



Increase in Intracellular Calcium Concentration Induced Differentiation of Hematopoietic Stem Cells

Aditi Singh¹, Elcim Eroglulari¹, Athar Aziz²

¹Laboratory of Translational Medicine, University of Salford, Manchester, UK

²Department of Biomedical Sciences, University of Salford, Manchester, UK

Email address:

a.singh6@edu.salford.ac.uk (A. Singh), e.eroglulari@edu.salford.ac.uk (E. Eroglulari), A.aziz@salford.ac.uk (A. Aziz)

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Abstract: Calcium (Ca^{2+}) is a key secondary messenger. It is responsible for the generation of intracellular signals which regulates the cellular division, differentiation and cell death. Intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) is maintained at a 10^5 -fold lower level than the extracellular calcium concentration. The rise in $[\text{Ca}^{2+}]_i$ induces differentiation in stem cells and this increased $[\text{Ca}^{2+}]_i$ also serves as an early indicator of cellular death by apoptosis. In haematological malignancies such as chronic myeloid leukaemia (CML), the cells are arrested in the megakaryocytic stage and are unable to differentiate into platelets. In this study, we treated two cell lines derived from CML patients-K562 and Marimo, with Ca^{2+} channel blockers (CCBs) - fendiline and BTP2. We examined the effects of CCBs on cellular differentiation and growth in the two cell lines. The $[\text{Ca}^{2+}]_i$ was found to increase with the increasing concentration of the CCBs. The morphology of the cells was then examined under a light microscope. It was observed that this increasing $[\text{Ca}^{2+}]_i$ subsequently induced differentiation in both the cell lines. Differentiation is closely linked with proliferation. At even higher concentrations (25 μM), it was observed that these CCBs led to a decline in the number of cells. Cell cycle analysis was then performed to verify if the CCBs had an apoptotic effect on the cell lines. On performing cell cycle analysis, it was concluded that these CCBs at a higher concentration triggered apoptosis in the cells. The results suggest that CCBs causes increase in the intracellular calcium concentration in the cell lines which leads to differentiation of the hematopoietic stem cells. In addition to this, these CCBs are toxic for the cells at high concentrations as they induce apoptosis in the cell lines.

Keywords: Calcium, Differentiation, Chronic Myeloid Leukaemia

1. Introduction

Ca^{2+} is an enormously versatile molecule. Ca^{2+} , a secondary messenger, generates signals intracellularly for regulation of cellular processes like apoptosis, cell proliferation and differentiation [1]. The underlying versatility of Ca^{2+} signalling mechanisms with respect to spatiotemporal patterning, amplitude and speed is responsible for regulating so many cellular processes [2]. Despite the importance of calcium in regulating vital cellular processes, this research has gained interest very recently [3]. Ca^{2+} concentration is maintained at a 20,000-100,000-fold lower concentration in the cytosol than the extracellular spaces. Ca^{2+} is pumped out of the cytosol to the endoplasmic reticulum (ER), extracellular spaces and mitochondria to maintain the low levels [4].

The rise in Ca^{2+} concentration initiates phosphoinositide metabolism which is crucial in the differentiation process [5]. The principal enzyme involved in this process is phospholipase C (PLC). It is responsible for hydrolysis of phosphatidylinositol biphosphate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3). Intracellular Ca^{2+} stores regulated by inositol 1,4,5-trisphosphate receptors (IP_3Rs) are present inside the cells. IP_3Rs are susceptible to signals from platelet-derived growth factor, histamine and ATP. They rely on store-operated channel entry (SOCE) for Ca^{2+} uptake and filling the intracellular stores of Ca^{2+} [6]. PLC is also a requirement for activation of store-operated channels which restores Ca^{2+} in intracellular stores. Ca^{2+} release from the intracellular stores is stimulated by DAG and IP_3 which consequently activates protein kinase C (PKC). The sustained rise in $[\text{Ca}^{2+}]_i$ along with transcription factors

regulated by PKC, increases the transcription of genes responsible for differentiation. Additionally, the Ca^{2+} receptors make a complex with Golgi Ca^{2+} pump and IP_3 receptors and control the release and uptake of Ca^{2+} from intracellular stores [7, 8].

A substantial increase in the concentration of cytosolic Ca^{2+} triggers apoptosis [9]. The apoptosis can then occur through two pathways-mitochondrial and ER-mediated pathways [10]. Intracellular Ca^{2+} stores regulated by Inositol 1,4,5-triphosphate receptors (IP_3Rs) are present inside the cells. IP_3Rs are susceptible to signals from a platelet-derived growth factor, histamine and ATP. They rely on store-operated channel entry for Ca^{2+} uptake and filling the intracellular stores of Ca^{2+} [6].

Several tumour suppressor genes and oncogenes affect the functioning of IP_3Rs and disrupt their capability to regulate mitochondrial Ca^{2+} influx. Thus, an increase in Ca^{2+} in the cytosol is an initial indication of apoptosis [11]. In the ER, foldases and chaperones are present on the surface which mediates around 30% of the protein folding and maturation. Ca^{2+} is required as a cofactor for the ideal functioning of chaperones [12]. Ca^{2+} depletion from ER alters protein folding processes and causes a condition of stress in the ER which initiates unfolded protein response (UPR) [13]. If the UPR is not able to restore the homeostasis in the cell, it alters its signalling and initiates cell death via apoptosis [14].

In this study, we used two CCBs- Fendiline and BTP2. Even at a very low concentration, fendiline has been shown to be an extremely effective CCB [15]. It binds with Sarco/endoplasmic reticulum calcium ATPase (SERCA) receptor and inhibits Ca^{2+} uptake by ER. BTP2 is a pyrazole derivative and it directly blocks the Ca^{2+} uptake by SOCE [16]. The effects of these CCBs were tested on two cell lines- K562 and Marimo. The cells were treated with different concentrations of CCBs and their effect on the $[\text{Ca}^{2+}]_i$ and differentiation of the cells were analysed. Also, cell cycle analysis was performed.

2. Method

Marimo (having CALR mutation) and K562 (having BCR-ABL genes) cell lines cultured in GIBCO® RPMI 1640 medium were used for the experiments. Medium (440ml) was supplemented with fetal bovine serum (50ml), streptomycin/penicillin (5ml) and L-glutamine (5ml). Splitting of cells was carried in aseptic conditions every 2-3 days. Cells were transferred in a test tube and centrifuged (1200 rpm, 5 mins). After discarding the supernatant, medium (1ml) was added in tubes with K562 cells and medium (3ml) was added in tubes with Marimo cells and mixed thoroughly. One-fourth of the medium containing cells were then transferred in two separate T75 flasks containing medium (10ml) and incubated at 37°C in a CO_2 incubator. The flasks were examined, before and after splitting, under a microscope for any contamination.

2.1. Treatment of Marimo and K562 for $[\text{Ca}^{2+}]_i$ Analysis

The cells were collected in two tubes by centrifugation after splitting for 24 hours. Medium (12ml) was added to each tube containing the cell lines and mixed thoroughly. Cells (1ml) was added to 24-well plate along with different concentrations of CCBs (0, 5 and 25µM of BTP2 and fendiline) and incubated for a day. Cells were then collected in centrifuge tubes and centrifuged (1500 rpm, 5 mins). After discarding the supernatant, 1X phosphate buffer saline (PBS) (500µl) was added to each tube. Fluo-8® (50µl) was mixed with 1X PBS (2.5ml) and 100µl of this mixture was mixed in each tube and tubes were incubated (30 mins, 37°C). FACSVerse BD® was used for analysis of $[\text{Ca}^{2+}]_i$.

2.2. Treatment of Marimo and K562 for Cell Cycle Analysis

The cells were collected in two tubes by centrifugation after splitting for 24 hours. Medium (25ml) was added to each tube containing the cell lines and mixed thoroughly. Cells (1ml) was added to 24-well plate along with different concentrations of CCBs (0, 1, 5 and 25µM of BTP2 and fendiline) and incubated for a day. Cells were then collected in centrifuge tubes and centrifuged (8000rpm, 3 mins). Ethanol (70%) was added to the tubes after discarding the supernatant and tubes were incubated on ice (15 mins). 1X PBS (500µl) was added and tubes were centrifuged (8000 rpm, 3 mins). PBS (100ml) was added upon discarding the supernatant. RNase (100µl) was mixed with PBS (10ml) and 100 µl of this mixture was added in each tube and incubated for 10 mins. PBS (100µl) was added to each tube and centrifuged (8000rpm, 3 mins). Propidium Iodide (100µl) was then mixed in each tube. Cell cycle analysis was then done using FACSVerse BD® machine.

2.3. Treatment of Marimo and K562 Cells for Microscopy

The cells were collected in two tubes by centrifugation after splitting for 24 hours. Medium (24ml) was added to each tube containing the cell lines and mixed thoroughly. Cover slips were placed in each well of a 12-well plate and collagenase (2ml) was added to each well and discarded after 15 mins. Cells (2ml) was added to 12-well plate along with different concentrations of CCBs (0, 5 and 25µM of BTP2 and fendiline) and phorbol 12-myristate 13-acetate (PMA) (1µM) and incubated for 48 hours. Medium was removed after incubation and fixative (2ml) was added and incubated for 5 mins. Giemsa stain (2ml) and 1X PBS (38 ml) was mixed and this mixture (2ml) was added to each well and incubated for 40 mins. Cover slips were washed under tap water and left to air dry until no visible residue was seen on cover slips. DBX was added to this cover slips and fixed on glass slides. The slides were observed under 40X magnification of a Leica light microscope (Leica, Heidelberg, Germany) for changes in the morphology and images were acquired using Leica air app.

3. Results

3.1. Higher Concentrations of Fendiline and BTP2 Results in Rise of Intracellular Calcium Concentrations

FACSVerse® was used to measure the $[Ca^{2+}]_i$ upon treating the cell lines with varying concentrations of CCBs- Fendiline and BTP2 (5 μ M and 25 μ M). The fluorescence indicator Fluo-8 was used which is known to emit green fluorescence upon binding with the free intracellular Ca^{2+} of the cell. This fluorescence is detected in the green fluorescent protein (GFP) channel in a FACS machine. The size of the cell is measured by forward scatter and granularity is determined by side scatter. Geometric mean of the fluorescence intensity of the FITC/GFP channel reflected the $[Ca^{2+}]_i$. The change in the fluorescence intensity from untreated to treated cells were analysed and Ca^{2+} concentrations along with various concentrations of BTP2 and fendiline are summarised in table 1. A graphical representation of this data was done (Figure 1). It was observed that for Marimo cell line, the $[Ca^{2+}]_i$ increased at 5

μ M concentrations and then decreased at 25 μ M concentrations for both BTP2 and fendiline. In K562 cell line, an increase in intracellular Ca^{2+} was observed at both 5 μ M and 25 μ M concentrations of BTP2 and fendiline. This change was not very significant despite the increase in $[Ca^{2+}]_i$.

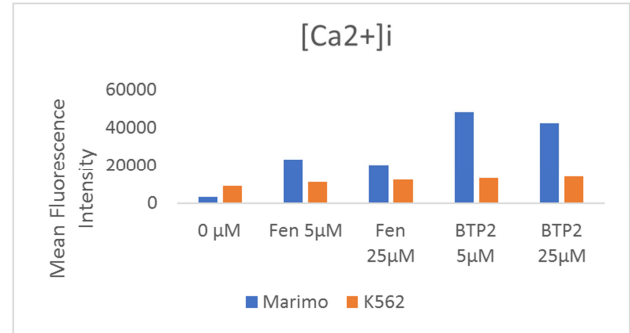


Figure 1. Increase in the $[Ca^{2+}]_i$ as seen from FACS analysis. Various concentrations of CCBs used are represented on x-axis and the y-axis represents mean fluorescence intensity which is indicative of the $[Ca^{2+}]_i$. Marimo and K562 cells are shown in blue and orange colour respectively.

Table 1. Mean fluorescence intensity with varying concentrations of CCBs in the two cell lines.

	0 μ M	Fen 5 μ M	Fen 25 μ M	BTP2 5 μ M	BTP2 25 μ M
Marimo	3428.33	23213.5	20200.5	48036.5	42408.75
K562	9117.25	11203.5	12470	13254	14155

3.2. CCBs Induced Differentiation in Hematopoietic Stem Cells

The consequences of treating the cells with CCBs on their ability to differentiate were examined. Microscope was used to examine the cellular morphology and determine the differentiation. Giemsa stain was used for staining after which the cells were examined under 40X magnification under a light microscope (Figures 2 & 3). Multi-nucleated and rather large cells known as megakaryocytes were viewed under the microscope. Multiple fields were taken into consideration and the number of megakaryocytic cells present in each field was expressed in percentage. Five and twenty-five μ M concentrations of CCBs was used for treating the cells along with a positive control- Phorbol 12-myristate 13-acetate (PMA), which is a known inducer of differentiation. The percentage of megakaryocytes along with the varying concentrations of CCBs were summarised (Table 2). The data was quantified and expressed in the form of graph (Figure 4). With fendiline in Marimo cells, it was observed that the number of megakaryocytes was higher with increasing drug concentration. However, with BTP2 in both cell lines and fendiline in K562 cell line, it was observed that at 5 μ M concentration, the percentage of megakaryocytes declined but at 25 μ M concentration, the number increased. An increase in number of

megakaryocytes was seen when cells were treated with PMA.

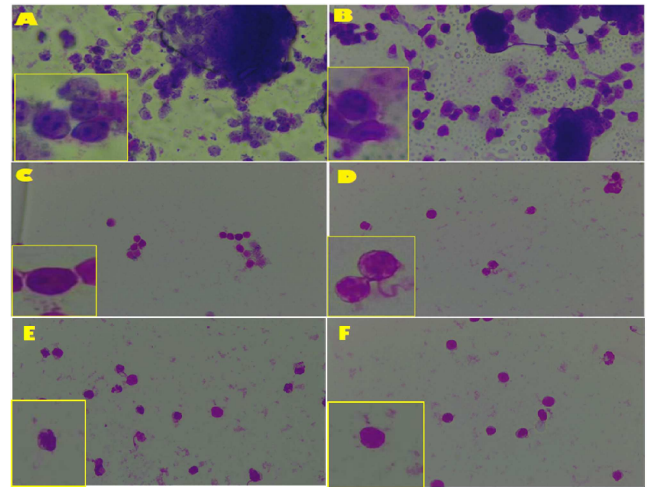


Figure 2. Differentiation induced by CCBs in the Marimo cell lines as seen under 40X magnification. Giemsa stain was used to stain the Marimo cells before analysing them under 40 X magnification of a light microscope. PMA induced differentiation is seen in Figure (A), the untreated cells are shown in Figure (B). Cells treated with 5 μ M and 25 μ M of fendiline are shown in Figure (C) and (D) respectively. Similarly, differentiation induced by 5 μ M and 25 μ M of BTP2 can be seen in Figure (E) and (F) respectively. A representation of the megakaryocytes which were being examined is shown in the small boxes present on each image. Leica Air software was used for image acquisition.

Table 2. The various concentrations of CCBs and PMA used along with the percentage of megakaryocytes are summarised below.

	PMA	Untreated	Fen 5 μ M	BTP2 5 μ M	Fen 25 μ M	BTP2 25 μ M
Marimo	45.07	40.9	52.9	37.36	69.38	57.57
K562	54.16	62.03	58.75	54.43	82.7	62.92

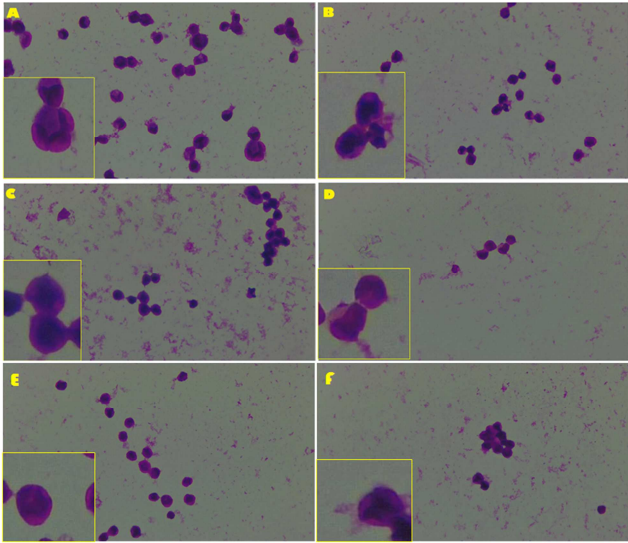


Figure 3. Differentiation induced by CCBs in the K562 cell lines as seen under 40X magnification. Giemsa stain was used to stain the K562 cells before analysing them under 40 X magnification of a light microscope. PMA induced differentiation is seen in Figure (A), the untreated cells are shown in Figure (B). Cells treated with 5 μ M and 25 μ M of fendiline are shown in Figure (C) and (D) respectively. Similarly, differentiation induced by 5 μ M and 25 μ M of BTP2 can be seen in Figure (E) and (F) respectively. A representation of the megakaryocytes which were being examined is shown in the small boxes present on each image. Leica Air software was used for image acquisition.

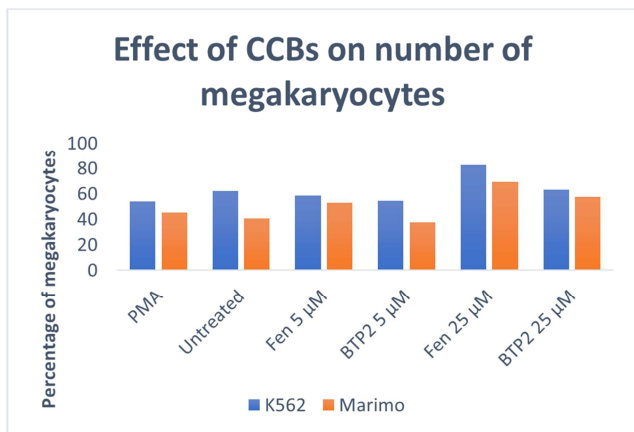


Figure 4. Effect of CCBs on the number of megakaryocytes. The x-axis of graph represents the varying concentration of the CCBs and positive control PMA and percentage of megakaryocytic cells are shown on y-axis. Blue colour represents the K562 cell lines whereas the Marimo cell lines are represented by orange colour.

3.3. Higher Concentration of CCBs Are Toxic for K562 Cell Lines

Proliferation and differentiation are closely related. While analysing the effects of CCBs on the cell lines, it was observed that cell number was declining. Cell cycle analysis was performed to determine if the cells were undergoing apoptosis. Propidium Iodide was used to stain the cells and cell cycle analysis was carried out in FACS machine. Dead cells take up the propidium iodide stain. The average percentage of parent population in each stage of the cell cycle

like Sub-G1, G2/M, S and G1 phase was obtained by FACS. Varying concentrations of the CCBs along with average percentage of parent population is summarized (Table 3). A graph was made upon quantification of data (Figures 5 & 6). The percent parent population in G1 phase increased with increasing concentrations of fendiline but decreased with increase in concentration of BTP2. A marked decrease in cell number was seen in S phase at 25 μ M concentration of both fendiline and BTP2. For G2/M phase, cell number decreased with increasing concentrations of CCBs. Cell number in Sub-G1 phase increased substantially with increasing concentrations of CCBs (1-25 μ M). It can be concluded that higher concentrations of CCBs (25 μ M) was toxic for cells as cells present in Sub-G1 phase are indicative of undergoing apoptosis.

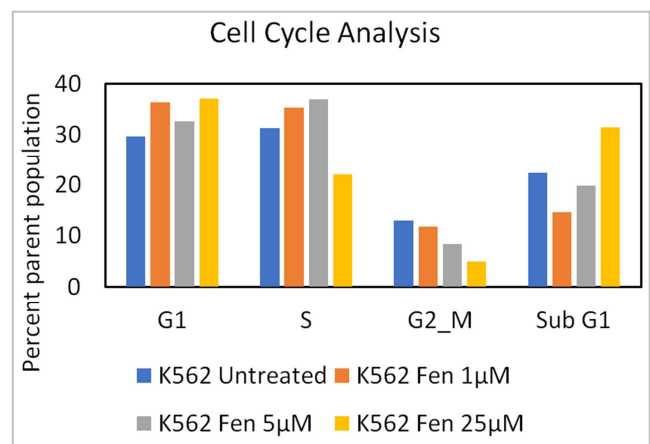


Figure 5. Depiction of percent parent population in each phase along with various concentration of BTP2. X-axis indicates the various phases of cell cycle. Y-axis indicates the percent parent population in each phase. Untreated cells are indicated by blue bar, orange, grey and yellow colour represents 1 μ M, 5 μ M and 25 μ M of BTP2 respectively.

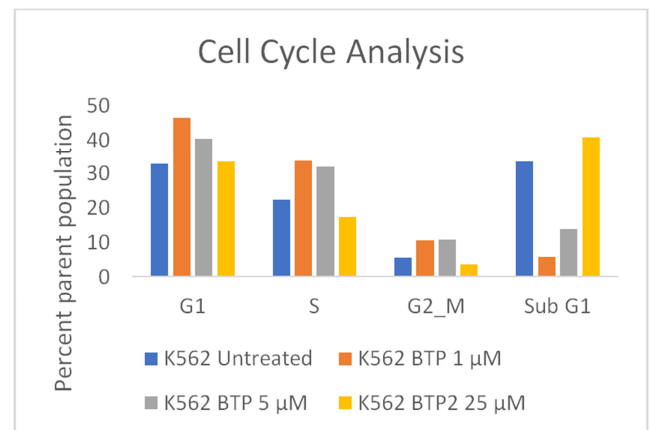


Figure 6. Depiction of percent parent population in each phase along with various concentration of BTP2. X-axis indicates the various phases of cell cycle. Y-axis indicates the percent parent population in each phase. Untreated cells are indicated by blue bar, orange, grey and yellow colour represents 1 μ M, 5 μ M and 25 μ M of BTP2 respectively.

Table 3. Various concentrations of CCBs and the per cent parent population in different phases.

	G1	S	G2_M	Sub G1
K562 Untreated	29.58	31.26	13.013	22.43
K562 Fen 1μM	36.30	35.26	11.74	14.68
K562 Fen 5μM	32.59	36.9	8.45	19.8
K562 Fen 25μM	37.03	22.05	4.91	31.4
K562 BTP2 1μM	46.361	33.83	10.56	5.66
K562 BTP2 5μM	40.38	31.98	10.79	13.83
K562 BTP2 25μM	33.55	17.27	3.673	40.75

4. Discussion

Elevated levels of intracellular calcium were detected in the cell lines upon treatment with CCBs. Fluo-8 was used to measure the effect of the CCBs in a concentration dependent manner. With an increase in the CCBs, a rise in mean fluorescence intensity was seen in both the cell lines. ER is the main store for intracellular calcium in a cell [17]. CCBs would block the Ca^{2+} receptors in the cell and hence it was expected to result in lowering of $[\text{Ca}^{2+}]_i$. However, as opposed to this, we found the levels of $[\text{Ca}^{2+}]_i$ to increase. This may be attributed to the fact that we measured the entire Ca^{2+} present in the cell and the CCBs used were blocking the receptors present on ER [15].

We used Fluo-8, which is a green fluorescence indicator. It uses a fluorescein core to measure the concentration of calcium inside a cell. It determines the total intracellular calcium content of a cell and it is not probable to measure the calcium concentration only inside ER (Lock *et al.*, 2015) [18]. It might be likely that the CCBs were decreasing the calcium content inside the ER but the overall concentration of calcium increased inside the cell.

Ca^{2+} concentration increase in the cell could be because of release of Ca^{2+} by IP_3Rs present on the ER which were not re-up taken by ER since CCBs were blocking the receptors. The increase in $[\text{Ca}^{2+}]_i$ could have also been because of activation of SOCE mechanism. Release of Ca^{2+} from ER activated the PLC pathway which consequently allowed Ca^{2+} influx from extracellular spaces by SOCE mechanism [7].

Hotchkiss *et al.*, suggested that $[\text{Ca}^{2+}]_i$ is a critical regulator between the differentiation and proliferation [19]. To assess the effect of increasing $[\text{Ca}^{2+}]_i$ on differentiation, we observed the morphology of cells under a microscope. We saw an increase in the number of megakaryocytes with increasing concentration of CCBs (5μM and 25μM). PMA is an identified inducer of differentiation and hence was selected as a positive control [20]. PMA induced differentiation in Marimo cells however, it failed to induce differentiation in K562 cell lines. This might be because PMA requires 48 hours for inducing differentiation and the number of cells killed in the meantime was more than the number of differentiated cells.

Concentration of intracellular calcium largely varies with the stage of the cell. In progenitor cells, calcium concentration is low. There is a slight increase in this concentration when the cell is in megakaryocytic stage.

However, a remarkable increase in the calcium content is noticed just before the differentiation of megakaryocytes into platelets. During differentiation the G-protein coupled receptors initiate calcium influx. Lowering the amount of calcium is associated with inhibition of differentiation [21]. Hence, the increase in calcium with increase in number of megakaryocytes could suggest that a changed intracellular calcium concentration is required for differentiation.

Even though differentiation was seen in cell lines, there was a marked decrease in number of cells with the increasing concentrations of CCBs. This could be reflective of cells undergoing apoptosis because higher concentrations of CCBs (25μM) are toxic for them. Cell cycle analysis was carried out to detect the changes induced by CCBs. A higher number of cells were found in S-phase when the concentration of CCBs were 0-5 μM which rapidly decreased when concentration was increased to 25 μM. Number of cells present in Sub-G1 phase were also found to increase with increase in concentration of CCBs.

Studies have shown that calcium dependent protein kinases play a major role in numerous cellular processes such as proliferation of HSCs, regulation of cell cycle and apoptosis [22]. Cells that do not proliferate such as HSCs are usually present in G0 phase whereas those present in Sub-G1 phase are indicative of undergoing apoptosis [23].

Apoptotic signalling inside a cell is induced under stress caused by increasing calcium concentration. Mitochondria and ER are two organelles where the earliest and most important events of apoptosis occur. ER-mitochondrial interactions that regulate apoptosis are directed by calcium. Higher concentrations of CCBs causes SERCA block in the ER. This block creates a calcium overload in the mitochondria. Mitochondria release a substantial amount of cytochrome c in response to the calcium overload. Apoptotic process starts when cytochrome c (a pro-apoptotic factor) activates the caspases and nucleases enzymes [24]. Increase in $[\text{Ca}^{2+}]_i$ can cause apoptosis by three pathways-mitochondrial, cytoplasmic and ER stress-related. Cancer cells are known to decrease their $[\text{Ca}^{2+}]_i$ to evade apoptosis [25]. Since CCBs caused an elevation in $[\text{Ca}^{2+}]_i$, it may have led to apoptosis of the cells.

Our results thus suggest that using CCBs increases the $[\text{Ca}^{2+}]_i$ which leads to differentiation of the cells. However, increase in $[\text{Ca}^{2+}]_i$ also led to cell death indicating that CCBs at a higher concentration (25μM) were toxic for the cells and induced apoptosis.

5. Conclusion

In CML, the cells are arrested in megakaryocytic state and are unable to differentiate into platelets. In our experiment, we attempted to induce differentiation in the cell lines K562 and Marimo which were derived from CML patients, using CCBs- fendiline and BTP2. Using the CCBs, we increased the calcium concentration inside the cells. This increased calcium concentration induced differentiation in the two cell lines and it was also seen that it resulted in reduced cell count. We carried a cell cycle analysis and found that though the CCBs induce differentiation in the CML cell lines, it also causes the cells to undergo apoptosis. We analysed the effect of varying concentrations of CCBs and found that CCBs in low concentration (5µM) induced differentiation. However, in higher concentrations (25µM), these CCBs are toxic for the cell lines as they induce apoptosis.

Authorship and Conflict-of-Interest Statements

1. Aditi Singh-Performed the experiments, collected the data, analysed the data, performed literature review and wrote the paper.
2. Elcim Eroglulari-Performed the experiments and collected the data.
3. Athar Aziz-Designed the experiments and critically reviewed the paper.

We know of no conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved for submission by all the named authors.

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