Antileukemic Activity and Mechanism of Drug Resistance to the Marine *Salinispora tropica* Proteasome Inhibitor Salinosporamide A (Marizomib)[§]

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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 (BTZ). 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 (IC_{50} = 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 (M45V) in CEM/S20 cells, salinosporamide A displayed a markedly impaired capacity to inhibit β_5 -associated catalytic activity. Last, compared with 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 *S. 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

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ABBREVIATIONS: AMC, 7-amino-4-methylcoumarin; BTZ, bortezomib; HDAC, histone deacetylase; MM, multiple myeloma; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; ONX 0914, (S)-3-(4-methoxyphenyl)-N-((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)-2-((S)-2-(2-morpholinoacetamido)propanamido)propanamide; WT, wild-type.

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 compared with 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 lymphoblastic leukemia cell lines, which is 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. (2011) discovered a redundant proteasome β -subunit (SalI) in the 20S proteasome machinery of *S. 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 (A49V and M45F) in these β -subunits as a result of point mutations in the SalI gene. Notably, the A49V mutation was defined as the major determinant of salinosporamide A resistance, since the M45F mutant largely retained its proteolytic activity and sensitivity to salinosporamide A inhibition. In vitro biochemical experiments, however, revealed that introduction of the A49V 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 the tumor cell growth inhibitory capacity of salinosporamide A in human CCRF-CEM acute lymphocytic leukemia cells and two of its bortezomib (BTZ)-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 C52F or both C52F and A49V in the β_5 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 *S. tropica*. Our findings reveal that salinosporamide A is a potent antileukemic 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 *S. tropica* CNB-440 cultures as previously described (Feling et al., 2003). Bortezomib (Velcade) was provided by Millennium Pharmaceuticals (Cambridge, MA). Antibodies to proteasome subunits β_1 , β_2 , β_5 , β_{1i} , and β_{5i} were purchased from Enzo Life Sciences (Farmingdale, NY); antiubiquitin (P4D1) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); and the IRDye infrared-labeled secondary antibodies were from LI-COR Biosciences (Lincoln, NE). In addition, antiactin (clone C4) was purchased from Millipore (Temecula, CA).

Cell Culture and Development of a Salinosporamide A-Resistant Cell Line. Human T-cell acute lymphoblastic leukemia CCRF-CEM cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium containing 2 mM L-glutamine (Invitrogen/Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (Greiner Bio-One, Alphen a/d Rijn, The Netherlands) and 100 μ g/ml penicillin/streptomycin (Invitrogen) in an atmosphere of 5% CO₂ and 37°C. Cell cultures were seeded at a density of 3×10^5 cells/ml and refreshed twice weekly. The bortezomib-resistant cell line CEM/BTZ200, resistant to 200 nM bortezomib, was developed by stepwise 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 stepwise increasing concentrations of salinosporamide A from 1 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- 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 (2 nM to 4 μ M) and bortezomib concentrations (1 nM to 2 μ M) for 4 days. The IC₅₀ value is defined as the drug concentration necessary to inhibit 50% of the cell growth compared with the growth of untreated control cells. For combination experiments, drugs were added simultaneously. Combinations were chosen starting from the IC₁₀ values of both drugs. CalcuSyn software (version 1.1.1 1996; Biosoft, Cambridge, UK) was used to calculate a combination index 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 using a Proteasome-Glo assay kit according to the manufacturer's instructions (Promega, Madison, WI). In brief, cells were exposed to a range of concentrations (1–100 nM) of salinosporamide A for 1 hour at 37°C in a white flat-bottomed 96-well plate (Greiner Bio-One) at a density of 10,000 cells per well in a total volume of 50 μ l. 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. β_5 , β_{5i} , and β_{1i} 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 IC₅₀ values that represent the drug concentration at which 50% maximal relative fluorescence was detected.

Proteasome Active Site Enzyme-Linked Immunosorbent Assay. An enzyme-linked immunosorbent assay-based assay (ProCISE) for quantitative assessment of active constitutive and immunoproteasome subunits was performed as previously described (Parlati et al., 2009). In brief, 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 horseradish peroxidase-conjugated secondary antibodies. A chemiluminescent substrate was used to generate signal associated with horseradish peroxidase 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).

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), corrected for background, and normalized to β -actin to correct for loading differences within blots.

DNA Sequencing Analysis. Sequencing analysis of proteasome subunits *PSMB6* (β_1), *PSMB7* (β_2), and exon 2 of *PSMB5* (β_5) was performed as previously described (Franke et al., 2012).

Results

Sensitivity of Bortezomib-Sensitive and -Resistant Hematologic Cell Lines to Salinosporamide A. To assess the antileukemic activity of salinosporamide A, its cell growth inhibitory effects were determined in the human T-cell acute lymphoblastic leukemia cell line CCRF-CEM, and its 10-fold (CEM/BTZ7) and 123-fold (CEM/BTZ200) bortezomib-resistant sublines, as compared with the sensitivity of these cell lines to bortezomib (Fig. 1, A and B; Table 1). Parental CCRF-CEM cells displayed a remarkable sensitivity to salinosporamide A (IC_{50} : 5.1 ± 1.7 nM). CEM/BTZ7 cells (harboring a single mutation C52F) with low levels of bortezomib resistance displayed 9-fold cross-resistance to salinosporamide A (IC_{50} : 47.4 ± 4.0 nM), whereas CEM/BTZ200 cells (harboring the mutations A49V and C52F) displayed 17-fold cross-resistance to salinosporamide A (IC_{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 (Fig. 2A). Conversely, CEM/BTZ200 cells exposed to minimally cytotoxic concentrations of each agent, e.g., 39.5 nM salinosporamide A (IC_{50} : 87.9 nM) and 158 nM bortezomib (IC_{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 four combinations that achieved more than 50% cell growth inhibition (Fig. 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 β_5/β_{5i} -associated chymotrypsin-like activity (EC_{50} : 1.1 nM) and equipotency for β_1 -associated caspase-like activity (EC_{50} : 15.7 nM), as well as for β_2/β_{2i} -associated trypsin-like activities (EC_{50} : 14.2 nM) (Fig. 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, 33.8, and >100 nM bortezomib, respectively (Supplemental Fig. 1). At equimolar concentrations of 100 nM salinosporamide A and bortezomib, the latter showed 54% residual trypsin-like activity as compared with 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 Fig. 1). In direct comparison with 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, 21.3, and 36.9 nM for chymotrypsin-like, caspase-like, and trypsin-like activities, respectively (Fig. 3B). Nonetheless, near complete inhibition of all three activities was accomplished in bortezomib-resistant cells at 100 nM salinosporamide A, similar to parental cells (Fig. 3B).

A closer examination of subunit-specific catalytic activities of the β_5 constitutive proteasome subunit and β_{5i} and β_{1i} immunoproteasome subunits showed that β_5 activity was less efficiently inhibited by salinosporamide A in cell extracts of bortezomib-resistant CEM/BTZ7 cells (EC_{50} : 1.6 nM) and CEM/BTZ200 cells (EC_{50} : 21.3 nM) as compared with parental CEM cells (EC_{50} : 0.96 nM). In contrast, inhibition of immunoproteasome subunit β_{5i} catalytic activity was only slightly less effective in bortezomib-resistant cell lines [EC_{50} CEM/wild-type (WT): 0.79 nM, CEM/BTZ7: 1.4 nM, CEM/BTZ200: 1.3 nM]. Last, the inhibitory capacity of salinosporamide

Fig. 1. Activity of salinosporamide A against bortezomib-sensitive and bortezomib-resistant human CEM T-cell acute lymphoblastic leukemia cells. Dose-response curves for salinosporamide A (A) and bortezomib (B) 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. (2012). Drug exposure was for 4 days. Dose-response curves represent the mean ($\pm$ S.D.) of at least three independent experiments.

TABLE 1

Characterization of salinosporamide-resistant CEM/S20 cells

Sensitivity profiling of CEM/S20 cells compared with 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.

	Salinosporamide A		Bortezomib		Mutations <i>PSMB5</i> Exon 2
	IC ₅₀ ± S.D.	Resistance Factor	IC ₅₀ ± S.D.	Resistance Factor	
	<i>nM</i>		<i>nM</i>		
Wild-type cell line					
CEM/WT	5.1 ± 1.7		3.4 ± 0.9		
Bortezomib-resistant sublines					
CEM/BTZ7	47.4 ± 4	9	12.4 ± 5.8 ^a	10 ^a	C52F
CEM/BTZ200	87.9 ± 12	17	419 ± 68	123	A49V and C52F
Sal A-resistant subline					
CEM/S20	23.2 ± 1.3	5	10.8 ± 2.7	3	M45V

Sal A, salinosporamide A.

^aAdapted from Franke et al. (2012).

A was equally potent in inhibition of β_{1i} 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 Fig. 2).

Altogether, 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 β_5 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 *S. tropica* harbors 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 stepwise gradual increments. Resistance emerged gradually over a period of 6 months with CEM cells adapting to growth in the presence of 20 nM salinosporamide A (CEM/S20). These cells exhibited an IC₅₀ value of 23.2 ± 1.3 nM salinosporamide A, being 5-fold resistant relative to parental cells (Fig. 4A). In addition, CEM/S20 cells displayed 3-fold cross-resistance to bortezomib (IC₅₀: 10.8 ± 2.7 nM vs. 3.4 ± 0.9 nM; Fig. 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 β_5) (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 (M45V) (Table 1). An identical point mutation was recently identified in bortezomib-resistant THP1 cells (Franke et al., 2012), bortezomib-resistant A549 nonsmall-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 M45V mutation showed 4.3-fold cross-resistance to salinosporamide A (data not shown), further confirming the impact of the M45V mutation in conferring salinosporamide A resistance. Notably, no mutations were found in the *PSMB6* gene (encoding β_1) and *PSMB7* gene (encoding β_2). Next, we characterized proteasome subunit expression by ProCISE analysis (Onyx Pharmaceuticals, South San Francisco, CA) 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 with their parental counterparts, whereas immunoproteasome subunit expression varied, being moderately increased for β_{5i} (up to 1.4-fold) but decreased up to 1.5-fold for β_{1i} and β_{2i} (Fig. 5A). These results were corroborated by Western blot analysis (Fig. 5B). Last, the inhibitory capacity of salinosporamide A against subunit-specific proteasome

Fig. 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 1:2 ratio for CEM/WT (A) and a 1:0.25 ratio for CEM/BTZ200 (B), based on a 4-day drug exposure and CalcuSyn analysis.

Fig. 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 parental CEM/WT cells (A) and bortezomib-resistant CEM/BTZ200 cells (B) after 1-hour exposure to a concentration range of salinosporamide A. Results are presented relative to untreated controls and represent the mean ($\pm$ S.D.) of three independent experiments.

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 β_5 activity in CEM/S20 cells compared with parental cells, whereas β_{5i} and β_{1i} immunoproteasome activities were similarly inhibited in CEM/S20 cells as in parental CEM cells (Fig. 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 Fig. 3) illustrate proficient accumulation of polyubiquitinated proteins in CEM/WT cells after 24-hour 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 β_5 catalytic activity inhibitory effect did these resistant cells reveal 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 *S. 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 β_5 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 nonsmall-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 C52F mutation in the β_5 subunit were 9-fold cross-resistant to salinosporamide A. A β_5 subunit-associated C52F mutation has been reported in bortezomib-resistant leukemia cells and lung cancer cells (de Wilt et al., 2012; Franke et al., 2012). Notably, a G52S mutation was observed in the Sall subunit of *S. 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. (2006a), amino acid position 52 did not emerge as being critical in proteasome inhibitor binding; however, studies from our laboratory identified that a C52F 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 (A49V) 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 β_5 subunit of CEM/BTZ200 and the Sall subunit of *S. tropica* harbor exactly the same A49V 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 C52F and A49V mutations both contribute to bortezomib and salinosporamide A resistance.

Crystal structure studies of salinosporamide A in complex with the 20S proteasome revealed that all three catalytic subunits (chymotrypsin-like/ β_5 , trypsin-like/ β_2 , and caspase-like/ β_1) were occupied by the compound (Groll et al., 2006b). Indeed, assessment of proteasome catalytic activity in parental and bortezomib-resistant CEM cells showed that

Fig. 4. Sensitivity of salinosporamide A-resistant CEM/S20 cells to salinosporamide A and bortezomib. Dose-response curves for salinosporamide A (A) and bortezomib (B) against salinosporamide A-resistant CEM/S20 cells compared with CEM/WT cells. Curves represent the mean ($\pm$ S.D.) of at least three independent experiments.

Fig. 5. Constitutive and immunoproteasome subunit expression of salinosporamide A-resistant CEM cells compared with CEM/WT cells. (A) Protein expression levels of constitutive proteasome subunits (β_1 , β_2 , and β_5) and immunoproteasome subunits (β_{1i} , β_{2i} , and β_{5i}) were determined by ProCISE and expressed in nanograms per micrograms of 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 ($\pm$ S.D.) of three separate experiments. (B) Western blot analysis of β_1 , β_2 , β_5 , and β_{5i} protein expression of CEM/WT, CEM/S5, CEM/S10, CEM/S15, and CEM/S20. One representative example of three separate experiments is depicted.

salinosporamide A inhibited all three proteasome activities at lower concentrations than bortezomib. In parental cells, caspase- and trypsin-like activities were similarly inhibited (ED_{50} 15.7 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_{50} 21.3 and 36.9 nM, respectively). 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 (Supplemental Fig. 1B), 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 is growing 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, as well as acquisition of a mutation (M45V) 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 β_5 is required for the growth inhibitory activity of proteasome inhibitors (Singh et al., 2011; Niewerth et al., 2014b), active site mutations in the β_5 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 β_5 -dependent catalytic activity in cell extracts of CEM/BTZ200 cells (Supplemental Fig. 2) and CEM/S20 cells (Fig. 6) versus parental cells. It is noteworthy that, other than for constitutive proteasome subunits, expression of immunoproteasome subunits β_{5i} , β_{2i} , and β_{1i} 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 β_{5i} and β_{1i} subunits, both of parental cells and CEM/S20 cells (Fig. 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 Parlatti 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 β_5 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 [(S)-3-(4-methoxyphenyl)-N-((S)-1-((R)-2-methyloxiran-2-yl)-1-oxo-3-phenylpropan-2-yl)-2-((S)-2-(2-morpholinoacetamido)propanamido)propanamide], a selective immunoproteasome inhibitor.

Surprisingly, whereas Met45 (compared with Ala49) mutations did little in contribution to the natural resistance to salinosporamide A in *S. tropica* (Kale et al., 2011), M45V 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 β_5 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

Fig. 6. Impact of salinosporamide A on proteasome catalytic activity in parental and salinosporamide A-resistant CEM/S20 cells. β_5 -, β_{5i} -, and β_{1i} -associated catalytic activity in cell extracts of CEM/WT and CEM/S20 cells was assessed after 1-hour exposure to salinosporamide A. Results depicted represent the mean ($\pm$ S.D.) of three separate experiments.

salinosporamide A binding, thus conferring resistance. Indeed, M45V mutations in CEM/S20 were implicated in a marked loss of the ability of salinosporamide A to inhibit β_5 -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 M45V mutation in salinosporamide A resistance

in CEM leukemia cells, we showed that the PR-924-resistant MM cell line 8226/PR7 harboring the M45V 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 β_5 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 *S. tropica* point 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 spliced X-box protein 1 (Leung-Hagesteijn et al., 2013), increased expression of the insulin growth factor receptor (Kuhn et al., 2012), or an impaired balance of constitutive versus 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 antileukemic 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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Authorship Contributions

Participated in research design: Niewerth, Riethoff, Jansen, Cloos, Kale, Moore.

Conducted experiments: Niewerth, Riethoff, van Meerloo, Anderl.

Contributed new reagents or analytic tools: Anderl, Moore, Kale.

Performed data analysis: Niewerth, Riethoff, van Meerloo, Anderl.

Wrote or contributed to the writing of the manuscript: Niewerth, Riethoff, Jansen, Kale, Moore, Assaraf, Zweegman, Kaspers, Cloos.

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