VR23: A Quinoline–Sulfonyl Hybrid Proteasome Inhibitor That Selectively Kills Cancer via Cyclin E–Mediated Centrosome Amplification

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Abstract

The proteasome is clinically validated as a target for cancer therapeutics. However, proteasome-inhibitory agents that are cancer selective have yet to be developed. In this study, we report the identification of a safe and effective proteasome inhibitor with selective anticancer properties. We screened a chemical library constructed using a hybrid approach that incorporated a 4-piperazinylquinoline scaffold and a sulfonyl phamarcophore. From this library, we identified 7-chloro-4-(4-(2,4-dinitrophenylsulfonyl)piperazin-1-yl)quinoline (VR23) as a small molecule that potently inhibited the activities of trypsin-like proteasomes (IC50 = 1 mmol/L), chymotrypsin-like proteasomes (IC50 = 50–100 mmol/L), and caspase-like proteasomes (IC50 = 3 mmol/L). Data from molecular docking and substrate competition assays established that the primary molecular target of VR23 was β2 of the 20S proteasome catalytic subunit. Notably, VR23 was structurally distinct from other known proteasome inhibitors and selectively killed cancer cells by apoptosis, with little effect on noncancerous cells. Mechanistic investigations showed that cancer cells exposed to VR23 underwent an abnormal centrosome amplification cycle caused by the accumulation of ubiquitinated cyclin E. In combinations with the clinically approved chymotrypsin-like proteasome inhibitor bortezomib, VR23 produced a synergistic effect in killing multiple myeloma cells, including those that were resistant to bortezomib. VR23 was effective in vivo in controlling multiple myelomas and metastatic breast cancer cells, in the latter case also enhancing the antitumor activity of paclitaxel while reducing its side effects. Overall, our results identify VR23 as a structurally novel proteasome inhibitor with desirable properties as an anticancer agent. Cancer Res; 75(19): 4164–75. ©2015 AACR.

Introduction

Centrosome duplication is strictly regulated to maintain genetic stability, and its deregulation may result in the development of tumors or microcephaly (1). Along with other proteins, cyclin E is essential for centrosome duplication; however, its overexpression can induce abnormal centrosome amplification, aneuploidy, and eventually tumorigenesis (2–5). Not surprisingly, the level of cyclin E is generally higher in cancer than in normal cells, which makes malignant cells more vulnerable to centrosome amplification (6, 7). Interestingly, however, cyclin E can be dispensable for cell proliferation (8), suggesting that centrosome duplication is governed by redundant mechanisms. Centrosome duplication in somatic cells is normally semiconservative, although centrosomes can also be synthesized by de novo assembly when residential centrioles are removed (9–11). In this case, the numerical control of the centrosome is usually lost, resulting in a variable number of centrosomes per cell (10).

Proteasomes play a critical role in regulating the level of cellular proteins and recycling damaged and misfolded proteins. Although essential for normal cell regulation, proteasome activity is especially critical for the proliferation and survival of cancer cells. Chen and Madura found that the level and activity of proteasomes are higher by >90% in primary tumors, compared with normal tissues (12). These authors also found that MCF10A, a nonmalignant breast cell line, did not show any increase in proteasome activity. High levels of proteasomes were also observed in acute lymphocytic leukemia, T-cell leukemia, and acute myelocytic leukemia cells (13). It is thought that the high level of proteasome activity in cancer cells provides survival benefits under the stress of continued cell proliferation (14, 15).

The inhibition of proteolysis can not only activate apoptosis but may also prevent angiogenesis and metastasis (16, 17). Since the first proteasome inhibitor bortezomib (Velcade) was approved by the FDA for the treatment of multiple myeloma in 2003, many proteasome inhibitors have been reported (18, 19). Although bortezomib and carfilzomib (Kyprolis) are quite effective for the treatment of multiple myeloma and other blood cancers, they either show considerable side effects or are ineffective against solid tumors (20–22).
We previously found that the antiproliferative effect of 4-piperazinylquinoline-isatin hybrid compounds is substantially more active for cancer than noncancer cells (23). Other groups showed that sulfonyl derivatives often possess effective antitumor activity (24). Therefore, we designed, synthesized, and examined the anticancer activities of novel 4-piperazinyl/amino quinoline–derived sulfonyl compounds on the assumption that the hybridization of a quinoline scaffold and a sulfonamide pharmacophore could lead to molecules with desirable anticancer property (Fig. 1A). We report here that 7-chloro-4-(4-(2,4-dinitrophenylsulfonyl)piperazin-1-yl)quinoline (VR23), one of these novel compounds, causes apoptosis in a cancer-specific manner through the inhibition of proteasome activity and cyclin E-mediated abnormal centrosome amplification.

Materials and Methods

Cell culture and synchronization

All the cancer cell lines used, including RPMI 8226-BR and ANBL6-BR (“BR” is for bortezomib resistance), were grown in RPMI1640 or DMEM supplemented with 10% FBS. RPMI 8226, KAS6/1, ANBL6, RPMI 8226-BR, and ANBL6-BR were kind gifts of Dr. R. Orlowski (MD Anderson Cancer Center, Houston, TX; refs. 25, 26). IL6–dependent cells (KAS6/1, ANBL6, ANBL6-BR) were further supplemented with 1 ng/mL of IL6. MCF10A and 184B5 cells were grown in DMEM/F12 supplemented with growth factors and 10% equine serum. Cell line authentication was performed by Genetica DNA Laboratories using short tandem repeat profiling (March 2015). Double thymidine–based cell synchronization at G1–S was carried out as described previously.
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(27). For synchronization at G1–M, cells were maintained in 5 ng/mL of nocodazole for 18 hours.

Proteasome assay

Exponentially growing cells on a 96-well clustered plate were treated with different concentrations of drugs or left untreated (control) for 6 hours. Proteasomes extracted with 0.5% NP40 buffer were mixed with equal amounts of samples in 100 µL total volume, and then incubated with 25 µmol/L of fluorogenic substrates (Boston Biochem; LRR-specific for trypsin-like activity, LLE-specific for caspase-like activity, and SUVY-specific for chymotrypsin-like activity) in black-bottom 96-well plates at 37°C. Fluorescence was monitored every 5 minutes at the wavelength of 360 nm (excitation) and 480 nm (emission).

The reaction was allowed to proceed for 30 minutes (chymotrypsin-like activity and chymotrypsin-like proteasome activities). Hydrolysis of substrate was added to the samples after they were preincubated for 1 hour at 37°C (e.g., 0, 25, 50, 100, and 200 µmol/L of LRR and Suc-LLVY, respectively, specific for trypsin-like activity and chymotrypsin-like proteasome activities). Hydrolysis reaction was allowed to proceed for 30 minutes (chymotrypsin-like substrate) and 1 hour (trypsin-like substrate) at 37°C. The fluorescence was measured at 380 nm excitation/460 nm emission at 30 minutes (for some experiments at 15, 30, 45, and 60 minutes). Data were analyzed with GraphPad PrismV using a linear regression curve analysis (Michaelis–Menten kinetics) as described previously (28).

Animal work

Five-week-old female ATH490 (athymic nude mice strain 490) were purchased from Charles River. Animals were monitored for food and water consumption every day, and twice per week for body weight and tumor weight/volume. Tumor volume, measured with a caliper, was determined using the following formula:\( V = \frac{1}{2} \times \text{length} \times \text{width}^2 \). Blood samples were collected (D15 or D29) by cardiac puncture and processed for alanine transaminase/aspartate aminotransferase (AST/ALT) measurements. Tumors and organs were harvested and fixed in 10% buffered formalin at 4°C overnight before processing for paraffin embedding. Each section of tumors and organs (4–5 µm thick) was stained with hematoxylin and eosin (H&E). Some tumor sections were subjected to immunohistochemistry with antibody specific for Ki-67, VEGF or CD31.

Animal Care Committee at Laurentian University (Ontario, Canada) reviewed and approved all aspects of animal experiments, including animal handling, care, treatments, and endpoint determination. The human MDA-MB-231 metastatic breast cancer cell line and RPMI 8226 multiple myeloma cells used were purchased from ATCC.

Anticancer activity of VR23 in xenograft mice

To establish tumors in mice, exponentially growing MDA-MB-231 or RPMI 8226 cells (10 × 10^6 cells in 0.2 mL of ice-cold 1× PBS) were subcutaneously injected into the flank of each animal. When tumor size reached 4 to 5 mm in diameter, mice were usually randomized into the following five groups (n = 4–6 per group): (i) untreated control, (ii) vehicle/sham control, (iii) VR23-treated sample, in which animals were administered intraperitoneally twice per week with VR23 (30 mg/kg body weight); (iv) paclitaxel-treated samples, in which animals were given intravenously with paclitaxel (20 mg/kg/week); and (v) combination group, in which paclitaxel (20 mg/kg) was given intravenously 24 hours prior to VR23 (30 mg/kg, i.p.) per week.

Statistical analyses

All values are mean ± S.E.M. of at least three independent experiments. Analyses were performed using GraphPad Prism software. Comparison between groups was made by P value using one-way ANOVA: P < 0.05 was considered to be statistically significant.

Results

VR23 is a novel proteasome inhibitor targeting β2 of the 20S proteasome subunit

Screening of the newly created chemical library (above) using three breast cancer cell lines (MDA-MB-231, MDA-MB-468, and MCF7) and two nonmalignant breast cell lines (184B5 and MCF10A) identified VR23 (Fig. 1B) as being a potentially effective and safe anticancer agent, as it killed/inhibited proliferation of cancer cells 2.6 to 17.6 times more effectively than noncancer cells (Fig. 1C). In addition, VR23 showed at least an additive effect when used in combination with paclitaxel (Supplementary Table S1).

Our preliminary data showed that VR23 slowed down cell-cycle progression, which is often observed in cells treated with proteasome inhibitors (29). Furthermore, a quinoline, from which the basic scaffold of VR23 was derived, is a weak proteasome inhibitor (28). Therefore, we postulated that VR23 would possess protease inhibition property. As expected, data from a fluorogenic assay using extracts from three cell lines (HeLa, MCF7, and MDA-MB-231) showed that VR23 is a very effective inhibitor of trypsin-like proteasome (IC_{50} 1 nmol/L; Fig. 1D). In addition, VR23 substantially inhibits chymotrypsin-like proteasome (IC_{50} 50–100 nmol/L) and caspase-like proteasome (3 µmol/L; Fig. 1D). As the target of trypsin-like proteasome inhibitors is β2 of the 20S proteasome catalytic subunit (19, 30), and as vinyl sulfones are known to covalently modify β peptide through the hydroxyl group of threonine and the double bond of the vinyl sulfone moiety (19, 31, 32), we carried out in silico docking analysis using MOE (see Supplementary Materials and Methods) to examine this possibility. As anticipated, VR23 fits into the pockets of the β2 subunit (both H and V chains) with low energy (−5.6288 kcal/mol and −5.5983 kcal/mol, respectively) and low root mean square deviation (RMSD, 0.6715 Å and 0.0035 Å, respectively; Fig. 1E and F). To confirm the interaction between VR23 and β2 subunit, we carried out a substrate competition assay. Our data plotted according to Lineweaver–Burk (28) confirm that VR23 effectively competes with substrates of trypsin-like proteasome. However, our data also indicate that VR23 inhibits trypsin-like proteasome by noncompetitive allosteric binding, as the x-axis interception point changes in the presence of VR23 (Fig. 1G).

As ubiquitination is an early step in the proteasome-mediated protein degradation, we examined the level of protein ubiquitination. As expected, ubiquitinated proteins accumulated in HeLa...
cells exposed to VR23 in a dose-dependent manner (Fig. 1H),
indicating that proteins are ubiquitinated, but not degraded by
proteasomes in the presence of VR23. The chemical structure of
VR23 does not show similarity with any known proteasome
inhibitors, suggesting that it is a novel class (18, 19).

When combined with bortezomib, VR23 shows synergistic
effects on RPMI 8226 and KAS 6/1 multiple myeloma cells
As proteasome inhibitors are often used for the treatment
of multiple myeloma, we determined IC_{50} values of VR23 on
RPMI 8226 and KAS 6 cells, which were found to be 2.94 and
1.46 μmol/L, respectively (Fig. 2A). The combination of 1.25
μmol/L VR23 and a low-dose bortezomib (e.g., 5 nmol/L) showed
highly synergistic effect on both RPMI 8226 (combinational index
(CI), 0.37) and KAS 6 cells (CI, 0.57; Fig. 2B and C). Interestingly,
VR23 is more effective on bortezomib-resistant RPMI 8226
than on naïve RPMI 8226 (Fig. 2D). VR23 is found to be equally
effective on both naïve and bortezomib-resistant ANBL 6/1 cell
(Fig. 2D). When combined with 10 nmol/L bortezomib, VR23
dramatically reduces the viability of two bortezomib-resistant
multiple myeloma cell lines examined (Fig. 2E and F).

VR23 causes apoptosis in a cancer-specific manner
A clonogenic assay showed that very few MCF7 cells survived
for 2 weeks at 1.5 μmol/L of VR23 (Supplementary Fig. S1A). Data
from flow cytometry showed that the treatment of MCF7 with
2.7 or 8.0 μmol/L of VR23 resulted in 23.7% or 38.1% sub-G_{1}
DNA content within 48 hours after treatment, respectively (Sup-
plementary Fig. S1B). In contrast, the flow cytometry profiles of
MCF10A cells were not notably different between the sham
control and VR23-treated samples, up to 8 μmol/L and 72 hours
after treatment (Supplementary Fig. S1C). These data demon-
strate that, unlike MCF7, the noncancer MCF10A is much more
resistant to VR23, which is consistent with data shown in Fig. 1C.

Figure 2.
Combination of VR23 and bortezomib (BTZ) shows synergistic effects. A, data shown are IC_{50} values of bortezomib and VR23 determined by the sulforhodamine
B assay (48 hours after treatment) on two multiple myeloma and two noncancer lines (184B5, MCF10A). B and C, the combination of 1.25 μmol/L VR23 and
RPMI 8226 (5 nmol/L) or KAS 6/1 (0.625 nmol/L) shows highly synergistic effects, as CI is 0.37 and 0.57, respectively. CI < 1.0, CI = 1.0, and CI > 1.0 are synergistic,
additive and antagonistic, respectively (46). D, VR23 is equally effective on both bortezomib (BTZ)-sensitive and -resistant cells. E and F, the combination of
bortezomib and VR23 is effective on bortezomib-resistant RPMI 8226 cells (E) and bortezomib-resistant ANBL 6 cells (F), RPMI 8226-BR cells were treated
with either 10 nmol/L bortezomib, 2.5 μmol/L VR23, or combination of the two. ANBL6-BR cells were treated with either 10 nmol/L bortezomib, 5 μmol/L VR23,
or combination of the two. Data, mean ± SEM (n ≥ 2; P < 0.005 for all cell lines).
To examine VR23-induced impediment of cell-cycle progression in more detail, HeLa cells arrested at G2–M by nocodazole were released into cell cycle at 0 hours in the absence (control) or presence of 5 μmol/L VR23 for 2, 4, or 8 hours. Under these conditions, over 30% of cells underwent DNA fragmentation (as manifested by sub-G1 DNA) within 4 hours in the presence of VR23, suggesting that cells are dying by apoptosis (Supplementary Fig. S2A). As expected, PARP1 was cleaved in response to VR23, confirming that VR23 induces apoptosis (Supplementary Figs. S1B, S1D, and S2A). It is known that the activation of apoptosis at mitosis often depends on the phosphorylation of p38 MAPK (33). Consistent with this previous observation, high levels of phosphorylated p38 are shown in cells treated with VR23 (Supplementary Fig. S2B). Thus, we conclude that VR23 induces apoptosis in mitotic cancer cells through the p38 MAPK-signaling pathway. It should also be noted that the level of cyclin E was much higher in cells treated with VR23 than control, even when over 30% of cells were already dead (Supplementary Fig. S2A and S2B).

To gain further insights into VR23 effects during G2–M, we examined the interactions of proteins involved in the regulation of metaphase progression. At 0 hours after nocodazole, cells were released into complete medium for 0.5 to 4 hours in the absence or presence of VR23 (5 μmol/L). The association of Cdc20 with Cdc27, which is required for the activation of anaphase promoting complex (APC; ref. 34), is peaked at 1 hour after nocodazole in control. In contrast, only a low level of the Cdc20–Cdc27 complex is detected in the VR23-treated sample at the same time point (Supplementary Fig. S2C). The spindle checkpoint protein BuBR1 is acetylated by kinetochores that are not fully attached to microtubules (35). Activated BuBR1 then inhibits the capability of APC to ubiquitinate securin and cyclin B and, thereby, prevents mitotic exit until cells are completely ready (36). As expected, high levels of BuBR1–Cdc20 association are shown in controls at all of the time points examined. In contrast, cells exposed to VR23 showed only a low level of BuBR1–Cdc20 association until 1 hour, after which the association is barely detectable. These data suggest that some cells in VR23-treated samples may progress through anaphase up to 1 hour after nocodazole; however, most cells do not activate mitotic spindle checkpoint. Emi1 (early mitotic inhibitor 1; FBX5; FBXO5) regulates progression of cells through early mitosis by inhibiting APC (37). Emi1 prevents APC activation through its binding to APC, Cdc20 or Fzr1/Cdh1. As expected, Emi1 strongly associated with APC during the first 30 minutes after release from nocodazole, followed by only low levels of association up to 2 hours in the control. These data suggest that cells released into drug-free medium have largely passed prometaphase–metaphase transition by 1 hour after nocodazole. In contrast, the Emi1–Cdc20 complex is not detected in cells treated with VR23, except 2 hours after nocodazole. As the associations of Cdc20 with Emi1 and with BuBR1 usually occur prophase and prometaphase, respectively (38), we conclude that cells exposed to VR23 are largely arrested at prometaphase. This interpretation is consistent with data obtained by confocal microscopy (Supplementary Fig. S3A, white arrows).

VR23 treatment resulted in the accumulation of cyclin E at centrosomes, leading to its amplification in a cancer cell–specific manner. Data obtained by confocal microscopy showed that VR23 causes abnormal amplification of microtubule organization centers (MTOC) in MCF7 (Supplementary Fig. S3A, yellow arrows). Under the experimental conditions, most chromosomes are condensed but not aligned at the equatorial plate, indicating that cells have not yet reached metaphase (Supplementary Fig. S3A, white arrows). To gain a better understanding, cells synchronized at G1–S were released into cell cycle for 6 hours, at which time most cells are around G2–M (Supplementary Fig. S3B). Cells were then maintained for additional 6 hours in the presence of VR23. As shown in Supplementary Fig. S3C, multiple centrosomes cause either cell-cycle arrest before metaphase (“no attachment”) or abnormally separate chromosomes if cells had already reached near metaphase when they were exposed to VR23 (triple and multiple MTOCs and skewed pull). Approximately 70% of cells showed “no attachment” phenotype: their chromosomes are either not completely condensed or not yet aligned at the equatorial plate. These data are consistent with that of Supplementary Fig. S2C (co-immunoprecipitation data).

As cyclin E positively regulates centrosome duplication (2, 39), we examined its centrosomal localization in early S-phase. We found that cyclin E was not localized to the centrosome during the first 2 hours after double thymidine, regardless of VR23 treatment (Fig. 3, 1–2 h after double thymidine). By 3 hours after double thymidine, however, cyclin E was localized to centrosomes in the presence (but not absence) of VR23. Unlike in HeLa cells, cyclin E was not localized to the centrosome in MCF10A regardless VR23 treatment (Fig. 3). Our data thus demonstrate that cyclin E and its localization to the centrosome may be responsible for centrosome amplification in cells exposed to VR23. To further confirm this, we examined centrosome amplification in cells whose cyclin E had been ablated with siRNA. As shown in Fig. 4, cyclin E ablation indeed resulted in the complete abrogation of VR23-induced centrosome amplification, although DNA replication still occurred (Fig. 4C). Together, our data demonstrate that cyclin E plays a critical role for abnormal centrosome amplification in cancer cells exposed to VR23.

Ubiquitinated cyclin E accumulates at centrosomes in response to VR23

As cyclin E is ubiquitinated in the process of degradation, we examined its ubiquitination status in cells exposed to VR23. As shown in Fig. 5A, centrosomally localized cyclin E is ubiquitinated in the presence of VR23 (inset box). Cyclin E ubiquitination was further confirmed by immunoprecipitation and subsequent Western blotting (Fig. 5B). Together, our data demonstrate that centrosomally localized cyclin E is ubiquitinated but not degraded in the presence of VR23. As this is directly correlated with centrosome amplification, we conclude that the high level of ubiquitinated cyclin E and its localization to the centrosome results in centrosome amplification. This conclusion is strengthened by the fact that VR23 causes neither ubiquitination nor centrosomal localization of cyclin E in MCF10A (Fig. 3 and Supplementary Fig. S4). In contrast, cyclin E is readily ubiquitinated in response to bortezomib in both cancer and noncancer cells (Fig. 5B and Supplementary Fig. S4).

VR23 shows effective antitumor and antiangiogenic activities in mice

We examined the antitumor activity of VR23 using AT1H490 athymic mice engrafted with cancer cells as described in Materials and Methods. The treatment of VR23 (30 mg/kg body weight, i.p.) twice per week for 3 weeks resulted in a 4.6-fold decrease in MDA-MB-231 tumor size, compared with
When animals were treated with 20 mg of paclitaxel and 30 mg of VR23 per week for 3 weeks, the tumor size was reduced by 29-fold (305.01 vs. 10.51 mm³; Fig. 6B, day 22). In this case, the tumor size shrank to approximately one-fifth of the original size (50.69 vs. 10.51 mm³). As expected, data from a Ki-67 uptake assay carried out at 15 days after treatment showed that VR23 substantially reduced cancer cell proliferation (Fig. 6C).

To examine VR23 antitumor activity on other cancers, we treated ATH490 mice (n = 4) engrafted with RPMI 8226 multiple myeloma cells. We found that the tumor volume of VR23-treated samples at 24 days after treatment was 19.1% of the control (Supplementary Fig. S5; 1,219.5 vs. 233.0); thus, the antitumor activity of VR23 is not limited to metastatic breast cancer.

As shown in Fig. 6D, substantial invasion of tumor cells into surrounding tissues (e.g., muscle) was observed in the controls (29 days after treatment). In contrast, tumor samples isolated from ATH490 mice treated with VR23 or paclitaxel did not show any evidence of tumor infiltration into surrounding tissues (Fig. 6D). Next, we determined the level of VEGF and CD31 in tumor samples at 15 days after treatment. As expected, the expression of VEGF and CD31 were not notable in tumors treated with either paclitaxel, VR23, or in combination (Fig. 6D and Supplementary Fig. S6A).

VR23 reduces adverse effects caused by paclitaxel

Treatment of ATH490 mice with VR23 (30 mg/kg), paclitaxel (20 mg/kg), or a combination of the two did not cause any notable ill-effects on animals, as determined by changes in body weights (Supplementary Fig. S6B). We next determined the toxic effects of VR23 and paclitaxel on the liver, spleen, kidney, and lung. As shown in Supplementary Fig. S7, organ weights did not change by the treatments, with one exception: the spleens of animals treated with 20 mg/kg of paclitaxel were enlarged by...
19.5% after 29 days of treatment. Although this is statistically not significant (P = 0.29), we consistently observed that paclitaxel treatment resulted in slight enlargement of spleen. Interestingly, spleen weight was not increased when animals were treated with both paclitaxel and VR23 in combination (Supplementary Fig. S7).

The ratio of serum ALT and AST is often used to determine liver injuries (40). Unlike VR23, paclitaxel treatment resulted in substantially higher AST/ALT ratio (Supplementary Table S2). To further examine the potential liver toxicity by paclitaxel, we counted mitotic cells in liver tissues (e.g., Supplementary Fig. S6C). Mice treated with VR23 (30 mg) showed similar liver mitotic index with controls (Fig. 7A). In contrast, samples treated with paclitaxel (20 mg) showed approximately 13 times more mitotic cells, again suggesting that paclitaxel causes liver damage. Intriguingly, the elevated level of mitotic index caused by paclitaxel was dramatically reduced when used in combination with VR23 (Fig. 7A).

We also found that VR23 did not cause any notable ill-effects on spleen (Fig. 7B). In contrast, paclitaxel caused several distinct abnormalities on spleen, including expanded germinal center, an increase in white pulp component, high cellularity, and capsule fibrosis. Similarly, VR23 did not cause any abnormality in kidney, while samples from paclitaxel-treated animals showed some morphologic changes similar to that of glomerulonephritis (Fig. 7C). Intriguingly, the level of paclitaxel-induced high cellularity was notably reduced when used in combination with VR23 (Fig. 7C). Taken together, our data from a serum AST/ALT test, the liver mitotic index count, and spleen and kidney studies strongly indicate that VR23 not only does not cause notable ill-effects but also can substantially reduce adverse effects caused by paclitaxel.

Discussion

Here we report the generation, functional mechanism analysis, and antitumor activity of VR23, a novel proteasome inhibitor. We...
have demonstrated that the primary target of VR23 is β2 of the S20 proteasome subunit, although chymotrypsin-like and caspase-like proteasome activities are also substantially inhibited. The proteasome inhibitory function appears to be a key mechanism how VR23 kills cells. An interesting point is that the combination of the β2-targeting VR23 and the β5-targeting bortezomib show strong synergistic effect (Fig. 2). Furthermore, the same combination also effectively kills BTZ-resistant multiple myeloma cells, raising the possibility that targeting multiple proteasome subunits can not only be more effective but also overcome the emergence of resistance to agents targeting single proteasome subunits.

An important point is that VR23 preferentially kill cancer over noncancer cells, mainly through the abnormal amplification of centrosomes in cancer but not in normal cells. Centrosome is normally duplicated only once per cell cycle in close collaboration with DNA replication and segregation (2, 41). However, cancer cells treated with VR23 continues to amplify their centrosomes even during G2–M, largely dissociating the centrosome regulation from DNA replication/segregation. This, in turn, leads to complete disarray of the cell division process and, eventually cell death by apoptosis.

In addition to its positive regulation for DNA replication, cyclin E is required for centrosome (re)duplication (2, 39, 42). Overall, our data are consistent with the idea that the failing of timely degradation of cyclin E is one of the key mechanisms how the centrosome is amplified in cells treated with VR23. This interpretation is strengthened by the observation that, unlike in controls or MG132-treated cells, cyclin E is localized to the amplified centrosomes in cells treated with VR23 (Fig. 3). As expected, cyclin E is largely ubiquitinated in the presence of VR23 (Fig. 5), indicating that the cyclin E degradation process is inhibited after the conjugation of ubiquitin molecules. The fact that the knockdown of cyclin E with siRNA completely suppresses VR23-induced centrosome amplification supports the notion that cyclin E plays a critical role in VR23-induced abnormal centrosome amplification (Fig. 4). Unlike in cancer cells, however, cyclin E is neither highly ubiquitinated up to 40 μmol/L of VR23 (Supplementary Fig. S4) nor localizes to the centrosomes in MCF10A (Fig. 3). This may be the major reason why VR23 neither induces centrosome amplification nor effectively kills noncancer cells. In contrast, bortezomib strongly induces cyclin E ubiquitination in both MCF10A and HeLa cells (Fig. 5B, Supplementary Fig. S4). This may be relevant to the fact that bortezomib does not exhibit selectivity for cancer over noncancer cells (43). We also found that MG132, another proteasome inhibitor, does not cause centrosome amplification (Fig. 5A). Thus, VR23 possesses a unique property in inhibiting proteasome activity and amplifying centrosomes in a cancer-specific manner.

Multiple reduplication of the centrosome usually results in the formation of a flower-petal configuration dubbed "rosette" (44). However, we did not observe a rosette shape cluster of centrosomes in cells treated with VR23. Therefore, the centrosome amplification caused by VR23 is not likely by multiple reduplications. Instead, de novo centrosome synthesis could be the primary mechanism of centrosome amplified in cancer cells exposed to VR23, as multiple centrosomes are often found throughout the cytoplasm as previously described for de novo centrosome synthesis (10). In this context, it is possible that the VR23-caused accumulation of (extremely bulky) ubiquitinated cyclin E molecules at discrete cytoplasmic sites can be the nidus to attract other proteins involving in centrosome synthesis (such as plk1, SAS6, centrin, and γ-tubulin), eventually leading to centrosome synthesis by a de novo assembly mechanism. Together, our data from...
*in vitro* studies support a model where *de novo* centrosome amplification by VR23 leads to mitotic abnormality including the lack of coordinated microtubule attachment to the kinetochore, chromosome breakage, and cell-cycle arrest at prometaphase, resulting in cell death by apoptosis.

When we screened our chemical library to identify potential anticancer agents, the most important criterion was selecting compounds that preferentially kill cancer over noncancer cells, on the assumption that this can be relevant to differential cancer cell killing *in vivo*. Tumor samples were taken 15 days after AT490 mice were treated as in A, followed by immunostaining with an anti-Ki-67 antibody. The samples were counterstained with hematoxylin. Pictures were taken with a Zeiss Epi-fluorescent microscope using a ×10 objective. D, VR23 inhibits angiogenesis and spreading of tumor cells to surrounding tissues. Tissue samples were either stained with H&E or immunostained with an antibody specific for CD31. See also Supplementary Fig. S6 for VEGF staining.

VR23 indeed shows substantial antitumor activity on both metastatic breast cancer (engrafted with MDA-MB-231) and RPMI 8226 multiple myeloma xenograft models. Furthermore, when used in combination, VR23 substantially increases the efficacy of paclitaxel. For example, paclitaxel alone could reduce tumor burden by 18.4-fold (i.e., 305.01 vs. 16.59 in Fig. 6B), while the combination of paclitaxel and VR23 could reduce the tumor burden by 29-fold (i.e., 305.01 vs. 10.51 in Fig. 6B). The advantage of VR23 as an anticancer agent is that it is not only nontoxic to animals but also can reduce toxicity caused by paclitaxel (and possibly other drugs). The examination of four vital organs (liver,
spleen, kidney, and lung) revealed that VR23 does not cause any visible morphologic changes in the organs examined. In contrast, paclitaxel at 20 mg/kg/week for 4 weeks caused a slight increase in spleen size and a substantial increase in liver mitotic index, while VR23 did not. Importantly, the liver mitotic index of animals treated with paclitaxel was dramatically reduced when used in combination with VR23. Furthermore, the AST/ALT ratio of VR23-treated mice is indistinguishable from that of the untreated control, while that of paclitaxel-treated animals was elevated by 1.5-fold (1.60 vs. 2.36; Supplementary Table S2). As paclitaxel can induce substantial hepatobiliary side effects in human (45), our data raise the possibility that VR23 may reduce paclitaxel-induced hepatobiliary effects in human.

Paclitaxel (20 mg/kg) caused a number of adverse effects on spleen, including the expansion of the germinal center and the hyperplasia of myeloid and lymphoid cells, none of which was shown in the mice treated with VR23. Similarly, VR23 did not cause any notable abnormality in the kidney (Fig. 7C). In contrast, kidneys from paclitaxel-treated mice showed increased glomerular cellularity and contracted Bowman’s space, reminiscent of the changes seen in endocapillary proliferative glomerulonephritis. Importantly, the paclitaxel-induced cellularity was substantially reduced when used in combination with VR23. As paclitaxel can cause renal failure in a certain subgroup of patients (45), our data raise the possibility that VR23 can be used along with paclitaxel to reduce renal toxicity. Thus, the potential of VR23 for combinational therapies is extremely promising, although it can certainly be used as a single anticancer agent.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: S. Pundir, H.-Y. Vu, V.R. Solomon, H. Lee

Development of methodology: H.-Y. Vu, V.R. Solomon

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.-Y. Vu, R. McClure

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Figure 7. VR23 reduces toxicity caused by paclitaxel. A, data from microscopy show that VR23 reduces mitotic index. Samples were taken at 29 days after treatment with 30 mg/kg VR23, 20 mg/kg paclitaxel (Tax), or combination of the two. At least 10 fields for each slide (one slide per mouse and 6 mice per group) were examined. P < 0.0001. B, VR23 does not cause any notable toxicity to spleen. GC, germinal center; white arrows show the presence of macrophages in the red pulp area (RP). H&E-stained images were taken with a x10 objective using a Zeiss EPI-fluorescent microscope. C, VR23 does not cause any adverse effects to kidney. Nephrotoxicity was analyzed after mice were treated with VR23, paclitaxel, or in combination. BS and GM denote Bowman space and glomerulus, respectively. Inset, another example of abnormal Bowman space in the paclitaxel-treated sample.
An analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Pundir, H.-Y. Vu, V.R. Solomon, R. McClure, H. Lee
Writing, review, and/or revision of the manuscript: S. Pundir, H.-Y. Vu, V.R. Solomon, R. McClure, H. Lee
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