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Combined Valproic Acid, Bezafibrate, and Medroxyprogesterone Acetate Inhibit Proliferation and Induce Death of Multiple Myeloma Cells in MSC Conditioned Medium

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Abstract: **Background:** Multiple myeloma is a B-cell malignancy that typically affects the elderly. The standard treatment relies on autosomal stem cell transplant with high-dose chemotherapy, which is not possible in most cases due to age and other comorbidities. Several novel therapies have been demonstrated to have improved the outcome; however, combination therapies are still required due to toxicity associated with high doses of individual drugs, which makes the treatment very expensive, yet not curable. This study aims to evaluate the efficacy of widely available and affordable redeployed drugs valproic acid, bezafibrate, and medroxyprogesterone acetate against P3U1 multiple myeloma cells. **Methods:** ST2 mouse bone marrow-derived mesenchymal stromal cell (BM-MSC) derived conditioned medium was used to mimic the bone marrow microenvironment, which facilitates myeloma cell growth. Different combinations of valproic acid, bezafibrate, and medroxyprogesterone acetate were tested against P3U1 mouse myeloma cells in terms of cell proliferation by colorimetric MTS assay, viability percentage by LIVE/DEAD staining, and induction of intracellular reactive oxygen species (ROS) in both the control medium and the conditioned medium. **Results:** In the control medium, valproic acid alone enhanced P3U1 myeloma cell proliferation on MTS assay but induced cell death observed in LIVE/DEAD assay. The combination of bezafibrate and medroxyprogesterone acetate (denoted BaP) inhibited cell proliferation and increased cell death in a dose-dependent manner. Adding valproic acid to Bap (denoted VBaP) further increased the efficacy in BaP concentration-dependent manner. Reactive oxygen species (ROS) were detected following VBaP treatment, suggestive of oxidative stress-mediated cell killing. In ST2 BM-MSC derived conditioned medium, VBaP significantly reduced P3U1 cell proliferation and enhanced P3U1 cell death at a clinically achievable dose. **Conclusion:** VBaP combination is an exciting prospect in developing an affordable and readily available novel drug treatment for multiple myeloma, particularly in the context of the bone marrow microenvironment in which these malignant cells prosper.

Keywords: Valproic Acid. Bezafibrate, Medroxyprogesterone Acetate.

INTRODUCTION

Multiple myeloma is the second most common hematological malignancy, which heavily depends on the bone marrow environment (Poczta, A. *et al*., 2021). It accounts for approximately 15% of hematological cancers and ~1% of all types of malignancies (Rosiñol, L. *et al*., 2021). The incidence and prevalence of this cancer are higher in the USA and Europe compared to countries in Asia (Niino, M. *et al*., 2021). It is estimated that over 130,000 new cases are diagnosed every year worldwide; where 30,000 new cases are diagnosed, along with ~13,000 deaths per year, only in the United States of America (Cowan, A.J. *et al*., 2018; Siegel, R.L. *et al*., 2019). An increasing trend in the incidence of multiple myeloma has been observed in the general population, which is expected to grow in the near future as well. It is because of the improvement of life expectancy overall since multiple myeloma is a disease typical of the elderly population (Hayden, R.E. *et al*., 2021). The usual age of diagnosis is between 50 to 70 years, where ~70% of cases are diagnosed at an age older than 65 and 40% older than 75 years (Niino, M. *et al*., 2021; Hayden, R.E. *et al*., 2021). However, rare incidences of multiple myeloma (3%) have been reported below 40 years of age as well (Niino, M. *et al*., 2021).

The therapeutic approach to multiple myeloma primarily depends on risk stratification and eligibility for autologous stem cell transplantation, which is affected by age, performance status, and comorbidities (Larocca, A. *et al*., 2018). A wide range of treatment options is available at present, which has improved the median overall survival from 2–3 years to 8–10 years (Poczta, A. *et al*., 2021). Melphalan, an alkylating agent, established as myeloma therapy in 1967, has served as the backbone of multiple myeloma treatment in combination with corticosteroids (prednisone, dexamethasone) for a long time (Rajkumar, S.V. *et al*., 2020; Barwick, B.G. *et al*., 2019). At present, the combination of newer drugs such as second-generation proteasome inhibitors are showing high efficacy, especially in the older population (Larocca, A. *et al*., 2018; Hoogstraten, B. *et al*., 1967). However, autologous stem cell transplantation remains the standard approach, if eligible, with high-dose chemotherapy before and after transplantation (Larocca, A. *et al*., 2018; Hoogstraten, B. *et al*., 1967; Pinto, V. *et al*., 2020). A study demonstrated that autologous stem cell transplantation increased the mean survival by 1.37 years, but the mean overall cost of care for a patient who underwent transplantation was

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approximately $100,000 USD greater ($299,554 USD overall cost for a transplant patient) compared to a patient who didn't receive a transplant ($199,973 USD) (Rajkumar, S.V., 2018). Moreover, the eligibility for autologous stem cell transplantation widely varies among countries and is restricted to those below 70 years in Europe (Shah, G.L. *et al*., 2015). Although the novel agents are showing great response in transplant-ineligible elderly patients, the overall cost of the combination regimens required for a prolonged period is very high, and the long-term outcome of these therapies (i.e., overall survival) remains uncertain. The cost of a single course of drug therapy is estimated to range from $75,000 to $250,000 USD, and very few regimens have been proven to be cost-effective where the threshold is

$150,000 USD per quality-adjusted life-years (QALY) gained (Carlson, J.J. *et al*., 2018). This suggests that although the advances in therapies are being proven beneficial in improving outcome, it remains confined to high-income countries mostly. There are marked discrepancies in both the availability and affordability of effective therapies like novel agents, stem cell transplantation and associated specialized healthcare for management of multiple myeloma in low- to middle-income countries (Cowan, A.J. *et al*., 2018). A study performed in Nigeria demonstrated a very poor outcome, with a 5-year survival of only 7.6%, due to lack of access to affordable health care, where the Black race itself is a risk factor for multiple myeloma (Nwabuko, O.C. *et al*., 2017; Hayden, R.E. *et al*., 2021). Moreover, despite the availability of several therapeutic strategies, multiple myeloma is not completely curable, and almost 100,000 cases end in death per year worldwide (Cowan, A.J. *et al*., 2018). Thus, there exists an unmet requirement for a therapy that is inexpensive, non-invasive, has low systemic toxicity, and is well tolerated by elderly people.

While developing a new drug requires a very expensive (costing an average of $2.6 billion USD) and time-consuming (10–17 years on average) series of events, there exists an alternative strategy known as drug redeployment or repositioning, which is beneficial both in terms of time and cost (Ho, M. *et al*., 2020). Drug redeployment is the application of an already synthesized drug from its original indication into a new indication (Ho, M. *et al*., 2020). Bezafibrate, medroxyprogesterone acetate, and valproic acid are the perfect examples of drug redeployment (Hossain, S., 2018; Southam, A.D. *et al*., 2015). The original indications for these drugs have been hyperlipidemia, contraception, and epilepsy respectively, and these drugs are cheaper and readily available (Maio, A. *et al*., 2022). However, several studies have proven the efficacy of different combinations of these drugs, especially bezafibrate and medroxyprogesterone acetate, against hematological malignancies such as acute myeloid leukemia (AML), Burkitt lymphoma, B cell non-Hodgkin lymphoma (B-NHL), chronic lymphocytic leukemia (CLL), and bone tumor osteosarcoma (Southam, A.D. *et al*., 2015; Maio, A. *et al*., 2022; Sheard, J.J. *et al*., 2021; Fenton, S.L. *et al*., 2003; Sant, T. *et al*., 2009; Hayden, R.E. *et al*., 2015). It has been demonstrated that valproic acid exerts an anti-cancer effect by increasing apoptosis-related genes, and bezafibrate, medroxyprogesterone acetate, both combined and alone, demonstrate antilipogenic action within cells and induce reactive oxygen species (ROS) mediated cell death without suppressing healthy bone marrow (Southam, A.D. *et al*., 2015; Maio, A. *et al*., 2022; Hayden, R.E. *et al*., 2015).

Multiple myeloma is also a B cell malignancy and is the commonest form of a distinct group of diseases known as plasma cell dyscrasias (Khanim, F.L. *et al*., 2009). Before the stage is diagnosed as multiple myeloma, the condition often progresses through an asymptomatic stage (Saikia, T.K., 2017). The condition gets characterized as multiple myeloma when there is evidence of at least 10% monoclonal plasma cells on bone marrow examination or biopsy from other tissue showing bony or extramedullary plasmacytoma, presence of M protein (monoclonal IgG) >3 g/dL in serum or urine, and evidence of end-organ damage by CRAB (hypercalcemia, renal failure, anemia, lytic bone lesion) criteria (Pinto, V. *et al*., 2020). M proteins are abnormally structured immunoglobulins produced by malignant plasma cells (multiple myeloma cells), which are incapable of producing an immune response to fight infection. Accumulation of a large amount of these abnormally structured proteins in different tissues results in end-organ damages, and their presence produces hyper-viscosity of blood (Hoogstraten, B. *et al*., 1967).

B cells (also known as B lymphocytes) are a type of white blood cells derived from hematopoietic stem cells, and Plasma cells are a type of immune cells capable of producing a large amount of a specific antibody that develops from activated B

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cells (Weiss, B.M. *et al*., 2009). Several cytogenetic errors have been identified in this differentiation process which ultimately gives rise to malignant plasma cells (multiple myeloma cells) (Pinto, V. *et al*., 2020).

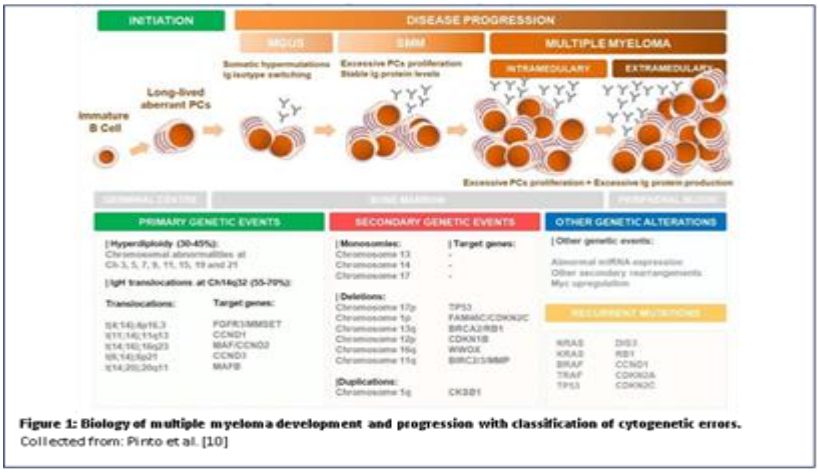

Figure 1: Biology of multiple myeloma development and progression with classification of cytogenetic errors.
Collected from: Pinto et al. [10]

Secondary cytogenetic abnormalities such as including gain(1q), del(1p), del(17p),del(13), and additional aberrant genetic events, such as RAS mutations, secondary translocations involving MYC, and microRNA (miRNA) abnormalities, Usually occur later along the disease course of multiple myeloma (see Figure 1) (Hoogstraten, B. *et al*., 1967; Pinto, V. *et al*., 2020). Identification of both these cytogenetic abnormalities has heavily influenced prognosis assessment, response to therapies, and modification of disease course and has helped understand the pathophysiology of multiple myeloma in a clearer way (Pinto, V. *et al*., 2020). Apart from these oncogenic genetic and epigenetic events, another factor that heavily influences the malignant plasma cells is the bone marrow microenvironment or niche (Manier, S. *et al*., 2012).

'Niche' refers to a specialized regulatory microenvironment composed of cells and other physical components, i.e., extracellular matrix, liquid milieu including cytokines, growth factors, and chemokines that collaborate to maintain, nurture, instruct, and control the fate specification of stem and progenitor cells (Manier, S. *et al*., 2012; Basak, G.W. *et al*., 2009). The cells that form the bone marrow microenvironment include hematopoietic and mesenchymal stromal cells, endothelial cells, osteoclasts, and osteoblasts (Manier, S. *et al*., 2012; Bernitz, J.M. *et al*., 2014). The mesenchymal stromal cells (MSC) have been proven crucial among them and have been demonstrated within the bone marrow (Méndez-Ferrer, S. *et al*., 2010). The mesenchymal stromal cells are essential for the maintenance of the bone marrow niche (González-González, A. *et al*., 2020). Roccaro *et al*. demonstrated that BM-MSCs influence the growth of multiple myeloma cells by mediating intercellular communication by transfer of exosomes in the bone marrow microenvironment (Roccaro, A.M. *et al*., 2013).

As redeployed drugs- bezafibrate, medroxyprogesterone acetate, and valproic acid have previously demonstrated anti-cancer properties against several bone marrow-related malignancies, and there is a need for a cost-effective, easily available treatment option for multiple myeloma, it was hypothesized that these drugs could be an exciting treatment option against multiple myeloma as well. Moreover, as BM-MSC-derived secretomes are essential for the bone marrow microenvironment, which potentiate multiple myeloma cell growth, BM-MSC-derived conditioned medium was used to represent the bone marrow microenvironment in vitro, and the efficacy of VBaP against mouse myeloma cells was examined within BM-MSC derived conditioned medium (representative of bone marrow microenvironment) by assessment of cell proliferation by MTS assay and cell viability by LIVE/DEAD staining.

MATERIALS AND METHODS:

Chemicals and Reagents:

The drugs- bezafibrate (BEZ, stock concentration of 0.5 M dissolved in Dimethyl Sulfoxide), medroxyprogesterone acetate (MPA, stock concentration of 5 mM dissolved in ethanol), and valproic acid (VPA, stock concentration of 0.6 M dissolved in distilled H_2O), and live/dead cell double staining kit were purchased from Sigma-Aldrich Merck, UK. CellTiter 96 AQueous One

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Solution Cell Proliferation Assay (MTS solution) was purchased from Promega, UK, and ab113851 DCFDA / H2DCFDA – cellular ROS assay kit was purchased from Abcam. All stocks were stored at -20°C.

Drug Treatments in Control Medium:

All sterile work was done in a biological safety cabinet. For each set of experiments (n=3), P3U1 cells were seeded at 25 x10^5 cells/mL in 10% DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Thermofisher Scientific, UK) in three different 96 well plates for three different sets of drug treatments at four different concentrations and corresponding solvent/carrier control (five replicates for each group) as follows:

- Valproic acid treatment concentrations: VPA- 0.15 mM, 0.3 mM, 0.6 mM, 1.2 mM, Carrier control: distilled H_2O at a dilution of 2 in 1000, which was equivalent to the highest concentration of VPA treatment.
- Combined bezafibrate and medroxyprogesterone acetate (BaP) treatment concentrations:

BEZ/MPA- 0.25mM/2.5µM, 0.5mM/5µM, 1mM/10µM,
2mM/20µM,
Carrier controls: Dimethyl Sulfoxide (DMSO) and ethanol combined at a final dilution of 4 in 1000, which was equivalent to the highest concentration of BaP treatment.

- Combined bezafibrate, medroxyprogesterone acetate and Valproic acid (VBaP) treatment concentrations: BEZ/MPA/VPA- 0.25mM/2.5µM/0.6mM,

0.5mM/5µM/0.6mM, 1mM/10µM/0.6mM,
2mM/20µM/0.6mM,

Carrier controls: Dimethyl Sulfoxide (DMSO), ethanol and distilled H_2O combined at a final dilution of 4 in 1000 for DMSO and ethanol, and 1 in 1000 for distilled H20, which was equivalent to the highest concentration of VBaP treatment.

For each well, 100µL cell suspension was treated with 100µL of either drug or carrier control at 2x final concentration at day 0 (to achieve the correct final concentration in the well) and incubated at 37°C for 3 days. The wells were further treated with 100µL of the same drug/carrier control at the final concentration on day 3 and incubated till day 5.

Drug Treatments in BM-MSC Conditioned Medium:

The conditioned medium was prepared by culturing ST2 mouse bone marrow-derived mesenchymal stromal cells (BM-MSC) in 10% DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Thermofisher Scientific, UK). The conditioned medium was harvested at three different time points of culture: 24 hours, 48 hours, and 72 hours. Following the same protocol mentioned above, cells were seeded in the conditioned mediums along with the control medium (10% DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin) and plated in a 96 well plate in two groups- a control group and combined bezafibrate, medroxyprogesterone acetate and Valproic acid (VBaP) treatment group (BEZ/MPA/VPA at 0.5 mM/5 µM/0.6 mM) and treated for 5 days. Three sets of experiments (n=3) were performed here as well, and four replicates were produced for each group (control vs. VBaP treatment in control medium, 24 hours, 48 hours, and 72 hours conditioned medium).

Cell Proliferation Assay (MTS Assay):

MTS assay was performed on day 5 of drug treatment to assess myeloma cell proliferation using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, UK) as per the manufacturer's protocol. It is a colorimetric assessment of cell growth that spectrophotometrically measures a biochemical marker to determine the metabolic activity of the cells (Kamiloglu, S. *et al*., 2020). It uses a tetrazolium reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)] that can be reduced by viable cells to produce a soluble formazan dye in the cell culture medium. This formazan dye can be absorbed at 490 nm wavelength (Fujihara, K. *et al*., 2005). 20µL of MTS solution was added to each well containing 200µL of cell suspension and incubated at 37° C. Absorbance was measured at 490 nm in a 96-well spectrophotometric plate reader (Thermofisher scientific, UK, with Skanit software 4.1 research edition) after an incubation period of 120 minutes. The values have been presented normalized to the mean control values.

Cell Viability (LIVE/DEAD assay):

Live/dead assay was performed on day 5 of drug treatment to assess the viability of the myeloma cells using live/dead cell double staining kit

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(Sigma-Aldrich Merck, UK). The staining kit contains calcein-AM and propidium iodide dye. The calcein-AM is a cell membrane permeable non- fluorescent dye that is converted into fluorescent calcein by intracellular esterase activity in live cells. Propidium iodide is membrane impermeable; therefore, it cannot enter viable cells. Its fluorescence property increases when it gets access to the nucleic acid compound of dead cells [Sanders, K. *et al*., 2012].

100µL of cell suspension from each well (single replicate per plate) was transferred to a new 96-well plate. 100µL of live/dead stain (5% Calcein-AM and 10% propidium iodide in DMEM/F12 medium) was added to each well and incubated at 37° C in a dark place for 30 minutes. The cells were then viewed under an inverted fluorescence microscope for manual scoring of the labeled cells (green stained for live cells, bright red stain for dead cells) at 10x and 20x magnification and images were captured using GXCapture imaging software. The viability percentage of the myeloma cells following drug treatments was calculated by dividing the number of live cells by the total number of both live and dead cells per well. The values have been presented normalized to the mean control values.

Detection of Reactive Oxygen Species (ROS):
Hydrogen peroxide (H2O2) and superoxide ions (O2-) are the vital reactive oxygen molecules that can be detected by H2DCFDA assay. H2DCFDA is a non-fluorescent compound that gets converted into highly fluorescent 2',7'- dichlorofluorescein (DCF) when it binds to reactive oxygen species (ROS) [Sanders, K. *et al*., 2016].

P3U1 cells were treated with VBaP at the following four concentrations with career control in a 96 well plate and incubated at 37°C for 24 hours (n=3):
Combined bezafibrate, medroxyprogesterone acetate, and valproic acid (VBaP) treatment concentrations: (BEZ/MPA/VPA- 0.25 mM/2.5 µM/0.6 mM, 0.5 mM/5
µM/0.6 mM, 1 mM/10 µM/0.6 mM, 2 mM/20 µM/0.6 mM), carrier controls: DMSO, ethanol and distilled H_2O combined at a final dilution of 4 in 1000 for DMSO and ethanol, and 1 in 1000 for distilled H20, which was equivalent to the highest concentration of VBaP treatment.

Following treatment, 200 µL of cell suspension per well in the 96 well plate was incubated at 37°C with 2',7'- dichlorofluorescein diacetate (ab113851 DCFDA / H2DCFDA
– cellular ROS assay kit; Abcam) as per manufacturer's instruction. ROS-positive cells were visually scored as bright cells under an inverted fluorescence microscope at 10x magnification, and relative ROS levels were calculated by dividing ROS-positive cells by the total number of cells; images were captured using GXCapture imaging software.

STATISTICAL ANALYSIS

Following drug treatment of P3U1 multiple myeloma cells with valproic acid only, BaP combination, VBaP combination in control medium, and drug treatment of P3U1 multiple myeloma cells with VBaP combination in conditioned medium harvested at 24 hours, 48 hours, and 72 hours, all data was collected as absorbance at 490 nm for MTS assay, and viability percentage of myeloma cells for LIVE/DEAD assay, and then normalized to the mean control values, to ensure that data was similar across all records. Percentages of relative reactive oxygen species (ROS) level following VBaP treatment were not normalized to control, as no ROS positive cells were detected in control. At least 3 independent experiments were performed for all analyses, and all data were pooled together. For either MTS assay, LIVE/DEAD assay, or ROS positive cell detection following each set of drug treatments, pooled data were first tested for normal distribution by the Shapiro-Wilk test using IBM SPSS statistics 27 software. Statistical analysis for assessment of significant differences was performed using IBM SPSS statistics 27 software in the following manner, where P values of <0.05 were considered significant:

- Detection of statistical significance between concentrations of each group of drug treatment:
- Valproic acid, BaP, VBaP in control medium MTS assay: data for each group were not normally distributed, independent samples Kruskal-Wallis tests were performed for significance.
- Valproic acid, Bap, VBaP in control medium viability assay: data for each group was normally distributed, one-way ANOVA with post-hoc Tukey tests were performed for significance.
- VBaP ROS detection: data were not normally distributed, independent samples Kruskal-Wallis test were performed for significance.
- Detection of statistical significance between Bap and VBaP treatments against the same

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concentrations (MTS assays):

- BEZ/MPA- 0.25mM/2.5µM, 0.5mM/5µM,
- 1mM/10µM: data for each group was normally distributed, independent sample T-tests were performed for significance.
- BEZ/MPA- 2mM/20µM: data were not normally distributed, independent sample Mann-Whitney U test was performed.
- Detection of statistical significance between Bap and VBaP treatments against the same concentrations (viability assays):
- BEZ/MPA- 0.25mM/2.5µM, 0.5mM/5µM, 1mM/10µM, 2mM/20µM: data for each group was normally distributed, independent sample T-tests were performed for significance.
- Detection of statistical significance between different conditioned mediums:
- Both control and VBap MTS assay
- Control viability assay
- VBaP viability assay
- Detection of statistical significance between control and VBaP in same conditioned mediums:
- 24-hour and 72-hour conditioned mediums
- 48-hour conditioned medium

The diagrams in the result section were generated using pooled data by Microsoft office excel software, and the statistical significances found in all the above-mentioned tests were denoted in the graphs. All data has been presented as mean±SD.

RESULTS

Valproic acid alone reduces viability but enhances cell proliferation of mouse multiple myeloma cells in control medium:

Valproic acid alone significantly enhanced P3U1 cell proliferation on MTS assay at 0.6mM and 1.2mM concentrations ($p<0.05$) (Figure 2). On the contrary, viability percentage was significantly reduced at 0.6mM and 1.2mM concentrations on LIVE/DEAD assay ($p<0.05$) (Figure 3).

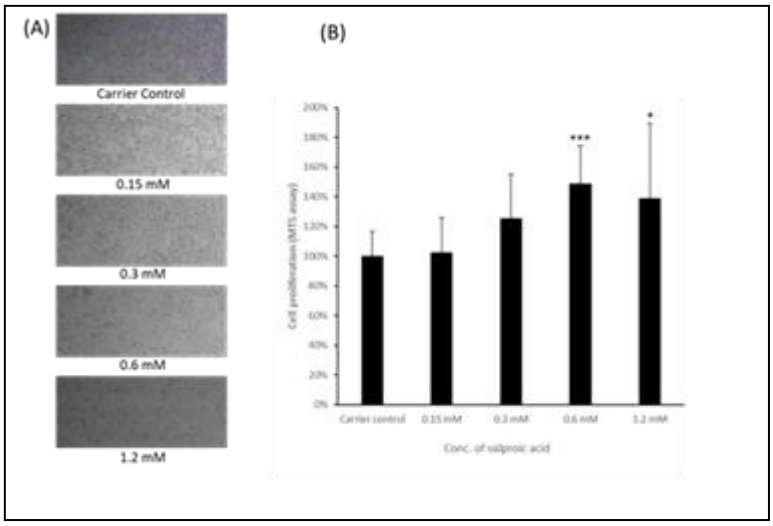

Figure 2: Valproic acid alone enhances proliferation of P3U1 mouse myeloma cells

(A) Images showing proliferation of P3U1 myeloma cells following different concentrations of valproic acid treatment under phase contrast microscope, images captured using GXCapture imaging software. (B) Bar chart showing increase in proliferation of P3U1 myeloma cells at different concentrations of valproic acid treatment, assessed by MTS assay (absorbance at 490 nm). Data shown as mean±SD (n=3 independent experiments); * P<0.05, **P<0.01, ***P<0.001.

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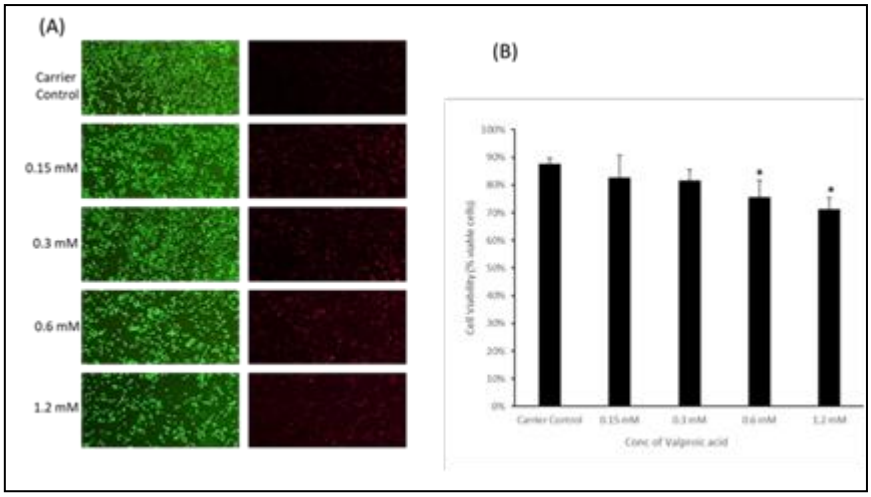

Figure 3: Valproic acid alone reduces viability of P3U1 mouse myeloma cells

(A) Images showing live (green) and dead (red) cells in LIVE/DEAD assay under inverted fluorescence microscope (10x magnification) depicting viability of P3U1 cells following valproic acid treatment; images captured using GXCapture imaging software. (D) Bar chart showing reduced viability percentage of P3U1 myeloma cells by different concentrations of valproic acid treatment compared to control, assessed by LIVE/DEAD assay. Data shown as mean±SD (n=3 independent experiments); * P<0.05.

BaP and VBaP (VBap>BaP) treatments inhibit proliferation and reduce viability of mouse multiple myeloma cells in a dose dependent manner, in control media:

Figure 4 shows dose-dependent inhibition of P3U1 cell proliferation by BaP and VBaP combination of drugs assessed by MTS assay. Statistically significant inhibition was seen for BaP at 1mM/10μM (BEZ/MPA) concentration ($p<0.01$), and at 1mM/10μM/0.6mM (BEZ/MPA/VPA) for VBaP ($p<0.001$) and higher. The greatest potency of VBaP had been seen at the concentration of 1mM/10μM/0.6mM (BEZ/MPA/VPA) and at 2mM/20μM (BEZ/MPA) for BaP. However, BaP didn't inhibit cell proliferation at lower concentrations, but VBaP showed statistically significant inhibition ($p<0.001$) compared to BaP at lower concentrations of 0.25mM/2.5μM/0.6mM and 0.5mM/5μM/0.6mM (BEZ/MPA/VPA).

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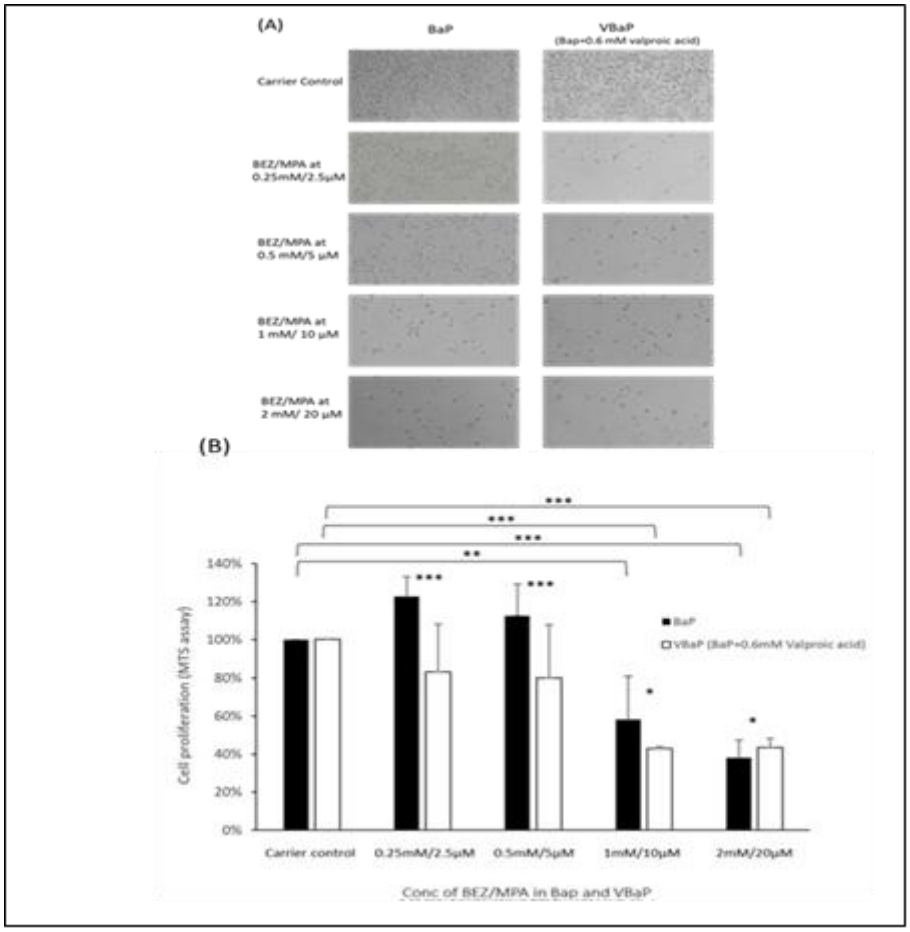

Figure 4: Both BaP and VBaP (VBaP>BaP) inhibit proliferation of P3U1 mouse myeloma cells

(A) Images showing proliferation of P3U1 myeloma cells following different concentrations of BaP and VBaP treatment under phase contrast microscope, images captured using GXCapture imaging software. (B) Bar chart showing higher efficacy of VBaP (compared to BaP) on inhibition of proliferation of P3U1 myeloma cells, assessed by MTS assay (absorbance at 490 nm). The concentrations of bezafibrate and medroxyprogesterone (BEZ/MPA) in VBaP are same as BaP (depicted in x-axis) and the concentration of valproic acid remains constant at 0.6mM in all four doses of VBaP. Data shown as mean±SD (n=3 independent experiments); * P<0.05, **P<0.01, ***P<0.001.

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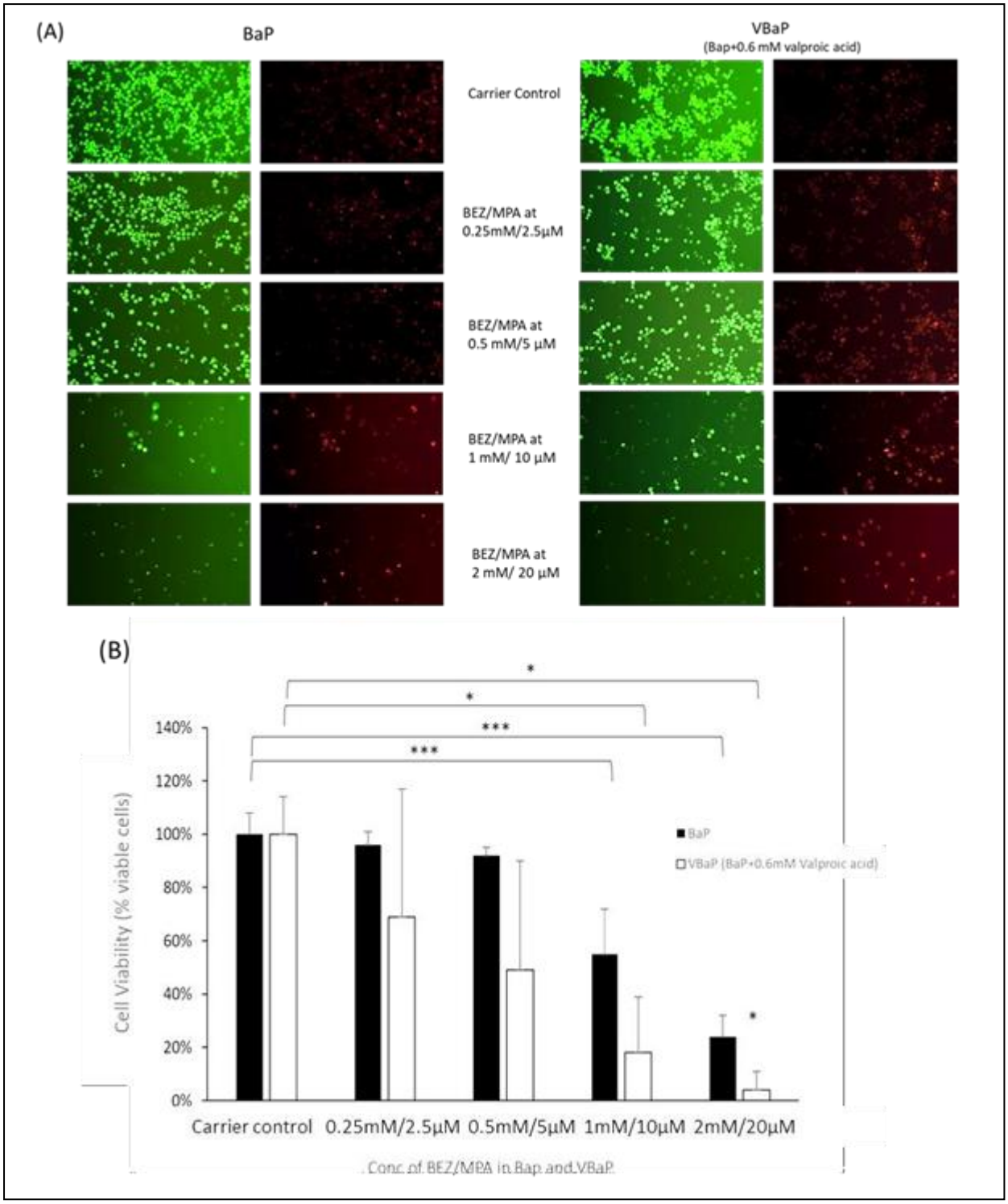

Figure 5: Both BaP and VBaP (VBaP>BaP) reduce viability of P3U1 mouse myeloma cells

(A) Images showing live (green) and dead (red) cells under inverted fluorescence microscope in LIVE/DEAD assay depicting viability of P3U1 cells following BaP and VBaP treatment, images captured using GXCapture imaging software. (B) Bar chart showing higher efficacy of VBaP (compared to BaP) on P3U1 myeloma cells in reducing viability, assessed by LIVE/DEAD assay. The concentrations of bezafibrate and medroxyprogesterone (BEZ/MPA) in VBaP are same as BaP (depicted in x- axis) and the concentration of valproic acid remains constant at 0.6 mM in all four doses of VBaP. Data shown as mean±SD (n=3 independent experiments); * P<0.05, **P<0.01, ***P<0.001.

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VBaP treatment induces the generation of intracellular reactive oxygen species (ROS) in P3U1 cells and correlates with dose dependent cell killing:

Following VBaP treatment of P3U1 cells for 24 hours, elevated levels of ROS-positive cells were detected in control medium. ROS% followed the similar dose-dependent trend observed in cell proliferation and viability percentage mentioned before (Figure 4,5). The highest level of ROS% was found at the concentration of 1mM/10μM/0.6mM (BEZ/MPA/VPA), which was statistically significant ($p<0.05$) (Figure 6). No evidence of ROS positive cell was found in the control group.

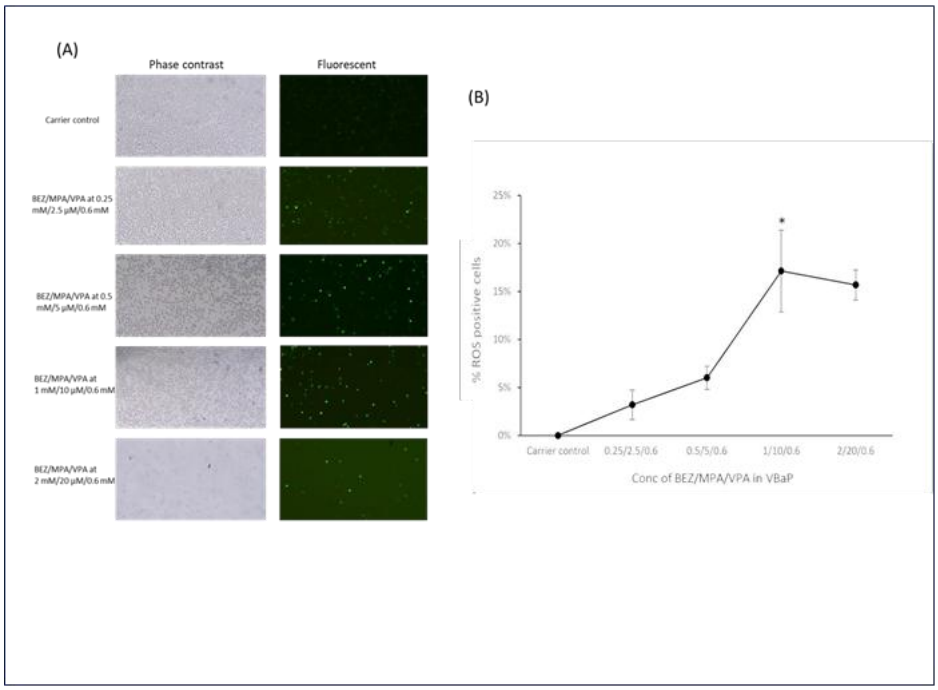

Figure 6: Dose dependent induction of intracellular ROS by VBaP treatment

(A) Images showing ROS positive cells (fluorescent green) relative to total number of P3U1 cells (phase contrast). 24 hours VBaP treated P3U1 cells were incubated with 2',7'-dichlorofluorescin diacetate (ab113851 DCFDA / H2DCFDA – cellular ROS assay kit; Abcam), relative ROS levels were assessed by visually scoring bright ROS positive cells under inverted fluorescence microscope (n=3), images were captured using GXCapture imaging software. (B) Line chart showing elevated levels of %ROS positive cells where highest level of ROS was found at the concentration of 1mM/10μM/0.6mM (BEZ/MPA/VPA). Data shown as mean±SD (n=3); *$p<0.05$.

VBaP inhibits cell proliferation and induces cell death of P3U1 myeloma cells in ST2 bone marrow-derived mesenchymal stromal cell conditioned medium:

P3U1 myeloma cells were found to proliferate more in ST2 bone marrow-derived mesenchymal stromal cell conditioned medium, most at 48 hours, compared to the control medium. However, the proliferation was significantly inhibited by the same conditioned medium harvested at 72 hours. When treated with VBaP (BEZ/MPA/VPA at 0.5mM/5μM/0.6mM) in these three conditioned mediums, it was seen that P3U1 cell proliferation (by MTS assay) was significantly inhibited against control ($p<0.001$). Most significant inhibition by VBaP was observed at 48 hours ($p<0.001$) (Figure 7).

A similar trend in efficacy was seen in killing the P3U1 cells by VBaP in ST2 bone marrow-derived mesenchymal stromal cell conditioned medium, assessed by LIVE/DEAD staining, where the most cell death was observed at 48 hours as well (Figure- 8). However, the results for viability percentage were not statistically significant, most likely due to the small sample size.

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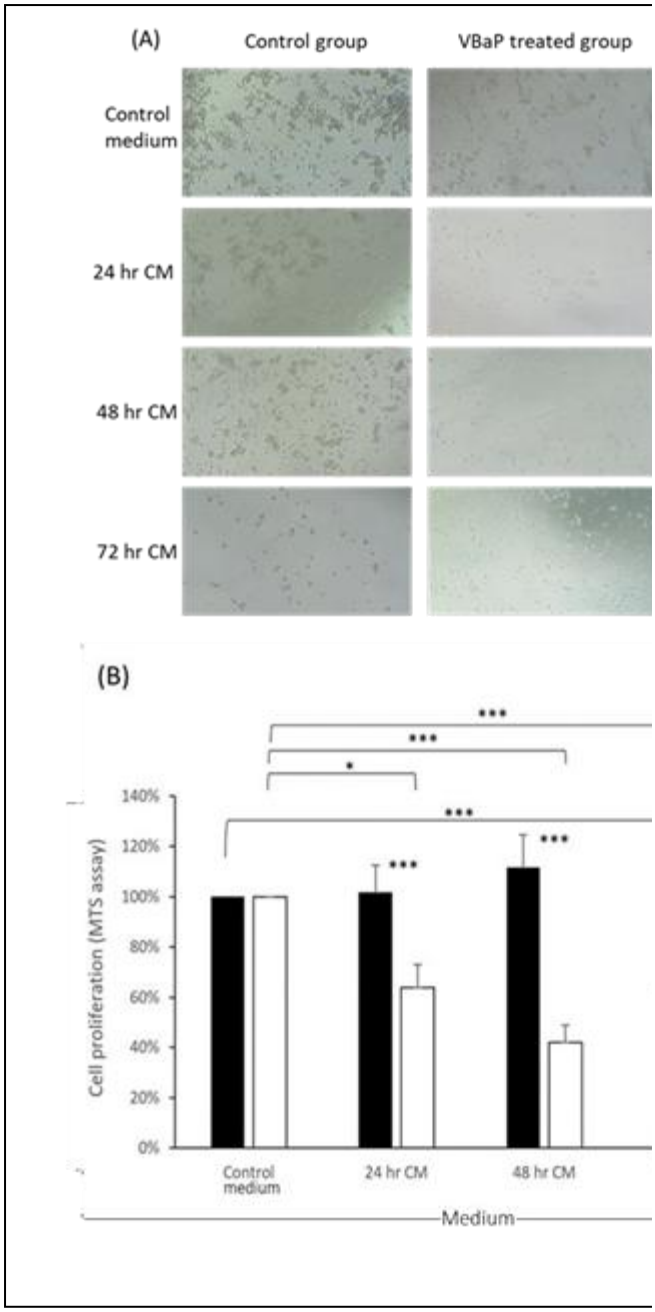

Figure 7: Efficacy of VBaP against P3U1 mouse myeloma cells in terms of inhibition of cell proliferation in ST2 bone marrow- derived mesenchymal stromal cell conditioned medium

(A) Images showing proliferation of P3U1 myeloma cells in control and ST2 bone marrow-derived mesenchymal stromal cell conditioned medium (at 24 hr, 48 hr, and 72 hr) following VBaP treatment (versus control) under phase contrast microscope, images captured using GXCapture imaging software. (B) Bar chart showing increased cell proliferation of P3U1 myeloma cells in ST2 bone marrow-derived mesenchymal stromal cell conditioned medium (at 24 hr and 48 hr) and statistically significant inhibition of the proliferation following VBaP (BEZ/MPA/VPA at 0.5 mM/5 μM/0.6 mM) treatment in conditioned mediums, assessed by colorimetric MTS assay. All data shown as mean±SD (n=3 independent experiments); * P<0.05, **P<0.01, **P<0.001.

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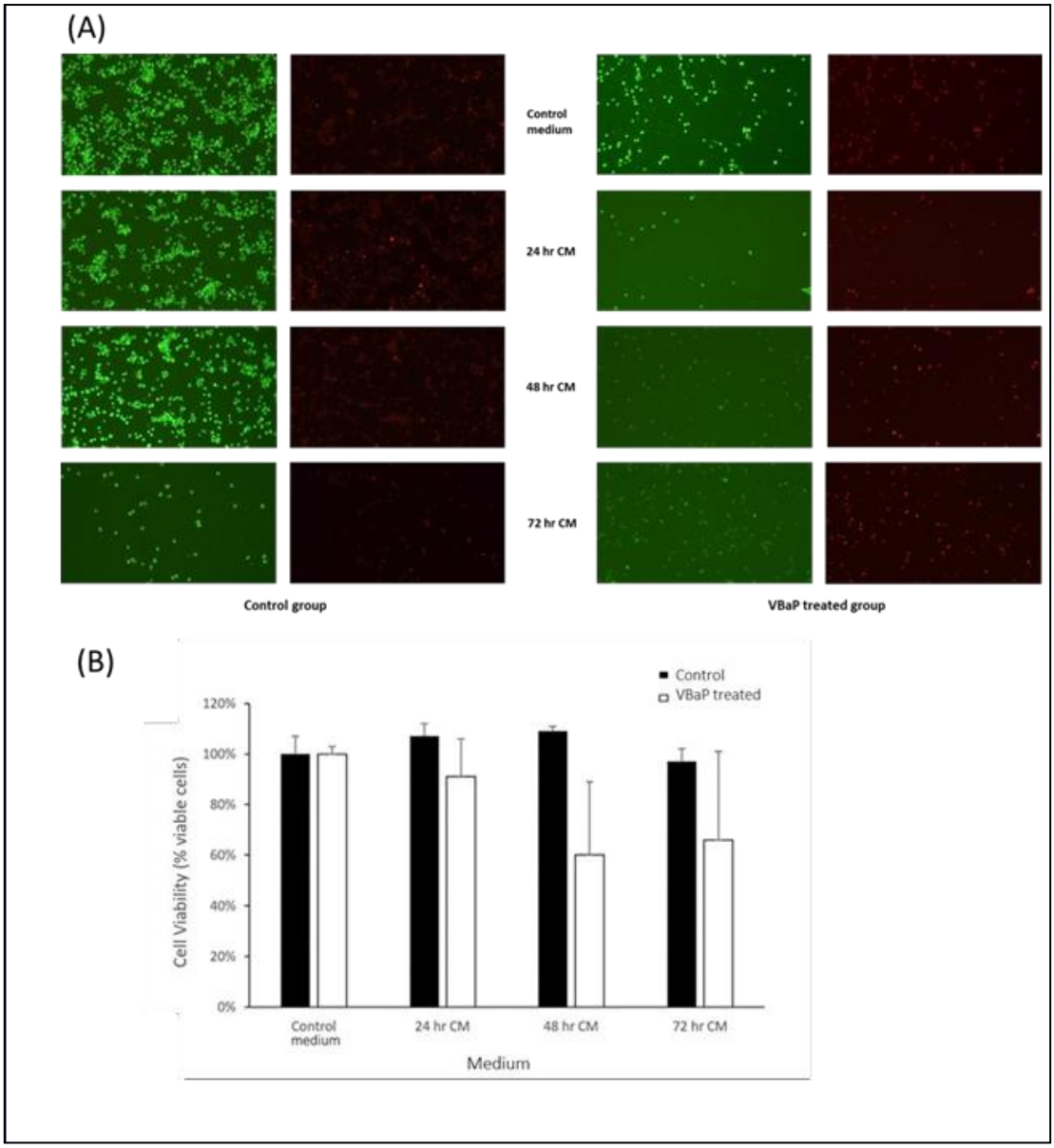

Figure 8: Efficacy of VBaP against P3U1 mouse myeloma cells in terms of induction of cell death (reduced viability) in ST2 bone marrow-derived mesenchymal stromal cell conditioned medium

Images showing live (green) and dead (red) cells under inverted fluorescence microscope in LIVE/DEAD assay depicting marked cell death of P3U1 cells following VBaP treatment (BEZ/MPA/VPA at 0.5mM/5µM/0.6mM) in ST2 bone marrow- derived mesenchymal stromal cell conditioned medium (at 24 hr, 48 hr, and 72 hr), images captured using GXCapture imaging software. (B) Bar chart showing reduced viability of P3U1 myeloma cells in ST2 bone marrow-derived mesenchymal stromal cell conditioned medium following VBaP (BEZ/MPA/VPA at 0.5mM/5µM/0.6mM) treatment, assessed by LIVE/DEAD assay. All data shown as mean±SD (n=3 independent experiments); no statistical significance found.

DISCUSSION

Several combination regimes of the drugs bezafibrate (BEZ), medroxyprogesterone acetate (MPA), and valproic acid (VPA) have been demonstrated to target hematological malignancies, i.e., acute myeloid leukemia, Burkitt lymphoma, B cell non-Hodgkin lymphoma (B-NHL), chronic lymphocytic leukemia (CLL) (Hossain, S., 2018; Southam, A.D. *et al*., 2015; Sheard, J.J. *et al*., 2021; Fenton, S.L. *et al*., 2003; Sant, T. *et al*., 2009; Hayden, R.E. *et al*., 2015). The efficacy of bezafibrate (BEZ) and medroxyprogesterone acetate (MPA) combination (BaP) has been found to demonstrate response against acute myeloid leukemia (AML), B cell

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non-Hodgkin lymphoma (B-NHL), and chronic lymphocytic leukemia (CLL) without any hematological toxicity in elderly patients in clinical trials (Murray, J.A. *et al*., 2010; Murray, J. *et al*., 2019). As multiple myeloma is a B-cell malignancy that typically affects the elderly, it was hypothesized that these drugs could be effective against multiple myeloma as well. In this study, several combinations of drugs in different doses were tested against P3U1 mouse multiple myeloma cells in both the control medium and ST2 mouse bone marrow-derived mesenchymal stromal cells conditioned medium (representative of bone marrow microenvironment). Although P3U1 cells grow in RPMI 1640 medium with 10% fetal bovine serum (FBS), P3U1 cells were seeded in 10% DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) for this drug treatment as the control medium because the conditioned medium was obtained by culturing ST2 cells in 10% DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS).

At first, P3U1 cells were treated with bezafibrate (BEZ) and medroxyprogesterone acetate (MPA) combination (BaP) and valproic acid (VPA) separately at several different doses in the control medium. Their efficacy against myeloma cells was assessed by MTS assay to see if cell proliferation was affected and LIVE/DEAD assay to assess the cell-killing potential of the drugs on the fifth day of individual drug treatments. It was found that BaP inhibited P3U1 cell proliferation and reduced viability at a minimum dose of 1mM/10μM (BEZ/MPA), and greater inhibition along with cell death was observed at 2mM/20μM (BEZ/MPA) (Figure 4,5). Valproic acid alone didn't reduce cell proliferation at any dose but rather increased. However, it reduced P3U1 viability at a minimum dose of 0.6mM (Figure 2,3). According to previous studies, 0.6 mM is the clinically safe concentration, and at this concentration, VPA has been demonstrated to exert an anti-cancer effect by inhibiting histone deacetylases (Gurvich, N. *et al*., 2004; Maio, A. *et al*., 2022). However, the minimum dose at which BaP displayed efficacy is not safe for elderly patients. It has been demonstrated in previous studies that a high dose of BEZ (1600–4800 mg/day) is not well tolerated by old patients, especially those with renal impairment (Murray, J.A. *et al*., 2010; Murray, J. *et al*., 2019). It is to be noted that one of the diagnostic criteria for multiple myeloma is renal impairment from the deposition of abnormally structured proteins (Pinto, V. *et al*., 2020). The maximum dose of BEZ that can be tolerated by this group of patients is 400 mg per day, which is equivalent to 0.5 mM BEZ (Maio, A. *et al*., 2022; Molyneux, E. *et al*., 2014). Hence, in the next phase of this study, different doses of BaP were combined with 0.6 mM valproic acid (VBaP) to see if VBaP could potentiate the efficacy of BaP in a lower dose. It was found that VBaP showed a significantly greater response in inhibiting cell proliferation at lower doses of 0.25 mM/2.5 μM/0.6 mM and 0.5 mM/5 μM/0.6 mM (BEZ/MPA/VPA) compared to BaP (Figure 4, 5). However, when compared to the control, a statistically significant reduction was seen at a minimum dose of 1 mM/10 μM/0.6 mM (BEZ/MPA/VPA) (Figure 4). VBaP reduced the viability percentage of P3U1 cells to a greater extent (compared to BaP) as well; however, statistical significance was not found (Figure 5), most likely due to the small sample size and high standard deviation. The P3U1 cells demonstrated ununiform distribution within the wells under the microscope, and a manual cell count of ununiformly distributed cells on LIVE/DEAD assay could be the reason behind this high standard deviation. A more automated quantitative analysis of viability, for example, Cell-IQ Imagen live-cell imaging platform, Cell-IQ Analyzer image analysis software, or colorimetric assay (i.e., MTT) with a bigger sample size could be used to confirm the findings observed in this study (Maio, A. *et al*., 2022; Hu, C. *et al*., 2022).

The mechanism of action on how VBaP acts against myeloma cells is not understood yet. However, in a previous study, it was demonstrated that, on addition of valproic acid to BaP, enhanced reactive oxygen species (ROS), lipid peroxidation, and inhibition of de novo fatty acid synthesis were the key factors for AML cell line killing by VBaP (Southam, A.D. *et al*., 2015). Chemotherapeutic agents such as cytarabine and daunorubicin facilitate leukemic cell killing by increasing oxidative stress within the cells by induction of ROS as well (Heasman, S.-A. *et al*., 2011). So, in the next phase of the study, ROS detection was performed to see if VBaP-mediated myeloma cell killing followed a similar mechanism. A dose-dependent elevation of ROS-positive cells was observed in P3U1 cells following VBaP treatment for 24 hours. Moreover, no ROS-positive cells were seen in the control group. This suggests that VBaP-mediated myeloma cell killing is also subject to oxidative stress. The dose-dependent

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elevation of ROS also correlates to the trend observed in proliferation and viability assays, where significant killing was seen at a minimum dose of 1 mM/10 μM/0.6 mM (BEZ/MPA/VPA) (Figure 6). Here a manual detection of ROS-positive fluorescent cells was performed in this study, and the proportion was calculated against the total number of cells. Flow cytometric quantitative analysis needs to be performed to confirm the capability of VBaP in producing enough oxidative stress responsible for myeloma cell death.

It has been proven in many studies previously that one of the key factors in the pathogenesis of multiple myeloma is the bone marrow microenvironment, which is maintained by cells like mesenchymal stromal cells, other hematopoietic progenitor cells, osteoblasts, osteoclasts, extracellular matrix, and a wide range of growth factors and cytokines (Bernitz, J.M. & Moore, K.A., 2014; Manier, S. *et al*., 2012). It has also been demonstrated that mesenchymal stromal cells secrete up to 1533 proteins that participate in different biological processes and help maintain the bone marrow niche (González-González, A. *et al*., 2020) (Figure 9). These can be obtained in vitro by culturing MSCs in the medium that supports growth and allows MSCs to secrete the soluble factors, which is then called MSC conditioned medium (Sagaradze, G. *et al*., 2019). So, bone marrow-derived mesenchymal stromal cell conditioned medium (harvested at 24 hours, 48 hours, 72 hours) was used in the next phase of this study to mimic the bone marrow microenvironment in vitro, and the efficacy of VBaP against P3U1 myeloma cells was then examined in this conditioned medium.

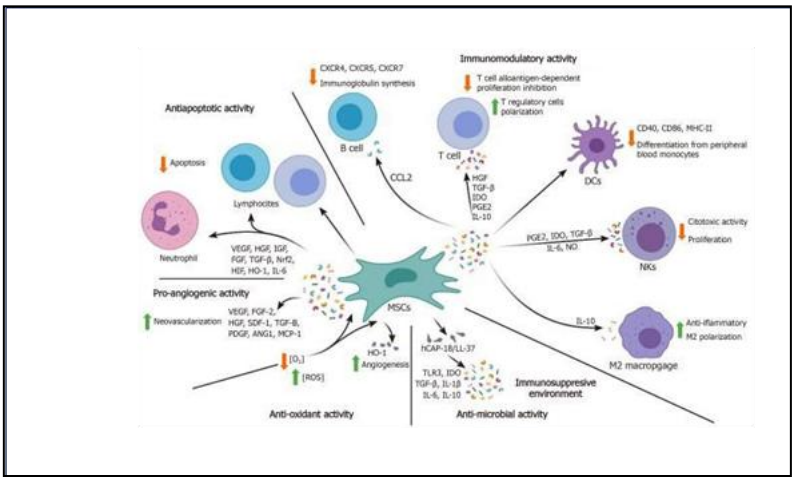

Figure 9: Summary of the various soluble factors secreted by mesenchymal stem cells and their functions

For this part of the experiment, only 0.5 mM/5 μM/0.6 mM (BEZ/MPA/VPA) dose of VBaP was used (against the control group), as this is the calculated maximum dose that an old patient with renal impairment can tolerate (Maio, A. *et al*., 2022). The control group displayed an increase in P3U1 myeloma cell proliferation and viability in 24 hr and 48 hr BM-MSC derived conditioned medium (MSC-CM), proving that MSC-CM promotes myeloma cell growth. However, the MSC-CM harvested at 72 hours significantly reduced both proliferation and viability for an unknown reason, which needs to be investigated in future studies. While the conditioned medium itself promoted P3U1 growth, VBaP at 0.5 mM/5 μM/0.6 mM (BEZ/MPA/VPA) dose significantly reduced the proliferation on MTS assay in the conditioned medium (compared to the control medium) (Figure 7). A reduction in viability percentage was observed as well; however, it was not statistically significant (Figure 8), most likely because of the same reason stated before for viability in the control medium.

The maximum dose for medroxyprogesterone acetate that can be tolerated by our target group of patients is 10 μM, which provides scope for a further study to see if VBaP at 0.5 mM/10 μM/0.6 mM (BEZ/MPA/VPA) could yield better efficacy (Maio, A. *et al*., 2022). The success of this study demands a future experiment on the screening of these drugs against human multiple myeloma cells in a 3D bone marrow microenvironment model. In this study, only detection of reactive oxygen species (ROS) was performed, which yielded positive findings. The

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other proposed mechanism of action of VBaP in leukemia cell killing, such as lipid peroxidation and inhibition of de novo fatty acid synthesis, needs to be studied for myeloma cell killing as well (Southam, A.D. *et al*., 2015). Moreover, although individual pharmacokinetics and pharmacodynamics of these drugs are well studied, there is not enough information available in terms of combination, which requires future attention.

The results obtained in this study suggest that VBaP has the potential to be established as an excellent treatment option for multiple myeloma. This cancer, unlike other hematological malignancies, often progresses through asymptomatic stages of monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (Saikia, T.K., 2017). Treatments are not usually offered at these stages as these are asymptomatic, do not always progress to the multiple myeloma stage, and the drugs used for the treatment of multiple myeloma (melphalan-prednisone, thalidomide) have a wide range of systemic toxicities while having demonstrated no difference or inferior outcomes with early treatment approach (Riccardi, A. *et al*., 2000; Landgren, O. *et al*., 2011). VBaP is a combination of redeployed drugs that are commonly used without any cancerous indication and has been proven not to have myelosuppressive properties (Maio, A. *et al*., 2022). This study suggests that VBaP has the potential to inhibit myeloma cell growth within the bone marrow microenvironment. Together, it can be hypothesized that VBaP has the potential to not only treat multiple myeloma but also has the potential to be used safely in high-risk asymptomatic MGUS and SMM cases to halt progression to multiple myeloma, which provides a wide scope for future studies. High-risk groups can be identified based on risk stratification models like the Mayo Clinic model and the Spanish model, which rely on cytogenetic analysis and general risk factors such as age, sex, race, cigarette smoking, and others (Riccardi, A. *et al*., 2000). Moreover, all three valproic acid, bezafibrate, and medroxyprogesterone acetate are widely available and affordable drugs that can help multiple myeloma treatment obtainable to patients of low and middle-income countries, if proven effective.

CONCLUSION

Multiple myeloma is a progressive B-cell malignancy that heavily relies on the bone marrow microenvironment. Despite recent advances in treatment options, it remains uncurable yet treatable at great expense to extend overall survival. As drug redeployment with valproic acid, bezafibrate, and medroxyprogesterone acetate has been proven beneficial against several hematological malignancies, this exciting combination was yet to be screened against multiple myeloma. It was found in this study that the VBaP combination of these three drugs inhibits proliferation and induces cell death of P3U1 mouse myeloma cells in a BaP dose-dependent manner. The generation of reactive oxygen species suggested that this action is mediated by the induction of oxidative stress. The need for understanding the mechanisms of action of VBaP against multiple myeloma claims the scope of future studies. Most interestingly, it was observed that VBaP inhibits proliferation and induces cell death of P3U1 mouse myeloma cells more effectively at a lower dose which is clinically achievable with minimum side effects in bone marrow derived MSC conditioned medium which mimics the bone marrow microenvironment in vitro. To conclude, this study shows that the VBaP combination possesses great potential for the development of an affordable treatment option for multiple myeloma.

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