harvested, washed twice with cold PBS. Upon suspension at a density of 1×10^6 cells/ml, 100 μ l of the cell suspension was transferred to a 5-ml culture tube and 5 μ l of Annexin V-FITC was added. After gentle mixing, the cells were incubated for 15 min at room temperature in the dark. Such cell samples were analyzed by flow cytometry within one hour and annexin V positive events were identified as apoptotic cells.

2.7. Caspase-3/-7 activation assay

Apo-ONE® Homogeneous Caspase-3/-7 Assay kit was used to measure the caspase-3/-7 activity according to the manufacturer's instructions. Briefly, Apo-ONE® Caspase-3/-7 reagent (50 μ l) was added to each well containing 50 μ l treated cell suspension (4×10^4 cells) in a 96-well plate. The suspension in the wells were mixed gently and incubated at 37 °C for 30 – 60 min. The fluorescence intensity of the sample in each well was measured with excitation at 485 nm and emission at 530 nm. Caspase-3/-7 activity in each well was normalized to the vehicle-treated control.

2.8. DNA fragmentation assay

DNA fragmentation was assessed by Apoptotic DNA Ladder Extraction Kit from Biovision. Briefly, Jurkat cells were treated by sHA 14-1 for 24 hours. 2.0×10^6 cells were harvested, washed with PBS, and pelleted by centrifugation for 5 min at $500 \times g$. The supernatant was removed and the cell pellet was suspended in 50 μ l DNA Ladder Extraction Buffer. After incubation at 23 °C for 10 seconds with gentle pipetting, the mixture was centrifuged for 5 min at $1600 \times g$. The supernatant was transferred to a fresh tube. The cell pellet was extracted again with DNA Ladder Extraction Buffer (50 μ l). The supernatants were combined and 5 μ l Enzyme A solution was added into the supernatant. The solution was mixed by gentle vortex and

incubated at 37 °C for 10 min. Enzyme B solution (5 µl) was then added into the mixture and further incubated overnight at 50 °C. Ammonium Acetate Solution (5 µl) was added to the sample and mixed well. Isopropanol (100 µl) was added and the solution was mixed well and kept at -20 °C for 20 min. DNA pellet was obtained by centrifugation at 13,000 × g for 10 min. The pellet was washed twice with cold 75% ethanol. The pellet was dried and re-suspended in 20 µl DNA Suspension Buffer. Samples were loaded on to a 1.2% agarose gel containing 0.5 µg/ml Ethidium bromide in both gel and running buffer. Electrophoresis was run at 50 V for 1 hour. DNA was visualized with UV light and photographed.

2.9. Fractionation of Jurkat cells into mitochondrial and cytosolic fractions (adapted from Korsmeyer et al.[32])

All the procedures of fractionation were performed at 4 °C. Briefly, the cells were collected by centrifugation at 100 × g for 5 min and washed twice with cold PBS. The pellets were re-suspended in 500 µl fractionation buffer A (1 mM HEPES-KOH, 0.1 mM EDTA, 1 mM EGTA, 250 mM sucrose, pH 7.4,) supplemented with protease inhibitor cocktail from Sigma (Saint Louis, MO). Cell disruption was performed by passing the cells through a 23-gauge needle 15 times. The lysate was centrifuged at 800 × g for 10 min at 4 °C to remove nuclei and unbroken cells. The supernatant was centrifuged at 10,000 × g for 10 min at 4 °C to pellet the mitochondrial fraction and the supernatant was the cytosolic fraction. The mitochondrial fraction was further washed with fractionation buffer B (500 µl, 10 mM HEPES-KOH, 5 mM KH₂PO₄, 5 mM succinate, 250 mM sucrose, pH 7.4) and suspended in fractionation buffer B (100 µl).

2.10 Western blot characterization of FADD and caspase-8 dominant-negative Jurkat cells

Cultured cells were harvested and washed with ice-cold phosphate-buffered saline (PBS). Cells pellets were suspended in RIPA buffer supplemented with protease inhibitor from Sigma. The cell suspension was incubated on ice for 30 min and centrifuged at 12000 × g for 10 min. The supernatant was collected and the protein concentration in the supernatant was quantified by Bradford method with BSA as the standard. Samples were separated at 150 V through electrophoresis for 1 hour on 12% SDS-PAGE. The proteins were blotted to PVDF membrane from Millipore (Boston, MA) and probed using the antibodies described under Materials and Methods. Bound antibodies were detected using appropriate peroxidase-coupled secondary antibodies followed by detection using the Supersignal chemiluminescence system from Pierce (Rockford, IL).

2.11. Statistical analysis

All biological experiments, including the binding assays, the *in vitro* cytotoxicity assays, caspase-3/-7 assays, ROS assays, and synergistic assays were performed at least twice with triplicates in each experiment. DNA fragmentation, cytochrome c release, and western blot analyses were performed at least twice. Representative results are depicted in this report. Data were analyzed and presented using the Prism program. Student's *t*-test was applied for comparison between groups using the Prism program. Significance was set at $P < 0.05$.

3. Results and discussion

3.1. Interaction of sHA 14-1 to Bcl-2 and Bcl-X_L

Mechanistically, anti-apoptotic Bcl-2 proteins and pro-apoptotic Bcl-2 proteins have been proposed to antagonize each other through heterodimerization in the apoptotic regulation. [33] Small-molecule antagonists against anti-apoptotic Bcl-2 proteins are expected to mimic the pro-apoptotic BH3-only proteins to bind the anti-apoptotic Bcl-2 proteins and release the pro-apoptotic Bax and Bak proteins for apoptosis initiation, thereby, antagonizing the anti-

apoptotic Bcl-2 proteins.[8,9] As a potential antagonist against anti-apoptotic Bcl-2 protein based on HA 14-1, sHA 14-1 was first assayed for its ability to disrupt the binding interaction of a Bak BH3 domain peptide with Bcl-2 and Bcl-X_L proteins respectively, a well-accepted model system for the heterodimer of anti-apoptotic and pro-apoptotic Bcl-2 proteins.[34] As shown in Fig. 1, compared to HA 14-1, sHA 14-1 demonstrated increased potency to disrupt the binding interaction of the Bak peptide with Bcl-2 and Bcl-X_L proteins, suggesting that sHA 14-1 is a potentially more potent antagonist against anti-apoptotic Bcl-2 proteins than HA 14-1.

3.2. The stability, cytotoxicity, and ROS generation of sHA 14-1

We next evaluated whether sHA 14-1, compared to HA 14-1, has improved stability and does not generate ROS intrinsically. In order to quickly determine whether sHA 14-1 was stable, we assayed the change of cytotoxicity of sHA 14-1 after it was incubated in cell culture medium. The rationale was that if sHA 14-1 was stable in cell culture medium, the incubated sHA 14-1 solution should have the same cytotoxicity as the freshly prepared solution. Briefly, we prepared a series of sHA 14-1 solutions of different concentrations in cell culture medium and incubated these solutions at 37 °C for 0 or 24 hours. These sHA 14-1 solutions were then evaluated for their *in vitro* cytotoxicity against Jurkat cells. As shown in Fig. 2, a 24-hour incubation of HA 14-1 in RPMI cell culture medium resulted in the total loss of its cytotoxicity (IC₅₀ increased ~ 60 fold, $t_{1/2}$ = 15 min) while such an incubation resulted in minimal decrease of the cytotoxicity of sHA 14-1 (IC₅₀ increased 1.7 folds, $t_{1/2}$ > 1 day), suggesting that sHA 14-1 has much better stability over HA 14-1. The stability of sHA 14-1 was further confirmed by HPLC analysis of a 24-hour incubated solution of sHA 14-1 in RPMI medium that resulted in no detectable decomposition product(s) (data not shown). Under the 24-hour treatment conditions, sHA 14-1 (IC₅₀ = 27.2 μM) had comparable cytotoxicity as HA 14-1 (IC₅₀ = 12.6 μM) toward Jurkat cells.

Upon establishing the improved stability of sHA 14-1, we next explored whether sHA 14-1 can induce ROS generation as HA 14-1 does. The potential generation of ROS was evaluated both in cell-free system and in the presence of Jurkat cells by following established procedures. [28]. Under both conditions, the sHA 14-1 treated samples did not induce significant increase of ROS as compared to background (Fig. 3). These results suggest that sHA 14-1 does not generate ROS even upon its interaction with cells. The lack of ROS from sHA 14-1 treatment further strengthens the argument that the ROS observed in HA 14-1 treatment is likely to derive solely from HA 14-1 decomposition, not through its antagonism against anti-apoptotic Bcl-2 proteins.

3.3. Apoptotic activity of sHA 14-1

Various Bcl-2 antagonists, including HA 14-1, have been demonstrated to induce apoptosis. [21,26,35] sHA 14-1 was therefore investigated for its ability to induce apoptosis as well, which was characterized in Jurkat cells with four assays that reflect different aspects of apoptosis. In all these assays, Jurkat cells were treated with sHA 14-1 for 24 hours over a range of concentrations. In the first assay, apoptosis was evaluated by flow cytometry for annexin-V FITC positive events. As shown in Fig. 4A, the percentage of annexin-V FITC positive events increased as the concentration of sHA 14-1 increased, suggesting that sHA 14-1 induced apoptosis in a dose-dependent manner. The induction of apoptosis by sHA 14-1 in Jurkat cells were further characterized by caspase-3/-7 activation, DNA fragmentation assays, and mitochondrial cytochrome *c* release. As shown in Fig. 4B, C, and D, sHA 14-1 induced caspase-3/-7 activation, DNA fragmentation, and the mitochondrial cytochrome *c* release, all in a concentration-dependent manner over the same range of sHA 14-1 concentrations. Taken together, these results clearly demonstrated that sHA 14-1 induced apoptosis in Jurkat cells.

3.4. Over-expression of Bcl-2 or Bcl-X_L induced no resistance to sHA 14-1

Our previous studies established that over-expression of Bcl-2 or Bcl-X_L in Jurkat cells could effectively induce such cells resistant to various standard cancer therapies (IC₅₀s increase > 5 folds, Fig. 5A – D).[29] In this study, we established that such over-expression rendered Jurkat cells resistant to cytotoxicity induced by Fas ligand as well (Fig. 5E). This is expected, as Jurkat cells undergo Type II extrinsic apoptotic regulation, which will activate caspase-8 to cleave Bid to tBid upon extrinsic apoptotic stress.[36] tBid will then induce the activation of caspase-3 through the mitochondrial intrinsic pathway, in which the anti-apoptotic Bcl-2 proteins are the key regulators to prevent apoptosis. Over-expression of the anti-apoptotic Bcl-2 proteins therefore can confer resistance to Jurkat cells against Fas ligand. In our previous study, we demonstrated that such over-expression in Jurkat cells, however, failed to induce resistance to HA 14-1.[29]

As an antagonist derived from HA 14-1, sHA 14-1 was assayed to determine whether it may nullify the drug resistance derived from the over-expression of anti-apoptotic Bcl-2 proteins as well. As shown in Fig. 5F, the Jurkat cell lines over-expressing Bcl-2 or Bcl-X_L showed very similar sensitivity to sHA 14-1 compared to the normal one. This result established that though the over-expression of Bcl-2 or Bcl-X_L can induce resistance to cancer therapies with varied mechanism of actions, it failed to induce resistance to sHA 14-1, further supporting that sHA 14-1 functions as an antagonist against anti-apoptotic Bcl-2 proteins. The ability of sHA 14-1 to abolish the drug resistance from over-expressing anti-apoptotic Bcl-2 proteins suggests that sHA 14-1 may be useful to treat cancers that are drug resistant through the elevation of those anti-apoptotic proteins.

3.5. Characterization of extrinsic apoptotic pathway deficient Jurkat cell lines and the sensitivity of these cell lines to sHA 14-1

HA 14-1 has been reported to synergize the anticancer activity of TRAIL – an extrinsic apoptotic stimulus,[14,18] implying that HA 14-1 may induce apoptotic stress through the extrinsic pathway, which would be valuable as a cancer therapy. Such synergism may also be mediated by the ROS generated through HA 14-1 decomposition.[37,38] We, therefore, evaluated whether the ROS-free sHA 14-1 was able to induce cell death through the extrinsic apoptotic pathway as well. We tested this by evaluating the sensitivity of two Jurkat cells with their extrinsic apoptotic pathway impaired – a FADD dominant-negative Jurkat cell and a caspase-8 dominant-negative Jurkat cell to sHA 14-1.[39,40] As caspase-8 could be activated by caspase-2 or caspase-3/-7 that are mediated through the intrinsic apoptotic pathway, [41-43] the caspase-8 dominant-negative Jurkat cells may be resistant to intrinsic apoptotic stimuli as well while the FADD dominant-negative Jurkat cells should be resistant only to extrinsic apoptotic stimuli. We hypothesized that if sHA 14-1 induced apoptosis independent of the extrinsic pathway, these two dominant-negative Jurkat cell lines, especially the FADD dominant-negative one, should have the same sensitivity to sHA 14-1 as the normal Jurkat cell line.

These two cell lines were first characterized for their expression of FADD and caspase-8. As shown in Fig. 6, western blot characterization confirmed that the FADD dominant-negative Jurkat cells are devoid of FADD and the caspase-8 dominant-negative Jurkat cells are devoid of caspase-8. These two Jurkat cell lines express similar levels of caspase-2 and caspase-9 compared to the normal Jurkat cells, suggesting that these two dominant negative Jurkat cell lines differ from the normal Jurkat cell line only with respect to FADD and caspase-8 respectively.

Next we evaluated the sensitivity of Fas ligand to these two Jurkat cells to establish whether the function of the extrinsic apoptotic pathway is impaired. We also evaluated the sensitivity

of doxorubicin and camptothecin to these two Jurkat cells to establish that the function of the intrinsic apoptotic pathway is intact as the control Jurkat cells. It was found that these three Jurkat cells showed the same sensitivity to doxorubicin and camptothecin (Fig. 7A and B). However, with all the concentrations tested, Fas ligand failed to induce any cytotoxicity to FADD dominant-negative Jurkat cells and induced much less toxicity to caspase-8 dominant negative Jurkat cells compared to the normal Jurkat cells (Fig. 7C). These functional studies further confirmed that the extrinsic apoptotic pathways are impaired in these two dominant negative Jurkat cell lines while they still retain normal intrinsic apoptotic pathway. The sensitivity of these two Jurkat cells to sHA 14-1 was then evaluated (Fig. 7D). Unlike Fas ligand, sHA 14-1 still induced obvious growth inhibition in both of these two Jurkat cells, suggesting that impairment of the extrinsic apoptotic pathway cannot fully block the *in vitro* cytotoxicity of sHA 14-1. This indirectly suggests that sHA 14-1 induces apoptotic stress through the intrinsic pathway, which is consistent with its antagonism against anti-apoptotic Bcl-2 proteins. However, both cells were less sensitive to sHA 14-1 compared to the normal Jurkat cells. The moderate resistance of these two dominant negative Jurkat cells, especially the FADD dominant-negative one, toward sHA 14-1 suggests that sHA 14-1 partially induces its cytotoxicity through the extrinsic pathway as well.

The above results suggest sHA 14-1 can target both the intrinsic and the extrinsic apoptotic pathways. Therefore, impairment of either pathway would fail to induce complete resistance to sHA 14-1, as demonstrated in the Jurkat cells with the over-expression of anti-apoptotic Bcl-2/Bcl-X_L or the removal of FADD/caspase-8.

3.6. Synergism of sHA 14-1

Since sHA 14-1 can induce apoptotic stress through the intrinsic and the extrinsic apoptotic pathways and HA 14-1 has been demonstrated to synergize apoptotic stimuli through both pathways, we speculated that sHA 14-1 may be able to synergize the anticancer activity of both intrinsic and extrinsic apoptotic stimuli. We, therefore, evaluated the synergistic potential of sHA 14-1 towards two different cytotoxic agents: dexamethasone (intrinsic apoptotic stimulus) and Fas ligand (extrinsic apoptotic stimulus). As shown in Table 1, sHA 14-1 synergized the anticancer activity of both candidates under all the concentration combinations tested. Comparatively, sHA 14-1 induced stronger synergism to Fas ligand than to dexamethasone.

4. Conclusion

HA 14-1, a small-molecule antagonist against anti-apoptotic Bcl-2 proteins, has been demonstrated to have exciting properties as a potential anticancer agent. Because of its instability and decomposition-associated generation of ROS, HA 14-1 is not an appropriate candidate for therapeutic development and biological application. We have developed a modified analog of HA 14-1 – sHA 14-1. The present study demonstrated that sHA 14-1 was much more stable than HA 14-1 ($t_{1/2}$ increases > 96 folds) and did not induce ROS generation. sHA 14-1 also demonstrated improved binding interaction with anti-apoptotic Bcl-2 proteins compared to HA 14-1, and displayed a similar *in vitro* cytotoxicity as HA 14-1. Given the fact that most of the cytotoxicity of HA 14-1 derives from ROS [28], sHA 14-1 has improved cytotoxicity derived from the antagonism against anti-apoptotic Bcl-2 proteins. As expected, sHA 14-1 effectively induces apoptosis in Jurkat cells, supported by Annexin V staining, caspase-3/-7 activation, DNA fragmentation, and mitochondrial cytochrome *c* release.

Drug resistance is one of the major obstacles to the successful treatment of malignancies. The mechanisms accounting for the drug resistance phenotype may be mediated through the over-expression of anti-apoptotic Bcl-2 proteins.[44] As an antagonist against anti-apoptotic Bcl-2 proteins, sHA 14-1 is insensitive to the drug resistance induced by the over-expression of the anti-apoptotic Bcl-2 proteins. Furthermore, knockout of the extrinsic apoptotic pathway only

induces moderate resistance to sHA 14-1. These studies suggest that sHA 14-1 induces apoptotic stress through both pathways and cancers may be unlikely to acquire significant drug resistance to sHA 14-1. sHA 14-1 also synergized the anticancer activities of both intrinsic and extrinsic apoptotic stimuli, further underscoring the potential of sHA 14-1 in cancer therapy.

Because of its improved stability, its ability to overcome drug resistance, and its synergistic property, we propose that sHA 14-1 is a promising anticancer candidate. Evaluation of its anticancer properties *in vivo* is undergoing.

Acknowledgments

This research was supported in part by the AACP New Investigator Program sponsored by the American Foundation for Pharmaceutical Education (D. T., S. D., J. D., C. X.) and NIH-R01GM062964 (J. P., J. L.).

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HA 14-1

sHA 14-1

Fig. 1. Competitive binding of HA 14-1 or sHA 14-1 and a fluorescent Bak BH3 peptide with anti-apoptotic Bcl-2 proteins monitored by the decrease of fluorescence polarization (FP, the more decrease indicates stronger binding). Each data point is the means $\pm$ S.D. of data obtained from two independent experiments (**: $P < 0.01$; ***: $P < 0.001$).

Fig. 2.

Changes of cytotoxicities of HA 14-1 and sHA 14-1 upon their incubation in RPMI cell culture medium. The cells were treated for 24 hours. Viability was normalized to that of the cells treated by 1% DMSO alone.

Fig. 3.

Induction of ROS generation by HA 14-1 or sHA 14-1. A: ROS generation by HA 14-1 (20 μM) and sHA 14-1 (5 – 40 μM) in PBS without Jurkat cells; B: ROS generation by HA 14-1 (20 μM) and sHA 14-1 (5 – 40 μM) in PBS with Jurkat cells. (Under both conditions, HA 14-1 induced significantly more ROS generation than the solvent control did, $P < 0.001$; sHA 14-1 did not induce ROS generation more than the solvent control did.)

Fig. 4.

Dose-dependent induction of apoptosis in Jurkat cells by 24-hour sHA 14-1 treatment. A. Flow cytometry analysis of Jurkat cells stained with Annexin-V FITC ($P < 0.01$ for [sHA 14-1] $> 10 \mu\text{M}$). Apoptotic cells were quantified as the Annexin-V FITC positive events. B. The activities of caspase-3/-7 that are normalized to the activity in the sample treated with 1% DMSO alone ($P < 0.01$ for [sHA 14-1] $> 20 \mu\text{M}$). C. The extent of DNA fragmentation. D. Western blot of cytochrome *c* in the mitochondrial and cytosolic fractions (actin was immunoblotted to ensure that equal amount of total cell lysate was analyzed for each treatment, not shown).

Fig. 5.

The sensitivities of the three Jurkat cell lines to sHA 14-1 and various anticancer agents (Inserts are the IC₅₀s of the individual agents against the corresponding Jurkat cells, no IC₅₀s for taxol and Fas ligand because the concentration range tested did not allow dose-response curve fitting to obtain the IC₅₀s). The cells were treated for 48 hours. The viability of the cells was then measured and normalized to that from the cells treated with 1% DMSO alone. $P < 0.001$ for the comparisons of the cytotoxicities of cisplatin, doxorubicin, thioguanine, taxol, and Fas between the normal Jurkat cell and each of the over-expressing ones at all the concentrations tested.

Fig. 6. Western blot characterization of Jurkat cell lines with antibodies indicated. Lanes from left to right: normal, caspase-8 dominant negative, and FADD dominant negative Jurkat cells.

Fig. 7.

In vitro cytotoxicity of doxorubicin (A), camptothecin (B), Fas ligand (C, $P < 0.001$ for the comparisons between the control Jurkat cell and each of the dominant negative ones at all the concentrations tested), and sHA 14-1 (D, **: $P < 0.01$; ***: $P < 0.001$, for the comparisons between the control Jurkat cell and each of the dominant negative ones) to various Jurkat cell lines. The cells were treated for 24 hours. The viability of the cells was then measured and normalized to that from the cells treated with 1% DMSO alone.

Table 1

The combination indexes (CI) for each cytotoxic agent combined with sHA14-1 in Jurkat cell line.

Treatment		CI
Fas	IC ₅₀	0.05 ± 0.02
	0.75 × IC ₅₀	0.24 ± 0.10
	0.5 × IC ₅₀	0.44 ± 0.13
Dex	IC ₅₀	0.78 ± 0.03
	0.75 × IC ₅₀	0.71 ± 0.02
	0.5 × IC ₅₀	0.89 ± 0.04

Dexamethasone (Dex) and Fas ligand (Fas) were combined with sHA 14-1 (sHA) at a fixed dose ratio (molar ratio of sHA 14-1: Dex = 1:10,000 and ratio of sHA 14-1: Fas = 20,000 M:1 g/ml respectively) to treat the cells and cell viability was assessed by Cell-Titer Blue Viability Assay or long-term survival colony assay. Data were analyzed as described. Data shown are means ± S.D. of the CI values obtained at the IC₅₀, 0.75 × IC₅₀, and 0.5 × IC₅₀.