A novel aryl-guanidinium derivative, VP79s, targets the signal transducer and activator of transcription 3 signaling pathway, downregulates myeloid cell leukaemia-1 and exhibits preclinical activity against multiple myeloma

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ABSTRACT

Aims: We have recently described a novel guanidinium-based compound, VP79s, which induces cytotoxicity in various cancer cell lines. Here, we aim to investigate the activity of VP79s and associated mechanisms of action in multiple myeloma (MM) cells in vitro and ex vivo.

Main methods: The effects of VP79s on cell viability and induction of apoptosis was examined in a panel of drug-sensitive and drug-resistant MM cell lines, as well as ex vivo patient samples and normal donor lymphocytes and platelets. Cell signaling pathways associated with the biological effects of VP79s were analysed by immunoblotting and flow cytometry. Gene expression changes were assessed by quantitative real-time PCR analysis.

Key findings: VP79s was found to rapidly inhibit both constitutively active and IL-6-induced STAT3 signaling with concurrent downregulation of the IL-6 receptors, CD130 and CD126. VP79s induced a rapid and dose-dependent downregulation of anti-apoptotic Bcl-2 family member, myeloid cell leukaemia-1 (MCL-1). VP79s enhanced bortezomib induced cell death and was also found to overcome bone marrow stromal cell induced drug resistance. VP79s exhibited activity in ex vivo patient samples at concentrations which had no effect on peripheral blood mononuclear cells, lymphocytes and platelets isolated from healthy donors.

Significance: As VP79s resulted in rapid inhibition of the key IL-6/STAT3 signaling pathway and downregulation of MCL-1 expression with subsequent selective anti-myeloma activity, VP79s may be a potential therapeutic agent with a novel mechanism of action in MM cells.

1. Introduction

Multiple myeloma (MM) is a plasma cell malignancy accounting for approximately 50,000 newly diagnosed cases in Europe each year \cite{1}. Treatment generally consists of drug combinations including a proteasome inhibitor, such as bortezomib, dexamethasone and an immunomodulatory drug or chemotherapeutic agent \cite{2}. MM remains a largely incurable disease despite the recent development and introduction of several next-generation proteasome inhibitors, immunomodulatory drugs and immunotherapies \cite{3}. These advances have significantly improved the depth and duration of MM remission \cite{2}; however, MM patients invariably relapse, eventually becoming refractory to treatment.

It is well established that MM cells have an intricate relationship with the bone marrow microenvironment (BMM) and that MM cells rely heavily on signals from the BMM in order to survive and proliferate, as well as contributing to drug resistance \cite{4}. The secretion of soluble factors by bone and stromal cells are important contributors to this...
such as STAT3 is frequently phosphorylated at serine 727 (S727) in cancer cells, results in dimerization and translocation to the nucleus. In addition, expression of myeloid cell leukaemia 1 (MCL-1) leads to apoptosis. Family proteins are key negative regulators of apoptosis and overexpression of target genes involved in regulating cell cycle progression, including MM, where it may have a role in regulating STAT3 activity [7]. Following translocation to the nucleus, STAT3 regulates the transcription of target genes involved in regulating cell cycle progression, such as MYC and CCND1, and anti-apoptotic members of B cell lymphoma 2 (Bcl-2) family, such as BCL2, BCL2L1 and MCL1 [8]. These Bcl-2 family proteins are key negative regulators of apoptosis and over-expression of myeloid cell leukaemia 1 (MCL-1) leads to apoptosis resistance and is associated with shorter patient survival in MM [9]. As STAT3 plays a critical role in cell survival, targeting the JAK/STAT3 pathway has emerged as a therapeutic target, not only in MM, but also in many hematological malignancies and solid cancers, such as breast and gastric cancer [10,11]. However, to date, targeting aberrantly activated STAT3 pathway has proved challenging.

We have previously described a 3,4'-bis-guainidinium diphenyl compound capable of inhibiting B Rapidly Accelerated Fibrosarcoma (BRAF) through a hypothetical type-III allosteric mechanism [12,13]. A derivative of this compound, VP79s, exhibits cytotoxicity in promyelocytic leukaemia, breast, cervical and colorectal carcinoma cell lines with IC50 values in the low micromolar range; however, it does not inhibit the Rat sarcoma (Ras)/BRAF/Extracellular Signal-Regulated Kinase (ERK) signaling pathway in promyelocytic leukaemia cells suggesting a novel mechanism of action [14].

In the present study, the anti-myeloma activity of VP79s was examined in a panel of drug-sensitive and resistant MM cell lines. We investigated the effect of VP79s on the IL-6/JAK/STAT3 signaling pathway and demonstrated a rapid downregulation in expression of the STAT3 target MCL-1. In addition, VP79s was capable of overcoming bone marrow stromal cell-induced resistance and enhancing bortezomib-induced cell death. Moreover, VP79s induced selective toxicity in ex vivo MM patient samples compared to normal donor lymphocytes and platelets. Based on these findings, we suggest that VP79s has potential for further investigation as a novel MM therapeutic agent with a mechanism of action targeting key survival and drug resistance pathways.

2. Materials and methods

2.1. Cell culture

Human multiple myeloma cell lines NCI-H929 (wildtype TP53, t (4;14)) and U266B1 (mutated TP53, t(11;14)) were obtained from DSMZ cell bank (Braunschweig, Germany). MM1.S and MM1.R (wildtype TP53, t(4;14)) and U266B1 (mutated TP53, t(11;14)) were obtained from the DSMZ cell bank (Braunschweig, Germany). MM1.S and MM1.R (wildtype TP53, t(4;14)) and U266B1 (mutated TP53, t(11;14)) were obtained from DSMZ (Brandeisen, Virginia, United States). HS5 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) GlutaMAX (Thermo Fisher Scientific) complete media and cultured at 37 °C containing 5% CO2. The bone marrow stromal cell line, HS5, was obtained from ATCC (Manassas, Virginia, United States). HS5 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 μg/mL streptomycin.

2.2. Reagents

Synthesis and characterization of VP79s have previously been described [14] and its purity was assessed to be >95% by HPLC. VP79s was dissolved in 100% ethanol at 10 mM and stored at 4 °C. Recombinant Human IL-6 (Biolegend, San Diego, California, United States), bortezomib (SelleckChem, Houston, Texas, United States), U0126 (Cell Signaling, Danvers, Massachusetts, United States), SP125600 (Fluorochem Ltd., Glossop, United Kingdom) and SB203580 (SelleckChem) were prepared and stored as per manufacturer’s instructions.

2.3. Primary cells

MM cells were isolated from patients presenting at the Department of Haematology, St James’s Hospital, Dublin following informed consent. This study was approved by the St James’s Hospital and Adelaide and Meath incorporating the National Children’s Hospital (AMNCH) Joint Ethics committee. In brief, bone marrow mononuclear cells (BMMCs) were isolated using lymphoprep® (Axis-Shield, Dundee, United Kingdom) density gradient centrifugation and CD138+ cells were isolated from the BMMCs by positive selection using the EasySep™ Human CD138 Positive Selection Kit II (Stemcell Technologies, Cambridge, United Kingdom) according to the manufacturer’s protocol. CD138+ cells were cryopreserved in liquid nitrogen in 90% FBS containing 10% DMSO until required. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors following informed consent using lymphoprep® density gradient centrifugation. PBMCs and CD138+ myeloma cells were cultured in RPMI 1640 media containing 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin. Platelets were isolated from peripheral blood collected from healthy volunteers who had abstained from taking any medications known to affect platelet function in the 14 days prior to blood donation. Washed platelet suspensions were prepared as described previously [16] and adjusted to 2.5 × 108 platelets/mL in Tyrode’s salt solution (Sigma-Aldrich Ireland Limited, Co. Wicklow, Ireland).

2.4. Cell viability assay

The effect of VP79s on MM cell viability and on HS5 bone marrow stromal cell viability was assessed using the AlamarBlue (Thermo Fisher Scientific) viability assay as previously described [17]. Briefly, 5 × 104 cells were seeded in 96 well plates and were treated as indicated, before 20 μL of AlamarBlue was added to each well. Plates were read on the Spectramax Gemini Plate Reader using SOFTmax Pro version 4.9 (Molecular Devices, Sunnyvale, California, United States) at excitation and emission wavelengths of 544 nm and 590 nm respectively. Vehicle treated cells were taken as 100% viability and wells treated with compounds were calculated as a percentage of the vehicle control.

The effect of VP79s on platelet viability was assessed using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega Corporation, Madison, Wisconsin, United States) by quantifying the release of lactate dehydrogenase (LDH) from platelets as per manufacturer’s protocol. In brief, 100 μL of washed platelets (2.5 × 108 platelets/mL) per well were treated, at increasing concentrations of the compound, in 96-well plates for 20 min. An equal volume of CytoTox-ONE™ Reagent was added to each well before fluorescence was recorded using a multiplate reader FLUOstar optima (BMG Labtech, Aylesbury, United Kingdom) with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Incubation with Triton X-100 (0.18% (w/v) final concentration) was utilised as a maximum LDH release control.

2.5. Cell death and cell cycle analysis

Apoptosis was analysed using annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) as previously described [17]. Briefly, following treatment cells were washed with annexin V binding buffer (5 mM HEPES, 70 mM NaCl, 1.25 mM CaCl2 pH 7.4) and stained with annexin V-FITC (IQ Corporation, Groningen, The Netherlands). Following washing with annexin V binding buffer, cells were
were fixed in 70% ethanol and incubated with 10 μg/mL propidium iodide (PI). After treatment, NCI-H929 and U266B1 cells were stained with DAPI exclusion (Pacific blue). Samples were analysed by flow cytometry on the BD FACS Canto II flow cytometer with cell viability determined by DAPI exclusion (Pacific blue).

Cell cycle was analysed by DNA quantification following staining by propidium iodide (PI). After treatment, NCI-H929 and U266B1 cells were fixed in 70% ethanol and incubated with 10 μg/mL of RNase A (Sigma-Aldrich) and 100 μg/mL PI (Sigma-Aldrich). Analysis was performed using a BD Accuri C6 flow cytometer and software.

2.6. Co-culture experiments

NCI-H929 cells were cultured on a confluent monolayer of HS5 bone marrow stromal cells. 10^5 HS5 cells were seeded in 12 well plates and left to adhere overnight. The media was removed and 3 × 10^5 NCI-H929 cells were seeded in co-culture with the HS5 cells or in monoculture in 1 mL of complete media. Cells were the treated with 5 μM VP79s for 24 h. Following treatment, NCI-H929 cells were recovered, stained with CD138^+ (CD138-PE, BD Biosciences, Franklin Lakes, New Jersey, United States), and apoptosis was assessed by annexin V/PI staining.

2.7. Intracellular staining of Phospho-STAT3

Following treatment cells were collected and washed with PBS containing 2% FBS and 1 mM EDTA (binding buffer) and fixed with Cytofix Fixation Buffer (BD Biosciences) and permeabilised using BD Phosflow Perm Buffer III (BD Biosciences). Cells were resuspended in 100 μL of binding buffer and 20 μL of each antibody (Phospho-STAT3 S727 Alexa Fluor 647 and Phospho-STAT3 Y705 PE, BD biosciences) was added and incubated in the dark at room temperature for 30 min. Following washing, samples were resuspended in binding buffer and were analysed on the BD FACs Canto II.

2.8. Cell surface staining of CD126 and CD130

U266B1 cells were seeded at 3 × 10^5 cells/mL in 12 well plates. Following treatment, cells were resuspended in 90 μL of PBS with 2% FBS and 1 mM EDTA containing 5 μL of anti-CD126-PE and anti-CD130-APC (BD biosciences). Cells were stained in the dark at room temperature for 15 min. Samples were analysed on the BD FACs Canto II.

2.9. Western blotting

Protein and phospho-protein expression were analysed by Western blotting as described previously [17]. Briefly, cell pellets were lysed in RIPA buffer (Merck KGAa, Darmstadt, Germany) supplemented with phosphatase inhibitor cocktail II and III (Merck KGAa) and complete ultra-protease inhibitor (Roche Applied Science, Penzberg, Germany). Protein concentration was determined using the BCA assay (Thermo Fisher Scientific) and lysates were incubated with Laemmli sample buffer supplemented with 50 μM DTT for 10 min at 90 °C. Equal amounts of protein were separated using polyacrylamide gel electrophoresis and transferred to an immobilisation-P PVDF membrane (Merck KGAa). The membrane was blocked in 5% non-fat dried milk and incubated overnight at 4 °C with the appropriate primary antibody. All antibodies were obtained from Cell Signalling Technologies except Bcl-2 (Calbiochem) and Bcl-xL (BD Pharmingen, Franklin Lakes, New Jersey, United States). Following probing with the relevant horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit or anti-mouse) (Promega) protein expression was detected with enhanced chemiluminescence detection reagent (Merck KGAa) using the BioRad Gel Doc™ XR + System with Image Lab software (Bio-Rad Laboratories, Inc, Hercules, CA, USA). All blots were reprobed with anti-GAPDH (Calbiochem) or anti-beta-actin (Merck KGAa) to ensure equal loading.

2.10. Gene expression assays

Gene expression was quantified using TaqMan Gene Expression Assays as described previously [18]. RNA was extracted using a RNeasy Mini Kit (Qiagen, Hilden, Germany) as per manufacturer’s instructions. RNA quality and quantity were then assessed by using a Nanodrop ND 1000 UV–Vis Spectrophotometer (Thermo Fisher Scientific). RNA was converted to cDNA using a mastermix containing Superscript First Strand Buffer, 25 mM dNTPs, 100 mM DTT and 3 μg/μL Random primers (Thermo Fisher Scientific). Samples were heated to 96 °C for 2 min and chilled on ice for 5 min. RNase out and superscript II (Thermo Fisher Scientific) were then added and samples were heated at 42 °C for 90 min, then to 96 °C for 2 min and chilled on ice for 5 min. The expression of STAT3 related genes MCL1, CDKN1, BCL2 and STAT3 was assessed using TaqMan Gene Expression Assays (Thermo Fisher Scientific) as per manufacturer’s instructions on an ABI 7500 Real Time PCR System (Applied Biosystems, Bleiswijk, The Netherlands). Beta-2-microglobulin (B2M) was used as an endogenous control.

2.11. Drug combination studies

For the analysis of bortezomib in combination with VP79s a constant ratio of 1:1 was utilised. Cell death induced by each drug alone and in combination was determined by annexin V/PI staining. Compusyn (Combosyn, Inc., Paramus, New Jersey, United States) was used to determine the interactions between drugs. The program utilises the Chou-Talalay method to determine Combination Index (CI) values for each drug combination. CI values <1, =1 and >1 indicate synergistic, additive and antagonistic effects of the drugs of interest respectively [19].

2.12. Statistical analysis

All statistical analysis was performed on Graphpad prism 5 (GraphPad Software, San Diego, California, United States). Results are displayed as the mean ± the standard deviation (S.D.). For comparisons of more than two groups, a one-way ANOVA followed by Tukey’s or Dunnett’s multiple comparison test was performed. For comparisons between two groups a paired t-test was performed where appropriate. p < 0.05 was considered to be significant.

3. Results

3.1. VP79s induces apoptotic cell death in a panel of MM cell lines

We have previously shown that VP79s, a novel aryl-guanidinium compound with a molecular weight of 421.81 Da (Fig. 1A), exhibits cytotoxicity in promyelocytic leukaemia, breast, cervical and colorectal carcinoma cell lines [14]. Here, we utilised the alamarBlue viability assay to evaluate the cytotoxic activity of VP79s in a panel of drug-sensitive and drug-resistant myeloma cell lines at various time points up to 72 h. The U266B1 cell line exhibits relative drug-resistance compared to the NCI-H929 cell line, possibly, in part, due to the presence of a TP53 mutation [15,18]. NCI-H929, U266B1, MM1.S and MM1.R cell lines are sublines of MM1 cells which differ only in their sensitivity to glucocorticoids, with MM1.R exhibiting dexamethasone resistance due to the loss of the glucocorticoid receptor [15]. NCI-H929, U266B1, MM1.S and MM1.R cells were treated with a range of VP79s concentrations (0.625–20 μM) for 24, 48 and 72 h. VP79s reduced the viability of myeloma cell lines in a dose- and time-dependent manner (Fig. 1B). The
A.  

![Chemical structure](image)

B.  

<table>
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<th>Timepoint</th>
<th>NCI-H929 (µM)</th>
<th>U266B1 (µM)</th>
<th>MM1.S (µM)</th>
<th>MM1.R (µM)</th>
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<td>24 h</td>
<td>4.3 ± 0.3</td>
<td>7.7 ± 0.9</td>
<td>7.3 ± 1.4</td>
<td>6.3 ± 0.7</td>
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<td>48 h</td>
<td>3.4 ± 0.2</td>
<td>3.8 ± 0.7</td>
<td>4.9 ± 0.7</td>
<td>4.3 ± 0.3</td>
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<tr>
<td>72 h</td>
<td>3.3 ± 0.2</td>
<td>3.9 ± 0.4</td>
<td>4.0 ± 1.0</td>
<td>3.6 ± 0.7</td>
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C.  

D.  

E.  

F.  

G.  

*(caption on next page)*
Fig. 1. VP79s elicits potent anti-myeloma activity. A. Chemical structure of VP79s. B. NCI-H929, U266B1, MM1.S and MM1.R cells were treated with various concentrations (0.625–20 μM) of VP79s for 24, 48 and 72 h. Cell viability was assessed using the AlamarBlue™ viability assay. \( n = 3 \). IC\(_{50}\) of VP79s in a panel of drug sensitive (NCI-H929, MM1.S) and resistant (U2661, MM1.R) myeloma cell lines at 24, 48 and 72 h. C. IC\(_{50}\) of VP79s in a panel of drug sensitive (NCI-H929, MM1.S) and resistant (U2661, MM1.R) myeloma cell lines at 24, 48 and 72 h. D. NCI-H929, U266B1, MM1.S and MM1.R cells were treated with various concentrations of VP79s (1.25–10 μM) for 24 h and apoptotic cell death was determined by annexin V/PI staining analysed by flow cytometry * \( p < 0.05 \), ** \( p < 0.01 \) and *** \( p < 0.001 \), one-way ANOVA with Dunnett’s post hoc test, \( n = 3 \). E. NCI-H929 and U266B1 cells were treated with vehicle (0.5% EtOH) or 10 μM VP79s for 8, 24, 48 and 72 h. Cells were fixed in 70% EtOH, stained with propidium iodide and percentage of cells in the subG1/G0 peak was determined by quantification of DNA content by flow cytometry. * \( p < 0.05 \), ** \( p < 0.01 \) and *** \( p < 0.001 \), one-way ANOVA with Tukey’s post hoc test, \( n = 3 \). F. NCI-H929 and U266B1 cells were treated with either vehicle (0.5% EtOH) or VP79s (1.25–10 μM) for 16 h. Caspase 3, caspase 8, caspase 9 and cleaved caspase 3 levels were analysed by Western blotting. Anti-GAPDH and anti-β-actin were used as loading controls. Results are representative of 3 independent experiments. G. HS5 bone marrow stromal cells were seeded at a density of 1 × 10^5 cells in 12 well plates and left to adhere overnight. NCI-H929 cells were seeded at a density of 3 × 10^5 cells/well either alone or in co-culture with the HS5 cells. Cells were treated with either vehicle (0.1% DMSO or 0.5% EtOH), 1.5 nM bortezomib or 5 μM VP79s for 24 h. Samples were collected and analysed by annexin/PI staining by flow cytometry, \( n = 3 \). NS = not significant and * \( p < 0.05 \) two-tailed paired \( t \)-test.

Fig. 2. VP79s activates the MAPKs ERK, p38 and JNK. A. NCI-H929 and U266B1 cells were seeded at a density of 3 × 10^5 cells/mL. Cells were treated with either vehicle [0.5% EtOH (v/v)] or VP79s (10 μM) for 30 min, 2, 4 or 8 h. Cells were lysed and analysed by Western blotting with the indicated antibodies. GAPDH was used as a loading control. Results are representative of 3 independent experiments. B. NCI-H929 cells were treated with 20 μM SB203580, U0126 or SP600125 for 30 min prior to treatment with either vehicle [0.5% EtOH (v/v)] or VP79s (10 μM) for 8 h. Cell death was analysed by annexin V/PI staining by flow cytometry. 10,000 cells were gated on vehicle treated cells. Columns and error bars represent the mean ± S.D. of cells in the viable gate of three independent experiments. Statistical analysis was performed using a paired two-tailed \( t \)-test. NS = not significant.
IC$_{50}$ values obtained were all in the low micromolar range with effects on viability observed as early as 24 h (Fig. 1C). Cell death was further investigated by flow cytometric analysis of annexin V/PI stained cells which allows for the detection and quantification of apoptosis. VP79s was found to induce apoptotic cell death in a dose-responsive manner in the panel of MM cell lines after 24 h with significant apoptosis detected in all cell lines treated with VP79s at 7.5 and 10 μM. The NCI-H929 cell line showed increased sensitivity to VP79s with over 90% of cells apoptotic compared to 50% of U266B1 cells following treatment with 10 μM VP79s. Of note, VP79s equipotently induced apoptosis in the isogenic dexamethasone-sensitive and -resistant MM1.S and MM1.R cells (Fig. 1D). Cell cycle analysis of the effect of VP79s on NCI-H929 and U266B1 cells demonstrated the induction of a subG1/G0 peak, indicative of apoptosis, after 24 h (Fig. 1E) with a concomitant decrease in cells in G1 and G2/M phases of the cell cycle (Supplementary Fig. 1).

Apoptosis is mediated by caspases and VP79s treatment resulted in the reduction of the pro-forms of caspase 3, 8 and 9, and induction of caspase 3 cleavage in NCI-H929 and U266B1 cells (Fig. 1E), indicating activation of both intrinsic and extrinsic apoptotic pathways.

The BMM plays a vital role in MM cell survival and drug resistance. The protective effect of the BMM on myeloma cells was assessed using co-culture with the HS5 bone marrow stromal cell line. Co-culture completely abrogated the apoptosis induced by the proteasome inhibitor bortezomib in NCI-H929 cells. Importantly, VP79s was found to overcome the pro-survival effects of HS5 co-culture in NCI-H929 cells while having minimal effect on HS5 cell viability (Fig. 1F and Supplementary Fig. 2).

3.2. VP79s activates mitogen-activated protein (MAP) kinase signaling pathways

We have recently shown that VP79s does not inhibit downstream BRAF signaling in promyelocytic leukaemia cells, unlike related compounds which exhibit type III allosteric inhibition of BRAF [14]. Here, we investigated the effect of VP79s on the activation of ERK, a MAP kinase which lies downstream of BRAF, in MM cells. Interestingly, VP79s was shown to increase ERK phosphorylation after 2 h in NCI-H929 cells and after 8 h in U266B1 cells. The effect of VP79s on the activation of MAP kinases JNK and p38 was also examined. As with ERK, a marked increase in phosphorylation of p38 and JNK was observed after 2 h in both myeloma cell lines (Fig. 2A). In order to determine if the activation of MAP kinases play a critical role in the anti-myeloma activity of VP79s, NCI-H929 cells were pre-treated with pharmacological inhibitors of ERK (U0126), p38 (SB203580) and JNK (SP600125). Inhibition of neither ERK, JNK nor p38 had any effect on VP79s induced cell death (Fig. 2B).

3.3. VP79s inhibits constitutively active and IL-6-induced STAT3 phosphorylation

The JAK/STAT3 signaling pathway is known to play an important role in the survival and proliferation of MM cells and is mediated by cytokines, in particular IL-6. However, STAT3 is commonly activated in MM cells, highlighting the importance of the JAK/STAT3 pathway in the pathobiology of MM [10]. U266B1 cells were shown to have constitutive STAT3 phosphorylation on tyrosine 705 and serine 727 (Fig. 3A). Treatment with VP79s was shown to completely abrogate STAT3 tyrosine phosphorylation in U266B1 cells after 2 h with limited effects on serine phosphorylation (Fig. 3A). This effect of VP79s on STAT3 phosphorylation in U266B1 cells was confirmed by flow cytometric analysis of intracellular staining of STAT3 phosphorylation on both tyrosine 705 and serine 727 (Fig. 3B). NCI-H929 cell exhibit constitutively phosphorylated STAT3 on serine 727; however, in contrast to U266B1 cells, minimal phosphorylation of tyrosine 705 was detected (Fig. 3B). In order to assess whether VP79s can inhibit induced as well as constitutive STAT3 phosphorylation, NCI-H929 cells were pre-treated with IL-6, inducing tyrosine 705 phosphorylation, and treatment with VP79s was shown to inhibit this IL-6-induced STAT3 phosphorylation after 4 h (Fig. 3C). Moreover, IL-6 did not protect NCI-H929 cells from VP79s-induced apoptosis (Fig. 3D). We investigated the mechanism of VP79s mediated inhibition of STAT3 phosphorylation by assessing the expression of upstream regulators of STAT3 activation. VP79s treatment resulted in a reduction of phosphorylation of JAK2 (Fig. 3E) but had no effect on phosphorylation of either Jak1 or Src (results not shown). Furthermore, cell surface staining of the IL-6 receptor CD130 and co-receptor CD126 demonstrated a decrease in expression after 30 min treatment with VP79s (Fig. 3F).

3.4. VP79s treatment results in a rapid downregulation of MCL-1

Activated STAT3 translocates to the nucleus where it regulates the expression of multiple genes, including its own expression, cell cycle regulatory genes such as CCND1 (cyclin D1) and anti-apoptotic Bcl-2 family members [21]; therefore, the effect of VP79s on these key downstream targets was assessed. Treatment of U266B1 cells with VP79s resulted in a decrease in the gene expression of MCL1 with a fold decrease of 0.68. An increase was observed in the expression of BCL2 (Fig. 4A). Western blot analysis demonstrated a dose responsive decrease in Cyclin D1 protein expression in both NCI-H929 and U266B1 cells. In addition, VP79s resulted in a decrease in the protein expression of the Inhibitor of Apoptosis family member, survivin, in U266B1 cells (Fig. 4B). Analysis of Bcl-2 family members, MCL-1, Bcl-2 and Bcl-xL demonstrated that treatment with VP79s led to a decrease in MCL-1 protein levels in both NCI-H929 and U266B1 cell lines. There was no significant change in either Bcl-2 or Bcl-xL detected in either cell line; however, a possible Bcl-2 cleavage product was observed following treatment with 10 μM VP79s in U266B1 cells (Fig. 4C). Moreover, treatment with VP79s led to a rapid and sustained decrease in MCL-1 expression after 2 h in both cell lines (Fig. 4D).

3.5. VP79 enhances bortezomib induced cell death

Proteasome inhibition has become a standard of care in MM treatment regimens; therefore, in order to assess the ability of VP79s to enhance bortezomib induced cell death, U266B1 cells were treated concurrently with VP79s and bortezomib for 16 h. VP79s was found to significantly enhance cell death induced by bortezomib upon co-treatments with 3 μM VP79s and 3 nM bortezomib and 7 μM VP79s and 7 nM bortezomib (Fig. 5A). However, drug dose-effects, analysed using Calcsyn software, showed that only combination treatment with 7 nM bortezomib and 7 μM VP79s synergistically increased cell death in U266B1 cells with a CI value of 0.76 (Fig. 5B).

3.6. VP79s selectively reduces the viability of ex vivo MM patient samples

Examination of the cytotoxic effects of VP79s in PBMCs isolated from 6 healthy donors demonstrated that treatment with VP79s for 24 h had a minimal effect at concentrations up to 5 μM (Fig. 6A). We further investigated any specific cytotoxicity in lymphocyte subsets in the donor PBMC samples. No effect was observed in T, B and NK cell populations at concentrations up to 5 μM for 24 h. (Fig. 6A). In addition, VP79s exhibited no cytotoxicity in platelets isolated from 3 healthy donors at concentrations up to 20 μM. (Fig. 6B).

We next assessed whether VP79s induced cell death in patient derived MM cells at concentrations which show no cytotoxicity in normal donor PBMCs or lymphocyte subsets. CD138$^+$ myeloma cells were isolated from bone marrow aspirates of 5 patients at diagnosis (3 males, 2 females; age range 55–83 years). These ex vivo myeloma cells were treated with VP79s (0.625–5 μM) for 24 h and cell viability was quantified by annexinV/PI assay. VP79s reduced myeloma cell viability in all patient samples (Fig. 6C). A dose response reduction in cell viability was observed in response to VP79s treatment, with a significant
A. U266B1

B. 10 µM VP79s

C. Phospho-STAT3 (Y705)

D. Phospho-STAT3 S727

E. Total STAT3

F. GAPDH

(caption on next page)
**Fig. 3.** VP79s inhibits constitutive and IL-6 induced STAT3 activation. A. U266B1 cells were treated with either vehicle (0.5% EtOH) or 10 μM VP79s for 30 min, 2, 4 or 8 h. STAT3 and phosphorylated STAT3 (pSTAT3 Y705 and pSTAT3 S727) levels were assessed by Western blotting. GAPDH was used as a loading control. Representative blot of 3 independent experiments shown. B. NCI-H929 and U266B1 cells were treated with either vehicle (0.5% EtOH) or 10 μM VP79s for 1 h. STAT3 phosphorylation on Y705 and S727 was determined by intracellular staining and flow cytometric analysis. Results shown are representative of 3 independent experiments. C. NCI-H929 cells were pre-treated with 1 ng/mL IL-6 for 30 min and were then treated with either vehicle (0.5% EtOH) or 10 μM VP79s in the presence or absence of IL-6 for the indicated time and phosphorylated STAT3 (Y705) and total STAT3 levels were determined by Western blotting. GAPDH was used as a loading control. Representative blot of 3 independent experiments shown. D. NCI-H929 cells were treated as indicated and apoptotic cell death at 16 h was determined by annexin V/PI staining by flow cytometry. Statistical analysis was performed using a paired two-tailed t-test. NS = not significant, n = 3. E. U266B1 cells were left untreated or treated with either vehicle (0.5% EtOH) or 10 μM VP79s for 4 h. JAK2 and phosphorylated JAK2 levels were assessed by Western blotting. GAPDH was used as a loading control. Representative blot of 3 independent experiments shown. F. U266B1 cells were treated with either vehicle (0.5% EtOH) or 10 μM VP79s as indicated. Following treatment, the cell surface expression of CD130 and CD126 was determined by flow cytometric analysis. The graph shows the fold change ± SEM in expression compared to vehicle control. **p < 0.01, one-way ANOVA with Dunnett’s post hoc test, n = 3.

4. Discussion

Despite recent advances in the treatment of MM, patients invariably relapse and eventually become refractory to therapy. Therefore, there is a need for novel agents targeting key oncogenic signaling pathways. VP79s derives from a class of 3,4'-bis-guanidinium pyridinophenyl ether compounds which we have previously shown to exhibit anti-cancer activity against a number of leukaemia and solid tumour cell lines [12–14]. Some of these compounds inhibit BRAF activity through a hypothetical type-III allosteric mechanism, although VP79s appears to induce cytotoxicity independent of Ras/BRAF inhibition in promyelocytic leukaemia cells [14].

In the present study, we have shown that VP79s elicits potent anti-myeloma activity and inhibits the JAK/STAT3 signaling pathway, a key pro-survival pathway in myeloma cells which has a role in microenvironment-dependent treatment resistance [21]. Our data show that VP79s exhibits potent anti-myeloma effect by inducing apoptotic cell death which is preceded by a rapid downregulation of the STAT3 target MCL-1, a critical pro-survival factor in MM.

STAT3 is an oncogenic transcription factor which promotes myeloma cell proliferation and survival [11] and STAT3 overexpression is...
Fig. 6. VP79s induces selective cell death in a dose-responsive manner in ex vivo MM patient samples. A. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors. 1 × 10⁷ cells/well were seeded in a 96 well plate and treated with either vehicle [0.5% (v/v) EtOH] or indicated concentrations of VP79s (0.625–10 μM) for 24 h. Cells were then collected, stained with anti-CD56-APC (NK cells), anti-CD19-PE (B cells) and anti-CD3-PerCp (T cells) antibodies and analysed by flow cytometry with gating on total PBMCs and lymphocytes (T, B and NK cells). Cell viability was determined by DAPI exclusion. Statistical analysis was performed using one-way ANOVA with Dunnett’s post hoc test. *p < 0.05 and **p < 0.01. (n = 6). B. Washed platelets were isolated from the peripheral blood of 3 healthy donors and incubated with the indicated concentrations of VP79s for 20 min. Cytotoxicity was assessed by quantifying the release of LDH from platelets. Resting platelets were used as untreated controls (NT). Bars represent the mean ± S.D. Statistical analysis performed using one-way ANOVA showed no difference between groups. C. Ex vivo MM patient samples were seeded at 5 × 10⁴ cells/well in 96 well plates and treated with either vehicle [0.25% EtOH (v/v)] or VP79s (0.625–5 μM) for 24 h. After incubation, cells were collected, stained with annexin V/PI and analysed by flow cytometry. 10,000 single cells were gated on vehicle treated cells excluding debris and doublets. (i) Relative cell viability following treatment with VP79s in individual ex vivo patient samples (MM1–5). (ii) Dose response of five MM patient samples (MM1–5) to VP79s. Bars represent the mean ± S.D. Statistical analysis was performed using one-way ANOVA with Dunnett’s post hoc test. *p < 0.05.

associated with poor prognosis in MM [22]. Constitutive activation of STAT3 has been reported in patient derived CD138+ myeloma cells but not in healthy controls [23], and contributes to resistance to apoptosis. STAT3 is activated in MM via autocrine and paracrine IL-6 signaling via JAK2 resulting in phosphorylation at Y705 which promotes constitutive STAT3 activation [21]. In addition, STAT3 is commonly phosphorylated in MM cells at S727 and, while Y705 phosphorylation has been shown to be essential for the transcriptional activity of STAT3, the role and regulation of S727 phosphorylation in MM is yet to be elucidated. Activated STAT3 controls the expression of numerous target genes, including those involved in cell cycle progression, cell survival and evasion of apoptosis. In the present study, we determined that VP79s inhibited constitutively active STAT3 phosphorylation on Y705 in U266B1 cells but had no significant effect on S727 phosphorylation in U266B1 and NCI-H929 cells. Constitutive phosphorylation of STAT3 on Y705 has been shown to confer resistance to apoptosis in U266B1 cells [24]. Tyrosine phosphorylation of STAT3 is mediated via JAK2 rather than JAK1 in MM [10,25] and we show that VP79s inhibits JAK2 phosphorylation in U266B1 cells. STAT3 is not constitutively phosphorylated on Y705 in NCI-H929 cells; however, IL-6-induced STAT3 Y705 phosphorylation in NCI-H929 cells was inhibited by treatment with VP79s and, in addition, VP79s induced apoptosis was not significantly affected in the presence of pro-survival IL-6. Previous studies have demonstrated that inhibition of STAT3 phosphorylation on Y705 induced cell death not only in MM cells but also in melanoma, breast, pancreatic and renal other cancer types [26–30]. In addition, the anti-biotic nifuroxazide has been shown to induce cell death in MM cells by selectively inhibiting STAT3 phosphorylation on Y705 but not S727 residues [31].

IL-6 signaling is mediated through the IL-6 receptor CD126 and its co-receptor, CD130 [11]. Treatment with VP79s led to a rapid down-regulation in the cell surface expression of CD126 and CD130. The constitutive activation of CD126 has been shown to be a key event in the induction of MM, as constitutive CD126/JAK/STAT3 signaling in a murine model has been shown to facilitate the development of MM [32]. Previous reports have also established the importance of CD130 in MM cell survival as inhibition of CD130/JAK/STAT signaling has led to the induction of apoptosis both in vitro and in vivo [33–35]. CD126 and CD130 surface expression can be regulated through shedding, down-regulation and internalisation [36]. Previous studies have demonstrated that IL-6 can upregulate expression of CD126 through a positive feedback loop [37], possibly suggesting that VP79s inhibition of the CD126/JAK/STAT3 pathway may prevent this positive feedback loop. In addition, CD130 has been reported to be a STAT3 target gene, suggesting a possible mechanism for VP79s mediated downregulation of CD130 expression [38]. However, further studies are required to elucidate the exact mechanism of VP79s mediated downregulation of CD126 and CD130 expression.

STAT3 can directly or indirectly regulate the expression of numerous genes involved in apoptosis and proliferation [10,39]. Here, VP79s was shown to cause a downregulation in gene expression of MCL1 as well as the protein levels of MCL-1, cyclin D1 and survivin in myeloma cells. Importantly, VP79s decreased the expression of MCL-1 after 2 h in both U266B1 and NCI-H929 cells. MCL-1 is considered a critical survival protein for MM and overexpression is associated with apoptosis resistance and shorter patient survival [40,41]. Recent reports have suggested that MM cells from relapsed patients and with poor prognostic markers have increased sensitivity to MCL-1 inhibition [42]. MCL-1 specific BH3 mimetics have been developed and are currently in clinical trials for MM [43]. This rapid downregulation of MCL1 expression following treatment with VP79s may indicate a novel mechanism of targeting MCL-1 in MM cells and our findings suggest that VP79s-induced downregulation of MCL-1 is mediated through inhibition of the IL-6/JAK2/STAT3 pathway. However, MCL-1 can undergo complex post-transcriptional regulation and modification which can affect protein expression and stability [44]. In addition to targeting the IL-6/JAK2/STAT3 pathway, VP79s was found to induce activation of the MAPK signaling pathways resulting in increased phosphorylation of ERK, JNK and p38. While JNK and p38 are activated by various environmental stresses, ERK is generally activated in response to survival and mitogenic signaling [45,46]. However, ERK activation in cell death mechanisms has previously been reported in a number of cancer cell types and by numerous chemotherapeutic drugs [47]. Furthermore, ERK activation has also been reported to be involved in TRAIL- and ROS-induced apoptosis [48]. JNK and p38 phosphorylation have also been shown to be involved in both intrinsic and extrinsic apoptosis [46,49].
While VP79s induced sustained phosphorylation of ERK, JNK and p38 in both the U266B1 and NCI-H929 cell lines, inhibition of these MAPKs had no effect on VP79s induced cell death, suggesting that these kinases do not play a critical role in VP79s-mediated cytotoxicity.

MM cells rely heavily on the BMM and in particular BMSCs in order to survive and proliferate, as well as for protection from both drug-induced and immunological cell death [4]. VP79s was shown to overcome stromal cell-mediated drug resistance in NCI-H929 cells when in co-culture with the HSS cell line. In contrast, HSS cells induced drug resistance to bortezomib in NCI-H929 cells. Co-culture of MM cells with BMSCs has been shown to protect MM cells from bortezomib-induced cytotoxic damage by BMSC-derived IL-6 activation of the JAK/STAT3 signaling pathway and subsequent upregulation of MUC-1, an oncogene associated with resistance to apoptosis and necrosis [50]. Our data suggests that VP79s may be able to overcome the resistance induced by BMSC through its ability to inhibit the JAK/STAT3 pathway. DCZ3301, an aryl-guanidinium compound which inhibits STAT3 signaling, as well as novel JAK inhibitors, have also been shown to overcome BMSC-mediated resistance in MM cells [51–53].

Despite the success of bortezomib in the treatment of MM, acquired resistance is a clinical problem. The BMM and MCL-1 have both been shown to play a role in this chemoresistance in MM [54–56] and, importantly, VP79s was found to enhance the activity of bortezomib in U266B1 cells. These results are consistent with several other studies which have demonstrated that inhibition of the JAK/STAT pathway in combination with bortezomib results in enhancement in cell death [57–59].

Crucially, VP79s was found to induce apoptotic cell death in patient derived CD138− MM cells at concentrations which had minimal effect on normal donor lymphocytes and platelets. VP79s treatment resulted in a dose-responsive increase in apoptosis with a significant induction of cell death. These results indicate that VP79s exhibits selectivity for MM cells compared to normal blood lymphocytes and thrombocytes, further supporting the potential of VP79s as an anti-myeloma agent.

5. Conclusion

Our data demonstrates for the first time the potent anti-MM activity of a novel guanidinium-based compound, VP79s. We show that VP79s induces selective cytotoxicity in MM cell lines and patient derived MM cells, overcomes bone marrow stromal cell induced drug resistance and exhibits synergy with bortezomib. This study also presents a molecular mechanism responsible for the effects of VP79s, by targeting the IL-6/JAK/STAT3 signaling pathway with subsequent modulation of MCL-1 expression and induction apoptosis. While further assessment of the toxicity of VP79s in normal cells, and the effects of tumour heterogeneity and microenvironmental interactions on the efficacy of VP79s are required, we have identified a promising potential therapeutic agent for MM that warrants further study into its translational capacity.

Ethics approval

This project was approved by the St James’s Hospital and Adelaide and Meath incorporating the National Children’s Hospital (AMNCH) Joint Ethics committee. Additional ethics approval for platelet studies was obtained from Trinity College Dublin School of Pharmacy and Pharmaceutical Sciences Research Ethics Committee. Fully informed and written consent was obtained from all individuals whose samples were used in this study.

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CRediT authorship contribution statement


Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2021.120236.

References

[7] K.L. Owen, N.K. Brockwell, B.S. Parker, JAK-STAT Signaling: A double-edged mechanism responsible for the effects of VP79s, by targeting the IL-6/JAK/STAT3 signaling pathway with subsequent modulation of MCL-1 expression and induction apoptosis. While further assessment of the toxicity of VP79s in normal cells, and the effects of tumour heterogeneity and microenvironmental interactions on the efficacy of VP79s are required, we have identified a promising potential therapeutic agent for MM that warrants further study into its translational capacity.

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S. Derenne, et al., Antisense strategy shows that mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells, Blood 100 (1) (2002) 194-199.


J. Li, et al., INCB16562, a JAK1/2 selective inhibitor, is efficacious against multiple myeloma cells and reverses the protective effects of cytokine and stromal cell support, Neoplasia 12 (1) (2010) 28-38.


