Biochemical Pharmacology 82 (2011) 600–609

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Novel proteasome-inhibitory syrbactin analogs inducing endoplasmic reticulum stress and apoptosis in hematological tumor cell lines

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ARTICLE INFO

Article history:
Received 7 May 2011
Accepted 20 June 2011
Available online 28 June 2011

Keywords:
Syrbactin
Proteasome inhibitor
Thapsigargin
Hematological tumors
Bortezomib

ABSTRACT

The proteasome has been recognized as a druggable target in cancer cells, and this has led to searches for pharmacologic agents that target this cellular organelle for cancer therapeutic purposes. Syrbactins are a group of microbial metabolites consisting of two related families, the glidobactins and the syringolins. Some members of this group have revealed cytotoxic efficacy in tumor cells, and more recently it was discovered that they exert proteasome-inhibitory function. Based on this therapeutic promise and to gain further understanding of their molecular modes of action, we chemically synthesized de-novo three novel syrbactin analogs and characterized their proteasome-inhibitory and in vitro anti-neoplastic activity in human cell lines representing multiple myeloma, Waldenström's macroglobulinemia, and lymphocytic leukemia. Our results show that two of these novel compounds are able to inhibit proteasome activity in the nanomolar range, reduce the expression of anti-apoptotic proteins survivin and Mcl-1, and cause severe endoplasmic reticulum (ER) stress, resulting in pronounced tumor cell death. These anticancer effects can be synergistically enhanced when the agents are combined with thapsigargin, which further aggravates ER stress by a different mechanism. Taken together, our findings support the notion that syrbactin analogs may provide a structural platform for the development of novel cancer therapeutics, and that their efficacy may be further increased when complemented with other agents that trigger ER stress.

1. Introduction

The mammalian ubiquitin–proteasome system represents a multi-protein complex responsible for the removal of unneeded or damaged proteins from the cell's inventory. At the center of this pathway is the 26S proteasome, which is composed of the proteolytically active 20S core particle (20S proteasome) and two 19S regulatory complexes. It has been recognized that pharmacological inhibition of this cellular machinery may lead to cell death, especially in cells with revved up protein synthesis, where accumulation of misfolded proteins may cause proteotoxicity [1–3]. As well, reduced turnover and stabilization of pro-apoptotic proteins may contribute to the cytotoxic outcome [4].

The first-in-class proteasome inhibitor to be tested in the clinic was the dipeptidyl boronic acid small molecule bortezomib (Velcade®), which has been approved for the treatment of patients with multiple myeloma (MM) and mantle cell lymphoma [5–7]. Recent clinical trials have also indicated some therapeutic benefit of bortezomib in other plasma cell disorders, such as Waldenström's macroglobulinemia [8,9]. Despite its clinical success, however, bortezomib presents several clinical challenges. For instance, not all MM patients respond to this drug, and most of those who do will ultimately relapse. As well, bortezomib causes significant side effects, such as thrombocytopenia and peripheral neuropathy (incidence ≥30%), which can be dose-limiting and may lead to discontinuation of treatment [7,10]. These serious drawbacks have spurred the search for novel proteasome inhibitors with improved therapeutic efficacy and greater tolerability. Several next-generation candidates are being investigated, including peptide boronic acid analogs MLN9708 and CEP-18770, peptide epoxyketones carfilzomib and PR-047, and NPI-0052, a beta-lactone compound. These compounds share the ability to inhibit proteasome activity in vitro, but show differences in enzyme binding kinetics, which

Abbreviations: ER, endoplasmic reticulum; GlbA, glidobactin A; SylA, syringolin A; SylB, syringolin B.

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doi:10.1016/j.bcp.2011.06.031

might affect their pharmacology and result in different efficacy and safety profiles (see reviews [6,11]).

Very recently, an entirely new class of proteasome inhibitors was identified that are structurally distinct from the established proteasome inhibitors and were shown to bind to the eukaryotic proteasome in a novel manner [12–14]. This new class has been termed the syrbactins, which represent a collection of two structurally related, but nonetheless distinct natural product families encompassing the syringolins and the glidobactins. Syringolins, such as syringolin A (SylA) and syringolin B (SylB), are virulence factors secreted by the plant pathogen *Pseudomonas syringae pv. syringae*. Glidobactins, such as glidobactin A (GlbA), are produced by a few species of the *Burkholderiales* order of proteobacteria.

Proteasome-inhibitory potency has been established for SylA, SylB, and GlbA, and the crystal structures of SylA or GlbA with the yeast proteasome revealed a novel mechanism of covalent, irreversible binding with strongest affinity for the β5 subunit, which confers chymotrypsin-like proteolytic activity [13,14]. As well, these compounds have revealed anticancer activity in preclinical studies. For example, SylA has been shown to induce apoptosis in human neuroblastoma, multiple myeloma, and ovarian cancer cell lines [15,16]; GlbA has revealed cytotoxic effects on human B16 melanoma and HCT-116 colon carcinoma cells, and was able to prolong the life span of mice implanted with P388 lymphocytic leukemia cells [17].

Encouraged by these earlier findings indicating that syrbactins may have the potential to become clinically useful anticancer agents, we de-novo synthesized three novel analogs with strong similarities to GlbA and SylB, and characterized their cytotoxic activity in hematological tumor cells in vitro.

2. Materials and methods

2.1. Materials

A detailed description of the de-novo chemical synthesis of three syrbactin analogs (T-02, T-03, T-04) is provided elsewhere (manuscript submitted). Each compound was dissolved in dimethyl sulfoxide (DMSO; Sigma–Aldrich, St. Louis, MO) to make a stock solution of 10 mM. Thapsigargin (Sigma–Aldrich) was dissolved in DMSO as well. Caspase inhibitor Z-VAD-FMK (in DMSO) was obtained from Promega (Madison, WI). All agents were added to the cell culture medium in a manner so that the final concentration of solvent (DMSO) did not exceed 1%, and mostly was well below this value.

2.2. Cell lines and culture conditions

Human RPMI/8226 multiple myeloma cells were obtained from the American Tissue Culture Collection (ATCC; Manassas, VA). The human Waldenström's macroglobulinemia cell line BCWM1 was kindly provided by Steven P. Treon [18], and human REH lymphocytic leukemia cells were a kind gift from Alan L. Epstein. All cells were propagated in RPMI 1640 (Mediatech, Manassas, VA) supplemented with 5% fetal bovine serum (Omega Scientific, Tarzana, CA), 100 U/mL penicillin and 0.1 mg/mL streptomycin (Gibco/Invitrogen, Carlsbad, CA), and kept in a humidified incubator at 37 °C and a 5% CO_2 atmosphere.

2.3. MTT assay

Cell survival in response to drug treatment was determined by conventional MTT assays in 96-well plates as described [19]. MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was purchased from Calbiochem/EMD Chemicals, Gibbstown, NJ. Each MTT assay was set up as triplicate treatments and was repeated independently several times at variable cell densities from 1.0 to 5.0×10^4 per well and with different drug treatment times from 48 to 72 h.

2.4. Immunoblots and antibodies

Total cell lysates were prepared and analyzed by Western blot as described earlier [20]. The primary antibodies were purchased from Epitomics (Burlingame, CA) (survivin: cat# 2565-1), from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (ubiquitin: sc-8017; actin: sc-1615; ATF3: sc-188) or from Cell Signaling Technology (Beverly, MA) (Mcl-1: #4572; PARP: #9542; CHOP: #2895; caspase 7: #9492; caspase 3: #9665) and were used according to the manufacturers' recommendations. The secondary antibodies were coupled to horseradish peroxidase and detected by chemiluminescence using the SuperSignal West substrate from Pierce (Rockford, IL).

2.5. Proteasome activity measurements

Chymotrypsin-like activity measurements of the 26S and 20S proteasomes from drug-treated cells were performed as described previously [21], with modifications as follows. Cell cultures were treated with drug analogs for various times and then collected and analyzed. To determine 26S proteasome activity, cells were first washed with buffer 1 (50 mM Tris, pH 7.4, 0.1 mM EDTA, 2 mM DTT, 5 mM $MgCl_2$, 2 mM ATP) (all chemicals from Sigma–Aldrich). Thereafter, cells were suspended in buffer 1 supplemented with 250 mM sucrose (Sigma–Aldrich) and 0.04% NP-40 (Sigma–Aldrich), and were lysed via multiple passaging (10×) through a syringe fitted with a 25-gauge needle. This was followed by centrifugation at 10,000 rpm for 10 min. The supernatant was collected and protein concentration was determined. Twenty microgram of protein was diluted with buffer 1 to a final volume of 200 μL, which was added to a well of a 96-well plate. Then, 1.6 μL of fluorogenic proteasome substrate Suc-LLVY-AMC (Sigma–Aldrich; stock solution: 10 mM) was added to each well (final concentration: 80 μM) to start the reaction. Proteolytic activity was measured by monitoring the release of the fluorescent 7-amido-4-methylcoumarin (AMC) group at 37 °C using the 2103 EnVision Multilabel Plate Reader (Perkin Elmer, Waltham, MA) with 360 nm excitation and 460 nm emission wavelength settings. Readings were taken at 15 min intervals up to 1 h.

For determination of 20S proteasome activity, cells were lysed in buffer 2 (50 mM Tris, pH 7.4, 0.1 mM EDTA, 20 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.03% SDS) supplemented with 0.04% NP-40, which was followed by centrifugation at 10,000 rpm. Twenty microgram protein from the supernatant was diluted to 200 μL in buffer 1, and the reaction was started by the addition of 1.6 μL Suc-LLVY-AMC substrate (Sigma–Aldrich) to a final concentration of 80 μM. Incubation in the 2103 Envision reader took place at 37 °C, and readings were taken at the settings described above. In all cases, the standard curve was established with the use of defined concentrations of free AMC in parallel.

2.6. Statistical analysis

Data are presented as mean ± SD. Comparisons between different treatments were performed using Student's *t*-test, and a probability value (p) of less than 0.05 was considered significant.

3. Results

3.1. Novel syrbactin analogs exhibit proteasome-inhibitory activity

The proteasome-inhibitory activity of syrbactins was discovered only very recently [13,14] and may explain at least part of

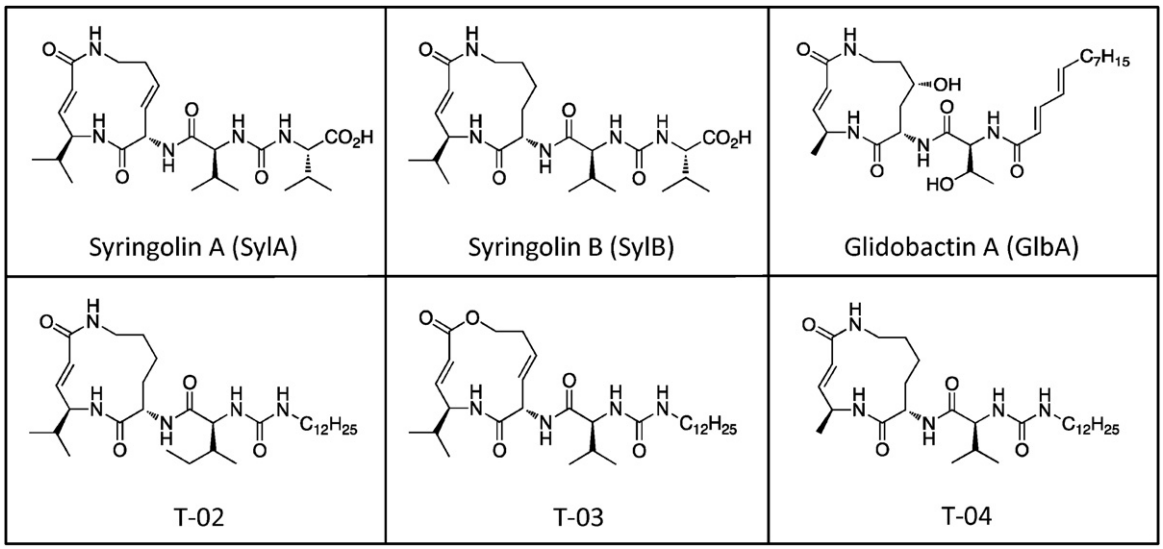

Chart 1. Chemical structures of naturally occurring and novel synthetic syrbactins.

their antitumor activity [15–17]. In an effort to further develop these types of promising anticancer molecules and gain understanding of their molecular modes of action, we de-novo synthesized three novel analogs with strong similarities to GlbA and SylA, named T-02, T-03, T-04 (Chart 1), to investigate their presumed anticancer activities.

All three synthetic syrbactin analogs were prepared with an aminoacyl dodecyl urea side chain (Chart 1). This choice was based on the work of Clerc et al. [13], who showed that a synthetic analog SylA-LIP (with the bis-valine urea side chain of SylA changed to valine decyl urea) has increased potency in proteasome inhibition. For T-02, the amino acid used was isoleucine rather than valine, a change aimed to exploit hydrophobic interactions in a binding pocket evident in the crystal structures of the proteasome inhibited by both SylA and GlbA [14]. For T-03, the α,β-unsaturated lactam of SylA was changed to a lactone. This was expected to increase the speed of nucleophilic addition of the proteasome Thr1 hydroxyl to the analog. For T-04, the isopropyl group of the syringolin series was changed to the methyl group of the glidobactins, which are known to have greater intrinsic anticancer activity. As is also apparent from the crystal structure of the proteasome inactivated by the syrbactins [14], the Thr1 hydroxyl adds to the drug from the same side of the ring as this alkyl group. Switching this group to the smaller methyl group should also enhance the speed of nucleophilic addition of the proteasome to the analog.

The measured proteasome-inhibitory activity of the novel compounds is shown in Fig. 1. Using the 26S proteasome as the target, we found strong inhibitory activity of T-02 and T-04 in the nanomolar range. In comparison, T-03 required higher concentrations to unfold its inhibitory effect and required low micromolar concentrations to achieve >50% inhibition (Fig. 1a). Inhibitory activity of T-02 and T-04 was also investigated with the 20S proteasome as the target. Here, their inhibitory activity was even more pronounced than in the case of the 26S proteasome, with an IC_{50} of about 65 nM for either compound (Fig. 1b). Fig. 1c shows a time course of these inhibitory effects and demonstrates that significant proteasome inhibition is achieved within the first 8 h of the addition of T-02 or T-04 to cells in culture, and nearly complete inhibition has taken place after 24 h. Combined, these results demonstrate time- and dose-dependent effects of our novel analogs, as well as a preference for more effective inhibition of the 20S subunit.

As an additional confirmation of proteasome inhibitory activity, we determined the accumulation of ubiquitinated proteins in cells treated with T-04. As shown in Fig. 2, treatment of RPMI/8226 multiple myeloma cells with increasing concentrations of this analog resulted in increasing amounts of ubiquitinated proteins, as determined by Western blot analysis with an antibody recognizing such modified proteins. This result confirms that inhibition of proteasome activity generates the expected cellular consequence, namely the blockage of degradation of proteins that are marked for removal from the cells' inventory. Also shown in this figure is that T-04 treatment resulted in proteolytic cleavage of PARP-1 (poly ADP-ribose polymerase-1), revealing that drug treatment triggered programmed cell death/apoptosis and providing initial evidence of anticancer activity (which we investigated in further detail below).

3.2. Syrbactin analogs are cytotoxic to hematological cancer cell lines

We next investigated the cytotoxic potential of our three syrbactin analogs in three cell lines representing different types of hematological cancers. RPMI/8226 multiple myeloma, BCWM1 Waldenström's macroglobulinemia, and REH lymphocytic leukemia cells were cultured in the presence of increasing concentrations of T-02, T-03, and T-04, and cell survival was determined by MTT assay. As shown in Fig. 3, T-02 and T-04 were similarly potent and effectively killed all cells in the nanomolar range. T-03 was noticeably less potent in RPMI/8226 and REH cells, but did exert stronger cytotoxicity in BCWM1 cells. Thus, cytotoxic potency of the three compounds was aligned with their proteasome-inhibitory activity as shown in Fig. 1, with T-02 ≈ T-04 > T-03.

Based on the above indication (i.e., PARP cleavage shown in Fig. 2) that cell death might involve apoptosis, we included the broad-spectrum caspase inhibitor Z-VAD-FMK in our analysis. As shown in Fig. 4a and b, this pan-caspase inhibitor effectively prevented cell death induced by analog T-04 in RPMI/8226 and BCWM1 cells, revealing that caspases played a major role in executing cell death in response to treatment with syrbactin analogs and thus, that cell death occurred through apoptosis.

As it is known that activation of caspases can lead to the cleavage of proteasome subunits and result in proteasome inhibition [22], we next sought to investigate whether proteasome inhibition after treatment with our syrbactin analogs possibly represented an indirect effect due to caspase activity. We analyzed cellular 20S proteasome activity in response to T-04 treatment in

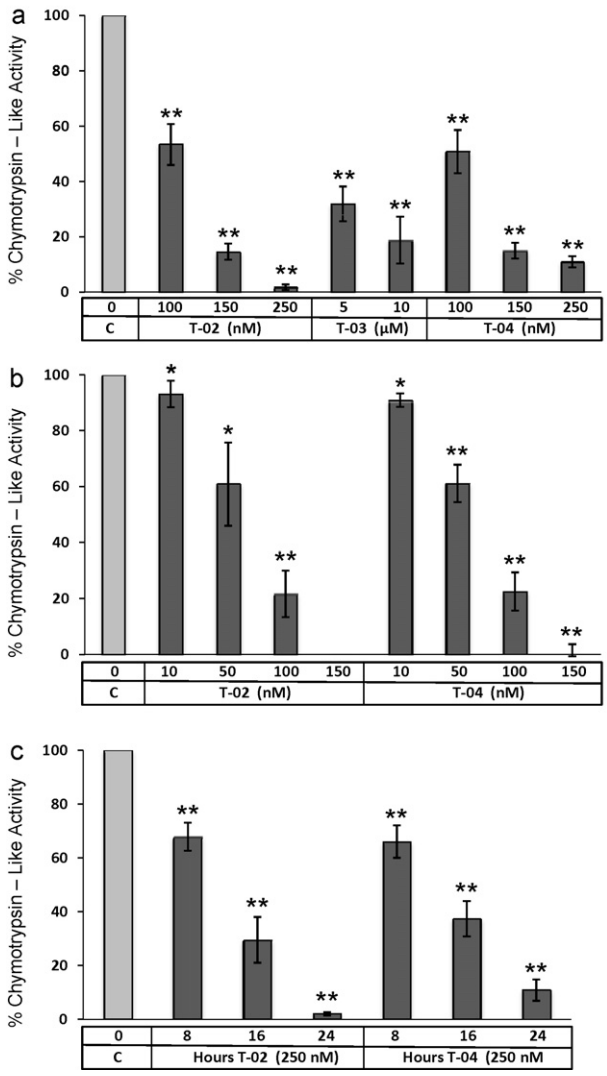

Fig. 1. Proteasome inhibition by syrbactin analogs. Cells were treated with individual syrbactin analogs and proteasome inhibition was determined. (a) 26S proteasome activity after treatment of cells with different concentrations of each analog for 24 h. (b) 20S proteasome activity after treatment of cells with different concentrations of analog for 16 h. (c) 20S proteasome activity after different time points of treatment. Shown is mean (±SD) percentage of proteasome activity, where untreated control (C) is set to 100%. $^{*}p < .05$, $^{**}p < .01$. Data were pooled from three independent experiments.

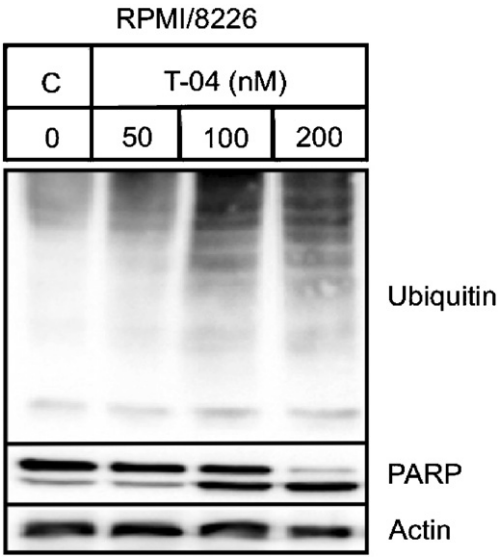

Fig. 2. Accumulation of ubiquitinated proteins after T-04 treatment. RPMI/8226 multiple myeloma cells were treated with increasing concentrations of T-04 for 24 h. Thereafter, total cell lysates were harvested and analyzed by Western blot with an antibody recognizing ubiquitinated proteins (as an indirect readout of proteasome inhibition) and with an antibody against PARP (as a direct readout of ongoing apoptosis). Each blot was stripped and re-probed with an antibody detecting actin levels (as a loading control; only one blot is shown). C, untreated control cells.

the presence or absence of Z-VAD-FMK. As shown in Fig. 4c, inclusion of the pan-caspase inhibitor had no effect at all on proteasome inhibition by T-04. This outcome excluded the possibility that proteasome inhibition might be a consequence of caspase activation, but rather was consistent with earlier reports that naturally occurring syrbactins bind to proteasome subunits and directly block their function [13,14].

3.3. Syrbactin analogs trigger pro-apoptotic ER stress

In order to gain insight into the molecular pathways that might be involved in mediating the cytotoxic effects of our proteasome-inhibitory compounds, we treated BCWM1, RPMI/8226, and REH cells with T-04 and investigated the impact on several key cellular targets known to control cell survival and cell death. We chose the following representative targets: survivin and Mcl-1 (myeloid cell leukemia-1), which are anti-apoptotic proteins known to support chemoresistance of cancer cells; CHOP (C/EPB homologous protein), a death-inducing transcription factor and prominent indicator of severe endoplasmic reticulum (ER) stress; ATF-3 (activating transcription factor 3), a component of the general cellular stress response; caspases 3 and 7, two representative proteases that execute apoptosis; and PARP-1, a substrate for caspase-3 and a marker for the late stages of apoptosis.

BCWM1 cells were exposed to increasing concentrations of T-04 for 20 h, and cellular lysates were analyzed by Western blot analysis. As shown in Fig. 5a, drug treatment resulted in down-regulation of survivin and Mcl-1 protein levels, and increased expression of CHOP and ATF-3. At the same time, there was pronounced conversion of inactive pro-caspases 3 and 7 to their activated effector counterparts, as well as proteolytic cleavage of PARP-1. To investigate the time course of these effects, BCWM1 cells were treated with 100 nM T-04 for various time periods. Fig. 5b shows that strong pro-apoptotic drug effects could be seen as early as 16 h, and by 32 h anti-apoptotic Mcl-1 and survivin proteins had disappeared and PARP-1 cleavage had reached completion. ER stress marker CHOP was strongly induced at 16 h, and high levels of this pro-apoptotic protein persisted until 32 h. Similarly, stress indicator ATF-3 presented with greatly elevated levels in response to drug treatment.

Similar effects of T-04 treatment could also be documented in RPMI/8226 cells, with down-regulation of Mcl-1, induction of CHOP and ATF-3, as well as activation of caspase-3 and cleavage of PARP-1 (Fig. 5c). In REH cells, prominent activation of caspase-3, cleavage of apoptosis marker PARP-1, and strong induction of CHOP were observed; however, no induction of ATF-3 expression and only modest effects on Mcl-1 and survivin expression were detected in these cells (Fig. 5d). Taken together, these results reveal desirable anticancer effects of T-04 at the molecular level, although there appeared to be some cell type-specific differences.

To further investigate the striking downregulation of Mcl-1 protein after treatment of BCWM1 cells with T-04, we exposed these cells to syrbactin analog in the presence or absence of Z-VAD-FMK. As shown in Fig. 6, the presence of the pan-caspase inhibitor completely prevented Mcl-1 downregulation by T-04. To control for caspase-inhibitory efficacy of Z-VAD-FMK, we also performed a

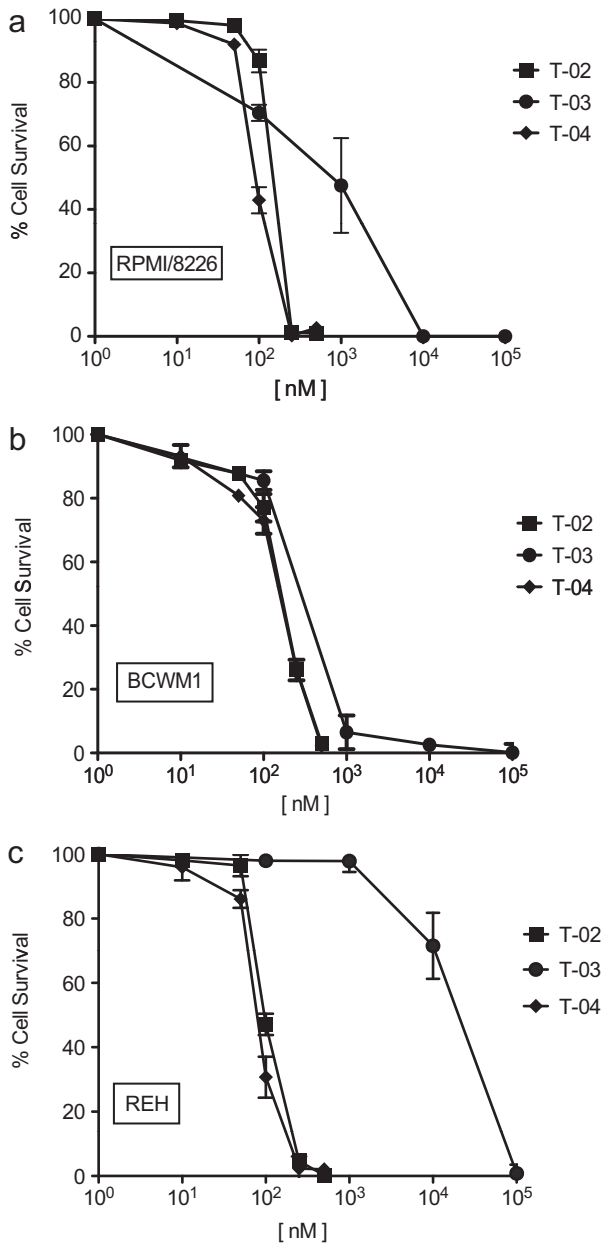

Fig. 3. Cytotoxic effects of syrbactin analogs. Various hematological tumor cell lines were treated with increasing concentrations of each syrbactin analog for 72 h. Thereafter, cell viability was measured with MTT assay. (a) RPMI/8226 multiple myeloma cells. (b) BCWM1 Waldenström's macroglobulinemia cells. (c) REH lymphocytic leukemia cells. Shown is mean (±SD) percentage of cellular viability, where untreated controls are set to 100%, and where each condition was analyzed in triplicate. The entire experiment was repeated several times at variable cell densities from 1.0 to 5.0 × 10^4 per well and consistently yielded similar results.

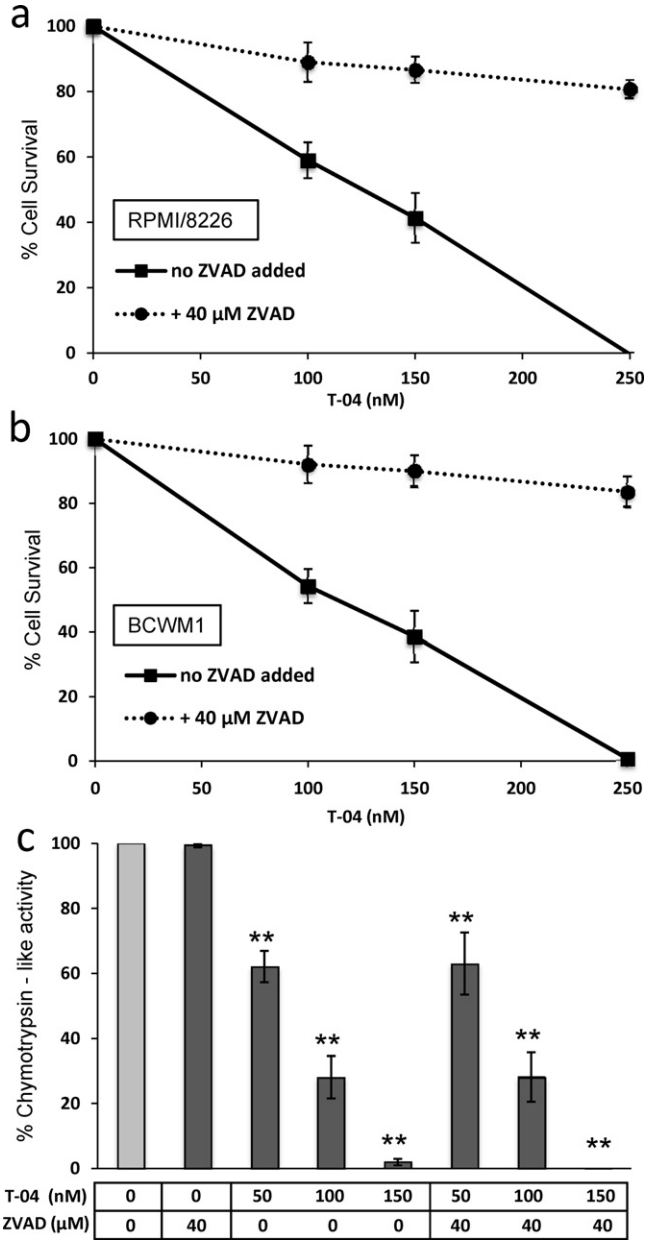

Fig. 4. Effects of pan-caspase inhibitor. The cell permeant caspase inhibitor Z-VAD-FMK (ZVAD) was included. (a) RPMI/8226 or (b) BCWM1 cells were incubated with increasing concentrations of T-04 in the presence or absence of 40 μM ZVAD, and cell viability was determined after 48 h by MTT assay. (c) 20S proteasome activity after treatment of BCWM1 cells with increasing concentrations of T-04 in the presence or absence of 40 μM ZVAD for 24 h. Shown is mean (±SD) percentage change, where untreated controls are set to 100%, and where each condition was analyzed in triplicate. $^{**}p < .01$ as compared to untreated control.

blot for caspase 3, which did confirm the absence of activated (proteolytically cleaved) caspase. Altogether, this result indicated that removal of Mcl-1 from the cell's inventory occurred through caspase activation by the syrbactin analog.

3.4. Thapsigargin enhances syrbactin analog-induced ER stress and cell death

The large increase in CHOP protein levels described above revealed that T-04 was able to trigger the ER stress response. We therefore asked whether the anticancer outcome could be enhanced if syrbactin analogs were combined with another compound that would trigger ER stress by a different mechanism. Thus, we used thapsigargin (Tg), a widely used inhibitor of SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase) that causes leakage of calcium from the ER into the cytosol, resulting in ER stress [23]. We combined different concentrations of T-02 or T-04 with different concentrations of Tg and determined cytotoxic efficacy in BCWM1 and RPMI/8226 cells. Relatively low concentrations of the syrbactin analogs were chosen, so that potential enhancing effects could emerge more prominently. As shown in Fig. 7, drug combination treatments were strikingly potent in both tumor cell lines. For example, 50 nM T-02, when administered alone, barely affected viability of RPMI/8226 cells, but when combined with 1 μM Tg, cell survival was reduced to nearly 0% (Fig. 7a). The overall efficacy of the different combination

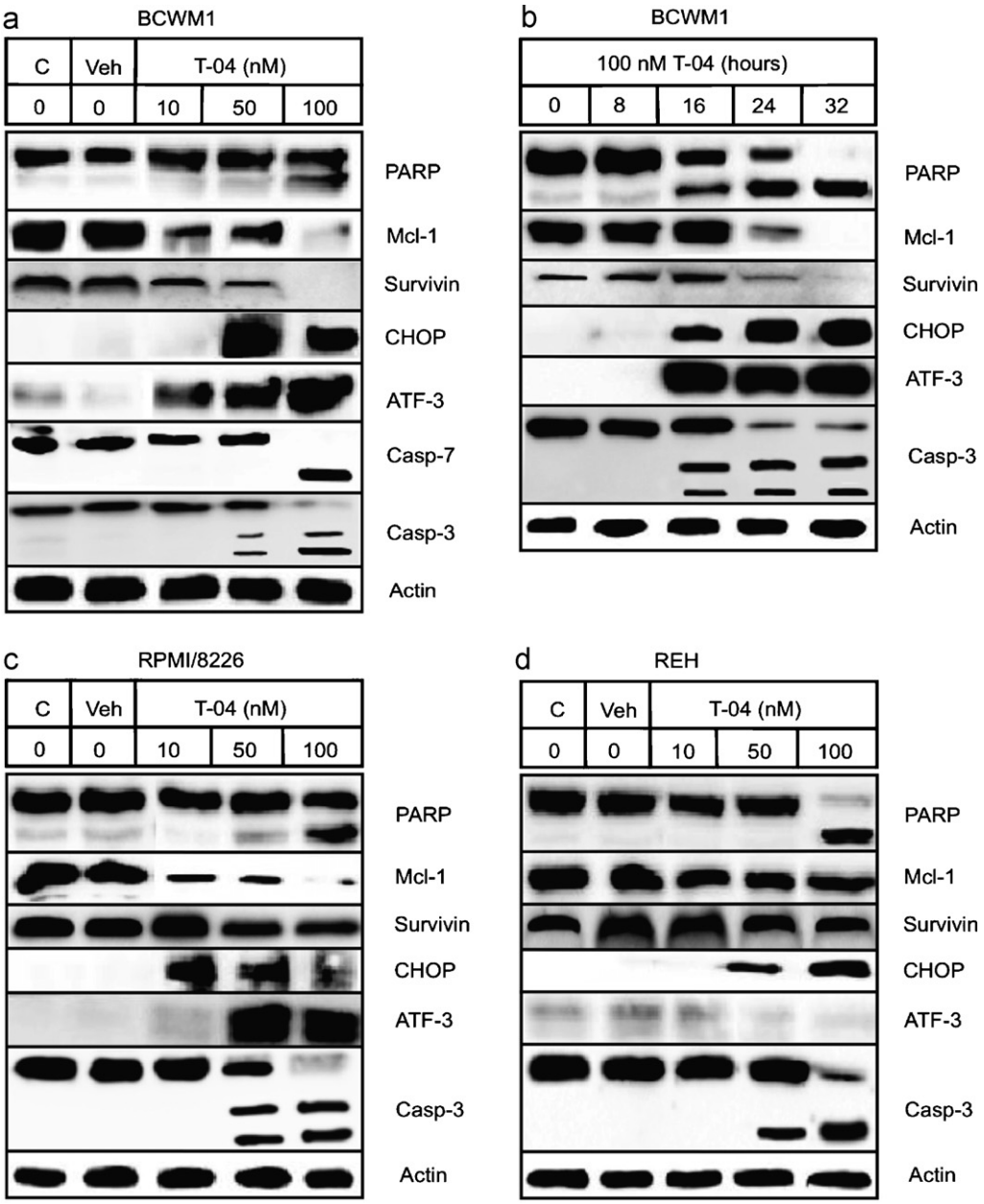

Fig. 5. Induction of markers for ER stress and apoptosis in cells treated with T-04. Various hematological tumor cell lines were treated with T-04 and expression levels of several cell survival-regulatory proteins were analyzed by Western blot. (a) BCWM1 cells were treated with increasing concentrations of T-04 for 20 h. (b) BCWM1 cells were treated with 100 nM T-04 for various time points. (c) RPMI/8226 cells and (d) REH cells were treated with increasing concentrations of T-04 for 20 h. Parallel cell cultures remained untreated (control, C) or received vehicle (Veh) only. For Western blot analysis, antibodies against the following targets were used: PARP, caspases 3 and 7 (apoptosis markers), Mcl-1 and survivin (anti-apoptotic proteins), CHOP (pro-apoptotic ER stress indicator), ATF-3 (component of the cellular stress response), and actin (loading control).

treatments was somewhat variable, depending on the exact concentrations chosen, but in all cases the effect consistently turned out to be synergistic. Table 1 lists the combination index (C.I.) for each combination, which was calculated according to Chou and Talalay [24], where C.I. < 1 is synergistic, C.I. $= 1$ is additive, and C.I. > 1 is antagonistic. Table 1 also presents a refined characterization of the C.I. by showing the degree of synergism according to Chou [25].

Because we suspected that enhancement of syrbactin analog-induced toxicity by Tg involved ER stress, we investigated the expression levels of the ER stress marker CHOP in BCWM1 cells after combination treatment with low dosages of T-04 and Tg. Indeed, as shown in Fig. 8, both agents together triggered much stronger CHOP expression than either agent alone. As well, apoptotic PARP cleavage was substantially greater in response to combination treatment as compared to individual treatments, and expression of the general stress marker ATF-3 followed a similar pattern. Thus, the enhancement of cytotoxic efficacy of T-04 by Tg, as shown in Fig. 7, was aligned with substantially aggravated ER stress (i.e., CHOP induction) and greater apoptosis (i.e., PARP cleavage), as shown in Fig. 8.

4. Discussion

Cancer therapy with bortezomib presents several clinical challenges, including significant side effects and development of treatment resistance [7,10], and therefore novel proteasome inhibitors with improved therapeutic efficacy and greater tolera-

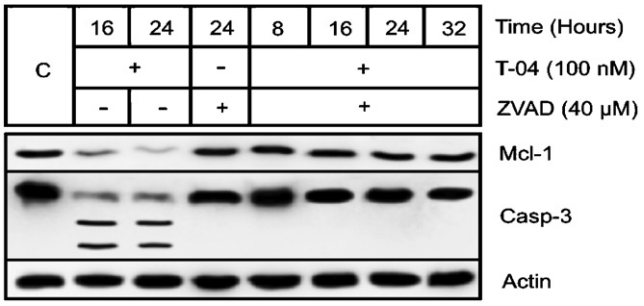

Fig. 6. Inhibition of Mcl-1 downregulation by pan-caspase inhibitor. BCWM1 cells were treated with 100 nM T-04 for various time points in the presence or absence of 40 μM Z-VAD-FMK (ZVAD). Expression levels of Mcl-1 and caspase 3 were analyzed by Western blot with specific antibodies. Actin was used as a loading control. Note that the addition of Z-VAD-FMK completely prevents proteolytic cleavage of pro-caspase 3 and there are no faster-migrating (i.e., activated) forms of this enzyme.

bility are needed. Syrbactins are microbial compounds that belong to one of two structurally related natural product families, the glidobactins (Glb) and the syringolins (Syl). From among these compounds, GlbA, SylA, and SylB have recently been shown to exert proteasome-inhibitory potency in a manner that is different from the established proteasome inhibitors [12–14], indicating that their clinical pharmacology, therapeutic efficacy, and safety profiles might turn out to be distinct. Whether this will translate into superior clinical efficacy remains to be explored, but pre-clinical studies with GlbA [17] and SylA [15,16] have shown anticancer activity and thus are promising.

Table 1
Combination index (C.I.) of cytotoxic potency.

T-02 (nM)[a]	T-04 (nM)[a]	Tg (μM)[a]	C.I.[b]	Effect[c] (degree of synergism)
RPMI/8226				
50	0	0.5	0.73	Moderate synergism
50	0	1.0	0.43	Synergism
100	0	0.5	0.76	Moderate synergism
0	50	0.5	0.97	Nearly additive
0	50	1.0	0.87	Slight synergism
0	100	0.5	0.42	Synergism
BCWM1				
50	0	0.5	0.63	Synergism
50	0	1.0	0.94	Slight synergism
100	0	0.5	0.75	Moderate synergism
0	50	0.5	0.74	Moderate synergism
0	50	1.0	0.88	Slight synergism
0	100	0.5	0.68	Synergism

[a] Concentrations used for combination treatments in MTT assays.
[b] Combination index according to Chou and Talalay [24], where C.I. < 1 indicates synergistic, C.I. = 1 indicates additive, and C.I. > 1 indicates antagonistic drug effect.
[c] Refined degree of synergistic drug cytotoxicity according to Chou [25].

In the current study, we have investigated the anticancer potential and molecular effects of three novel, synthetic syrbactin analogs, T-02, T-03, and T-04, which are based on modified structural features derived from naturally occurring syrbactins. T-02 and T-04 display a macrocyclic lactam core that is also present in GlbA, SylA, and SylB, whereas this core structure was changed to a macrolactone in T-03. In addition, the exocyclic side chain was modified and harbored an extended lipophilic side chain (Chart 1).

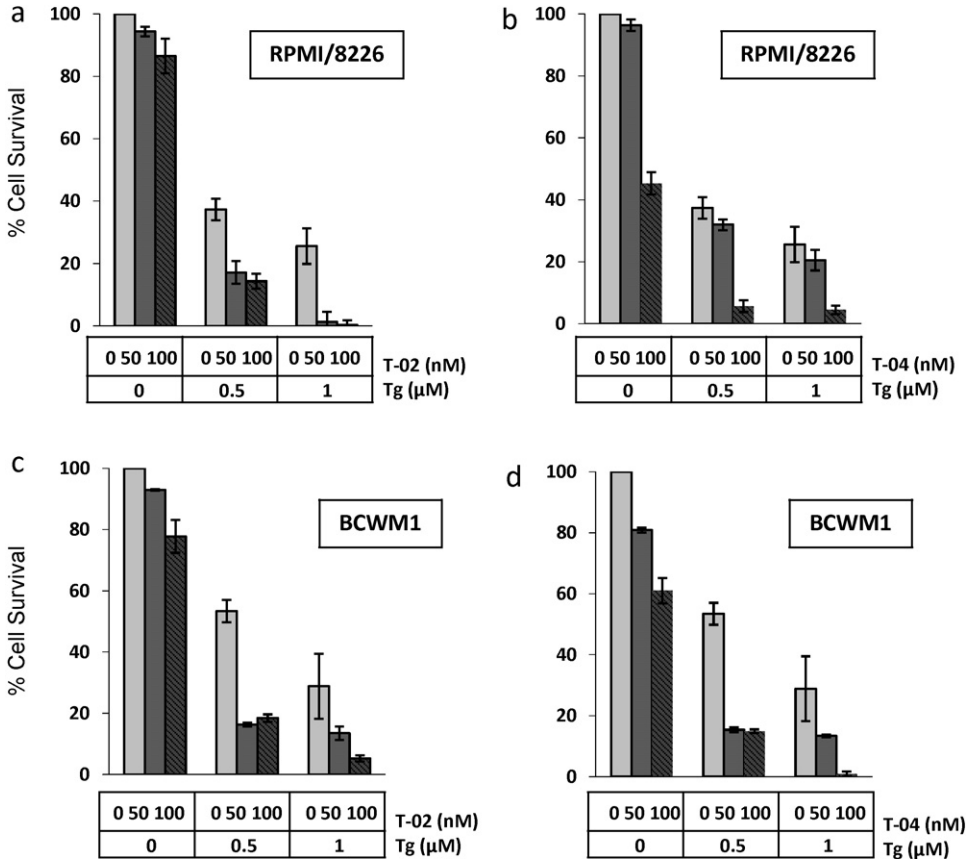

Fig. 7. Enhanced cytotoxic outcome in combination with thapsigargin. Cells were treated with different concentrations of syrbactin analogs in the presence or absence of thapsigargin (Tg), and after 72 h cell viability was measured by MTT assay. (a) RPMI/8226 cells treated with T-02 ± Tg. (b) RPMI/8226 cells treated with T-04 ± Tg. (c) BCWM1 cells treated with T-02 ± Tg. (d) BCWM1 cells treated with T-04 ± Tg. Shown is mean (±SD) percentage of cellular viability, where untreated controls are set to 100%. These experiments were repeated several times with similar outcomes.

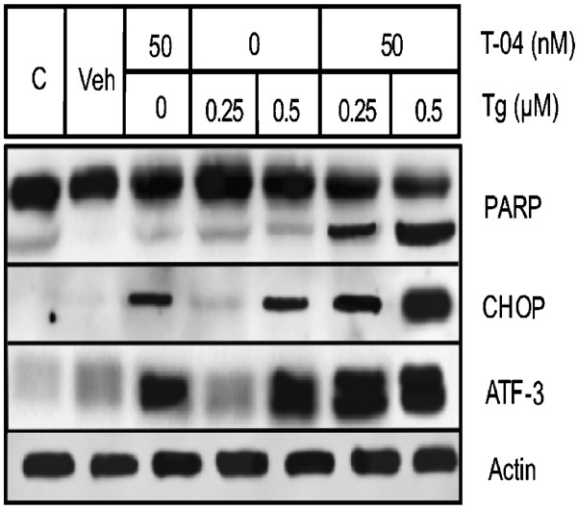

Fig. 8. Enhanced expression of markers for ER stress and apoptosis after combination treatment. BCWM1 cells were treated with 50 nM T-04 in the presence or absence of 0.25 or 0.5 μM thapsigargin (Tg). Parallel cell cultures remained untreated (control, C) or received vehicle (Veh) only. After 20 h, cell lysates were harvested and analyzed by Western blot as described in the legend to Fig. 5.

Previous studies have demonstrated that GlbA, SylA, and SylB display by far the strongest inhibitory potency towards the chymotrypsin-like activity of the proteasome, whereas trypsin-like and caspase-like activities are substantially less affected [13,14]; for this reason, we focused the investigation of our novel compounds on the proteasomal chymotrypsin-like activity. Our results demonstrate that all three of these synthetic compounds harbor proteasome-inhibitory activity. The IC_{50} of T-02 and T-04 towards the 20S proteasome was approximately 65 nM, whereas T-03 required low micromolar concentrations for effective inhibition. In comparison, the previously published IC_{50}s for GlbA, SylA, and SylB were 49 nM, 843 nM, and 8 μM, respectively [13,14]. Thus, the inhibitory potency of our novel compounds compared very favorably with their naturally occurring counterparts. (The overall comparability of our experimental system with previously published systems was verified by using SylA side-by-side with our novel analogs [not shown], which demonstrated that our SylA readouts were very similar to earlier studies reporting on the potency of this particular compound.)

All three synthetic compounds were able to reduce the viability of three cell lines that were chosen to represent hematological malignancies: multiple myeloma (RPMI/8226), Waldenström's macroglobulinemia (BCWM1), and lymphocytic leukemia (REH). T-02 and T-04 were similarly potent in the sub-micromolar range, whereas T-03 was less cytotoxic and displayed pronounced cell-type specific differences in its cell killing ability. The reasons for T-03's greater cell type specificity are unclear, but conceivably could be related to the presence of an ester group in its macrocyclic core (as opposed to amides in T-02 and T-04), which might provide greater susceptibility to hydrolysis (i.e., inactivation) by esterase activity, which is known to be variable between cell lines [26]. Otherwise, it emerged that the proteasome-inhibitory potency of these compounds (T-02 ≈ T-04 > T-03) was aligned with their cytotoxic activity in cell culture.

With regards to the structural requirements for proteasome inhibition and tumor cell killing, our results indicate that the macrocyclic lactam core (T-02, T-04) provides for greater potency than the corresponding macrolactone (T-03). Besides generating potential differences in metabolic turnover, as alluded to above, it is also likely that this alteration in the macrocyclic core may influence binding to the proteasome. For example, previous analyses of SylA, SylB, and GlbA have indicated that structural composition of their macrocyclic cores represents a critical determinant of proteasomal subsite selectivity and preferentially targets the proteasome in complex proteomes [13,27]. This aspect, however, was not further investigated in our study, because our primary focus was to learn more about the molecular pathways that might be affected by these compounds and might be involved in mediating their anticancer effects at the cellular level.

Cytotoxic effects of SylA, SylB, and GlbA have been reported for a variety of human tumor cell lines, such as neuroblastoma (SK-N-SH and LAN-1), ovarian carcinoma (SKOV-3), multiple myeloma (MM1 and U266), melanoma (B16), and colon carcinoma (HCT-116), in vitro [15–17]. In addition, GlbA was shown to prolong the life span of mice implanted with P388 lymphocytic leukemia cells [17]. However, with regards to the molecular pathways that are affected by syrbactins, there is very little information available. Two prior studies from the Bachmann lab have shown that treatment of human neuroblastoma cells with GlbA increases the expression of the tumor suppressor protein p53, and GlbA or SylA trigger apoptosis, as indicated by activation of caspase 3 and cleavage of PARP protein [15,16].

In the present study, we demonstrate that our de-novo synthesized novel syrbactin analogs also trigger apoptosis, as evidenced by the activation of caspase 3, cleavage of PARP protein, and inhibition by Z-VAD-FMK (only shown for T-04). In addition, T-04 exerts pronounced effects on several other critical controllers of cell survival and chemoresistance. For instance, survivin and Mcl-1 are anti-apoptotic proteins that are commonly found over-expressed in tumor cells and known to confer increased resistance of such cells towards various types of conventional cancer therapies [28,29]. The striking downregulation of these proteins in BCMW1 and RPMI/8226 cells by T-04 suggests the possibility that this analog might display chemosensitizing properties, which is an aspect that we currently investigate.

Earlier studies with bortezomib have shown that, at least initially, proteasome inhibition prevents Mcl-1 turnover and leads to the accumulation of this short-lived pro-survival protein; however, prolonged exposure to bortezomib triggers caspase activation, which results in the degradation and removal of Mcl-1 [30,31]. In comparison, we did not observe pronounced accumulation of Mcl-1 protein in response to treatment with syrbactin analogs, but we did note its potent downregulation by caspase activity. Because Mcl-1 accumulation has been shown to slow the pro-apoptotic effects of bortezomib [32], the lack of its accumulation in response to T-04 may bode well for potential future clinical applications of syrbactin analogs, as perhaps greater cytotoxic potency and/or chemosensitizing effects could be provided early on during treatment. However, additional studies are required to clarify this hypothetical aspect.

T-04 was able to strongly increase expression of the transcription factor CHOP in all three hematological cancer cell lines investigated. CHOP is a reliable indicator of severe ER stress [33] and it has been shown that cells are unable to tolerate prolonged high-level expression of this pro-apoptotic protein [34]. In view of its strongly increased expression in response to T-04 treatment, which was maintained for at least 32 h (Fig. 4b), we surmise that the ER stress response might play a critical role in mediating the cytotoxic effects of our syrbactin analogs. This was also indicated by our observation that T-04 strongly activates caspase 7, which is known to be an ER stress-associated event [35].

The involvement of ER stress was further substantiated by our finding that thapsigargin (Tg) synergistically enhanced the cytotoxic efficacy of T-02 and T-04. This synergy (i.e., combination index <1.0, as calculated according to Chou and Talalay [24]) was obtained with a range of different concentrations and thus was

quite robust. Tg is a model ER stress inducer that functions via the inhibition of the ER transmembrane calcium pump SERCA (sarcoplasmic/endoplasmic reticulum calcium ATPase), which results in the leakage of calcium from the ER storage space into the cytoplasm [23]. Our results show that combining Tg with T-04 caused much greater CHOP induction (i.e., ER stress) and PARP cleavage (i.e., apoptosis) than either agent alone. Over the past few years, the ER stress response system has been recognized as a target that might be exploitable for cancer therapy, where the intention is to aggravate this system (and induce pro-apoptotic CHOP) to the point where efficient apoptosis and tumor cell death is initiated [36,37]. It was proposed that the combination of pharmacologic agents that trigger ER stress by different mechanisms might result in synergistic anticancer outcomes, and several examples to this effect have indeed been published (see detailed Refs. in reviews [36,38]). In this context, our current study suggests the intriguing possibility that combining naturally occurring or synthetic syrbactins with other agents that impact the ER stress response might represent a novel pharmacological approach worthy of further evaluation for purposes of establishing optimized anticancer regimens in the future. As well, since proteasome inhibition in general has been shown to synergize with several other apoptosis-inducing drugs [39,40], many additional combinations of syrbactin analogs with other pro-apoptotic agents may be worthy of exploring for their potential cancer therapeutic value.

In summary, our study introduces novel synthetic syrbactin analogs with proteasome inhibitory potency in the nanomolar range. Among their desirable anticancer features is the down-regulation of anti-apoptotic proteins in combination with the aggravation of ER stress and elevated expression of pro-apoptotic CHOP. Altogether, these compounds should become useful in further characterizing the detailed mechanisms that mediate the potent anticancer properties of syrbactins at the molecular and cellular level. As well, these synthetic analogs can provide a platform for the synthesis of additional structural variants that could further improve on the biological and potentially therapeutic properties of the parent natural products.

Conflict of interest

None declared.

Acknowledgements

We thank Steven P. Treon (Dana Farber Cancer Institute, Boston, MA) and Alan L. Epstein (University of Southern California, Los Angeles, CA) for generously providing cell lines. The USC Glioma Research Group is acknowledged for stimulating discussions. Financial support was provided by UC-MEXUS and CONACYT (TIR) and the University of California Cancer Research Coordinating Committee (MCP).

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