Archives of Pharmaceutical Sciences and Biotechnology Journal

Volume 2 Issue 1 January, 2022 Page 29 - 42 ISSN: 2971_611X
APSB-03

APSBJournal

DETERMINATION OF APOPTOTIC EFFECT OF ASPIRIN AND ITS ANALOGUES ON AN ADHERENT CELL LINE USING FLOW CYTOMETRY: PROBLEMS AND POSSIBLE SOLUTIONS

***Bashir AIJ[a], Perry CJ[b], Nicholl ID[b] and Safrany ST[c]**

[a]Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Kaduna State University, Kaduna-Nigeria.
[b]Research Institute in Healthcare Science, University of Wolverhampton, Wolverhampton, WV1 1LY, UK.
[c]RCSI Bahrain, P.O.Box 15503, Adliya, Kingdom of Bahrain.
***Corresponding Author:** Bashir AIJ **E-mail:** asmau.junaidu@yahoo.com **Phone**: +2348100348071

ABSTRACT

Aim: To determine the apoptotic effect of aspirin and its analogues on an adherent cell line using Flow cytometry and whether a change in the isomeric positions of the acetyl group to the benzene ring in these compounds might be responsible for any change in behaviour. Also, to observe the problems encountered together with possible solutions
Study design: Qualitative and Quantitative.
Place and Duration of Study: This study was conducted in the Research Institute in Healthcare Sciences, University of Wolverhampton, UK.
Methodology: Flow cytometry and immunocytochemistry (ICC) were used to determine the apoptotic effects of aspirin and its analogues on SW480 CRC cell line. Annexin-V dye was used to detect apoptotic cells and PI to detect necrotic cells in flow cytometry, while YO-PRO®-1 and propidium iodide (PI) dyes were employed to detect apoptosis and necrosis respectively in ICC, using confocal microscopy to view cells.
Results: Using flow cytometry, 30±8.9% of the cell population were necrotic after 16 h and this did not change even after 40 h of treatment with *meta*- (PN548) and *para*- (PN549) aspirin. On the other hand, the percentage of cell population treated with *ortho*-thioaspirin (PN590) were significantly necrotic with a small population apoptotic. But it's *meta*- (PN591) and *para*- (PN592) isomers did not significantly cause apoptosis or necrosis with most of the cell population live/healthy even after 40 h of treatment. It was observed that flow cytometry showed more "necrotic" cells than ICC when treated with staurosporine (apoptotic control), H_2O_2 (necrotic control) and aspirin analogues.
Conclusion: It was concluded that aspirin and its analogues with the exception of PN591 and PN592 had some apoptotic effect after 16 h. However, a high necrotic effect was observed after 40 h, which was not observed using ICC. In order to obtain unambiguous and reliable results, it was concluded that flow cytometry, a popular method in the detection of apoptosis in various cell lines, is less suitable for adherent cell lines, unless additional measures are taken. Unfortunately, these additional measures will result in additional costs. Further research using ICC with the inclusion of DAPI (4',6-diamidino-2-phenylindole), a blue fluorescence dye that stains the nucleus is required for accurate quantitative results.
Keywords: Apoptosis, Adherent cell line, Flow cytometry, Immunocytochemistry (ICC), Necrosis, SW480 CRC cells.
Abbreviations: Colorectal cancer (CRC), immunocytochemistry (ICC), programmed cell death (PCD), propidium iodide (PI), 4', 6-diamidino-2-phenylindole (DAPI).

Archives of Pharmaceutical Sciences and Biotechnology Journal

Volume 2 Issue 1 January, 2022 Page 29 - 42 ISSN: 2971_611X

APSB-03

APSBJournal

INTRODUCTION

Studies have shown Aspirin, a non-steroidal anti-inflammatory drug (NSAID) known for its anti-inflammatory actions, can also be used as prophylaxis against development and reoccurrence of CRC [1]. Due to the side effect of gastrointestinal bleeding associated with the prolonged use of aspirin especially in the elderly [2], there has been the hunt for analogues of aspirin with same or greater effects but a safer profile than aspirin itself [3]. Given the reported apoptotic effects of aspirin on cancer cell lines, we sought to investigate the effects of synthesized aspirin analogues on SW480, an adherent colorectal cell line. Amongst the aspirin analogues synthesized [4], the *meta*- and *para*- isomers of aspirin were found to exhibit the same biological profiles as the *ortho*- isomer accompanied with safer profiles in regard to gastrointestinal (GI) toxicity [5]. This study investigates whether these compounds [aspirin, meta-aspirin (PN548), para-aspirin (PN549), thioaspirin (PN590), meta-thioaspirin (PN591) and para-thioaspirin (PN592)], cause cell death via apoptosis, necrosis or a mixture of both; and whether a change in the isomeric positions of the acetyl group to the benzene ring might be responsible for this change in behaviour.

PCD (Programmed Cell Death) studies were carried out using and comparing two different methodologies - Flow cytometry and Immunocytochemistry (ICC).

Definitions:

***Cell/Tissue culture*:** This is the growth of cells derived from living tissue in an artificial medium. It was discovered over a 100 years ago by Ross Harrison and modified a few years after by Alexis Carrel [6]. Cell/tissue culture is commonly used *in vitro* in a lot of fields for improving our understanding of cell biology, tissue morphology and mechanisms of diseases and action of drugs, which could be in form of 2D or 3D models [7].

***Adherent cell line*:** Adherent cell lines are *in vitro* model systems that are extensively used in different areas of medical research for drug discovery and cancer research [8]. They are sometimes referred to as monolayers and are cells which must be attached to a surface to grow.

Programmed Cell Death: Programmed cell death was classified as occurring via two separate morphological patterns, one of which is known as 'coagulative necrosis' where there is swelling and rupture of organelle membranes and disintegration of their structure while the other pattern is known as apoptosis. In apoptosis, the cell organelles maintain their integrity with the membrane forming different bulges that eventually separate and form membrane-bound globules ready to be phagocytized [9]. However, it is now believed that programmed cell death (PCD) occurs via different pathways with more than one of these pathways triggered simultaneously [10,11]. Cell death could be as a result of apoptosis, necrosis or autophagy [10,11]. PCD is particularly important in the life cycle of a cell as a balance between cell proliferation and death, which is essential for the maintenance of homeostasis [12]. During apoptosis, the phosphatidylserine (PS) normally found in the inner leaflet of the phospholipid cell membrane flips and is exposed [10, 13].

As opposed to necrosis, apoptosis is the preferred method for drug-induced cell death, otherwise the leaked cell contents cause

Archives of Pharmaceutical Sciences and Biotechnology Journal

Volume 2 Issue 1 January, 2022 Page 29 - 42 ISSN: 2971_611X
APSB-03

APSBJournal

damage to neighbouring cells in an uncontrolled manner. However, more often these patterns of cell death go hand in hand [10]. Staurosporine, a potent kinase inhibitor [14], has been shown to induce apoptosis in a number of cell lines [15,16,17] while hydrogen peroxide (H_2O_2), a reactive oxygen species is produced naturally during metabolism and high levels causes necrosis [17,13]. Thus, Staurosporine and H_2O_2 were used as positive controls for apoptosis and necrosis respectively.

Importance of Cell Culture in Research:

The first cell cultures were carried out in 1907 by Harrison during his research into the origin of nerve fibres [19]. These days, primary cells isolated from human donors or an established cell line from bioresource centres, such as the ATCC (American Type Culture Collection) are used to carry out experiments because they closely mimic the *in vivo* genetic characteristics of tumours, thus, making it an important model for cancer research [7,20].

Cell culture provides a model system for the study of the physiology and biochemistry of selected cells [21]. Through this, the metabolic rates and mechanisms of action of drugs can be discovered. Generally, 2D cell culture is preferred due to its simplicity and low cost, however, 3D cell culture is increasingly being adopted for its ability to mimic *in vivo* conditions [22]. Cell culture in today's day and age is the stepping stone in drug discovery research and also minimizes the unnecessary use of live animals in scientific research, therefore contributing to the welfare of these laboratory animals. Unfortunately, this mode of research has not been fully adopted in the Nigerian research world due to high cost of equipment, lack of stable power supply and high maintenance of these cell lines.

MATERIALS AND METHODS

The materials used include BD Accuri™ C6 flow cytometer (Becton Dickinson, UK), Non-vented tissue culture flasks (Sarstedt Ltd., UK), Square cover glass [22 X 22 mm] (AmScope), Zeiss LSM 880 Confocal Microscope (Carl Zeiss Ltd., UK), Annexin-V-FLUOS Staining kit (11 858 777 001, Roche Diagnostics Ltd., UK), Vybrant® Apoptosis Assay Kit #4, YO-PRO®-1/Propidium Iodide (V13243, Life Technologies Corporation), Vectashield® Mounting Medium (Vector Laboratories Inc., UK).

Detection of apoptotic and necrotic cells on SW480, an adherent CRC cell line using Flow Cytometry and ICC was carried out and the two methods compared. Annexin-V dye was used to detect apoptotic cells and PI to detect necrotic cells in Flow Cytometry while YO-PRO®-1 and PI dyes were employed to detect apoptosis and necrosis respectively in ICC, using confocal microscopy to view cells.

Tissue culture

The SW480 CRC cell line (ECACC, Salisbury, UK) was cultured in Leibovitz's L-15 medium (Thermo Fisher Scientific) supplemented with 10% (v/v) FBS (Fetal Bovine Serum) and 1% (v/v) penicillin-streptomycin. The cells were cultured at 37°C in a humidified incubator and passaged at approximately 80% confluency.

Compounds

Aspirin, Staurosporine and H_2O_2 were purchased from Sigma® Sigma-Aldrich

Archives of Pharmaceutical Sciences and Biotechnology Journal

Volume 2 Issue 1 January, 2022 Page 29 - 42 ISSN: 2971_611X

APSB-03

APSBJournal

Company Ltd., UK. PN548, PN549 and PN590 were a gift from Dr. Christopher Perry and Dr. Iain Nicholl.

Thioaspirin analogues were synthesised as previously published [4,23].

Flow cytometry

Flow cytometry was used to sort out the population of cells (Figure 1) into apoptotic cells that are stained with Annexin V due to interaction with phosphatidylserine exposed by the flipped cell membrane [24] and necrotic cells that are stained by Propidium Iodide (PI) due to its ability to penetrate the nucleus of dead cells [25].

2 X 10^5 of SW480 cells per well were plated out in a 6-well tissue culture plate and left in the incubator for 24 h after which they were treated with either aspirin analogues, Staurosporine as a positive control for apoptosis [26] or H_2O_2 as a positive control for necrosis [27]. The cells were washed with PBS and then dissociated from the wells using trypsin 0.25% (w/v) after 16 h and 40 h, and centrifuged at 200 X g for 5 min to separate cell pellets from the supernatant media. 0.25% (w/v) trypsin lacking EDTA was used in order to minimize damage to the cell membrane. The cell pellets were then washed in PBS and centrifuged again. The cells were then re-suspended in binding buffer containing the dyes FITC-Annexin V and PI. After 15 minutes of incubation at room temperature, each sample was then analysed using the BD Accuri™ C6 Flow Cytometer installed with the BD Accuri™ C6 software (Version 1.0.264.21). Cells were treated with compounds at 0.5 mM, 0.3 mM and 0.1 mM, staurosporine at 500nM [28] and H_2O_2 at 5 mM. 10,000 cells were acquired for each experimental condition.

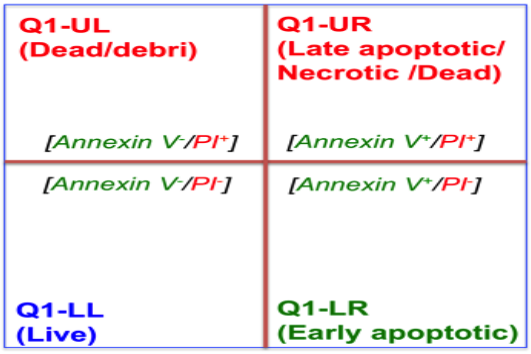

Figure 1. Gating of Cells Undergoing Apoptosis and Necrosis using Flow Cytometry

The cytogram (Figure 1) is divided into four different quadrants in which cell populations are grouped according to the fluorescence dye it is stained with. Cell populations that do not take up any of the dyes are the live healthy cells and are grouped in the lower-left quadrant (Q1-LL) as shown by the BD Accuri™ C6 software. Cells that take up the Annexin V dye are those undergoing early apoptosis and are grouped in the lower-right quadrant (Q1-LR). Cell populations that take up both dyes are undergoing late apoptosis, necrosis or considered dead and fall in the upper-right quadrant (Q1-UR). Populations that only take up the PI dye are considered either dead cells or debri and fall in the upper-left quadrant (Q1-UL) [29].

Immunocytochemistry (ICC) YO-PRO®-1

Apoptotic and necrotic cells were differentiated using YO-PRO®-1 and PI dyes respectively.

YO-PRO®-1 is a green fluorescent dye that selectively passes through the membrane of apoptotic cells whereas dyes like the red fluorescent PI cannot [30]. Thus, a

Archives of Pharmaceutical Sciences and Biotechnology Journal

Volume 2 Issue 1 January, 2022 Page 29 - 42 ISSN: 2971_611X
APSB-03

APSBJournal

combination of these two dyes provides a sensitive assay for apoptosis.

SW480 CRC cells were plated on glass cover slips in 6-well-plates to obtain 70% confluency after 24 h. These cells were then treated with the compounds of interest for 16 and 40 h after which they were washed with cold PBS once and then treated with 1 µl each of YO-PRO®-1 and PI stock solution in 1 ml of PBS per well on ice for 30 min. This step was then followed by two washes with cold PBS, left on ice in PBS for a further 30 minutes and then coverslips placed onto a drop of VectaShield® mounting-medium. Microscopy was performed using a Zeiss LSM 880 confocal microscope, equipped with 405 and 561 nm excitation lasers using 40X/1.30 oil immersion DIC M27 objective. The Zeiss: ZEN2 (blue edition) software was used to process images.

SW480 CRC cells were treated with the isomers of these aspirin analogues at 0.5 mM with the exception of PN590, which was treated at 0.1 mM because of its high cytotoxic effect, all for 16 h and 40 h, using staurosporine and H_2O_2 as positive controls for apoptosis and necrosis respectively.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 7 software (GraphPad software Inc., San Diego, CA, USA). The test used to determine statistical significance was ordinary one-way ANOVA. Differences between control and test groups were considered as statistically not significant at $p>0.05$, and significant at $p\leq0.05$, $p\leq0.01$. Where a significant difference was obtained, Dunnett's Post hoc analysis was carried out.

RESULTS & DISCUSSION

Studies have shown aspirin to have an apoptotic effect on colorectal cancer cells both *in vitro* and *in vivo* [1,31]. We investigate the effects of these aspirin analogues, Staurosporine and H_2O_2 on SW480 CRC cell line using Flow cytometry and ICC.

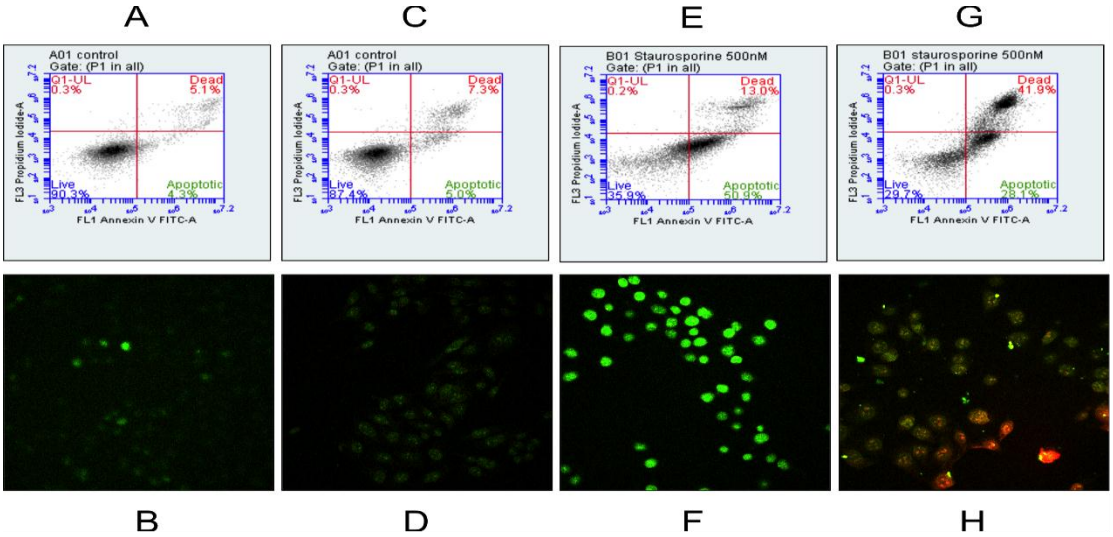

Figure 2. Representation of flow cytometric analysis and ICC for negative control (untreated) and positive control for apoptosis in SW 480 CRC cell line

Archives of Pharmaceutical Sciences and Biotechnology Journal

Volume 2 Issue 1 January, 2022 Page 29 - 42 ISSN: 2971_611X
APSB-03

APSBJournal

(A) Flow cytogram of negative control [untreated cells] after 16 h (B) ICC slide of negative control [untreated cells] after 16 h (C) Flow cytogram of negative control after 40 h (D) ICC slide of negative control after 40 h (E) Flow cytogram of cells treated with staurosporine for 16 h (F) ICC slide of cells treated with staurosporine for 16 h (G) Flow cytogram of cells treated with staurosporine for 40 h (H) ICC slide of cells treated with staurosporine for 40 h. The fluorescent dyes used for flow cytometry are Annexin-V-FITC (green) for apoptotic cells and PI (red) for necrotic cells. The fluorescent dyes used for ICC were YO-PRO®-1 (green) for apoptotic cells and PI (red) for late apoptotic/necrotic cells. Representative confocal images were taken at 40X oil/1.30 oil immersion objective. *n=3.*

Flow cytometric analysis of untreated cells detected the presence of dead cells after 16 h (Figure 2A) and the percentage increased after 40 h (Figure 2C) of incubation. However, using ICC, dead cells were not detected in the untreated cells after 16 h (Figure 2B) and 40 h (Figure 2D). The treatment with staurosporine in both flow cytometry (Figure 2E) and ICC (Figure 2F) yielded a significant amount of apoptotic cells after 16 h. However, a small percentage, 10.7±1.2% (*n=3*) of dead cells was seen with flow cytometry (Figure 2E). Both methods detected a combination of apoptotic and late apoptotic/necrotic cells after 40 h of treatment (Figure 2G and 2H).

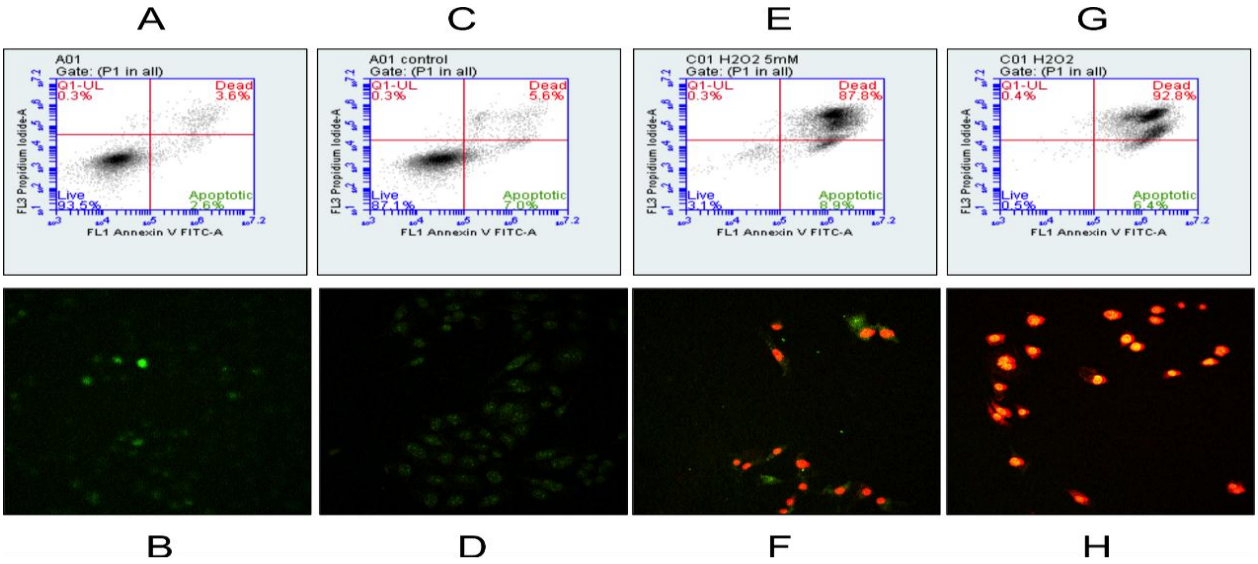

Figure 3: Representation of flow Cytometric Analysis and ICC for Negative control (untreated) and Positive control for Necrosis in SW 480 CRC Cell Line

(A) Flow cytogram of negative control [untreated cells] after 16 h (B) ICC slide of negative control [untreated cells] after 16 h (C) Flow cytogram of negative control after 40 h (D) ICC slide of negative control after 40 h (E) Flow cytogram of cells treated with H_2O_2 for 16 h (F) ICC slide of cells treated with H_2O_2 for 16 h (G) Flow cytogram of cells treated with H_2O_2 for 40 h (H) ICC slide of cells treated with H_2O_2 for 40 h. The fluorescent dyes used for flow cytometry are Annexin-V-FITC (green) for apoptotic cells and PI (red) for necrotic cells. The fluorescent dyes used for ICC were YO-PRO®-1 (green) for apoptotic cells and PI (red) for late apoptotic/necrotic cells. Representative confocal images were taken at 40X oil/1.30 oil immersion objective. *n=3.* *(Figure 2A-D are same as Figure 3A-D)

Archives of Pharmaceutical Sciences and Biotechnology Journal

Volume 2 Issue 1 January, 2022 Page 29 - 42 ISSN: 2971_611X
APSB-03

APSBJournal

The flow cytometric analysis (Figure 3E) showed 11.5±3.7% apoptotic cells with 82.1±6.1% being late apoptotic/necrotic in samples treated with H_2O_2 for 16 h, which correlates with the ICC slides (Figure 3F) showing most of the cells fluorescing red-yellow and few fluorescing green. 90.9±4.0% of the SW480 cells turned necrotic (Figure 3G) fluorescing red (Figure 3H) after 40 h of treatment with 5 mM H_2O_2.

Sequel to staurosporine and H_2O_2 producing very similar outcomes from both analysis, they were deemed fit to serve as reliable controls for detecting apoptotic and late apoptotic/necrotic cells respectively in SW480 CRC cell line. The cells were then treated with the compounds of interest.

The Flow cytometric analysis detected a large number of necrotic cells after treatment with 0.5 mM of aspirin for 16 h and a larger percentage of late apoptotic/necrotic cells after 40 h of treatment. 4.7±1.6% of the cell population was apoptotic after treatment with aspirin for 16 h. This increased to 16.6±9.6% after 40 h of treatment with aspirin. The level of apoptotic cells detected agrees with the qualitative data from the ICC method, however, necrotic cells were not seen after 16 h and 40 h. 5.3±1.3% of the SW480 cells became apoptotic after treatment with 0.5 mM of PN548 for 16 h, which increased to 14.2±6.6% after 40 h of treatment and 34.1±10.9% necrotic cells. Qualitatively, most of the cells were seen to be apoptotic after 16 h of treatment with some of the cells necrotic after 40 h of treatment.

With flow cytometry, apoptotic cells increased from 5.3±1.5% after being treated with 0.5 mM of PN549 for 16 h to 19.4±9.4% after 40 h of treatment. After 16 h and 40 h of treatment with PN549, 33.2±8.0% and 34.5±7.3% of cells in the late apoptotic/necrotic region were respectively detected. Although this appears like a large number of late apoptotic/necrotic cells detected by this method, the same is observed from slides analyzed by ICC method. This qualitative image was also similar to the positive control, staurosporine image.

Flow cytometric analysis detected 51.1±18.6% and 45.3±11.7%, a large percentage of late apoptotic/necrotic cells after treatment with 0.1 mM PN590 for 16 h and 40 h respectively. The apoptotic cells remained the same 10.0±6.1% after 16 h of treatment and 11.5±2.6% after 40 h. The ICC slides after 40 h treatment appear yellow in fluorescence unlike the brownish/red fluorescence seen when cells were treated with H_2O_2.

Treatment with 0.5 mM PN591 after a duration of 16 h resulted in 4.0±1.9% of apoptotic cells using flow cytometry. The presence of more apoptotic cells with an increase in duration of treatment was reflected by the ICC confocal qualitative images as the apoptotic cells emitted a bright green fluorescence.

Apoptotic cells increased after treatment with 0.5 mM PN592 from about 6.3±1.6% after 16 h to about 10±3.2% after treatment for 40 h (Figure 4) as shown by Flow Cytometric analysis. About 16.3±7.1% of necrotic cells was detected using Flow Cytometry after 16 h and 21.3±6.2% after 40 h (Figure 5). However, necrotic cells were not detected using the ICC method.

Archives of Pharmaceutical Sciences and Biotechnology Journal
Volume 2 Issue 1 January, 2022 Page 29 - 42 ISSN: 2971_611X
APSB-03
APSBJournal

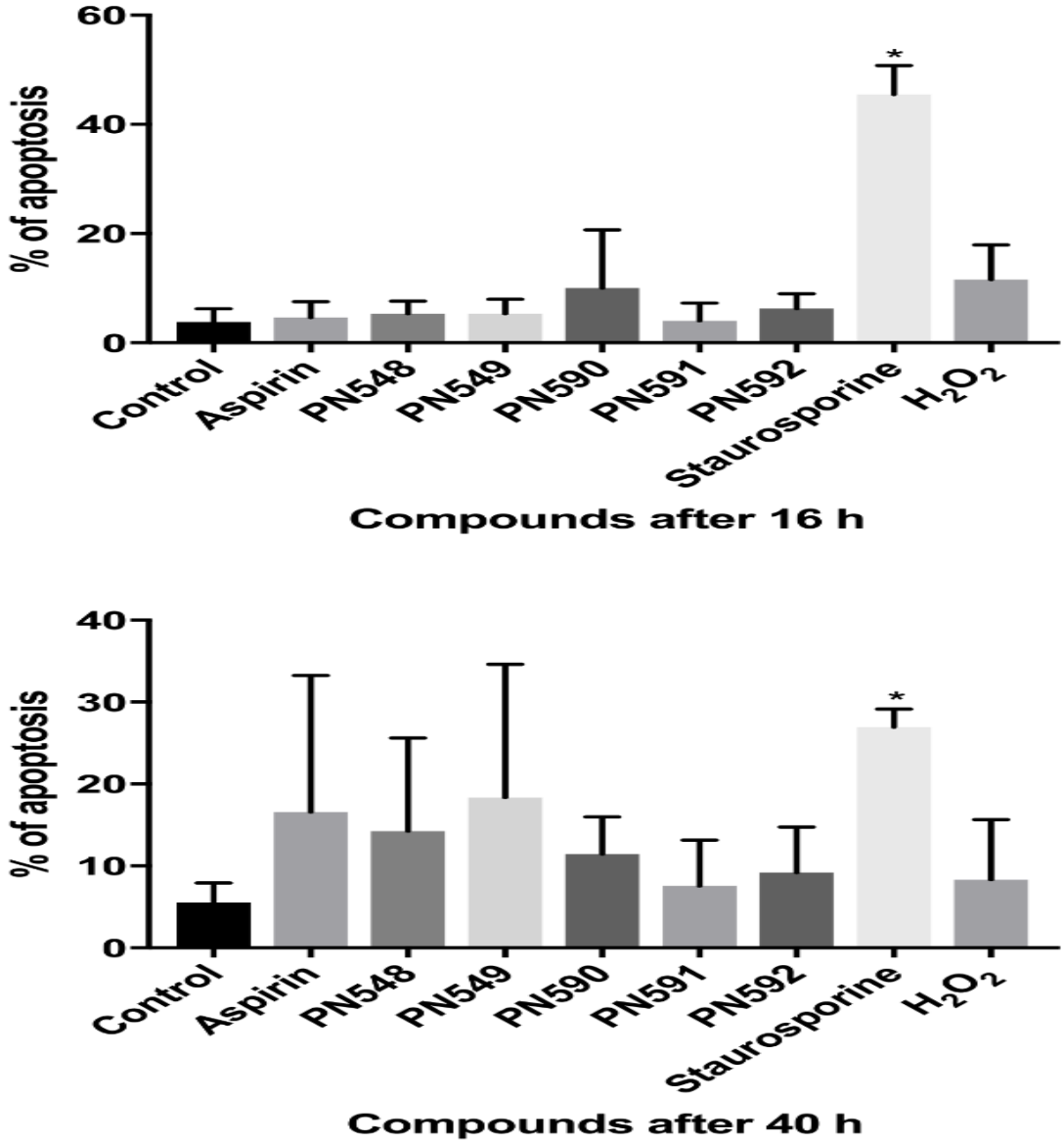

Figure 4: Flow Cytometric Analysis of Aspirin Analogues showing Induced Apoptosis in SW480 CRC cell line

SW480 CRC cells were treated with compounds for 16 h and 40 h, stained with Annexin-V-Fluos and counterstained with PI fluorescence dye and analysed using flow cytometry. Data plotted as mean ± SEM (*n=3*), $^{\star}P \leq 0.05$

Archives of Pharmaceutical Sciences and Biotechnology Journal
Volume 2 Issue 1 January, 2022 Page 29 - 42 ISSN: 2971_611X
APSB-03
APSBJournal

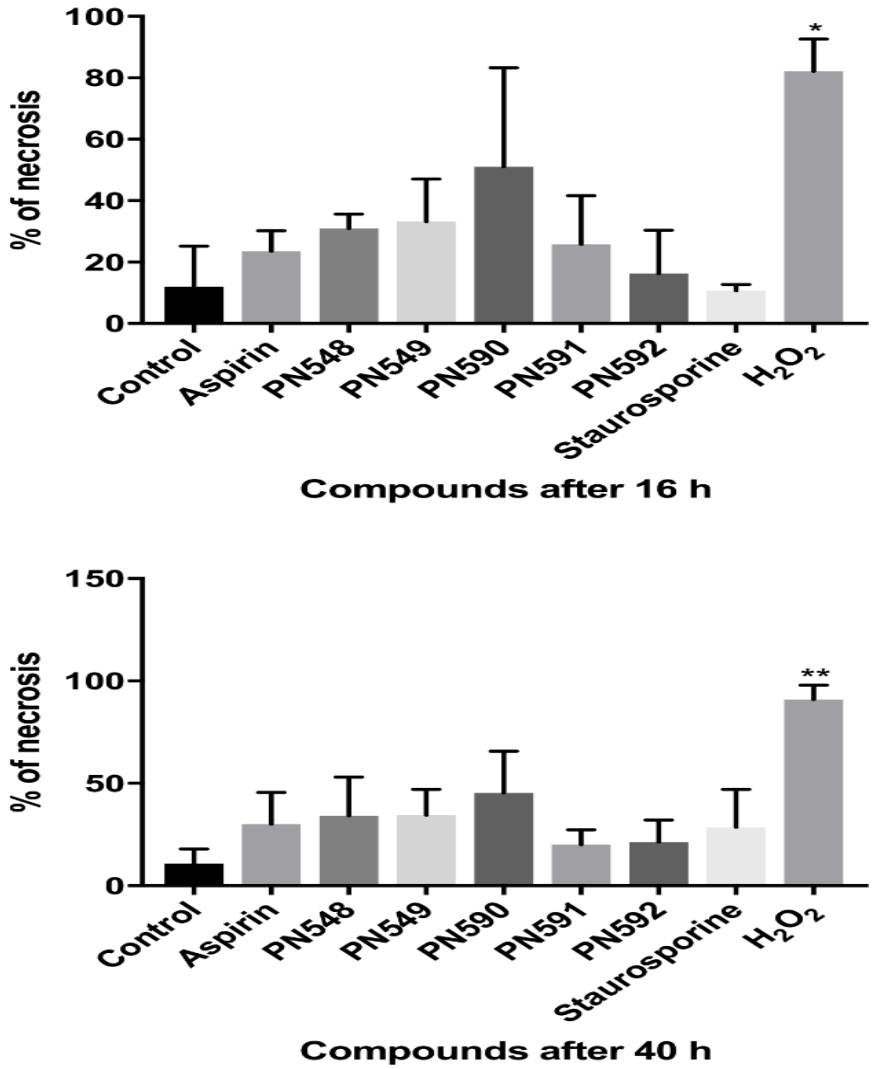

Figure 5. Flow cytometric analysis of aspirin analogues showing induced late apoptosis/necrosis in SW480 CRC cell line

SW480 CRC cells were treated with compounds for 16 h and 40 h, stained with Annexin-V-Fluos and counterstained with PI fluorescence dye and analysed using flow cytometry. Data plotted as mean ± SEM (*n=3*) $^{\star}P \leq 0.05, ^{\star\star}p \leq 0.01$.

Archives of Pharmaceutical Sciences and Biotechnology Journal

Volume 2 Issue 1 January, 2022 Page 29 - 42 ISSN: 2971_611X
APSB-03

APSBJournal

The pooled Flow cytometric data revealed the percentage of induced apoptosis in SW480 cells to be quite low after treatment with all aspirin analogues for 16 h. However, the percentage apoptotic cells did increase after 40 h of treatment with the exception of Staurosporine, the compound used as a positive control to apoptosis (Figure 4). Necrotic/dead cells also increased in population after 40 h of treatment. It appeared that the longer the cells were under treatment, the more necrotic cells appeared (Figure 5). This effect was also seen with untreated cells, which could be attributed to the cells being an adherent cell line.

Flow cytometry and ICC were used to compare results obtained when searching for the route of cell death induced by various drugs and chemicals. Membrane damage during the harvesting process of adherent cell lines by trypsin or mechanical scraping has been found to produce false positives in apoptosis analysis, and is therefore an inadequate harvesting method [32]. However, the ICC method does not involve the use of trypsin thereby excluding possible damage to the cell membrane.

The comparison between the quantitative apoptotic status of cell populations using Flow cytometry and ICC led to the rise of two main concerns about the use of Flow cytometry in the quantitative determination of adherent cells. The first concern is false PI staining as a result of damaged cells due to trypsinization in order to make a suspension out of the monolayers ready for analysis using the Flow cytometer. Trypsin damages the cell membrane, thereby allowing the cells to take up PI [13] and thus giving a false result for necrotic/dead cells. Further investigation revealed that all manufacturers of apoptosis kits use cells that are in suspension by nature as sample cells for quantitative analysis of their products found on the protocol sheets (Table 1 in Appendix).

We propose that the manufacturers should highlight the experimental artefacts that can arise when using these kits on adherent cells. In addition, methods should be devised to reduce damage to cells whilst harvesting them for these analyses. Some protocols suggest harvesting the cells by incubation in standard trypsin with [13] EDTA, trypsin without EDTA, EDTA without trypsin or mechanical scraping with a rubber policeman. However, all these methods result in damage of the cell membrane [13]. Such damage is likely to happen when attempts are made to harvest adherent cells from the surface of tissue culture plates and was thus suggested to allow for Annexin V binding to cells before harvesting [13]. The second concern is that late apoptotic cells also appear in the upper-right (UR) quadrant positive for PI staining (Figure 2). This means that the analysis is unable to differentiate between late apoptotic, necrotic and dead cells. Part of this issue is raised by Reiger *et al*., [34] in that about 40% of PI stain results could be due to staining of RNA within the cytoplasm and thereby producing false positive events [33]. They suggest a protocol, which includes the addition of 16 µl of 1:100 diluted RNase A (Sigma, R4642) to give a final concentration of 50 µg/ml and incubation before analysis [34]. This enables the removal of cytoplasmic RNA and thus eliminates any false necrotic values due to PI staining.

Another suggestion to reduce the production of false positive events is by adopting a two-step protocol [35], which excludes washing, centrifugation and transfer of cells between plates, thereby reducing cell death. Although cost effective, EDTA is also found to damage

Archives of Pharmaceutical Sciences and Biotechnology Journal

Volume 2 Issue 1 January, 2022 Page 29 - 42 ISSN: 2971_611X
APSB-03

APSBJournal

the cell membrane [13] and is not effective in detaching strongly adherent cell lines such as CRC cells.

Another possible solution to the problem of false positives is the exposure of cells to ultrasonic waves in combination with diluted trypsin. This is done by the vibration of the culture surface using ultrasonic waves propagated from an ultrasonic transducer placed under the flask in combination with diluted trypsin. It was found out that the viability and proliferation of the detached cells were higher in this method compared to conventional methods [36].

These suggestions to standard protocols of apoptotic assays for adherent cell lines will surely lead to additional costs and prolong experiments, which could easily be avoided if manufacturers of these apoptotic assay kits test and adjust quantifications specifically for an adherent cell line.

Results from ICC confocal images after treatment with isomers of aspirin and thioaspirins revealed that SW480 CRC cells treated with 0.5 mM of aspirin, PN591 and PN592 for 16 h caused few cells to undergo early apoptosis, which increased substantially after 40 h of treatment. This could be because a dominant number of the cells analysed had undergone early apoptosis and a negligible number of necrotic cells were present.
Aspirin has previously been reported to cause cell death in SW480 cells via apoptosis [37]. Quantitatively, using Flow cytometry, it was observed that a small number of the cell population treated with aspirin and its analogues, PN548 and PN549 became apoptotic after 16 h, which significantly increased after 40 h. However, about 30% of the population were necrotic after 16 h and this did not change even after 40 h of treatment. On the other hand, the percentage of cell population treated with *ortho*-thioaspirin significantly caused necrosis with a small population apoptotic. But, its *meta*-(PN591) and *para*- (PN592) isomers did not significantly cause apoptosis or necrosis with most of the cell population live/healthy even after 40 h of treatment.

CONCLUSION

It was thus concluded that using Flow cytometry, aspirin and its analogues with the exception of PN591 and PN592 had some apoptotic effect after 16 h. However, a high necrotic effect was observed after 40 h, which was not observed using ICC. Further investigation will be needed to compare these results while adapting the suggestions made by [13], [33], [34] and [36]. The inclusion of DAPI fluorescent dye in order to stain the nucleus for accurate quantitative results for the ICC method is also vital.

It was also observed that isomers have similar effects in relation to PCD on SW480 cell line regardless of the change in isomeric position of the acetyl group attached to the benzene ring. PCD by the isomers of aspirin followed a particular manner and the isomers of thioaspirin followed another.

Competing Interests: Authors have declared that no competing interests exist.

Authors' Contributions: BAIJ designed the study, wrote the protocol and first draft of the manuscript, performed the statistical analysis. PCJ, NID and SST managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

Archives of Pharmaceutical Sciences and Biotechnology Journal
Volume 2 Issue 1 January, 2022 Page 29 - 42 ISSN: 2971_611X
APSB-03
APSBJournal

REFERENCES

[1] Belayneh YM, Amare GG, Meharie BG. Updates on the molecular mechanisms of aspirin in the prevention of colorectal cancer. J Oncol Pharm Pract. 2021; 27(4): 954-961 https://doi.org/10.1177/1078155220984846

[2] Perisetti A, Goyal H, Tharian B, Inamdar S, Mehta JL. Aspirin for prevention of colorectal cancer in the elderly: friend or foe? Ann Gastroenterol. 2021; 34(1): 1-11.

[3] Bashir AIJ, Perry CJ, Safrany ST. Effect of aspirin analogues in a colorectal cancer cell line. KASU Journal of Pure and Applied Life Sciences. 2021; 1(1): 9-16.

[4] Bashir AIJ, Kankipati CS, Jones S, Newman RM, Safrany ST, Perry CJ, et al. A novel mechanism for the anticancer activity of aspirin and salicylates. International Journal of Oncology. 2019; 54(4): 1256-1270 (https://doi.org/10.3892/ijo.2019.4701)

[5] Kodela R, Chattopadhyay M, Goswami S, Gan ZY, Rao PP, Nia KV, et al. Positional isomers of aspirin are equally potent in inhibiting colon cancer cell growth: Differences in mode of cyclooxygenase inhibition. J Pharmacol Exp Ther. 2013; 345(1): 85-94.

[6] Mather JP and Roberts PE. Introduction to Cell and Tissue Culture. 1st ed. Plenum Press New York: Springer, Boston, MA; 1998.

[7] Kaapalczynska M, Kolenda T, Przybyla W, Zajaczkowska M, Teresiak A, Filas V, et al. 2D and 3D cell cultures-a comparison of different types of cancer cell cultures. Archives of Med Science. 2018; 14(4): 910.

[8] Mirabelli P, Coppola L, Salvatore M. Cancer cell lines are useful model systems for medical research. Cancers. 2019; 11(8): 1098.

[9] Wyllie AH, Kerr JF, Currie AR. Cell death: The significance of apoptosis. Int Rev Cytol. 1980; 68: 251-306.

[10] Leist M and Jaattela M. Four deaths and a funeral: From caspases to alternative mechanisms. Nat Rev Mol Cell Biol. 2001; 2(8): 589-598.

[11] Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N. Crosstalk between apoptosis, necrosis and autophagy. Biochim Biophys Acta. 2013; 1833(12): 3448-3459.

[12] Schwartzman R and Cidlowski J. Apoptosis: the biochemistry and molecular biology of programmed cell death. Endocrine reviews. 1993; 14(2): 133-151.

[13] van Engeland M, Ramaekers FC, Schutte B, Reutelingsperger CP. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry. 1996; 24(2): 131-139.

[14] Kiyoto I, Yamamoto S, Aizu E, Kato R. Staurosporine, a potent protein kinase c inhibitor, fails to inhibit 12-o-tetradecanoylphorbol-13-acetate-caused ornithine decarboxylase induction in isolated mouse epidermal cells. Biochem Biophys Res Commun. 1987; 148(2): 740-746.

[15] Bertrand R, Solary E, O'Connor P, Kohn KW, Pommier Y. Induction of a common pathway of apoptosis by staurosporine. Exp Cell Res.1994; 211(2): 314-321.

[16] del Solar V, Lizardo DY, Li N, Hurst JJ, Brais CJ, Atilla-Gokcumen GE. Differential regulation of specific sphingolipids in colon cancer cells during staurosporine-induced apoptosis. Chem Biol. 2015; 22(12): 1662-1670.

[17] McKeague AL, Wilson DJ, Nelson J. Staurosporine-induced apoptosis and hydrogen peroxide-induced necrosis in two human breast cell lines. Br J Cancer. 2003; 88(1): 125-131.

[18] Miyoshi N, Oubrahim H, Chock PB, Stadtman ER. Age-dependent cell death and the role of atp in hydrogen peroxide-induced apoptosis and necrosis. Proc Natl Acad Sci U S A. 2006; 103(6): 1727-1731.
[19] Harrison RG, Greenman MJ, Mall FP, Jackson CM. Observations of the living developing nerve fiber. The Anatomical Record. 1907; 1(5): 116-128.
[20] Jacoby W and Pasten I. Methods in enzymology. Cell Culture. 1979; 58. New York: academic Press.
[21] UKEssays. (November 2018). Importance of Cell Cultures. Retrieved from https://www.ukessyas.com/essays/biology/importance-cell-cultures-5193.php?vref=1.
[22] Duval K, Grover H, Han L, Mou Y, Pegoraro AF, Fredberg J, et al. Modeling physiological events in 2D vs. 3D cell culture. Physiology. 2017; 32: 266-277.
[23] Bordwell FG and Boutan PJ. Conjugative effects in divalent sulphur groupings. J Am Chem Soc. 1956; 78: 854-860.
[24] Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin v. J Immunol Methods. 1995; 184(1): 39-51.
[25] Chou WY, Marky LA, Zaunczkowski D, Breslauer KJ. The thermodynamics of drug-DNA interactions: Ethidium bromide and propidium iodide. J Biomol Struct Dyn. 1987; 5(2): 345-359.
[26] Jacobson MD, Burne JF, King MP, Miyashita T, Reed JC, Raff MC. Bcl-2 blocks apoptosis in cells lacking mitochondrial DNA. Nature. 1993; 361(6410): 365-369.
[27] Teramoto S, Tomita T, Matsui H, Ohga E, Matsuse T, Ouchi Y. Hydrogen peroxide-induced apoptosis and necrosis in human lung fibroblasts: Protective roles of glutathione. Jpn J Pharmacol. 1999; 79(1): 33-40.
[28] Maciel E, Neves BM, Santinha D, Reis A, Domingues P, Teresa Cruz M, et al. Detection of phosphatidylserine with a modified polar head group in human keratinocytes exposed to the radical generator aaph. Arch Biochem Biophys. 2014; 548: 38-45.
[29] Wlodkowic D, Telford W, Skommer J, Darzynkiewicz Z. Apoptosis and beyond: Cytometry in studies of programmed cell death. Methods Cell Biol. 2011; 103: 55-98.
[30] Idziorek T, Estaquier J, De Bels F, Ameisen JC (1995) Yopro-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. J Immunol Methods. 1995; 185(2): 249-258.
[31] Shimura T, Toden S, Komarova NL, Boland C, Wodarz D, Goel A. A comprhensive *in vivo* and mathematic modeling-based kinetic characterization for aspirin-induced chemoprevention in colorectal cancer. Carcnogenesis. 2020; 41(6): 751-760 (https://doi.org/10.1093/carcin/bgz195)
[32] Bundscherer A, Malsy M, Lange R, Hofmann P, Metterlein T, Graf BM, et al. Cell harvesting method influences results of apoptosis analysis by Annexin V staining. Anticancer Research. 2013; 33(8): 3201-3204.
[33] Rieger AM, Hall BE, Luong le T, Schang LM, Barreda DR. Conventional apoptosis assays using propidium iodide generate a significant number of false positives that prevent accurate assessment of cell death. J Immunol Methods. 2010; 358(1-2): 81-92.

[34] Rieger AM, Nelson KL, Konowalchuk JD, Barreda DR. Modified annexin v/propidium iodide apoptosis assay for accurate assessment of cell death. J Vis Exp. 2011; (50): 2597.

[35] Kaur M and Esau. Two-step protocol for preparing adherent cells for high-throughput flow cytometry. Biotechniques. 2015; 59(3): 119-126.

[36] Tauchi H, Imashiro C, Kuribara T, Fujii G, Kurashina Y, Totani K, et al. Effective and intact cell detachment from a clinically ubiquitous culture flask by combining ultrasonic wave exposure ans diluted trypsin. Biotechnology and Bioprocess Engineering, 2019; 24(3): 536-543.

[37] Stark LA, Din FV, Zwacka RM, Dunlop MG. Aspirin-induced activation of the nf-kappab signaling pathway: A novel mechanism for aspirin-mediated apoptosis in colon cancer cells. FASEB J. 2001; 15(7): 1273-1275.

APPENDIX

Table 1: List of various Apoptosis Detection kits from Different Companies with the type of Cell used in the Documentation/Protocol Sheet

Product	Company	Type of cells	Culture properties
⋆Annexin V-FLUOS kit	Roche® Life Sciences	U937 lymphocyte cells	Suspension
FITC Annexin V/PI	invitrogen® Molecular probes®	Jurkat cells (T-cell Leukaemia,human)	Suspension
Annexin V Reagents for apoptosis	IncuCyte®	Jurkat cells	Suspension
FITC Annexin V apoptosis detection kit with 7-AAD	Biolegend®	Jurkat cells	Suspension
PE Annexin V apoptosis kit I	BDPharminogen™	Jurkat cells	Suspension
Annexin V-CF Blue 7-AAD apoptosis staining/detection kit	Abcam	Jurkat cells	Suspension
Annexin V apoptosis detection kit PE	e Bioscience™	Mouse thymocytes	Suspension
Annexin V/7-AAD apoptosis kit	Abnova	Jurkat cells	Suspension

(⋆Product used for this study).