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Combined Therapeutic Effects of Arsenic Trioxide and Poly (Adp-Ribose) Polymerase Inhibitor on Chronic Myeloid Leukaemia Cell Line

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Abstract
Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder that takes its origin from haematopoietic pluripotent stem cells and the difficulty in its treatment has been well documented. Arsenic trioxide (ATO) is currently the most effective single first line anti-cancer agent in the treatment of acute promyelocytic leukaemia (APL). Its use in the treatment of other leukaemias including CML and solid cancers has been widely reported. However, different levels of dose-dependent cell toxicity and resistance of cancer cells have been observed. Combination treatment with other cancer therapeutic drugs can sometimes aid in overcoming resistance to anticancer drugs. This study aimed to explore the therapeutic effect of ATO in combination with 3, 4-dihydro-5-[4-(1-piperidinyl) butoxy]-1(2H)- isoquinolinone (DPQ), a poly (ADP-ribose) polymerase-1 (PARP-1) inhibitor, on apoptosis of CML cell line, K562. Annexin-V/FITC and (propadium iodide) PI dual staining by flow cytometry was used to detect and quantify apoptosis after 48 h incubation of cells treated with ATO and DPQ singly and combined. Also, flow cytometry analysis for cell cycle distribution was performed after staining cell nuclei with DRAQ7 stain. Confocal fluorescence microscopy analysis for apoptosis was carried out on cells stained with triple staining solution of Annexin-V/FITC, PI and DAPI. Results obtained from these experiments showed that ATO alone could induce the cells to undergo early apoptosis. Although the combination therapy induced slightly increased apoptosis than ATO alone (D = 0.02), it was not significant enough to be considered an additive or synergistic effect. Results of the cell cycle analysis showed that ATO failed to induce cell cycle arrest in any of the phases whereas DPQ as well as ATO in combination with DPQ induced G2/M arrest. Visual examination of the drug treated cells under confocal microscopy revealed that ATO alone and combination of 1.25 µM ATO and DPQ 40 nM induced early apoptosis in K562 cells. Taking all the experimental results together, ATO alone induced apoptosis in K562 cells whereas combination of DPQ and ATO failed to significantly enhance ATO-induced apoptosis. However, continued study of the combination of ATO and DPQ at varied concentrations will give a better insight into the potential of this combination as a promising strategy in the treatment of CML.
Keywords: *Therapeutic effect, arsenic trioxide, Adp-ribose, polymerase inhibitor, chronic myeloid leukemia*.

Introduction

Leukaemia otherwise known as blood cancer is a somatic disease characterized by uncontrolled proliferation of abnormal clone of early haemopoietic cells which leadto presence of these malignant cells in the peripheral blood and bone marrow[1][2][3][4]. Leukaemia was first recognized in 1845 when a report was published about a patient who died of a disease that manifested abnormally amplified number of blood cells[5]. It is one of the most common forms of cancer worldwide. In a study by[6] it was found that in the year 2000, about 2, 056, 000 children and adult worldwide developed leukaemia and 2,009, 000 of these patients died of the disease. Previous report shows that over 4300 people die from leukaemia annually in the UK[7]. Notwithstanding the effective treatment available for certain types of leukaemia, it has been rated high as a major cause of morbidity and mortality.

Chronic myeloid leukaemia is a clonal myeloproliferative disorder that takes its origin from haematopoietic pluripotent stem cells[8][9].

The pioneer treatment of CML occurred in the 19th century with the use of compounds containing arsenic[10]. Splenic irradiation was introduced in early 20th century which successfully reduced the rate of splenomegaly. However, this was replaced by alkylating agents in the 1960s, after the first ever randomised study of CML revealed better survival in patients treated with busulfan[9]. It was later discovered that busulfan lacked the ability to normalize blood count and may also cause mutation that might induce blast crisis. This discovery led to its replacement with hydroxycarbamide, a ribonucleotide reductase inhibitor. Unfortunately, although busulfan and hydroxycarbamide had a positive effect on the high blood count and also provided symptomatic relief, both drugs failed to slow down the progression of the chronic phase of the disease to accelerated phase[11]. Also, cytogenetic investigations revealed that patients remained 100% Philadelphia chromosome positive after treatment with these agents[9].

Two completely different treatment strategies, interferon α and allogeneic stem cell transplantation evolved in the 1970s. Several research studies are on-going to determine the best treatment for CML [8] reported that imatinib mesylate (Glivec) as the first line therapy for CML has been found to induce remission in more than 75% of patients with CML. However, its inability to eradicate CML stem cells and resistance of dormant CML stem cells to imatinib has been widely reported [12][13][14][15][16].

Arsenic is a naturally occurring substance that is available as toxic, chemically unstable oxides and sulfides. [17] shared this view and[18] also reported its use as medicinal agent for over 2400 years in the treatment of a variety of illnesses including ulcers, the plague and malaria. Arsenic trioxide (ATO), a form of inorganic arsenic was reported to be the main therapeutic agent used successfully for the treatment of acute promyelocytic leukaemia (APL), and has also been used in treating other leukaemias including CML and cancers such as multiple myeloma, breast, prostate and ovarian cancers as well as various solid tumours from the 1700s through the early 1900s prior to the invention of modern chemotherapy and radiotherapy in the mid-1900s[19][20][21][22][23][24]. It was reported that ATO has been effectively used in the teatment of APL with a complete remission rate of up to 90%[25].

Poly (adenosine diphosphate, ADP-ribose) polymerases (PARPs) are nuclear proteins which play a critical role in deoxyribonucleic acid (DNA) repair through the base excision repair pathway (BER) in response to genomic DNA damage caused by environmental exposures and cell replication [26][27].

The difficulties in treating CML have been well documented. Although recent advances in CML therapy have substantially improved patient's quality of life and improved survival, current treatments still suffer the limitations of some side effects and resistance.

This research aims to assess the effect of arsenic trioxide in combination with PARP inhibitors on inducing apoptosis in K562 cells, a chronic myelogenous leukaemia cell line. This study is based on developing an improved ATO-mediated anti-cancer treatment strategy aimed at improving

the sensitivity of CML cells to ATO, reducing ATO induced cell toxicity, overcoming the high intrinsic resistance of CML cells as well as improving ATO's efficacy in the treatment of CML.

Materials and Methods

Materials

Cells, Drugs and Chemicals

The human CML cell line K562 cells was purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 U/mL penicillin/ 100µg/mL streptomycin and 1-glutamine at 37^0C in 5% CO_2. ATO was purchased from Sigma chemical Co. (St. Louis USA). 2.5 µM of ATO was prepared from 3.2 mM by adding 15.6 µL of 3.2 mM ATO to 20 mL of media. 1.25 µM of ATO was also prepared. RPMI 1640 medium, phosphate buffered solution (PBS), fetal bovine serum (FBS), streptomycin, penicillin, propadine iodide, Annexin V, 4' 6-diamidino-2-phenylindol (DAPI), trypan blue were all purchased from Sigma chemical Co. PARP inhibitor 3, 4-dihydro-5-[4-(1-piperidinyl) butoxy]-1(2H)- isoquinolinone (DPQ) was also purchased from Sigma chemical Co. DRAQ7 DNA dye was purchased from Beckman Coulter Inc.

Flow cytometer (BD FACS Calibur) and Inverted confocal microscope (Leica TCS SP2 Leica microsystems Heideberg Germany) were used for analysis. Other equipment used include; Laminal hood (SCANLAF), haemocytometer, automatic pipettes, cover slips, hand tally counter, bucket centrifuge, Eppendorf centrifuge (DJB Labcare UK) and optical microscope (Optech microscopes Ltd).

Methods

Cell Culture

All procedures were performed in aseptic condition to prevent contamination.

Sample Preparation

Two flasks of K562 cells were grown for three days in 5% CO_2 and 95% air humidified incubator at 37^0C. The cells were then collected into 50 mL falcon tubes. The tubes were centrifuged for 3 min at 1500 rpm at 24^0C ambient temperature and the supernatant decanted. To prepare the cell suspension, 5 mL of RPMI culture media was added into each falcon tube containing the cell pellets.

Cell Counting

Cell counting was performed using the haemacytometric method. Using 10X objective, the lens was focused on the grid lines of the haemocytometer.

Since the haemocytometer is designed in such a way that the number of cells in one set of 16 squares is equivalent to the number of cells x 10/ mL, result is written as;

Number of cells counted X 10^4/ mL

The average number of cells was multiplied by 2 which is the dilution factor for the cell suspension – trypan blue dilution (1:1). Percentage viable cells were calculated using the formular below;

$$\frac{\text{Live cell count}}{\text{Total cell count}} = \text{Percentage viability}$$

Treating Cells with ATO and PARP Inhibitors

After growing the K562 cells for 48 h, cells from both the flasks and plates were spun down in separate tubes. After decanting the supernatant, cells were suspended in fresh complete media. The flasks were properly labelled. The first flask functioning as the negative control contained only the cells in suspension with no drug added to it while flasks 2 to 4 contained cells treated with 2.5 µM ATO, 40 nm DPQ, and 2.5 µM ATO with 40 nM DPQ respectively.

Also, K562 cells were treated with ATO and DPQ in the 6-well plate in the following order; the first well-functioning as the negative control contained only cells without drug treatment, cells in wells 2 to 6 were treated with ATO 2.5 µM, DPQ 40 nM, ATO 2.5 µM with DPQ 40 nM, ATO 2.5 µM with DPQ 20 nM and ATO 1.25 µM with DPQ 40 nM respectively. The treated cells were grown in complete media containing RPMI 1640 supplem-ented with FBS and 1% penicillin-streptomycinand incubated at 37^0C in 5% CO_2 for 48 and 72 h.

Flow Cytometry Analysis for Apoptosis

Cells were harvested after the 48 h treatment with different concentrations of ATO and DPQ both

separately and combined by spinning the cell suspension in separate falcon tubes at 1500 rpm for 3 min at 24^0C. The supernatant was decanted and the cell pellets washed once with PBS. 1X binding buffer solution was prepared by adding 2.25 ml of deionized water to 250 µL of 10X binding buffer. The washed cells were suspended in 500µL of 1X binding buffer. 10 µL of propadine iodide (PI) working solution and 5 µL of Annexin V – FITC were added to each cell suspension. The experiment was performed in the dark since PI is sensitive to light. The tubes containing the mixtures were wrapped with foil to protect the cells and prevent PI deterioration. The cells were incubated at room temperature for 10 min. Stained cells were then analysed by flow cytometry, measuring the fluorescence emission at 530 nm and 575 nm using an excitation wavelength of 488 nm.

Flow Cytometry Analysis for Cell Cycle

The control cells and ATO and DPQ treated cells were spun in separate falcon tubes at 1500 rpm for 3 min at 24^0C ambient, after which the cells were harvested. 1 mL of PBS was added to each tube to make a cell suspension. 500 µL of each cell suspension was transferred to a separate Eppendorf tube and 500 µL of PBS added to make up to 1 mL. After resuspension, the cells were washed with PBS and fixed by gently adding 70% ethanol (perforates cells effectively to permit the dye to permeate the cells) in drops while vortexing. The fixed cells were stored in -20^0C before analysis. Cells were thawed and washed with PBS to remove ethanol, after which the cell nuclei were stained with DRAQ7 solution. The cells were analysed with FACS Calibur flow cytometry (Becton Dickinson) for relative DNA content based on fluorescence. Each of the G1, S, G2/M phases of the cell cycle was calculated using the RFIT program/Multicycle software, Phoenix Flow System USA.

Confocal Microscopy Analysis for Apoptosis

K562 cells that were treated with varied concentrations of ATO and DPQ both singly and combined for 48 h were washed once with PBS. A triple dye solution was prepared with 20µL of Annexin V, 15 µL of PI and 20 µL of 10X binding buffer in 130 µL of distilled water. 25 µL of the dye solution was added to the washed cell pellets. Cell nuclei were stained by the PI while the cell membrane was stained by the Annexin V/ FITC. After thorough mixing, smears of the stained cells were made on clean dry slides and allowed to air dry. Coverslips were carefully mounted centrally onto microscope slides using a mounting reagent containing DAPI. A drop of immersion oil was placed over the coverslip and the characteristics of cell morphology as related to apoptosis were evaluated by observing under a laser scanning microscope using 400X objective.

Statistical Analysis

The Kolmogorov-Smirnov Statistics was carried out to perform analysis of difference in distribution apoptotic cells of the control group and the drug treated groups. However, given the large sample size used for this study, the p-Value was not considered since any difference in distribution will seem significant. Therefore, the D-value will be considered I evaluating the effect size. The strength of the difference (D= 0 – 1) was rated as; $D < 0.1$ (negligible), $D = 0.1 – 0.3$ (weak), $D \geq 0.3$ (strong).

Results

Flow Cytometry Analysis for Apoptosis

The response of K562 CML cell line to treatment with 2.5 µM ATO only, 40 nM DPQ, and 2.5 µM ATO combined with 40 nM DPQ was determined using flow cytometry analysis are as shown in fig. 3.1.

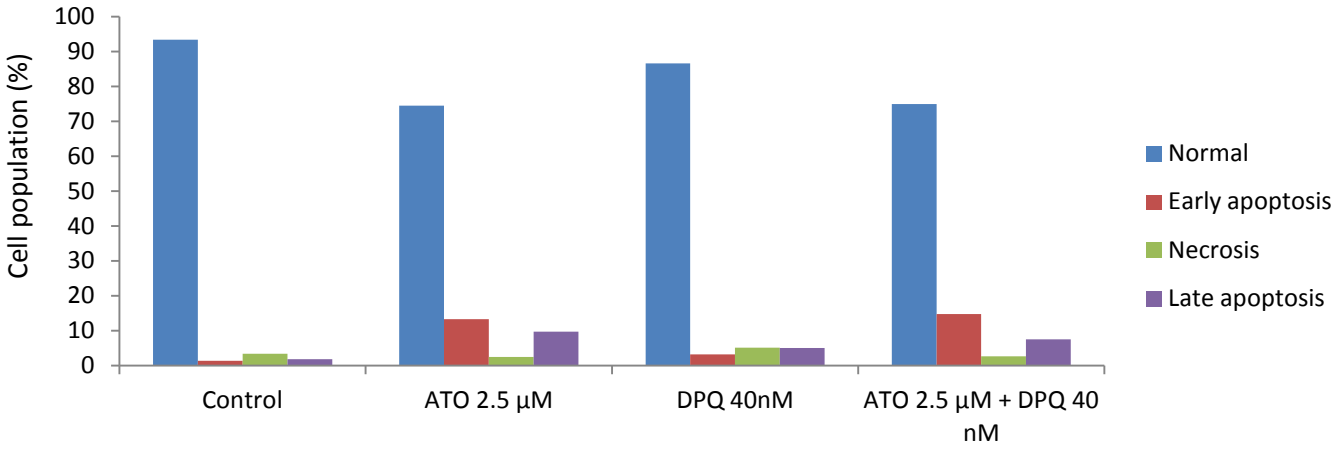

Fig 3.1.Cell apoptosis analysis using Flow cytometry after staining cells with Annexin V and PI apoptosis detection Kit

Cell Cycle analysis by flow cytometry

Annexin V- FITC/ PI dual staining flow cytometry is capable of distinguishing early apoptotic cells, late apoptotic cells as well as necrotic cells as presented in fig 3.2.

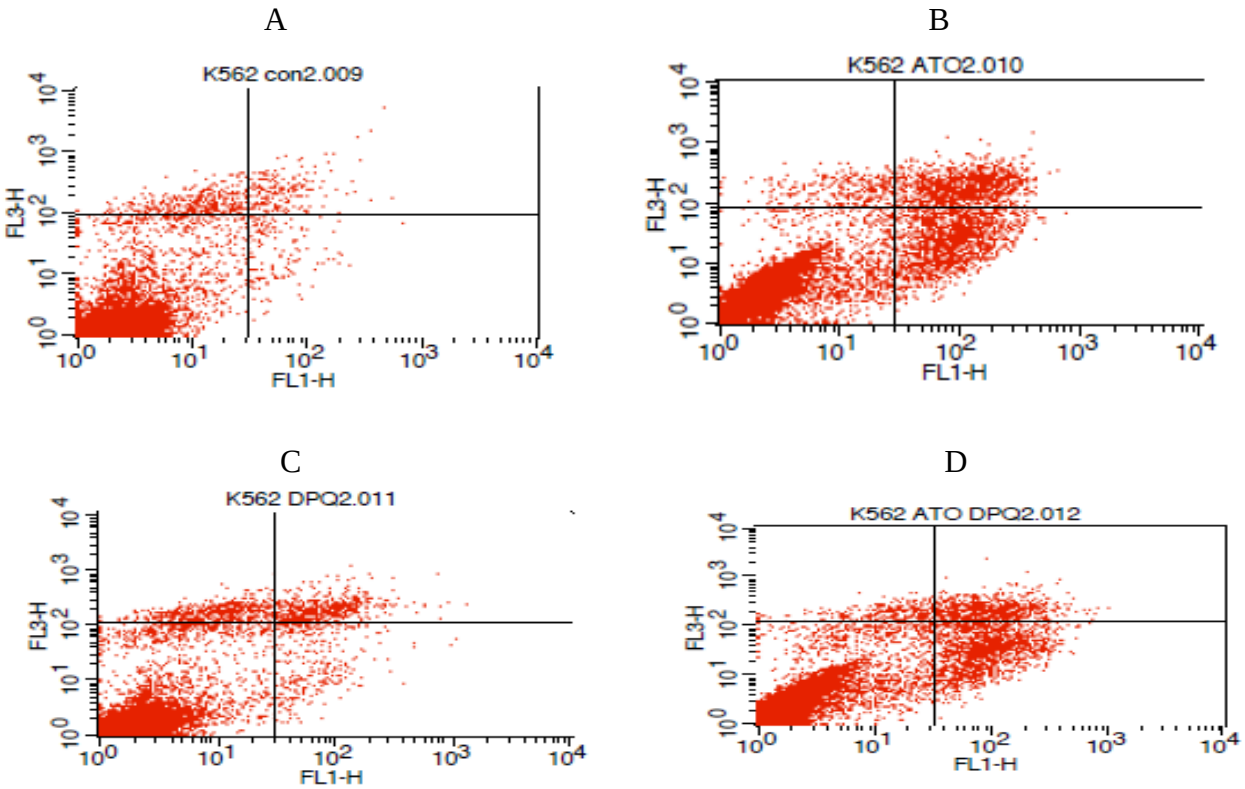

Fig 3.2 Dot plots showing distribution of unstained K562 CML cells and apoptotic cell population detected by AnnexinV/FITC and PI dual staining using flow cytometry.

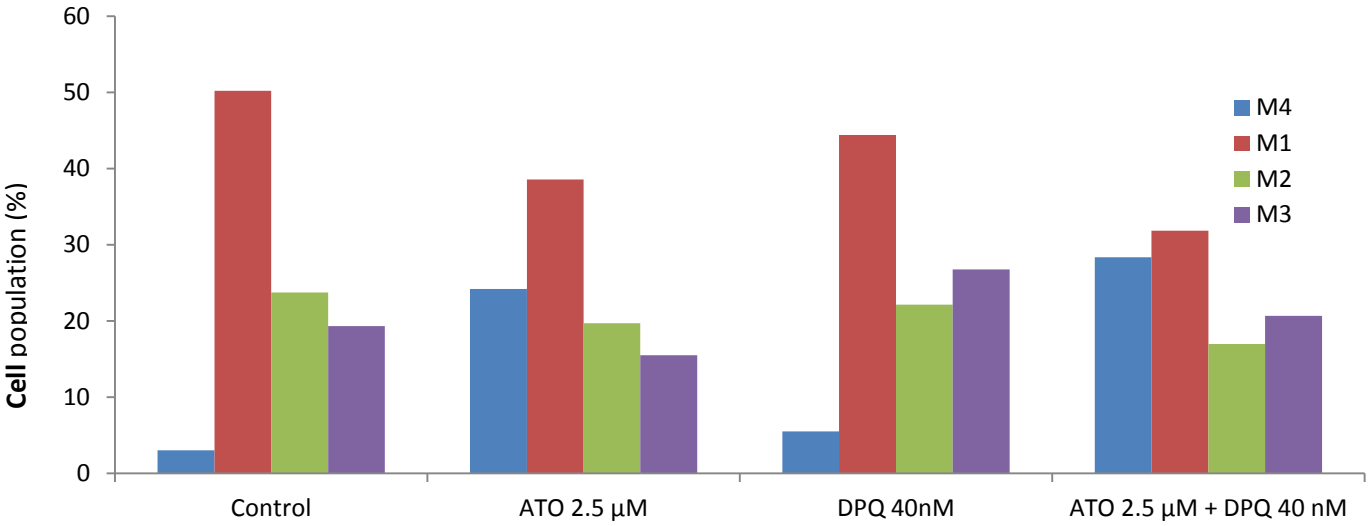

Fig 3.3 Cell cycle analysis (gated cells) Histogram of column charts representing effect of ATO and DPQ singly and combined on cell cycle of varying cell population.

The effects of ATO and DPQ or their combination on cell cycle distribution of K562 CML cells were examined. With the non-gated cells, (data not shown) the control cells with no drug treatment had 3.84% of the cell population in the sub-G1 phase, 53.31%, 27.31% and 14.14% cell population in G1, S and G2/M phases respectively. Histograms showing changes in cell cycle phase distribution of K562 CML cells (gated cells) when treated with ATO, DPQ and combination of DPQ to ATO are shown in fig. 3.4.After cells were gated, a slightly different result (figs 3.3& 3.4) was obtained compared to that of non-gated cells. For the control cells, the population of cells in the sub-G1 and G1 phases were 3.03% and 50.21% which are slightly lower than that in the same phase for non-gated cells. On the other hand, the cell population in the S and G2/M phases increased slightly compared to that of non-gated cells in the same phases to 23.73% and 19.33% respectively. In the ATO treated cell group, similar proportion of cells were observed in the sub-G1, G1 and S phases for both gated and non-gated cells.

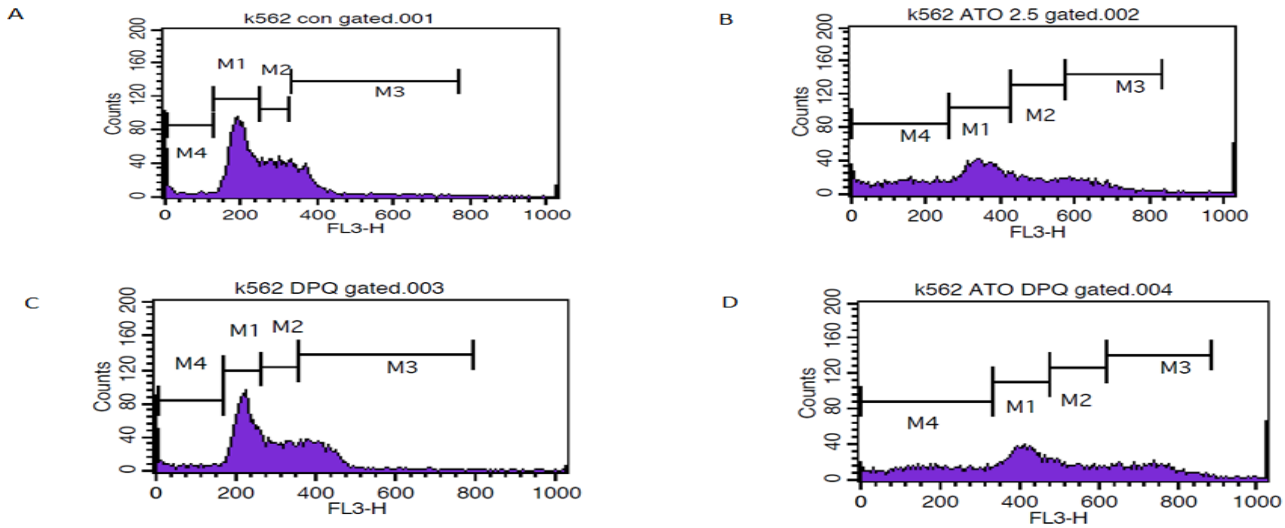

Fig 3.4 Histograms showing changes in cell cycle phase distribution of K562 CML cells (gated cells) when treated with ATO, DPQ and combination of DPQ to ATO.

Confocal microscopy analysis for apoptosis

As apparent on the micrograph representing control cells without drug treatment (fig. 3.5A), almost all the cells were stained blue by DAPI. Very few cells were stained green by Annexin V-FITC and red by PI. As shown on panel B (fig 3.5), a greater number of the cells treated with 2.5 µM ATO were stained green by Annexin V-FITC, while the others exhibited dual staining by both Annexin V-FITC and PI. Most of the cells appeared broken with visible nucleus. Majority of the cells treated with 40nM DPQ were stained red by PI (fig 3.5C). A few cells were stained green by Annexin V.

The cells treated with combined 2.5 µM ATO and 40 nM DPQ appeared damaged, looking rod-like and bizarre (fig. 3.5D). Very few cells were stained green by Annexin V-FITC whereas most of the cells were stained red by PI. The PI staining was more evident than the Annexin V-FITC. The cells treated with 2.5 µM ATO and 20nM DPQ shown in fig. 3.5E were stained by mostly green by Annexin V-FITC. Some cells were stained red by PI while a few cells were stained pink by Annexin V-FITC and PI combined. Finally, cells treated with half dose of ATO and full dose of DPQ had greater numbers of cells stained green by Annexin V-FITC (fig. 3.5F). Lesser population of cells was stained red by PI whereas few cells showed dual staining by both stains.

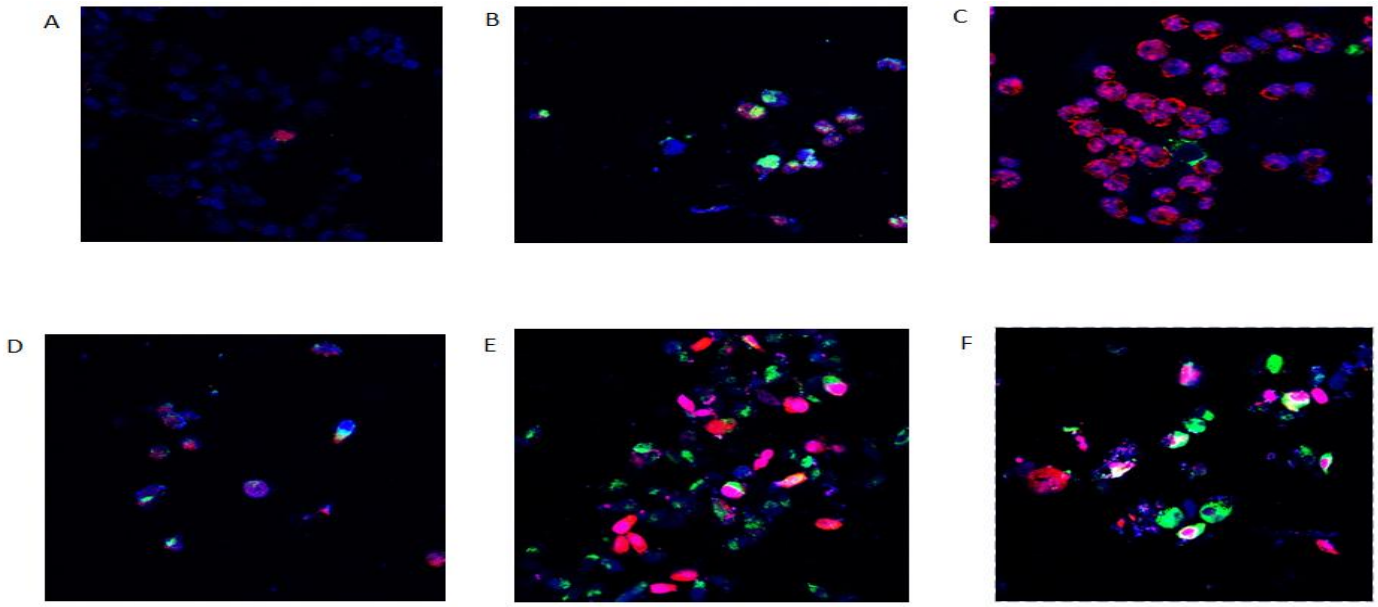

Fig. 3.5 Micrographs of morphological study of apoptotic cells induced by ATO and DPQ both singly and combined.

Discussion

Flow Cytometry Analysis for Apoptosis

Accumulated evidence has revealed that combination treatment with other therapeutic drugs helps in overcoming resistance to anticancer drugs [29]. This research aimed to explore whether PARP inhibitor, DPQ, was able to enhance ATO-induced apoptotic cell death in the K562 CML cell line. Firstly, results from the flow cytometry analysis of apoptosis showed that majority of the control cells were normal (viable), which was expected since the cells were not treated with any of the drugs. The few cells that died either by necrosis or early apoptosis in the control cell group may have been due to starvation or natural causes. In comparison to 1.36% of early apoptotic cells observed in control cell group after 48 h incubation, 13.31% of cells presented in early apoptosis with 2.5 µM ATO treatment, indicating that ATO alone induced early apoptosis in K562 CML cells. This is consistent with previous studies where ATO alone has been found to effectively induce cell apoptosis in CML cells but in a dose-dependent manner[30]. In a previous study, 2 µM ATO induced early apoptosis in 9.44% of K562 CML cells after 48 h incubation [31], which is comparable to the result obtained from

this study.[32] reported ATO's dose-dependent dual effect on cancer cells. Their report revealed that at low concentration ≤ 0.5 µM ATO induced cell differentiation but apoptosis at relatively high concentrations. In another study, 0.5 µM ATO was reported to induce cell differentiation in NB4 APL cells[33]. It was also reported that the level of ATO induced apoptosis in CML cells increased with concentration and in some cases, the drug induced apoptosis more effectively at concentrations up to 10 µM[34]. At such high concentrations, ATO induced many side effects including significant toxicity to normal cells and tissues[35].

Nonetheless, at clinically achievable concentrations like 0.5-2.5 µM, the effectiveness of ATO in inducing apoptosis is significantly reduced[36][37]. This often resulted in prolonged pro-apoptotic periods up to 7-10 days[38]. ATO's ability to effectively induce apoptosis in CML cells was also reported by[39] and[40]. ATO has been used effectively in the treatment of acute leukaemias especially APL, and its mechanism of action is mainly induction of apoptotic cell death[8]. It has been reported that anti- cancer drugs including ATO are capable of inducing DNA lesions in form of oxidized DNA adducts and DNA strand breaks which in turn affects normal DNA synthesis resulting in cell death[41]. Meanwhile ATO has been applied widely in the treatment of CML[42][43]. ATO was reported to be a potential drug in treatment of CML by[8], after their work confirmed that ATO effectively induced apoptosis in CML cell lines. On the other hand, the difference between the population of apoptotic cells of the cell group treated with DPQ alone and control cells was negligible (D = 0.08). Majority of the cells were normal and viable after DPQ only treatment indicating that DPQ alone did not have significant apoptotic effect on K562 CML cell line. Numerous studies have revealed that PARP inhibitors evaluated in clinical trials as single agents were effective mostly on cells carrying BRCA mutations owing to their mechanism of action[44]. PARP inhibitors exploit the concept of synthetic lethality in exerting their anti-cancer effect as a single agent, where combination or synthesis of mutations such as BRCA-1 or BRCA-2 results in cell death[45][46]. This may be the reason why the PARP inhibitor, DPQ did not have significant apoptotic effect on K562 CML cell line, since these cells lack BRCA germline mutation. However, research is on-going on the prospects of applying this concept to target non- BRCA mutant cancers. Again, these agents have been evaluated in clinical trials in combination with DNA damaging therapies[47][48][49]. The rationale for this is that PARP inhibitors prevent PARP-1 from repairing DNA damage induced by second DNA damaging drug, therefore, accumulation of damaged DNA results in cell death. Further, the flow cytometry analysis of apoptosis showed a slight increase (ATO alone –13.31%, ATO + DPQ- 14.83%) in cells presented in early apoptosis after cells were treated with a combination of ATO and DPQ indicating that the combination therapy induced slightly more apoptosis in K562 CML cell line than either agent alone. However, the difference was not of enough significance (D = 0.02) to be considered as either a synergistic or additive effect. Although this result does not support the hypothesis of this study that combination of DPQ with ATO may have the potential to enhance ATO-induced cell apoptosis in K562 CML cell lines, the combination can still be considered to have a positive outlook. A number of issues may have been responsible for the failure to achieve the desired results. Firstly, it can be inferred that using limited concentrations of each experimental drugs may have contributed to failure to obtain a positive result.[50] reported different results obtained from their research after treating MTLn3 cells with a series of ATO concentrations ranging from 20 µM to 1.22nM both singly and in combination with radiation.[25] reported that their study provided evidence that PARP- 1 inhibitor, 4AN synergistically improved ATO- induced apoptotic cell death via abrogation of ATO-induced G2/M checkpoint and prevention of DNA damage repair. As previously mentioned, the activation of PARP functions as a protective mechanism for cells in response to DNA damage, however, this action

contributes to drug resistance in cancer treatment [51][52]. Both in vitro and in vivo studies have agreed that PARP-1 activity is a crucial determinant of sensitivity of cells to chemotherapeutic drugs. Therefore, inhibiting its activity provides a positive therapeutic outlook[53][54]. Although the data from this research shows that combination of PARP inhibitor, DPQ failed to significantly enhance the apoptotic effect of ATO, this strategy can be considered to be a promising opportunity in developing a combination therapy in the treatment of CML but will require more studies. Further studies exploring the combination of varied concentrations of ATO and DPQ as well as incubating drug treated cells for different durations of time may yield a better outcome in terms of developing more efficient treatment strategies with improved efficacy and reduced cell toxicity.

Flow Cytometry Analysis for Cell Cycle

The cell cycle distribution was analysed using DRAQ7 staining to stain the cell DNA and determine accumulation of cells in various phases of the cell cycle after treatment with ATO, DPQ and ATO in combination with DPQ. DRAQ7stain labels dead as well as permeabilized cells. Further, DRAQ7 offers the advantages of chemical stability, easy compatibility with Annexin V/FITC/PI stained samples as well as specific binding to nuclear DNA with high affinity, thus requiring no further procedure before flow cytometry analysis[55]. Cells were gated to remove doublets, triplets and clumped cells in order to generate better information about the cells since single cells are assessed. The results showed that treatment of K562 cells with ATO alone resulted in accumulation of cells in the sub-G1 region reflecting apoptotic cells. The difference in population of accumulated cells in this region compared to the control indicated that ATO alone induced apoptosis in these cells. This is consistent with the result of a previous study by[55] who reported accumulation of sub-G1 phase cells containing hypodiploid amounts of DNA and increased apoptotic rate after treating K562 cells with ATO.

In contrast to the result obtained with ATO only treatment, no significant increase in cell population in the sub-G1 region was observed with DPQ only treatment compared to the control. This indicates no significant apoptotic effect of DPQ on the CML cell lines. Further, the population of DPQ treated cell group in the G1 and S phases were less than that in the same phases of control cells indicating progression to the next phase whereas accumulation of cells were observed in the G2/M phase meaning that DPQ induced G2/M phase arrest. DPQ potentially inhibits PARP-1 to impair DNA repair.

Furthermore, k562 cells accumulated slightly more in the sub-G1 region after treatment with the combination of ATO and DPQ compared to treatment with either drug alone, reflecting more apoptotic effect. However, the cell population in this region was not significantly higher than that seen with the solely ATO treated cell group indicating that DPQ failed to significantly promote ATO-induced sub-G1 phase cells. This augments the result earlier obtained from the analysis of apoptosis that DPQ did not significantly promote ATO-induced cell death. There was no accumulation of cells in the G1 and S phases of cell cycle compared to the control cells indicating absence of G1 and S phase arrest. Partial cell cycle arrest was observed in the G2/M phase with the combined drug treatment. The population of cells in the combined therapy induced G2/M arrest were less than that in the DPQ induced G2/M checkpoint (figure 3).

Confocal Microscopy Analysis for Apoptosis

The confocal fluorescence microscopy visual result showed that individual treatment with ATO induced early apoptosis in K562 CML cell line. However, the combination treatment failed to show significant additive effect in inducing cell apoptosis. Annexin V- FITC and PI stain cells only when they have undergone apoptosis while DAPI stains nuclei of all cells without differentiating between live or dead cells. Earliest detectable apoptotic events involve the cell membrane flipping open with the inner cell protein, phosphatidyl serine (PS) being translocated to the outer leaflet of the plasma membrane[56].

Annexin-V/FITC labelled antibodies bind to PS to identify early apoptotic cells whereas PI binds to nucleic acids to stain the nuclear material in late apoptosis[57][58]. Almost all the control cells showed homogenous staining of cell nuclei with DAPI and were devoid of both red and green fluorescence which is an indication of intact plasma membrane (fig. 5A). This is not surprising since these cells were not treated with any of the drugs. The minority of cells stained red by PI indicating necrosis. It is not unreasonable to infer that the few cells that died were probably as a result of starvation due to congestion and insufficient nutrients or by natural programmed cell death. Treatment of cells with ATO alone induced early apoptosis in majority of the cells as evident with increased green fluorescence emitted by a greater number of cells (fig. 5B). This correlates with results obtained in previous studies on the effect of ATO on CML cell lines. It has been reported that ATO induced cell apoptosis in K562 CML cell line in a time and dose dependent manner.

In contrast, almost all the cells treated with DPQ 40nM were stained red by PI (fig 5C), indicating necrosis. According to[59], apoptosis and necrosis are two processes that often occur independently, sequentially and simultaneously.

More cells had intense Annexin V-FITC staining indicating early apoptosis. However, a few living cells were present, indicated by blue staining only. Finally, cells treated with half dose of ATO and full dose of DPQ had large numbers of early apoptotic cells compared to that observed with other combination concentrations (figure 5F). There is clearly membrane staining with Annexin V as expected. Cells were not distorted. This is consistent with previous studies that proposed low dose combination therapy. Several studies have adopted low-dose combination therapies with different levels of breakthroughs recorded[60].

Comparison of flow Cytometry and Confocal Analysis for Apoptosis

Taken together, results of analysis of apoptosis obtained from flow cytometry is consistent with that of confocal fluorescent microscopy showing that ATO alone induced apoptosis in K562 cells while treatment of cells with DPQ only had a necrotic rather than apoptotic effect on the cells. In addition, data from both experimental techniques concurred that combination of DPQ to ATO failed to significantly enhance apoptosis in K562 CML cell line.

Conclusion

Numerous studies have explored the therapeutic effects of combining arsenic trioxide with conventional chemotherapeutic agents mostly in the treatment of APL and other haematological malignancies including CML. Its use in the treatment of CML both singly and in combination with other anti-cancer agents is well documented. Taken together, the results of this research showed that ATO alone induced apoptosis in K562 CML cell line. DPQ alone had a necrotic rather than apoptotic effect on the leukaemia cell line. Unexpectedly, targeting PARP-1 with DPQ, a potent PARP-1 inhibitor failed to significantly enhance the apoptotic effect of ATO. In addition, the cell cycle analysis results showed that ATO treated cells were not arrested in any of the cell cycle phases whereas DPQ alone and combined ATO and DPQ treated cells did show G2/M arrest. Furthermore, combining ATO with DPQ at different concentrations induced different degrees of apoptosis on K562 CML cells. For instance, combination of ATO 1.25 µM and DPQ 40 nM had a better apoptotic effect than combination of ATO 2.5 µM and DPQ 40 nM as well as ATO 2.5 µM and DPQ 20 nM. Although no significant synergistic effect of combination of ATO and DPQ on apoptosis of K562 CML cell line was observed in this study, continued research on combination therapy using varied concentrations of these two anti-cancer agents at different incubation periods will allow the identification of the most effective and safest treatment regimen for CML treatment.

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