Antileukemic activity and mechanism of drug resistance to the marine *Salinispora tropica* proteasome inhibitor salinosporamide A (marizomib)

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Nonstandard abbreviations
BTZ : bortezomib ; MM : multiple myeloma ; ALL : acute lymphoblastic leukemia;
AML : acute myeloid leukemia, HDACi : histone-deacetylase inhibitor ; MTT: 3-[4,5-
dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; IC: inhibitory concentration,
EC: effective concentration
Abstract

Salinosporamide A (NPI-0052, marizomib) is a naturally-occurring proteasome inhibitor derived from the marine actinobacterium *Salinispora tropica* and represents a promising clinical agent in the treatment of hematologic malignancies. Recently, these actinobacteria were shown to harbor self-resistance properties to salinosporamide A by expressing redundant catalytically-active mutants of the 20S proteasome β-subunit, reminiscent of *PSMB5* mutations identified in cancer cells with acquired resistance to the founding proteasome inhibitor, bortezomib. Here, we assessed the growth inhibitory potential of salinosporamide A in human acute lymphocytic leukemia CCRF-CEM cells, and its 10-fold (CEM/BTZ7), and 123-fold (CEM/BTZ200) bortezomib-resistant sublines harboring *PSMB5* mutations. Parental cells displayed sensitivity to salinosporamide A (IC₅₀= 5.1 nM), whereas their bortezomib-resistant sublines were 9- and 17-fold cross-resistant to salinosporamide A, respectively. Notably, combination experiments of salinosporamide A and bortezomib showed synergistic activity in CEM/BTZ200 cells. CEM cells gradually exposed to 20 nM salinosporamide A (CEM/S20) displayed stable 5-fold acquired resistance to salinosporamide A and were 3-fold cross-resistant to bortezomib. Consistent with the acquisition of a *PSMB5* point mutation (Met45Val) in CEM/S20 cells, salinosporamide A displayed a markedly impaired capacity to inhibit β5-associated catalytic activity. Lastly, compared to parental CEM cells, CEM/S20 cells exhibited up to 2.5-fold upregulation of constitutive proteasome subunits, while retaining unaltered immunoproteasome subunit expression.

In conclusion, salinosporamide A displayed potent antileukemic activity against bortezomib-resistant leukemia cells. β-subunit point mutations as a common feature
of acquired resistance to salinosporamide A and bortezomib in hematologic cells and *Salinispora tropica* suggest an evolutionarily conserved mechanism of resistance to proteasome inhibitors.
Introduction

The proteasome has emerged as an important clinical target for the treatment of hematologic malignancies. A decade ago, the reversible proteasome inhibitor bortezomib was approved for the treatment of relapsed/refractory and newly-diagnosed multiple myeloma (MM) and mantle cell lymphoma (Kane et al., 2003), and is an emerging treatment strategy for acute leukemia (Messinger et al., 2012; Niewerth et al., 2013a). However, relevant clinical disadvantages of bortezomib relate to its unsuitability for oral administration, its toxicity profile comprising peripheral neuropathy, and the emergence of drug resistance phenomena (Kale and Moore, 2012). Salinosporamide A (NPI-0052, marizomib) is a naturally-occurring proteasome inhibitor derived from the marine sediment actinomycetes *Salinispora tropica* and *Salinispora arenicola* (Feling et al., 2003; Gulder and Moore, 2010). This inhibitor, which belongs to the class of β-lactones, is a novel cancer treatment option since it is orally bioactive (Chauhan et al., 2005) and irreversibly inhibits all three proteolytic activities of the proteasome. In contrast, the founding proteasome inhibitor bortezomib reversibly and preferentially inhibits chymotrypsin-like activity and, to a lesser extent the caspase-like activity, but not the trypsin-like activity (Miller et al., 2007) of the proteasome. Consistent with this inhibition profile, the enhanced potency of salinosporamide A over bortezomib was shown in human MM cell lines (Chauhan et al., 2005) and in chronic lymphocytic leukemia patient specimens (Ruiz et al., 2006). Salinosporamide A is currently being evaluated in Phase I clinical trials as monotherapy and in combination with dexamethasone in patients with relapsed/refractory MM. A combined interim analysis of two dose-escalation studies showed a distinct toxicity profile to that of bortezomib, with no peripheral neuropathy.
Furthermore, out of 15 evaluable patients, three bortezomib-refractory patients reached partial remission (Richardson et al., 2011). Therefore, salinosporamide A may be attractive as a proteasome inhibitor for further combination chemotherapy studies in several malignancies, including acute leukemia. Building on this premise, combination therapy studies bearing minimally-cytotoxic concentrations of bortezomib and salinosporamide A exhibited synergistic effects in MM, leukemia, and lymphoma cell lines. Furthermore, this combination significantly decreased viability in tumor cells from five relapsed MM patients (two being bortezomib-resistant) and reduced tumor growth in a human MM xenograft mouse model without any noticeable toxicity (Chauhan et al., 2008). Moreover, a combination of salinosporamide A and histone deacetylase (HDAC) inhibitors showed a synergistic effect in human acute lymphocytic leukemia (ALL) cell lines, being superior to bortezomib combined with an HDAC inhibitor (Miller et al., 2007, 2009). Notwithstanding these promising results, it is conceivable that acquired resistance to salinosporamide A may evolve upon prolonged drug treatment. In this respect, recent studies by Kale et al (Kale et al., 2011) discovered a redundant proteasome β-subunit (SalI) in the 20S proteasome machinery of Salinispora tropica, which conferred 30-fold resistance to salinosporamide A in this species. This mechanism of naturally-occurring resistance was explained by molecular alterations involving amino acid substitutions (Ala49Val and Met45Phe) in these β-subunits as a result of point mutations in the SalI gene. Notably, the Ala49Val mutation was defined as the major determinant of salinosporamide A resistance, since the Met45Phe mutant largely retained its proteolytic activity and sensitivity to salinosporamide A inhibition. In vitro biochemical experiments, however, revealed that introduction of the Ala49Val point mutation did not recapitulate the full salinosporamide A resistance phenotype as the original SalI
subunit (Kale et al., 2011). Since hematoLOGic tumor cell lines with in vitro acquired resistance to bortezomib have also displayed similar mutations in exon 2 of PSMB5 (Oerlemans et al., 2008; Ruckrich et al., 2009; Ri et al., 2010; Balsas et al., 2012; de Wilt et al., 2012; Franke et al., 2012; Verbrugge et al., 2012), this points to a common mechanism of resistance to proteasome inhibitors.

In the present study, we examined tumor cell growth inhibitory capacity of salinosporamide A in human CCRF-CEM acute lymphocytic leukemia cells and two of its bortezomib-resistant sublines, CEM/BTZ7 (10-fold resistant to bortezomib) and CEM/BTZ200 (123-fold resistant). Bortezomib resistance in these lines is due to well-established PSMB5 mutations introducing amino acid substitutions Cys52Phe or both Cys52Phe and Ala49Val in the β5 subunit, respectively (Franke et al., 2012). We further determined whether salinosporamide A cytotoxic activity may be compromised by the development of resistance in hematologic tumor cells in a similar fashion as the naturally-occurring resistance mechanism to salinosporamide A in Salinispora tropica. Our findings reveal that salinosporamide A is a potent anti-leukemic agent to both parental and bortezomib-resistant leukemia cells, hence warranting further clinical evaluation of its suitability as a novel antitumor agent.

Materials and methods

Drugs and antibodies

Salinosporamide A was extracted and purified from Salinispora tropica CNB-440 cultures as previously described (Felting et al., 2003). Bortezomib (Velcade®) was provided by Millennium Pharmaceuticals (Cambridge, MA, USA). Antibodies to
proteasome subunits β1, β2, β5, β1i, and β5i were purchased from Enzo Life Sciences (Farmingdale, NY, USA), anti-ubiquitin (P4D1) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the IRDye infrared-labeled secondary antibodies were from LI-COR Biosciences (Lincoln, NE, USA). In addition, anti-actin (clone C4) was purchased from Millipore (Temecula, CA, USA).

Cell culture and development of a salinosporamide A-resistant cell line

Human T-cell acute lymphoblastic leukemia (T-ALL) CCRF-CEM cells (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 medium containing 2 mM L-glutamine (Invitrogen/Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) and 100 µg/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) in an atmosphere of 5% CO₂ and 37°C. Cell cultures were seeded at a density of 3x10⁵ cells/ml and refreshed twice weekly. The bortezomib-resistant cell line CEM/BTZ200, resistant to 200 nM bortezomib, was developed by step-wise drug selection as described previously (Franke et al., 2012).

Development of the salinosporamide A-resistant cell line, CEM/S20, was achieved by exposing CCRF-CEM cells to step-wise increasing concentrations of salinosporamide A from 1 nM to 20 nM (i.e., 4-fold the IC₅₀ concentration for parental cells) over a period of 6 months.

Cell growth inhibition assay

In vitro drug sensitivity was determined using the 4-day MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) cell growth inhibition and cytotoxicity assay (van Meerloo et al., 2011). Prior to these experiments, bortezomib-resistant and
salinosporamide A-resistant cells were cultured in drug-free medium for at least 4 days. Cells were then exposed to a series of salinosporamide A concentrations (range: 2 nM – 4 µM) and bortezomib concentrations (1 nM - 2 µM) for 4 days. The IC$_{50}$ value is defined as the drug concentration necessary to inhibit 50% of the cell growth compared to the growth of untreated control cells. For combination experiments, drugs were added simultaneously. Combinations were chosen starting from the IC$_{10}$ values of both drugs. CalcuSyn (Version 1.1.1 1996, Biosoft, Cambridge UK) software was used to calculate a combination index (CI) based on the median-effect principle for each drug combination tested (Chou and Talalay, 1984).

Proteasome activity

An intact cell-based assay was used to determine caspase-like, trypsin-like, and chymotrypsin-like proteasome activities and was conducted by using a Proteasome-Glo assay kit according to the manufacturer’s instructions (Promega, Madison, WI). Briefly, cells were exposed to a range of concentrations (range 1 - 100 nM) of salinosporamide A for 1 hour at 37°C in a white flat-bottomed 96-well plate (Greiner Bio-one, Alphen aan den Rijn, The Netherlands) at a density of 10,000 cells per well in a total volume of 50 µl. After a 15-minute incubation period with luminogenic substrates, luminescence was determined with an Infinite 200 Pro microplate reader (Tecan, Giessen, The Netherlands). Background signal from cell-free medium plus substrate was subtracted from cell measurements. β5, β5i, and β1i subunit-specific activities in cell extracts were performed with subunit specific peptide-AMC (7-amino-4-methylcoumarin) probes as previously described (Niewerth et al., 2014a). The
extent of inhibition of catalytic activity was indicated by IC\textsubscript{50} values that represent the drug concentration at which 50% maximal relative fluorescence was detected.

**Proteasome active site ELISA**

An ELISA-based assay (ProCISE) for quantitative assessment of active constitutive and immunoproteasome subunits was performed as previously described (Parlati et al., 2009). Briefly, cell lysate was incubated with a biotinylated proteasome active site binding probe. The lysate was then denatured, and subunits bound to probe were isolated with streptavidin-conjugated sepharose beads. Individual subunits were probed with subunit-specific primary antibodies, followed by HRP-conjugated secondary antibodies. A chemiluminescent substrate was used to generate signal associated with HRP binding, which was read on a GENios plate reader (Tecan, Männedorf, Switzerland). Absolute values of nanograms of subunit per microgram of total protein were based on a purified proteasome standard curve. Protein quantification was performed utilizing the Pierce BCA Protein Assay (Thermo Scientific, Rockford, IL, USA).

**Western blotting**

Protein expression of proteasome subunits was determined by Western blotting, as previously described (Franke et al., 2012). Protein bands were quantified by Odyssey software (Li-cor biosciences, Lincoln, NE, USA), corrected for background, and normalized to \(\beta\)-actin to correct for loading differences within blots.

**DNA sequencing analysis**
Sequencing analysis of proteasome subunits \textit{PSMB6} (\(\beta_1\)), \textit{PSMB7} (\(\beta_2\)), and exon 2 of \textit{PSMB5} (\(\beta_5\)) was performed as previously described (Franke \textit{et al.}, 2012).
Results

Sensitivity of bortezomib-sensitive and -resistant hematologic cell lines to salinosporamide A

To assess the anti-leukemic activity of salinosporamide A, its cell growth inhibitory effects were determined in the human T-ALL cell line CCRF-CEM, and its 10-fold (CEM/BTZ7) and 123-fold (CEM/BTZ200) bortezomib-resistant sublines, as compared to the sensitivity of these cell lines to bortezomib (Figure 1A-B; Table 1). Parental CCRF-CEM cells displayed a remarkable sensitivity to salinosporamide A (IC\textsubscript{50}: 5.1 ± 1.7 nM). CEM/BTZ7 cells (harboring a single mutation Cys52Phe) with low levels of bortezomib resistance displayed 9-fold cross-resistance to salinosporamide A (IC\textsubscript{50}: 47.4 ± 4.0 nM), whereas CEM/BTZ200 cells (harboring the mutations Ala49Val & Cys52Phe) displayed 17-fold cross-resistance to salinosporamide A (IC\textsubscript{50}: 87.9 ± 12 nM). Thus, given the 123-fold resistance to bortezomib in CEM/BTZ200 cells, salinosporamide A retained appreciable activity in these highly bortezomib-resistant cells.

Synergistic activity of salinosporamide A and bortezomib drug combinations in bortezomib-resistant cells

Next, combined exposure to a wide range of salinosporamide A and bortezomib concentrations showed no synergistic effects for these two drugs in parental CEM cells (Figure 2A). Conversely, CEM/BTZ200 cells exposed to minimally cytotoxic concentrations of each agent, e.g., 39.5 nM salinosporamide A (IC\textsubscript{50}: 87.9 nM) and 158 nM bortezomib (IC\textsubscript{50}: 419 nM), achieved 82% growth inhibition, whereas no significant growth inhibition was observed using either of these agents alone at these
low concentrations. Consistently, combination indices indicated synergism for all 4 combinations that achieved more than 50% cell growth inhibition (Figure 2B).

**Proteasome subunit catalytic activity inhibition profiles of salinosporamide A**

To examine the inhibitory potency of salinosporamide A against the β subunit-associated catalytic activities of the proteasome in hematologic cells, parental CEM cells and CEM/BTZ200 cells were exposed to a range of salinosporamide A concentrations for 1 hour. Salinosporamide A was effective in inhibiting all three proteolytic activities in both parental CEM and CEM/BTZ200 cells. For parental CEM cells, a most pronounced inhibition by salinosporamide A was observed for β5/β5i-associated chymotrypsin-like activity (EC$_{50}$: 1.1 nM) and equipotency for β1-associated caspase-like (EC$_{50}$: 15.7 nM), as well as for β2/β2i-associated trypsin-like activities (EC$_{50}$: 14.2 nM) (Figure 3A). Notably, the inhibitory potential of salinosporamide A was superior to that of bortezomib, which achieved 50% inhibition of chymotrypsin-like activity, caspase-like activity and trypsin-like activity at 7.3 nM, 33.8 nM and >100 nM bortezomib, respectively (supplemental Figure S1). At equimolar concentrations of 100 nM salinosporamide A and bortezomib, the latter showed 54% residual trypsin-like activity as compared to 3% by salinosporamide A. Similarly, chymotrypsin-like activity was completely abolished by 10 nM salinosporamide A, whereas 50 nM bortezomib was required to establish the same inhibitory effect (supplemental Figure S1). In direct comparison to parental CEM cells, 1.4-2.5-fold higher concentrations of salinosporamide A were necessary to inhibit the three catalytic proteasome activities in the bortezomib-resistant CEM/BTZ200 cells, with EC$_{50}$ values of 1.6 nM, 21.3 nM and 36.9 nM for chymotrypsin-like, caspase-like and trypsin-like activities, respectively (Figure 3B).
Nonetheless, near complete inhibition of all 3 activities was accomplished in bortezomib-resistant cells at 100 nM salinosporamide A, similar to parental cells (Figure 3B).

A closer examination of subunit-specific catalytic activities of β5 constitutive proteasome subunit, and β5i and β1i immunoproteasome subunits showed that β5 activity was less efficiently inhibited by salinosporamide A in cell extracts of bortezomib-resistant CEM/BTZ7 cells (EC50: 1.6 nM) and CEM/BTZ200 cells (EC50: 21.3 nM) as compared to parental CEM cells (EC50: 0.96 nM). In contrast, inhibition of immunoproteasome subunit β5i catalytic activity was only slightly less effective in bortezomib-resistant cell lines (EC50 CEM/WT: 0.79 nM, CEM/BTZ7: 1.4 nM, CEM/BTZ200: 1.3 nM). Lastly, the inhibitory capacity of salinosporamide A was equally potent in inhibition of β1i immunoproteasome subunit catalytic activity in parental and CEM/BTZ7 cells (EC50 CEM/WT: 4.1 nM, CEM/BTZ7: 4.7 nM), but 2-fold less potent for CEM BTZ200 cells (EC50: 8.4 nM) (supplemental Figure 2).

Together, salinosporamide A showed greater potency over bortezomib in inhibiting all proteolytic activities, in particular trypsin-like activity, for both parental cells and bortezomib-resistant cells. This property was retained in CEM/BTZ7 cells with low levels of bortezomib-resistance and CEM/BTZ200 with high levels of bortezomib resistance, with the exception of β5 catalytic activity being 22-fold less efficiently inhibited by salinosporamide A.

**Characterization of salinosporamide A-resistant cells**

We, as well as others, have previously reported that chronic exposure of leukemic cells to proteasome inhibitors induces acquired drug resistance (Oerlemans et al.,
2008; Franke et al., 2012; Niewerth et al., 2014b). Given that actinomycete Salinispora tropica harbor intrinsic resistance to salinosporamide A by a similar mechanism as acquired resistance to proteasome inhibitors including bortezomib in leukemic cells (Kale and Moore, 2012), we set out to explore the acquisition of resistance to salinosporamide A in cultured CEM leukemic cells by step-wise gradual increments to salinosporamide A. Resistance emerged gradually over a period of 6 months with CEM cells adapted to grow in the presence of 20 nM salinosporamide A (CEM/S20). These cells exhibited an IC\textsubscript{50} value of 23.2 ± 1.3 nM salinosporamide A, being 5-fold resistant relative to parental cells (Figure 4A). In addition, CEM/S20 cells displayed 3-fold cross-resistance to bortezomib (IC\textsubscript{50}: 10.8 ± 2.7 nM vs. 3.4 ± 0.9 nM; Figure 4B). Resistance to salinosporamide A in CEM/S20 cells was stable when cells were cultured in drug-free medium for a month (not shown).

In keeping with previous studies from our laboratory, establishing that chronic exposure of leukemic cells to bortezomib results in the acquisition of point mutations in exon 2 of PSMB5 (encoding the S1 pocket of β5) (Oerlemans et al., 2008; Franke et al., 2012), PSMB5 of CEM/S20 cells was also sequenced, revealing a point mutation at nucleotide position A310G that introduced a methionine to valine shift at position 45 (Met45Val) (Table 1). An identical point mutation was recently identified in bortezomib-resistant THP1 cells (Franke et al., 2012), bortezomib-resistant A549 non-small lung cancer cells (de Wilt et al., 2012), and PR-924 resistant 8226/PR8 cells (Niewerth et al., 2014b). The PR-924 resistant cell line 8226/PR8 harboring the Met45Val mutation showed 4.3-fold cross-resistance to salinosporamide A (data not shown), further confirming the impact of the Met45Val mutation in conferring salinosporamide A resistance. Notably, no mutations were found in the PSMB6 gene (encoding β1) and PSMB7 gene (encoding β2). Next, we characterized proteasome
subunit expression by ProCISE analysis in consecutive salinosporamide A-resistant CEM cells. Remarkably, within series of CEM/S5 until CEM/S30 cells, expression of constitutive proteasome subunits was significantly increased (up to 2.5-fold) compared to their parental counterparts, whereas immunoproteasome subunit expression varied to be moderately increased for β5i (up to 1.4-fold), but to be decreased up to 1.5-fold for β1i and β2i (Figure 5A). These results were corroborated by Western blot analysis (Figure 5B). Lastly, the inhibitory capacity of salinosporamide A against subunit-specific proteasome catalytic activities in cell extracts of salinosporamide A-resistant CEM cells revealed a 22-fold lower potency of salinosporamide A in achieving half-maximal inhibition of β5 activity in CEM/S20 cells compared to parental cells, whereas β5i and β1i immunoproteasome activities were similarly inhibited in CEM/S20 cells as in parental CEM cells (Figure 6). To substantiate these results, we analyzed the accumulation of polyubiquitinated proteins in CEM/WT cells and CEM/S30 cells upon exposure to salinosporamide A. These results (supplemental Figure S3) illustrate proficient accumulation of polyubiquitinated proteins in CEM/WT cells after 24 hours exposure to 10 nM salinosporamide A, whereas CEM/S30 displayed minimal accumulation of polyubiquitinated proteins when exposed to their selective concentration. Only upon addition of salinosporamide A concentrations that exert a β5 catalytic activity inhibitory effect, also these resistant cells revealed accumulation of polyubiquitinated proteins.
Discussion

The present study is the first to show that PSMB5 mutations underlie a common molecular mechanism of in vitro acquired resistance of hematologic tumor cells to the marine proteasome inhibitor, salinosporamide A, as the intrinsic natural resistance mechanism of the salinosporamide A-producing actinomycete, Salinispora tropica (Kale et al., 2011). This finding demonstrates that PSMB5 mutations have an evolutionary ancestor in conferring proteasome inhibitor resistance. Point mutations in the PSMB5 gene introducing single amino acid substitutions in the bortezomib-binding pocket of the β5 subunit were previously found in bortezomib-resistant leukemic cell lines (Lu et al., 2008; Oerlemans et al., 2008; Ri et al., 2010; Franke et al., 2012; Verbrugge et al., 2012), bortezomib-resistant JY lymphoblastoid cells (Verbrugge et al., 2013), and in non-small cell lung cancer cells (de Wilt et al., 2012), all pointing to this subunit as a key determinant in mediating drug resistance to proteasome inhibitors.

Human CEM/BTZ7 leukemia cells with low level (10-fold) bortezomib-resistance due to a Cys52Phe mutation in the β5 subunit were 9-fold cross-resistant to salinosporamide A. A β5 subunit-associated Cys52Phe mutation has been reported in bortezomib-resistant leukemia cells and lung cancer cells (de Wilt et al., 2012; Franke et al., 2012). Notably, a Gly52Ser mutation was observed in the Sall subunit of Salinispora tropica with intrinsic resistance to salinosporamide A, but its exact contribution to the resistance phenotype was not further defined (Kale et al., 2011). Based on crystallography studies by Groll et al (Groll et al., 2006a), amino acid position 52 did not emerge as being critical in proteasome inhibitor binding; however,
studies from our laboratory identified that a Cys52Phe mutation did result in repulsion of bortezomib from the S1 pocket, which negatively affects binding affinity (Franke et al., 2012). Acquisition of an additional mutation (Ala49Val) was found in CEM/BTZ200 cells, which display high level (123-fold) resistance to bortezomib and 17-fold cross-resistance to salinosporamide A. Notably, both the β5 subunit of CEM/BTZ200 and the Sall subunit of *Salinispora tropica* harbor exactly the same Ala49Val substitution to confer acquired resistance to bortezomib and intrinsic resistance to salinosporamide A, respectively (Kale et al., 2011; Franke et al., 2012). These results indicate that Cys52Phe and Ala49Val mutations both contribute to bortezomib and salinosporamide A resistance.

Crystal structure studies of salinosporamide A in complex with the 20S proteasome revealed that all 3 catalytic subunits (chymotrypsin-like/β5, trypsin-like/β2, and caspase-like/β1) were occupied by the compound (Groll et al., 2006b). Indeed, assessment of proteasome catalytic activity in parental and bortezomib-resistant CEM cells showed that salinosporamide A inhibited all three proteasome activities at lower concentrations than bortezomib. In parental cells, caspase and trypsin-like activities were similarly inhibited (ED<sub>50</sub> 15.7 nM and 14.2 nM, respectively), whereas in CEM/BTZ200 cells, trypsin-like activity was even better inhibited by salinosporamide A than caspase-like activity (ED<sub>50</sub> 21.3 nM and 36.9 nM, respectively). Kisselev et al (Kisselev et al., 2003) established that inhibition of the caspase-like sites stimulates the trypsin-like activity. Since trypsin-like activity in CEM/BTZ200 cells lacks targeting by bortezomib (Figure S1B), it is conceivable that residual trypsin-like activity is a compensatory mechanism, which preserves sufficient proteasome activity. The synergistic effect of bortezomib in combination with
salinosporamide A in these bortezomib-resistant cells may therefore be explained by complementary inhibition of trypsin-like activity by salinosporamide A in an irreversible and more prolonged fashion. In this respect, salinosporamide A and bortezomib were reported to trigger different apoptosis pathways, which may also add to their synergistic effect (Chauhan et al., 2008).

Although emergence of bortezomib resistance in myeloma patients grows as a clinically-relevant issue (Petrucci et al., 2013), the onset of acquired resistance to salinosporamide A is largely unexplored, let alone any underlying molecular basis. Here, we delineated the mechanism underlying acquired resistance to salinosporamide A in CEM/S20 cells that were 22-fold resistant to salinosporamide A. Strikingly, salinosporamide A resistance in CEM/S20 cells shared common features with mechanisms of acquired resistance to bortezomib (Oerlemans et al., 2008; Franke et al., 2012) and immunoproteasome inhibitor PR-924 (Niewerth et al., 2014b). These include: upregulation of constitutive proteasome β-subunits (Franke et al., 2012) as a first compensatory mechanism to relieve proteasome inhibitor selective pressure, and second, acquisition of a mutation (Met45Val) in PSMB5. Consistent with a genetic alteration in CEM/S20 cells, salinosporamide A resistance was stable when CEM/S20 cells were cultured in the absence of salinosporamide A for more than a month. Since inhibition of constitutive β5 is required for the growth inhibitory activity of proteasome inhibitors (Singh et al., 2011; Niewerth et al., 2014b), active site mutations in the β5 subunit are the most evident to be induced after chronic exposure to proteasome inhibitors. The mutation found in the CEM/S20 cells is likely to inflict a negative impact on the binding affinity of salinosporamide A, as was previously observed for bortezomib (Franke et al., 2012). Indeed, this notion is
supported by the marked loss of salinosporamide A inhibitory potency of β5-dependent catalytic activity in cell extracts of CEM/BTZ200 cells (supplemental Figure S2) and CEM/S20 cells (Figure 6) versus parental cells. It is noteworthy that other than for constitutive proteasome subunits, expression of immunoproteasome subunits β5i, β2i, and β1i was essentially unaltered in salinosporamide A-resistant CEM cells. None of these subunits harbored mutations. Remarkably, salinosporamide A displayed great potency in inhibiting the catalytic activities of the β5i and β1i subunits, both of parental cells and CEM/S20 cells (Figure 6). Nonetheless, proficient immunoproteasome inhibition by salinosporamide A had no apparent impact on preventing the onset of salinosporamide resistance in CEM/S20 cells. This notion is in line with the observations by Parlati et al (Parlati et al., 2009) and our laboratory (Niewerth et al., 2014b), that growth inhibitory effects of selective immunoproteasome inhibitors require concomitant inhibition of constitutive proteasome subunits. When, for example, constitutive β5 subunits harbor mutations hampering bortezomib or salinosporamide A active site binding, this property will largely dictate the resistance level. This also implies that by upregulating the immunoproteasome at the expense of the constitutive proteasome, cells may gain sensitivity to proteasome inhibition. Indeed, recent studies by our laboratory (Niewerth et al., 2014a) showed that upregulation of cellular immunoproteasome subunit content, e.g., by exposure to interferon-γ, allowed partial sensitization of bortezomib-resistant hematologic cells to ONX 0914, a selective immunoproteasome inhibitor.

Surprisingly, whereas Met45 (compared to Ala49) mutations did little in contribution to the natural resistance to salinosporamide A in Salinispora tropica (Kale et al.,

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2011), Met45Val emerged as the dominant mutation conferring resistance to salinosporamide A in CEM/S20 cells. In fact, Met45 has been characterized to undergo conformational changes upon binding of bortezomib (Groll et al., 2006a) or immunoproteasome inhibitor ONX 0914 (Huber et al., 2012) to the β5 active site binding pocket. Within the S1 pocket, Met45 facilitates favorable hydrophobic interactions with the P1 side chain of salinosporamide A (Groll et al., 2006b; Kale and Moore, 2012). Hence, Met45 mutations may impact these interactions by restricting salinosporamide A binding, thus conferring resistance. Indeed, Met45Val mutations in CEM/S20 were implicated in a marked loss of the ability of salinosporamide A to inhibit β5-associated catalytic activity, and a markedly impaired capacity to accumulate polyubiquitinated proteins in resistant cells upon exposure to salinosporamide A. To further pinpoint the impact of the Met45Val mutation in salinosporamide A resistance in CEM leukemia cells, we showed that the PR-924 resistant MM cell line 8226/PR7 harboring the Met45Val mutation (Niewerth, et al., 2014b) was markedly cross-resistant to salinosporamide A.

Collectively, the current and other studies (Ruckrich et al., 2009; Franke et al., 2012; Niewerth et al., 2014b) point to upregulation of β5 subunit expression as the primary response mechanism to proteasome inhibition, which may set the stage for acquisition of mutations following prolonged bortezomib exposure. PSMB5 mutations have not yet been recorded in human clinical specimens derived from patients receiving proteasome inhibitor-based therapy (Politou et al., 2006; Shuqing et al., 2011; Lichter et al., 2012). This might be explained by the fact that current treatment strategies do not implement maintenance therapy with proteasome inhibitors, however, with the introduction of orally-available proteasome inhibitors without emerging peripheral neuropathy, this will likely be investigated soon. Indeed, in the
Netherlands, the role of ixazomib citrate as maintenance therapy will be explored. However, common PSMB5 mutations in human in vitro-derived proteasome inhibitor-resistant leukemia cells and in Salinispora tropica points toward an evolutionarily-conserved resistance mechanism. It should be emphasized that PSMB5 mutations do not represent the sole mechanism of proteasome inhibitor resistance. In fact, multiple mechanisms can be accountable in contributing to either intrinsic or acquired resistance, e.g., drug efflux transporters (Verbrugge et al., 2012), suppression of XBP1s (Leung-Hagesteijn et al., 2013), increased expression of IGFR (Kuhn et al., 2012), or an impaired balance of constitutive vs. immunoproteasome subunit expression (Niewerth, et al., 2013b). These mechanisms were mostly revealed in relation to bortezomib resistance and certainly warrant further investigation with respect to salinosporamide A.

In conclusion, salinosporamide A displayed potent anti-leukemic activity, even in bortezomib-resistant cells and was synergistic with bortezomib in these cells. The oral availability and promising results from early phase clinical trials may hold potential for therapeutic interventions in leukemia. Given the potential emergence of acquired resistance to salinosporamide A, its optimal application will most likely come in the form of combination therapy with bortezomib or other agents, e.g., lenalidomide (Chauhan et al., 2010) and HDAC inhibitors (Miller et al., 2009).

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Author contributions

Participated in research design: DN, LFVR, GJ, JC, AJK, BSM

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Performed data analysis: DN, LFVR, JM, JLA

Wrote or contributed to the writing of the manuscript: DN, LFVR, GJ, AJK, BSM, YGA, SZ, GJLK, and JC
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Groll M, Huber R, and Potts BCM (2006) Crystal structures of Salinosporamidine A (NPI-0052) and B (NPI-0047) in complex with the 20S proteasome reveal


Footnotes

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Figure legends

Figure 1. Activity of salinosporamide A against bortezomib-sensitive and bortezomib-resistant human CEM T-ALL leukemia cells. Dose-response curves for (A) salinosporamide A and (B) bortezomib against parental CEM cells (CEM/WT), 10-fold bortezomib-resistant CEM/BTZ7 cells, and 123-fold bortezomib-resistant CEM/BTZ200 cells. *Data for CEM/BTZ7 were from Franke et al. (Franke et al., 2012). Drug exposure was for 4 days. Dose response curves represent the mean (± standard deviation) of at least three independent experiments.

Figure 2. Combination indices for salinosporamide A and bortezomib combinations in bortezomib-sensitive CEM/WT cells and bortezomib-resistant CEM/BTZ200 cells. Combination indices were calculated on the indicated concentrations of bortezomib and salinosporamide A in (A) a 1 : 2 ratio for CEM/WT and (B) a 1 : 0.25 ratio for CEM/BTZ200, based on a 4-day drug exposure and CalcuSyn analysis.

Figure 3. Impact of salinosporamide A on proteasome catalytic activity in parental and bortezomib-resistant CEM cells. Chymotrypsin-like, caspase-like, and trypsin-like proteasome activity was analyzed by Proteasome-Glo assay in intact (A) parental CEM/WT cells, and (B) bortezomib-resistant CEM/BTZ200 cells after 1-hour exposure to a concentration range of salinosporamide A. Results are presented relative to untreated controls and represent the mean (± standard deviation) of three independent experiments.
Figure 4. Sensitivity of salinosporamide A-resistant CEM/S20 cells to salinosporamide A and bortezomib. Dose-response curves for (A) salinosporamide A and (B) bortezomib against salinosporamide A-resistant CEM/S20 cells compared to CEM/WT cells. Curves represent the mean (± standard deviation) of at least three independent experiments.

Figure 5. Constitutive and immunoproteasome subunit expression of salinosporamide A-resistant CEM cells compared to CEM/WT cells. (A) Protein expression levels of constitutive proteasome subunits (β1, β2, and β5) and immunoproteasome subunits (β1i, β2i, and β5i) were determined by ProCISE and expressed in ng/μg protein for CEM/WT and CEM cells adapted to growth in the presence of 5-30 nM salinosporamide A (CEM/S5, CEM/S10, CEM/S15, CEM/S25, and CEM/S30). Results depicted represent the mean (± standard deviation) of 3 separate experiments. (B) Western blot analysis of β1, β2, β5, and β5i protein expression of CEM/WT, CEM/S5, CEM/S10, CEM/S15, and CEM/S20. One representative example of 3 separate experiments is depicted.

Figure 6. Impact of salinosporamide A on proteasome catalytic activity in parental and salinosporamide A-resistant CEM/S20 cells. β5, β5i, and β1i-associated catalytic activity in cell extracts of CEM/WT and CEM/S20 cells were assessed after 1-hour exposure to salinosporamide A. Results depicted represent the mean (± standard deviation) of 3 separate experiments.
Table 1. Characterization of salinosporamide-resistant CEM/S20 cells.

Sensitivity profiling of CEM/S20 cells compared to parental CEM cells and bortezomib-resistant CEM/BTZ7 and CEM/BTZ200 cells was performed for salinosporamide A and bortezomib. An overview of PSMB5 mutations (exon 2) in the indicated cell lines is provided.

<table>
<thead>
<tr>
<th></th>
<th>Salinosporamide A</th>
<th>Bortezomib</th>
<th>Mutations PSMB5 exon 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC_{50} ± SD (nM)</td>
<td>Resistance factor</td>
<td>IC_{50} ± SD (nM)</td>
</tr>
<tr>
<td><strong>Wildtype cell line</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CEM/WT</td>
<td>5.1 ± 1.7</td>
<td>3.4 ± 0.9</td>
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<td><strong>Bortezomib-resistant subline</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEM/BTZ7</td>
<td>47.4 ± 4</td>
<td>9</td>
<td>12.4 ± 5.8*</td>
</tr>
<tr>
<td>CEM/BTZ200</td>
<td>87.9 ± 12</td>
<td>17</td>
<td>419 ± 68</td>
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<tr>
<td><strong>Sal A-resistant subline</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CEM/S20</td>
<td>23.2 ± 1.3</td>
<td>5</td>
<td>10.8 ± 2.7</td>
</tr>
</tbody>
</table>

* Adapted from Franke et al. Leukemia 2012
Figure 1

A

Growth (% of control) vs. Salinosporamide A [μM]

- CEM/WT
- CEM/BTZ7
- CEM/BTZ200

B

Growth (% of control) vs. Bortezomib (μM)

- CEM/WT
- CEM/BTZ7
- CEM/BTZ200
Figure 2

(A) Combination Index vs. Bortezomib (nM) for antagonism and synergism.

(B) Combination Index vs. Bortezomib (nM) for antagonism and synergism.
Figure 3

A  CEM/WT

B  CEM/BTZ200

Proteasome activity (%) vs. Salinosporamide A (nM)

- Caspase-like
- Trypsin-like
- Chymotrypsin-like
Figure 4

**A**

Growth (% of control) vs. Salinosporamide A [μM]

**B**

Growth (% of control) vs. Bortezomib [μM]
Figure 5

A

B

WT     S5      S10     S15     S20

β-actin

ng subunit / μg total protein

0 5 10 15

WT S5 S10 S15 S20

β5

β5i

β2

β1

β1i

β2i
Figure 6

Proteasome activity (%)

β5

Proteasome activity (%)

β5i

Proteasome activity (%)

β1i

Salinosporamide A (nM)