Structure−Activity Relationship Studies of Salinosporamide A (NPI-0052), a Novel Marine Derived Proteasome Inhibitor

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Abstract: Salinosporamide A (**1**, NPI-0052) is a potent proteasome inhibitor in development for treating cancer. In this study, a series of analogues was assayed for cytotoxicity, proteasome inhibition, and inhibition of NF-κB activation. Marked reductions in potency in cell-based assays accompanied replacement of the chloroethyl group with unhalogenated substituents. Halogen exchange and cyclohexene ring epoxidation were well tolerated, while some stereochemical modifications significantly attenuated activity. These findings provide insights into structure−activity relationships within this novel series.

The proteasome, a multicatalytic proteolytic complex that regulates protein degradation in cells, is receiving considerable attention as a target for treating cancer.[1] This approach was validated by FDA approval of the proteasome inhibitor bortezomib (Velcade) for the treatment of multiple myeloma (MM).[2] Emerging clinical resistance to bortezomib[3] suggests the need for alternative therapies from structurally unique proteasome inhibitors such as salinosporamide A (**1**, NPI-0052), a product of a marine actinomycete *Salinispora tropica* that exhibits nM potency against the 20S proteasome[4,5] and overcomes bortezomib-resistance in MM cells.[5]

The 26S proteasome contains one or two 19S regulatory subunit caps and a 20S proteolytic core. Three distinct catalytic functions within the 20S proteasome [chymotrypsin-like (CT-L), trypsin-like (T-L), and PGPH or caspase-like (CA-L)] regulate the proteolysis of many substrates, including damaged proteins and cell signaling molecules. The 20S proteasome also regulates the activity of the transcription factor NF-κB.[1] NF-κB promotes cell survival by regulating genes encoding cell-adhesion molecules, proinflammatory cytokines, and antiapoptotic proteins.[6] In its inactive form, NF-κB complexes with its inhibitor IκB, but upon stimulation, IκB is degraded by the proteasome, leading to NF-κB activation.[1] NF-κB is constitutively active in many malignancies including MM, thereby enhancing MM cell survival and resistance to cytotoxic agents.[7] Interference with the activity of NF-κB by proteasome inhibition results in enhanced chemosensitivity and increased apoptosis in cancer cells.[8] Salinosporamide A inhibits all three catalytic activities of the proteasome,[4,5] NF-κB activation (vide infra), and the growth of human tumor cell lines.[4,5]

A number of important proteasome inhibitors are of microbial origin, including lactacystin,[9] the precursor to *clasto*-lactacystin β-lactone.[10] Also known as "omuralide" (**2**),[11] this β-lactone forms a covalent adduct with the catalytic N-terminal Thr1O$^{\gamma}$ residue of the β5-subunit of the 20S proteasome.[12,13] The β-lactone-γ-lactam bicyclic ring system of omuralide is present in salinosporamide A; however, each molecule is uniquely substituted such that **1** bears a methyl group and a cyclohex-2-enyl moiety at C-3 and C-5, respectively, while **2** bears a hydrogen and an isopropyl group. In addition, the chloroethyl group alpha to the lactam carbonyl of **1** is replaced with a methyl group in **2**. While the β-lactone is recognized as a key pharmacophore of omuralide, the enhanced potency of salinosporamide A suggests that certain substituents of the bicyclic ring system are also critical; thus, their significance was evaluated in a SAR program utilizing analogues generated through fermentation or derivatization of **1**. The SAR was based on proteasome mediated activities, including inhibition of CT-L, T-L, and CA-L activities of the rabbit 20S proteasome, inhibition of NF-κB activation in HEK293 cells, and cytotoxicity against the MM cell line RPMI 8226.

Prior to studying the substituents of the bicyclic ring system, the result of hydrolyzing the β-lactone ring was evaluated. When aqueous solutions of **1** were monitored by LC-MS, a species with a molecular ion (*m/z* 332, $[M + H]^+$) consistent with the β-lactone hydrolysis product **3** was observed. This product is analogous to the hydroxy acid reportedly formed from omuralide in aqueous solution.[10] Uniquely, however, β-lactone hydrolysis of **1** was followed by intramolecular nucleophilic addition to the chloroethyl group, forming cyclic ether **4** (Scheme 1). Compound **4** was independently obtained by base hydrolysis of **1** (0.5 N LiOH in THF/H_2O; complete conversion in 10 min) or by very slow conversion under acidic conditions. While the instability of **3** precluded isolation in sufficient purity for assay, **4** was inactive in all assays at the highest concentrations tested ($IC_{50} > 20\ \mu M$). These data suggest that, as with omuralide, the β-lactone is a critical pharmacophore of **1**, but further implicate the chloroethyl group as a reactive "trigger".

As noted, the β-lactone-γ-lactam bicyclic ring system is common to both **1** and **2**, yet **1** exhibits superior potency across key bioassays (Table 1). It was therefore of interest to modify each substituent (R_1 (cyclohex-2-enyl); R_2 (chloroethyl); R_3 (methyl), and C-5(OH); Formula **I**) and assess activity. Several naturally occurring "R_2 analogues" were obtained from "*S. tropica*" fermentation extracts or via directed biosynthesis of related secondary metabolites, including methyl analogue **5**, ethyl analogue **6** (salinosporamide B), bromoethyl analogue **7**, and C-2 epimer **8**. In addition, compound **9**, in

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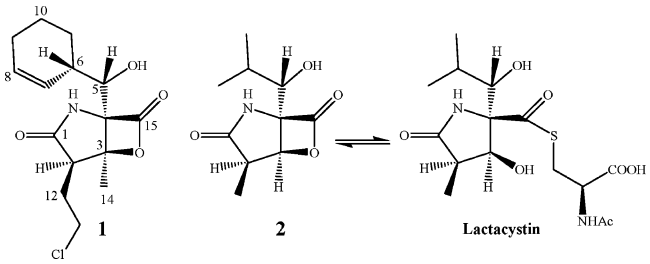
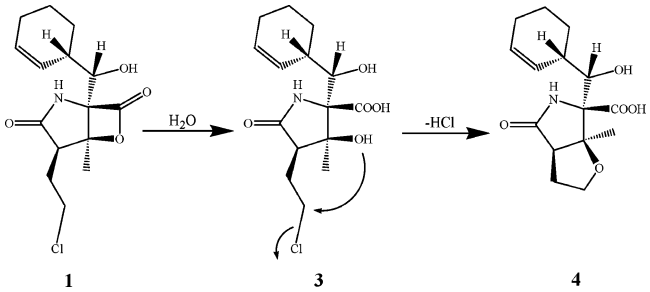
Chart 1



Scheme 1. Proposed Mechanism of Degradation of **1** in Aqueous Solution



which the methyl substituent at the C-3 ring junction (R_3) is replaced with an ethyl group, was obtained. Although these analogues provided preliminary SAR data, a more systematic study was warranted, and the analogue series was expanded through derivatization of the natural products. The presence of the potentially labile β-lactone ring presented unique challenges given the necessity to preserve this functionality in the derivatives.

To complement the analogues available through fermentation, four additional R_2 analogues were obtained as follows. Treatment of **1** with sodium iodide in acetone yielded iodoethyl analogue **10** after slow conversion at room temperature. This derivative allowed for more complete evaluation of halogen substitution and served as an excellent substrate for nucleophilic displacement reactions to produce additional analogues. Reaction of **10** with sodium azide in DMSO gave desired azido derivative **11**. Hydroxyethyl analogue **12** was obtained after briefly exposing **10** to a sodium hydroxide solution in acetone, which resulted in a complex mixture of products from which **12** was isolated in low yield. Finally, to extend the analogue series characterized by a hydrocarbon substituent at C-2 (i.e., **5**, R_2 = methyl; **6**, R_2 = ethyl), propyl analogue **13** was prepared by addition of **10** to a lithium dimethylcuprate (Gilman reagent) solution in THF at −78°C.

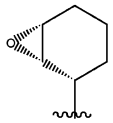
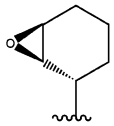
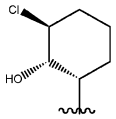
The impact of modification to the cyclohexene ring (R_1) was of particular interest in light of SAR reports on omuralide, which indicated that an isopropyl group at this position was optimal for proteasome inhibition and that replacement with a phenyl ring resulted in complete loss of activity.[11] Modification of the cyclohexene ring of **1** was first achieved through catalytic hydrogenation (Pd/C) to produce cyclohexyl analogue **14**. Other derivatizations included mCPBA epoxidation of **1**, which gave rise to diastereomers **15** and **16** in a 9:1 ratio. Subsequent epoxide ring opening of **15** with HCl (5% in CH_3CN) gave chlorohydrin **17**. To assess the significance of C-5(OH), the secondary alcohol was oxidized using Dess−Martin periodinane to obtain the corresponding ketone **18** as a mixture of rotamers. The C-5 epimer of **1** could be prepared by sodium borohydride reduction of the ketone; however, the sensitivity of the β-lactone ring to hydrolysis precluded the use of polar protic solvents. A procedure reported for reduction of keto-omuralide was considered,[12] and by modifying the temperature, the desired 5(*R*) diastereomer was obtained. Specifically, treatment with sodium borohydride in 1,2-dimethoxyethane/1% H_2O at −78 °C gave 5(*R*) isomer **19** in 90% ee, whereas reactions at higher temperatures gave increasing amounts of **1**.

The rank order of the active compounds in each assay was established based on their IC_{50} values. In terms of cytotoxicity against RPMI 8226 cells, the following activities were defined: **1** ≅ **7** ≅ **10** > **15** > **14** > **16** > **8** ≅ **9** > **2** > **13** ≅ **6** ≅ **11** > **5**. The rank order of the active compounds based upon their inhibitory effect of NF-κB-mediated luciferase reporter gene activity was: **1** ≅ **7** ≅ **10** > **15** > **14** > **16** > **5** ≥ **6** ≥ **11** ≥ **2** > **13** > **12** > **9** ≥ **8**. Thus, cytotoxicity was well correlated with inhibition of NF-κB activation, particularly with respect to the most potent compounds in the series (**1**, **7**, **10**, **15**, **14**, and **16**). The four most potent compounds as determined against the three protease-like activities of rabbit 20S proteasomes were **1**, **7**, **10**, and **15**, which were also the most potent compounds in the cell-based assays. As discussed below, however, several potent proteasome inhibitors in the series were only weakly cytotoxic. The results are summarized in Table 1 and were evaluated according to the functional group modified (Formula **I**) to help define the preferred substitution pattern for these novel proteasome inhibitors.

Modifications to the cyclohexene ring (R_1) resulted in varying degrees of attenuated biological activity. Cyclohexyl analogue **14**, in which the double bond and one chiral center are removed, was 3- to 12-fold less potent than **1**. Epoxidation gave two analogues with unique stereochemistry, including **15**, the most potent analogue in the R_1 series. This epoxide was only 2- to 4-fold less potent than **1** across most assays and comparable with respect to inhibition of CA-L activity. In contrast, epoxide **16** was 25- to 40-fold less potent than **1** across most assays and over 7-fold less potent in its inhibition of CA-L activity. Halohydrin **17** weakly inhibited CT-L activity with an IC_{50} value in the μM range, and no other activity was observed. The decreased potency of **16** and **17** indicate that steric bulk on this face of the cyclohexane ring is not well tolerated.

The only analogue that reported on the impact of R_3 was **9**, in which the ring junction methyl is replaced with an ethyl group. This simple modification attenuated the inhibition of CT-L activity by nearly 3 log units, while cytotoxicity and inhibition of NF-κB activation and T-L activities decreased by over 2 logs. The weak cytotoxicity and poor inhibition of NF-κB activation by **9** may stem from poor proteasome inhibition and an unfavorable steric effect and/or hydrophobic interaction. With respect to C-5(OH), epimerization (**19**) resulted in complete loss of activity. Oxidation of **1** to the corresponding ketone **18** also resulted in a dramatic loss in activity, with retention of only μM inhibition of the CT-L and T-L activities and no other activity observed over the concentration range tested. These results are similar to those reported for *epi*- and keto-omuralide, for which

Table 1. Biological Activities (IC_{50} (nM)) of **1** and Its Analogues and Omuralide (**2**)[a]

Str #	Modification from **1**	Cytotoxicity RPMI 8226	Inhibition of NF-κB Activation	Rabbit 20S Proteasome Inhibition		
				CT-L	T-L	CA-L
1	None	8.2 ± 2.0	11 ± 3	2.6 ± 0.2	21 ± 3	430 ± 60
14	R_1 = Cyclohexyl	52 ± 13	71 ± 20	20 ± 3	250 ± 30	1400 ± 200
15	R_1 = 	32 ± 3	34 ± 4	6.3 ± 0.6	92 ± 6	570 ± 160
16	R_1 = 	320 ± 5	300 ± 60	91 ± 8	770 ± 50	3200 ± 500
17	R_1 = 	>20000	>20000*	8200 ± 3000	>20000*	>20000*
7	$R_2 = CH_2CH_2Br$	7.4 ± 1.6	14 ± 4	2.6 ± 0.4	14 ± 2	290 ± 60
10	$R_2 = CH_2CH_2I$	6.8 ± 0.8	10 ± 3	2.8 ± 0.5	13 ± 3	410 ± 230
5	$R_2 = CH_3$	8500 ± 3300	850 ± 230	7.7 ± 3.0	340 ± 40	1200 ± 500
6	$R_2 = CH_2CH_3$	6100 ± 3300	1600 ± 700	27± 4	640 ± 60	1200 ± 100
13	$R_2 = CH_2CH_2CH_3$	6300 ± 3100	1900 ± 500	24 ± 5	1100 ± 200	1200 ± 200
11	$R_2 = CH_2CH_2N_3$	5500 ± 1300	1000 ± 500	7.7 ± 1.5	210 ± 40	560 ± 60
12	$R_2 = CH_2CH_2OH$	>20000*	2600 ± 700	8.0 ± 1.0	800*	2500*
8	R_2 = *epi*-CH_2CH_2Cl	1900 ± 300	3800 ± 800	330 ± 20	2500 ± 500	>20000*
9	$R_2 = CH_2CH_3$	1900 ± 400	3600 ± 1200	2100 ± 100	3300 ± 200	>20000*
18	C-5 Ketone	>20000*	>20000*	8200 ± 600	10000 ± 2200	>20000*
19	*epi*-C-5(OH)	>20000*	>20000*	>20000*	>20000*	>20000*
2	$R_1 = CH(CH_3)_2$; $R_2 = CH_3$; R_3 = H	3300 ± 1600	1600**	57 ± 6	540*	>10000*

[a] IC_{50} values represent the mean ± standard deviation of three or more experiments except where indicated *, the average of two experiments is shown. ** IC_{50} value determined in a single experiment.

very weak or no inhibition of the CT-L activity of the proteasome were reported.[11]

Modifications to R_2 provided particularly insightful SAR trends. Substitution of chlorine (**1**) with bromine (**7**) or iodine (**10**) resulted in equipotent analogues across all assays. The halogenated analogues were 10- to 80-fold more potent inhibitors of T-L activity than other analogues in the R_2 series when the 2*S* stereochemistry was retained. With respect to inhibition of CT-L activity, most 2*S* R_2 analogues exhibited single-digit nM IC_{50} values. The presence of a heteroatom at the 3-position of the R_2 chain (**11** and **12**) was well tolerated in this assay. In contrast, substitution with a hydrocarbon at this position (**13**; R_2 = propyl) resulted in a 9-fold reduction in potency. While omuralide analogues reportedly follow the trend: propyl > ethyl ≅ butyl > methyl (omuralide) > H with respect to inhibition of CT-L activity,[11,14] suggesting an optimal hydrocarbon chain length of three, salinosporamide A analogues did not follow this pattern. Methyl analogue **5** was more potent than ethyl and propyl analogues **6** and **13**, and was ∼7-fold more potent than omuralide, which also contains a methyl group at C-2, demonstrating the collective impact of the cyclohexyl and ring junction methyl substituents on inhibition of CT-L activity. In terms of stereochemistry, C-2 epimer **8** was over 2-log units less potent against the CT-L and T-L activities of the proteasome than **1** and exhibited only μM cytotoxicity. Omuralide is also a more potent inhibitor of CT-L activity than its C-2 epimer.[11]

Perhaps most striking with respect to R_2 analogues was the observation that replacement of halogenated ethyl groups (**1**, **7**, and **10**) with methyl (**5**), ethyl (**6**), propyl (**13**), azidoethyl (**11**), or hydroxyethyl (**12**) resulted in a dramatic reduction in potency in cell-based assays. In fact, a ∼3-log decrease in potency was observed with respect to cytotoxicity, while a ∼2-log decrease in potency was observed with respect to inhibition of NF-κB activation. Several factors may contribute to the disparity between potent inhibition of purified proteasomes and weak activity in cell-based assays. (i) Proteasome-mediated events, such as inhibition of NF-κB activation and cytotoxicity, may be dependent upon inhibition of specific proteasomal activities. Weakly cytotoxic R_2 analogues were less potent inhibitors of T-L activity (IC_{50} 210 nM (**11**) to 1.1 μM (**13**)) than their halogenated counterparts (IC_{50} 13 to 21 nM). This rationale is challenged, however, by R_1 analogue **14**, which inhibits T-L activity with an IC_{50} value of 250 nM but retains nM potency in cell-based assays. (ii) Analogues of **1** may exhibit differences in their abilities to permeate the cell membrane and/or inhibit the proteasome once inside the cell. Experimental evidence of cell permeability was obtained by exposing RPMI 8226 cells to **1**, **2**, and **5**, lysing the cells, and measuring inhibition of CT-L activity. Inhibition was

observed in each case, indicating that all compounds cross the cell membrane.[15] Interestingly, while 85% inhibition of CT-L activity was achieved upon exposure to **1** at a concentration of 5 nM, the same concentrations of **2** and **5** resulted in only 8% and 31% inhibition, respectively. Higher levels of inhibition (80% and 93% for **2**; 73% and 87% for **5**) could only be achieved by increasing the concentrations to 1 μM and 10 μM, respectively, values of similar magnitude to those required to achieve cytotoxicity. (Increased serum concentrations had little effect on the cytotoxicity of compounds **1** and **5** (data not shown; compound **2** not tested)). (iii) It is tempting to consider the possibility that the role of the R_2 halogen is mechanistic. As noted previously, the β-lactone ring of **1** is hydrolyzed in aqueous solutions with subsequent formation of cyclic ether **4** (Scheme 1). An analogous cyclic ether may exist in the drug−enzyme complex as a stabilized entity covalently bound to the catalytic threonine. Given that β-lactone **2** can be generated from its corresponding thioester lactacystin (Chart 1),[10] it is conceivable that an ester, such as the one that tethers **2** to the proteasome, could be similarly cleaved through regeneration of the β-lactone ring, potentially allowing **2** to be eliminated from its binding site. The cyclic ether form of **1** would preclude regeneration of the β-lactone and the potential for subsequent elimination. Alternative to cyclic ether formation is the possibility that a second nucleophile on the enzyme displaces chlorine to form a two-point covalent drug−enzyme adduct. This would not be unprecedented, as the X-ray crystal structure of epoxomicin complexed with the 20S proteasome revealed a two-point addition product.[13] Although the leaving group ability of the halogen provides potential alkylating power, the observation that both **1** (data not shown) and **2**[12] irreversibly inhibit the proteasome in vitro suggests that the β-lactone ring and other structural features common to **1** and **2** are sufficient for irreversible inactivation. While the structure of salinosporamide A is well optimized, analogues with alternative functional groups at R_2 may provide further insight into the significance of the substituent at this position.

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Supporting Information Available: Experimental details on biological assays, compound preparation, spectral characterization of compounds **4** and **10**−**19** (including ^{1}H NMR spectra), and HPLC purity data for compounds **1** and **4**−**19** are available free of charge via the Internet at http://pubs.acs.org.

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