Title

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Syrbactin proteasome inhibitor TIR-199 overcomes bortezomib chemoresistance and inhibits multiple myeloma tumor growth *in vivo*

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Abstract

Multiple myeloma (MM) and mantle cell lymphoma (MCL) are blood cancers that respond to proteasome inhibitors. Three FDA-approved drugs that block the proteasome are currently on the market, bortezomib, carfilzomib, and ixazomib. While these proteasome inhibitors have demonstrated clinical efficacy against refractory and relapsed MM and MCL, they are also associated with considerable adverse effects including peripheral neuropathy and cardiotoxicity, and tumor cells often acquire drug resistance. TIR-199 belongs to the syrbactin class, which constitutes a novel family of irreversible proteasome inhibitors. In this study, we compare TIR-199 head-to-head with three FDA-approved proteasome inhibitors. We demonstrate that TIR-199 selectively inhibits to varying degrees the sub-catalytic proteasomal activities (C-L/β1, T-L/β2, and CT-L/β5) in three actively dividing MM cell lines, with Ki_{50} (CT-L/β5) values of 14.61 ± 2.68 nM (ARD), 54.59 ± 10.4 nM (U266), and 26.8 ± 5.2 nM (MM.1R). In most instances, this range was comparable with the activity of ixazomib. However, TIR-199 was more effective than bortezomib, carfilzomib, and ixazomib in killing bortezomib-resistant MM and MCL cell lines, as

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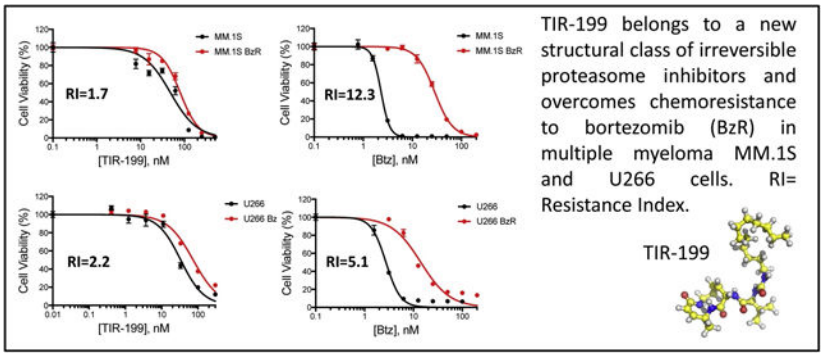
Conflicts of Interest: A.S.B is a named inventor of a United States patent (US 8597904, December 3, 2013) that relates to pharmaceutical compositions for the treatment of conditions responsive to proteasome inhibition. M.C.P is a named inventor of a United States patent (US 9221772 December 29, 2015) that relates to the synthesis of TIR-199. M.C.P and A.S.B are founders and shareholders of Hibiskus Biopharma, Inc. (Kalamazoo, MI, USA). N.G.D is founder and shareholder of Leukogene Therapeutics, Inc. (Charleston, SC, USA). No potential conflicts of interest were disclosed by the other authors.

judged by a low resistance index (RI) between 1.7 and 2.2, which implies that TIR-199 indiscriminately inhibits both bortezomib-sensitive and bortezomib-resistant MM and MCL cells at similar concentrations. Importantly, TIR-199 reduced the tumor burden in a MM mouse model ($p < 0.01$) confirming its potency *in vivo*. Given the fact that there is still no cure for MM, the further development of TIR-199 or similar molecules that belong to the syrbactin class of proteasome inhibitors is warranted.

Graphical Abstract



Keywords

Chemoresistance; multiple myeloma; mantle cell lymphoma; TIR-199; syrbactin; proteasome inhibitors

1. Introduction

The eukaryotic proteasome is a large multi-protein assembly. Its proteolytic activity is involved in a vast amount of functions in normal cells and is heavily relied on in the pathologic progress of several disease states including hematological cancers, autoimmune disorders, and inflammatory diseases [1–5]. The proteasome can vary in its composition of sub-catalytic units and activities giving rise to alternative forms including the immunoproteasome, thymoproteasome, and spermatoproteasome [6, 7]. As some form of the proteasome is present in all eukaryotic cells, it is heavily relied upon for DNA repair, cell-cycle progression, antigen presentation, cell survival, apoptosis and other functions.

Compared to normal cells, cancer cells have an increased sensitivity to proteasomal inhibition. There are 7 classes of proteasome inhibitors that block the proteasomal sub-catalytic activities (*i.e.*, caspase-like activity (C-L/β1), trypsin-like activity (T-L/β2), and chymotrypsin-like activity (CT-L/β5)) to varying degrees resulting in proteolytic stress and ultimately antiproliferative effects in several tumor cell types [8]. These proteasome inhibitor classes are comprised of both natural product-inspired and synthetic molecules. The FDA-approved proteasome inhibitors bortezomib (BTZ), carfilzomib (CAR), and ixazomib (IXA) have had clinical success in hematological cancers such as multiple myeloma (MM) and mantle cell lymphoma (MCL). Both BTZ and IXA are reversible inhibitors that predominantly inhibit CT-L activity and weakly inhibit C-L activity[3, 9–14].

Meanwhile, CAR irreversibly (covalently) inhibits the CT-L activity and, at higher concentrations, also the T-L activity [10]. While clinically important, chemoresistance of patients and considerable adverse effects including peripheral neuropathies, thrombocytopenia, and cardiotoxicities are associated with these drugs, which in part is due to off-target effects on proteasome-independent proteases [15–19].

The syrbactins represent the latest (seventh) class of mammalian proteasome inhibitors that irreversibly (covalently) bind to the catalytic Thr1 residue of the proteasome [8, 20]. Since the discovery of these microbe-derived natural products, which include the syringolins [20–25], glidobactins [23, 26–30], and cepafungins [23, 31, 32], the total synthesis of SylA and SylB [33–35] and a number of syrbactin-inspired structural analogs and their biological activities have been reported [33, 36–47]. TIR-199 is the most potent syrbactin analog to date and selectively inhibits the CT-L and T-L activities of both the constitutive proteasome and immunoproteasome *in vitro*. It is superior with regard to potential off-target effects on proteasome-unrelated proteases and exhibits significant anti-cancer activities *in vitro* as well as *in vivo* using hollow fiber assays [38].

In this study, we evaluate the biological activity of TIR-199 head-to-head against three FDA-approved proteasome inhibitors using BTZ-resistant and BTZ-sensitive MM and MCL cell lines and assess the anti-tumor efficacy of TIR-199 *in vivo* in MM tumor-xenografted mice.

2. Materials and methods

2.1. Chemicals

TIR-199 was synthesized as previously described [38] and the chemical structure is shown in Fig. 1. BTZ was purchased from LC Laboratories (Woburn, MA, USA). CAR and IXA were purchased from Ubiquitin-Proteasome Biotechnologies (Aurora, CO, USA). All drug solutions were prepared at 10 mM in DMSO, sterile-filtered, and stored frozen at −80°C. At the beginning of each experiment, aliquots were thawed and diluted to the final concentration.

2.2. Mammalian cell culture and reagents

Authenticated human MM cell lines were obtained from certified suppliers between 2014 and 2016. ARD and U266 were a gift from Dr. David Monsma (Van Andel Institute). MM.1R and MM.1S were purchased from the American Type Culture Collection (ATCC). MM.1R cell line derived from a patient who had become resistant to steroid-based therapy, dexamethasone. All cell lines were maintained in Roswell Parke Memorial Institute (RPMI) 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and supplemented with penicillin (100 U/ml) and streptomycin (100 μg/mL). Cells were cultured in 37°C in a humidified atmosphere containing 5% CO_2 and plated 24 h before drug treatment. Control cells were treated with 1% DMSO in culture media, equivalent to the maximum amount of DMSO present at the highest doses of drug. MM.1S BzR and U266 BzR, and 8226 BzR cell lines were a gift from Dr. Brian Van Ness (University of Minnesota) and were generated as previously described [48–50]. Granta BzR

and Mino BzR cell lines were selected for BTZ resistance by exposure to gradually increasing concentrations of BTZ over 6 months starting at 1 nM BTZ [50].

2.3. Proteasome activity assay

The cell-based proteasome activity assay to determine the sub-catalytic proteasomal activities in cells was performed as previously described[45]. Cells were seeded in solid white 96-well plates 24h prior to treatment. Cells were then treated with the indicated drug (0–50 nM) for 24 h. Cells were incubated for 15 min the proteasome Glo™ reagents according to the manufacturer's instructions (Promega), Proteasome inhibition of the caspase, trypsin and chymotrypsin activity sites were measured by addition of luminogenic substrates Z-nLPnLD-aminoluciferin, Z-LRR-aminoluciferin and Suc-LLVY-aminoluciferin, respectively.

2.4. Cell viability assay

The Cell Titer-Glo Assay (Promega) was performed as previously described [48] and used to determine the viability of cancer cells after 24h treatment with indicated drugs and concentrations. Luminescence was measured on a SpectraMax L Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at 470 nm with a 1s integration time. After the addition of Cell Titer-Glo solution to each well, the plate was allowed to equilibrate at 37°C for 5 min prior to reading. The data is expressed in percent (%) cell survival relative to control (untreated) cells.

2.5. Animal studies

Seven-week old male NSG-Scid mice (NOD.CB17-Prkdc^{scid}/J) were obtained from the Van Andel Research Institute (VARI) breeding colony and animal studies performed in accordance with approved IACUC protocols PIL-16-09-013 and XPA-17-09-014. Mice were subcutaneously injected with 1×10^6 ARD cells suspended in a 1:1 mixture of PBS/matrigel (R&D Systems, RGF BME PathClear®, #3434-005-02, MM, USA) in the right flank. Tumors were allowed to grow until they reached 200 mm^3 and the mice were randomized into treatment groups. Treatment included an initial dose of 25 mg/kg TIR-199 followed by additional doses of 12.5 mg/kg for the remainder of the study, or vehicle, (n=4 per group). TIR-199 drug formulation for the *in vivo* studies were 33% ethanol, 33% PEG-300, 33% PBS. Drug was stored at −80°C in aliquots for single use and prepared fresh. First day of treatment is considered day 0, when TIR-199 or vehicle was administered i.p. twice a week. The VARI preclinical therapeutics core staff monitored mice daily and measured tumor volume with digital calipers three times a week. Blood was collected each week via retro-orbital bleeding at 30 min, 1, 2, 6, or 24 h post injection. When the tumor volume reached 1500 mm^3, the mice were euthanized with CO_2 and the tumors were harvested and processed. All mouse work was performed by staff of the VARI pre-clinical therapeutics core facility and complied with the requirements set forth in the Guide for the Care and Use of Laboratory Animals.

2.6. Statistical analyses

GraphPad Prism v8.1.2 was used to generate EC_{50} curves, proteasome inhibition (K_i) curves, tumor growth regression analysis and Kaplan-Meier plots. The EC_{5o} values were determined from a non-linear regression fit with three or four parameters, depending on if values plateaued at higher doses. Similarly, K_i values were determined from a non-linear regression fit. For *in vivo* data, day zero was set when tumor size was palpable, at least 30 mm^3, and resulted in asynchronous measurement intervals for tumor. Tumor volume data are represented with the last value carried forward and standard error bars. To determine if tumor growth rates between TIR-199 and vehicle-treated animals differed, a linear mixed-effects model with a random slope and intercept for each mouse was fit via the R v3.6.0 (https://cran.r-project.org/) package 'lme4' (https://github.com/lme4/lme4/). The difference in survival was determined by a Kaplan-Meier Log-Rank analysis with euthanasia or death as the endpoint.

3. Results

3.1. TIR-199 shows selectivity for the CT-L sub-catalytic activity in MM cells

Our previous studies have revealed that the natural product syringolin A (SylA) and the syrbactin analog TIR-199 inhibit the constitutive proteasome and the immunoproteasome *in vitro* using purified proteasomes as a substrate [20, 38]. TIR-199 emerged as the most potent inhibitor with Ki_{50} values of 18 nM and 194 nM for the CT-L and T-L activity sites of the constitutive proteasome, respectively. Higher Ki_{50} values of 300 nM and 250 nM were observed for the CT-L and T-L activity sites of the immunoproteasome, respectively, with only minimal activity against the C-L activity site [38].

In the present study, we compared the anti-proteasomal activity of TIR-199 in MM cell lines head-to-head against three FDA-approved proteasome inhibitors; BTZ, CAR, and IXA. To accomplish this, we used a cell-based assay that accurately measures the three sub-catalytic proteasomal activities in actively dividing cells. As shown in Fig. 2, TIR-199 most prominently inhibited the CT-L activity in three MM cell lines (ARD, U266, and MM.1R) in a dose-dependent manner, with more moderate effects on the T-L and C-L activities in ARD and U266 cells. The three FDA-approved drugs BTZ, CAR, and IXA inhibited the proteasomal activities to various degrees (Fig. 2). While BTZ and CAR almost indiscriminately inhibited all three sub-catalytic activities, TIR-199 followed more closely the CT-L and T-L inhibition pattern of IXA. However, unlike TIR-199, IXA also strongly inhibited the C-L activity in the three MM cell lines. The Ki_{50} values of the four proteasome inhibitors for each of the three sub-catalytic activities are listed in Table 1. TIR-199 inhibited the CT-L activity with Ki_{50} values of 14.61 ± 2.68 nM (ARD), 54.59 ± 10.4 nM (U266), and 26.8 ± 5.2 nM (MM.1R). In most instances, this Ki range was comparable with the activity of ixazomib in these MM cell lines. TIR-199 generally had lower effects on both C-L and T-L sub-catalytic activities, with Ki_{50} values > 100 nM, except for the T-L activity of MM.1R cells with a Ki_{50} of 53.7 ± 15.9 nM. BTZ and CAR generally inhibited the CT-L, C-L, and T-L sub-catalytic activities with Ki_{50} values ranging between 0.53 ± 0.09 nM and 45.8 ± 17.5 nM (Table 1).

3.2. TIR-199 overcomes bortezomib chemoresistance in MM and MCL cells

To determine if TIR-199 can overcome BTZ chemoresistance in MM and MCL cells, the viability of MM cells (MM.1S, U266) and MCL cells (Granta, Mino) that either are resistant or sensitive to BTZ was assessed. TIR-199 reduced the viability of BTZ-resistant MM.1S (MM.1S BzR) and U266 (U266 BzR) cells in a dose-dependent manner (Fig. 3A). Most strikingly, the concentration of TIR-199 that induces 50% cell death (EC_{50}) in BTZ-resistant cell lines was comparable to the concentration required for BTZ-sensitive MM cell lines (Table 2). For example, the EC_{50} for TIR-199 in MM.1S and MM.1S BzR cells was 48.1 ± 8.7 nM and 80.5 ± 5.8 nM, respectively. In contrast, the treatment of BTZ-resistant cell lines MM.1S BzR and U266 BzR cells with BTZ, CAR, and especially with IXA required significantly higher drug concentrations to achieve similar cell inhibition results. EC_{50} values of IXA in MM1. S and MM.1S BzR cells were 25.5 ± 0.9 nM and 449 ± 23 nM, respectively (Table 2). Comparable observations were made with BTZ-resistant versus BTZ-sensitive MCL cell lines (Granta and Granta BzR; Mino and Mino BzR) (Fig. 3B and Table 2). To better quantify this observation, we calculated the resistance index (RI) for TIR-199, BTZ, CAR, and IXA in each cell line. As shown in Table 3, the RI for TIR-199 was between 1.7 ± 0.3 and 2.2 ± 0.4 for MM cell lines compared to an RI range of 5.1 ± 0.8 and 12.3 ± 0.3 (BTZ), 1.6 ± 0.2 and 5.1 ± 0.4 (CAR), and 4.9 ± 0.5 and 17.6 ± 1.1 (IXA). Less significant RI differences between these drugs were observed in MCL cell lines but TIR-199 still maintained the lowest RI value of 2.0 ± 0.1. These findings demonstrate that MM and MCL cells with an acquired resistance to the FDA-approved proteasome inhibitor BTZ maintain their sensitivity to TIR-199.

3.3. TIR-199 suppresses tumor growth and prolongs survival in MM mouse tumor xenografts

To determine the *in vivo* tumor efficacy of TIR-199, we used a xenograft tumor model of NSG-SCID mice that had been subcutaneously injected with MM (ARD) cells. The mice were treated twice a week with TIR-199 (one-time dose of 25 mg/kg followed by a dose of 12.5 mg/kg for remainder of treatment schedule) and tumor volume was measured. TIR-199 suppressed MM tumor growth compared to untreated control mice (Fig. 4A, $p < 0.01$) and mice treated with TIR-199 showed a 30% increase in median survival using this aggressive MM model (Fig. 4B). Moreover, on day 12, all vehicle mice had reached the survival endpoint whereas 75% of the mice treated with TIR-199 were still alive. Although a number of syrbactin analogs have been synthesized and tested in the cell culture settings, this is the first study to evaluate a syrbactin class proteasome inhibitor (TIR-199) *in vivo*, using a mouse tumor xenograft model.

4. Discussion

Syrbactins are derived from the natural product SylA and represent a novel class of irreversible proteasome inhibitors [8, 20–25]. Similar to CAR, the syrbactin analog TIR-199 is an irreversible inhibitor that covalently binds its target [38]. In this study, we compared the anti-proteasomal activity of TIR-199 head-to-head with the three FDA-approved proteasome inhibitors, BTZ, CAR, and IXA, we measured the anti-tumor effect of these drugs in a panel of MM and MCL cells that are resistant to BTZ, and we tested *in vivo* efficacy of TIR-199 in

a MM tumor xenograft mouse model. We found that in MM cell-based proteasome activity assays, TIR-199 predominantly inhibits the CT-L activity (Fig. 2 and Table 1), which is in agreement with our previous findings *in vitro* using purified proteasomes [38]. We further found that TIR-199 is effective against BTZ-resistant MM and MCL cells (Fig. 3). Finally, we showed for the first time that TIR-199 impedes tumor growth *in vivo* and slightly extends survival in 75% of TIR-199-treated mice (Fig. 4). While the potency of TIR-199 has significantly improved if compared with SylA, additional rounds of modifications will be necessary to further optimize this new lead analog.

The identification and development of novel, structurally diverse drugs able to overcome drug chemoresistance is important because many patients relapse or develop resistance to current treatments including BTZ, and there is still no cure for MM. The underlying molecular mechanisms for such resistance may include: (1) proteasome subunit (*PSMB5*, CT-L/β5) mutations, (2) alterations in redox homeostasis (protection from proteasome inhibitor-induced oxidative damage) (3), alterations in protein folding machinery, and (4) changes in energy regulation [51–53]. In our studies, the proteasome inhibitor-resistant MM cell lines did not have any mutations in the CT-L/β5 proteasome subunit, thus excluding this mechanism of resistance. However, they have heightened levels of mitochondrial respiration and increased mitochondrial biomass associated with proteasome inhibitor resistance [49]. Further studies are warranted to elucidate the underlying mechanisms engaged during TIR-199-induced cell death in BTZ-, CAR-, and IXA-resistant cells and MM tumor mouse models.

Proteasome inhibitors BTZ, CAR, and IXA are approved by the FDA for the treatment of MM and MCL. These drugs are now also investigated for other cancer and non-cancer indications and immunoproteasome-tailored inhibitors have become available [4, 5, 7, 54, 55]. We have recently synthesized such novel compounds called thiasyrbactins that are expected to find wide applications also outside of the cancer field, for example, in the treatment of various autoimmune and inflammatory diseases including rheumatoid arthritis and lupus [39]. Of note, sub-catalytically targeted constitutive proteasome or immunoproteasome inhibitors that specifically and only inhibit the β2 or β2i subunits, respectively, are being developed with the goal to elicit therapeutic responses that differ from the traditional effects of proteasome inhibitors [4, 54]. Notably, proteasome inhibitors may also find an application in the treatment of parasitic diseases including African sleeping sickness (trypanosomiasis) [56]. Finally, β subunit- or ubiquitin ligase-independent targets to inhibit the proteasome have recently emerged. Guo *et al* showed that phosphorylation of Rpt3 at Thr25 by dual-specificity tyrosine phosphorylation-regulated kinase 2 (DYRK2) is necessary for proteasome activity and DYRK2 inhibitors might present an alternative strategy to block the proteasome [57]. We recently showed that the natural product harmine can bind to DYRK2 and inhibits neuroblastoma tumor growth [58] and others found that curcumin impedes proteasome activity by direct inhibition of DYRK2 [59]. The combination of TIR-199 with DYRK inhibitors is currently under investigation to determine potential drug synergisms.

In summary, we show for the first time that the irreversible proteasome inhibitor TIR-199 overcomes BTZ resistance and exhibits anti-tumor activity *in vivo*. Importantly, this is the

first study to test the anti-tumor efficacy of a syrbactin class proteasome inhibitor using a mouse tumor xenograft model. Given the fact that there is still no cure for MM, the further development of TIR-199 or similar molecules that belong to the syrbactin class of proteasome inhibitors is warranted.

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Highlights:

- TIR-199 is a syrbactin natural product analog and belongs to a new structural class of irreversible proteasome inhibitors.
- TIR-199 overcomes chemoresistance to bortezomib in multiple myeloma and mantle cell lymphoma
- TIR-199 reduced the tumor burden in a multiple myeloma mouse model

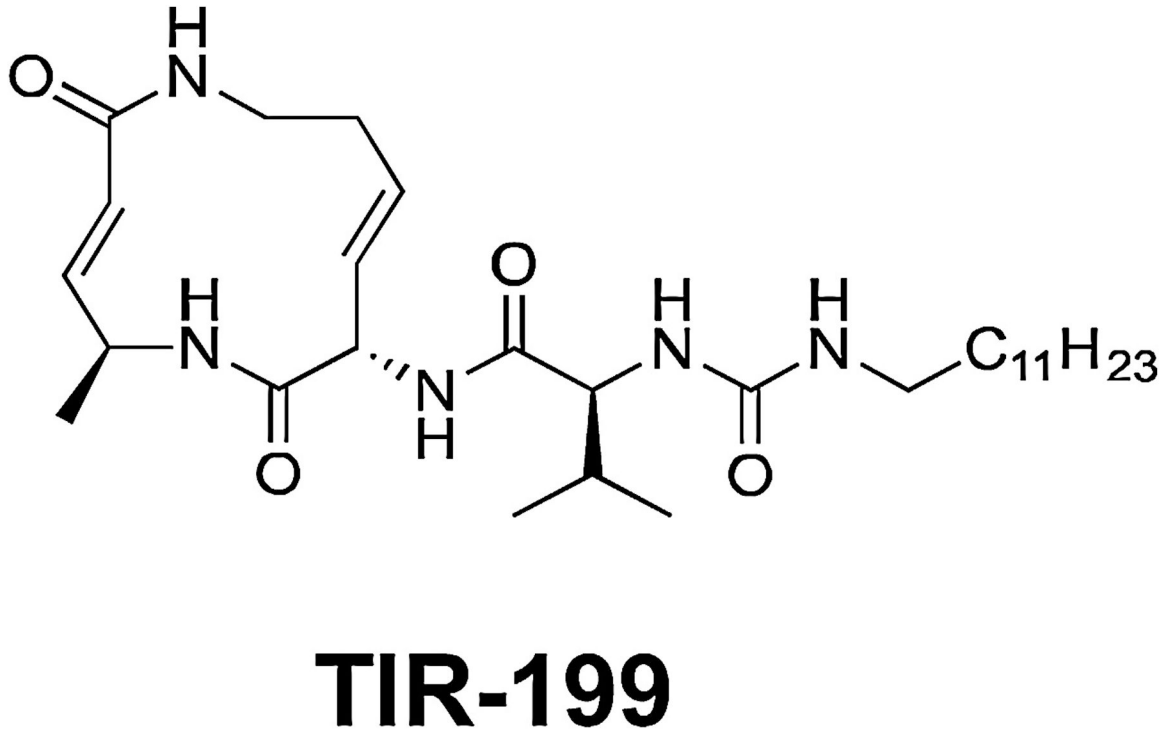

Fig. 1.

Chemical structure of proteasome inhibitor TIR-199, an analog of natural product syringolin A (SylA). Molecular weight of TIR-199 is 534.

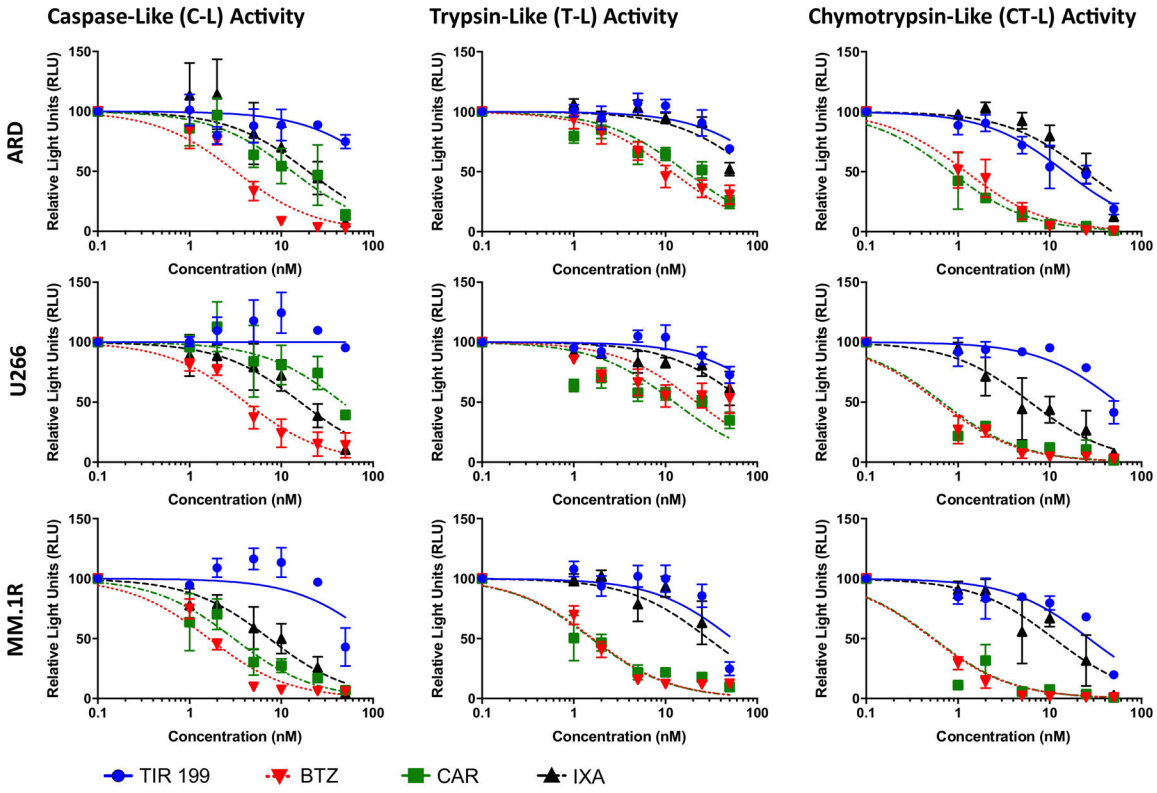

Fig. 2.

Cell-based proteasome activities for each sub-catalytic site in response to proteasome inhibitors TIR-199, bortezomib (BTZ), carfilzomib (CAR), and ixazomib (IXA). Multiple myeloma (MM) cell lines, ARD, U266, and MM.1R were used in this study. Data represent the average of three independent experiments (n=3) ± S.D. See Ki_{50} values in Table 1.

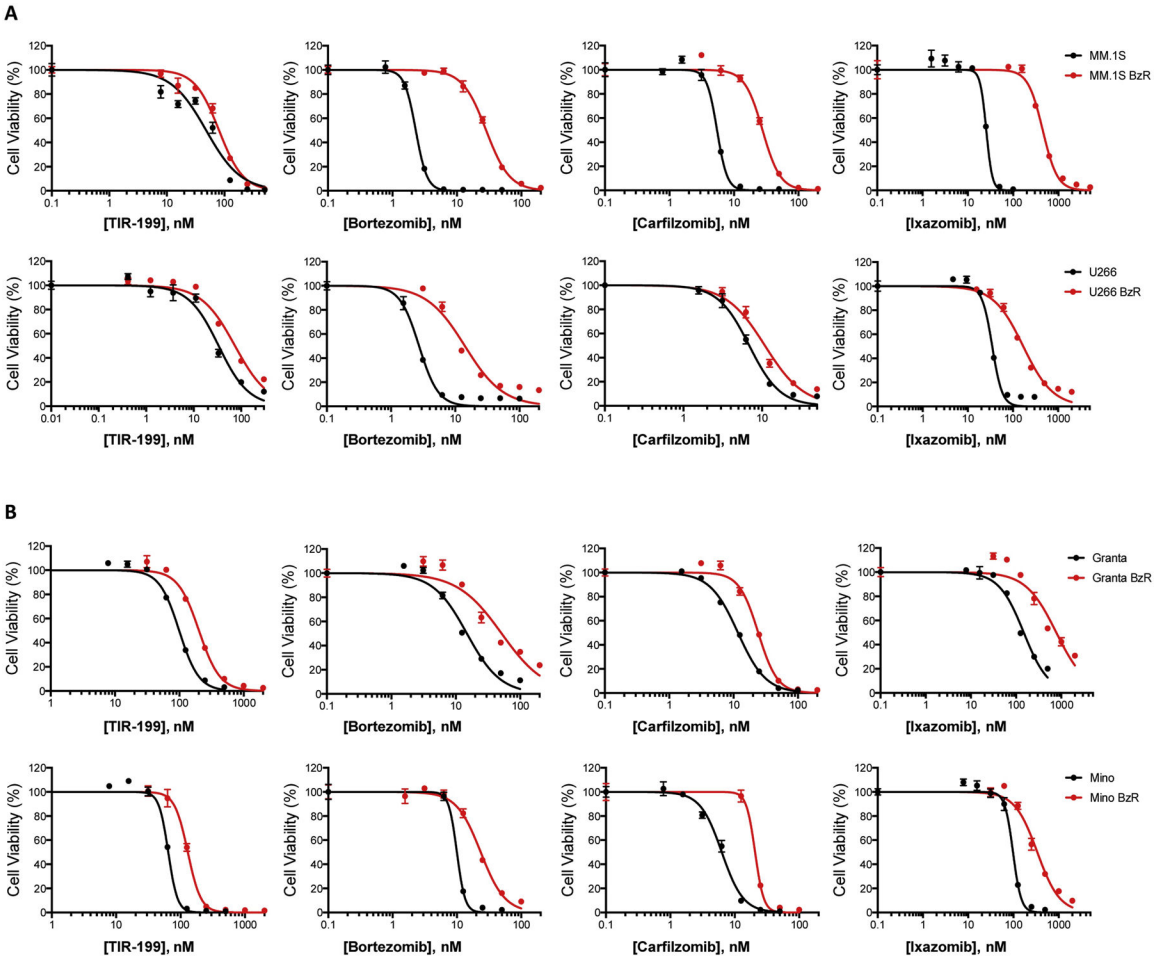

Fig. 3.

Cell viability for (A) multiple myeloma (MM) cell lines MM.1S, MM.1S-BzR, U266 and U266-BzR and (B) mantle cell lymphoma (MCL) cell lines Granta, Granta-BzR, Mino, and Mino-BzR after treatment with proteasome inhibitors TIR-199, bortezomib (BTZ), carfilzomib (CAR), and ixazomib (IXA). BzR = BTZ resistant. Data represents the average of three independent experiments (n=3) ± S.E. See EC_{50} values in Table 2 and corresponding resistance index (RI) values in Table 3.

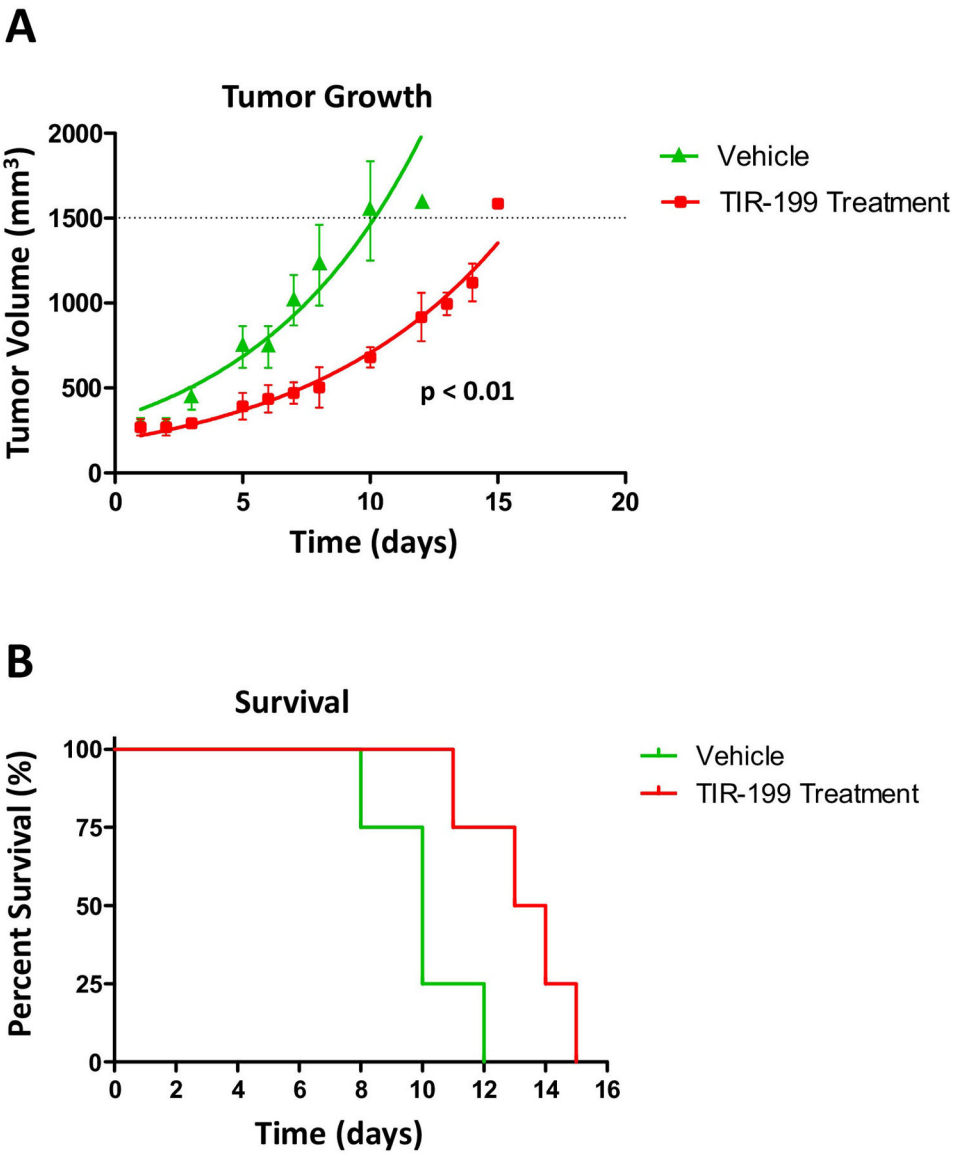

Fig. 4.

In vivo activity of TIR-199 in a multiple myeloma tumor mouse model. (A) Tumor growth curve and (B) Kaplan-Meier survival plot in ARD-xenografted mice with and without TIR-199 treatment. The mice were treated twice a week. The first dose of TIR-199 was given i.p. at 25.0 mg/kg, followed by a reduced dose of 12.5 mg/kg for remainder of the experiment. Tumor volume was measured using a caliper three times a week ($p < 0.01$). Data represent the average of 4 mice per group (n=4).

Table 1

Ki_{50} values of proteasomal sub-catalytic activities in MM cells

		TIR-199	BTZ	CAR	IXA
Cell line	**Site**	**Ki_{50}, (nM)**	**Ki_{50} (nM)**	**Ki_{50} (nM)**	**Ki_{50} (nM)**
ARD	CT-L (β5)	14.61 ± 2.68	1.22 ± 0.24	0.83 ± 0.18	24.3 ± 4.76
	C-L (β1)	137.9 ± 52.0	3.17 ± 0.57	13.2 ± 3.77	19.6 ± 8.32
	T-L (β2)	161.9 ± 50.4	11.6 ± 1.90	16.6 ± 2.77	92.8 ± 19.1
U266	CT-L (β5)	54.59 ± 10.4	0.62 ± 0.11	0.67 ± 0.13	6.12 ± 1.46
	C-L (β1)	-	4.06 ± 0.70	45.8 ± 17.5	16.1 ± 4.01
	T-L (β2)	170.2 ± 47.9	21.7 ± 5.64	12.5 ± 3.01	74.9 ± 16.8
MM.1R	CT-L (β5)	26.8 ± 5.2	0.53 ± 0.09	0.54 ± 0.15	11.1 ± 2.72
	C-L (β1)	104.4 ± 46.6	1.6 ± 0.24	3.03 ± 0.72	7.41 ± 1.59
	T-L (β2)	53.7 ± 15.9	1.6 ± 0.21	1.56 ± 0.31	29.3 ± 7.83

Values were determined from data shown in Fig. 2.

Table 2

EC_{50} values of MM and MCL cells

	TIR-199	BTZ	CAR	IXA
Cell line	**EC_{50}, (nM)**	**EC_{50}, (nM)**	**EC_{50}, (nM)**	**EC_{50}, (nM)**
Multiple Myeloma				
MM.1S	48.1 ± 8.7	2.31 ± 0.03	5.5 ± 0.2	25.5 ± 0.9
MM.1S BzR	80.5 ± 5.8	28.4 ± 0.6	27.7 ± 1.6	449 ± 23
U266	33.5 ± 4.5	2.7 ± 0.2	6.9 ± 0.5	34.7 ± 2.1
U266 BzR	74 ± 10	14.1 ± 2.0	10.9 ± 1.4	169 ± 15
Mantle Cell Lymphoma				
Granta	99.3 ± 4.8	14.9 ± 1.6	11.7 ± 0.3	147 ± 12
Granta BzR	202 ± 10	51.5 ± 9.3	24.2 ± 1.4	774 ± 150
Mino	64.4 ± 2.1	10.0 ± 0.4	6.3 ± 0.3	97.6 ± 4.6
Mino BzR	129 ± 2	23.4 ± 1.1	20.7 ± 0.7	336 ± 30

Values were determined from data shown in Fig. 3.

Table 3

Resistance index for proteasome inhibitors

	TIR-199	BTZ	CAR	IXA
Cell line	**Ratio**	**Ratio**	**Ratio**	**Ratio**
Multiple Myeloma				
MM.1S BzR : MM.1S	1.7 ± 0.3	12.3 ± 0.3	5.1 ± 0.4	17.6 ± 1.1
U266 BzR : U266	2.2 ± 0.4	5.1 ± 0.8	1.6 ± 0.2	4.9 ± 0.5
Mantle Cell Lymphoma				
Granta BzR : Granta	2.0 ± 0.1	3.5 ± 0.7	2.1 ± 0.1	5.2 ± 1.1
Mino BzR : Mino	2.0 ± 0.1	2.3 ± 0.1	3.3 ± 0.2	3.4 ± 0.3

Resistance index (RI) for each proteasome inhibitor was calculated by dividing the EC_{50} value of bortezomib-resistant (BzR) cell lines with the EC_{50} value of their isogenic control (Table 2).