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Apoptosis induced anticancer potential of aspirin on A549 Carcinoma Cell

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***Abstract*---**Recent research has discovered that using aspirin for a long time lowers the long-term risk of certain cancers, particularly colon cancer. However, the mechanism of anti-cancerous activity of aspirin against lung cancer is less studied. A molecular docking strategy was employed to identify the possible targets of aspirin while5-Fluorouracil (5-FU) was used as a positive control against lung cancer cell line A459. The *In-silico* analysis suggested that Caspase-3, Bax, andBcl-2could be potential targets for aspirin. The estimation of binding energies for these proteins resulted in -5.2, -5.8, and -5.7 Kcal/mol, respectively, which were better than 5FU (-4.8, -4.6, and -4.4, respectively).Trypan blues dye exclusion test exhibited a reduction in cell viability with the increase in Aspirin concentration. The IC50 values of Aspirin were calculated as 2.79 mM by MTT assay. The treatment of A459 cells with aspirin enhanced the levels of apoptotic genes at mRNA as well as at protein levels. The effect on the A549 lung cancer cell line, this study contributes to a better understanding of how Aspirin and 5-FU work in lung cancer.

***Keywords*---**Aspirin (ASA), A549 lung cancer cells, 5-Fluorouracil (5-FU).

Introduction

Now a days, Cancer remains one of the major causes of death worldwide [1].This disease has troubled millions of people. Despite recent breakthroughs in therapeutic techniques, such as targeted medicines, the five-year mortality rate for lung cancer is still around 16 percent [2]. Resistance to numerous therapies is one of the key causes for this cancer's poor prognosis. Cancer stem cells can be

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define as subpopulation of cancer cells with treatment-resistant potential that have the ability to self-renew, differentiate, and disseminate [3]. It is critical for developing particular medicines that target cancer stem cells to enhance lung cancer clinical outcomes. Aspirin (Acetylsalicylic acid, $C_9H_8O_4$) (ASA) is a regularly prescribed medication for the alleviation of fever and pain caused by a variety of illnesses. Both anti-inflammatory and antipyretic effects are found in aspirin. By decreasing platelet aggregation, this drug also helps to prevent blood clots, myocardial infarction and strokes. Long-term usage of ASA has anti-cancer action against a range of malignancies, including colorectal, breast, esophageal, prostrate, lung, liver, and skin cancer, according to several research studies [4].The evidence on aspirin's impact on lung cancer is more divided, though largely beneficial. Lung cancer incidence was reduced by 19 percent in case-control studies, but not in cohort studies [5,6]. Inflammation is well understood to play a key role in the genesis and spread of cancer [7-10]. COX-2, which is significantly expressed in several human malignancies including lung, colorectal, prostate, and breast cancer, as well as HCC, might be one of the putative linkages between inflammation and malignancy [11-13]. COX-2 transforms arachidonic acid into prostaglandins, such as prostaglandin E2 (PGE2), which contributes to cell proliferation and apoptosis suppression [14-16]. Furthermore, by inhibiting cell migration and inducing cell death in human HCC cells, the COX-2 inhibitor has been demonstrated to decrease cell migration and cause cell apoptosis.

Combination treatment is becoming more used in modern medicine, particularly in the treatment of cancer [17-19]. As a result of developed drug resistance, it is unavoidable to boost therapeutic efficacy by indefinitely boosting dosages; as a result, a significant number of anti-cancer treatments would produce substantial toxic side effects and even put the patient's life in jeopardy. The goal of combining two or multiple medications is to look into the potential uses of classic anti-cancer therapies and non-toxic chemical compounds that might operate on several therapeutic targets to treat cancer, decreasing drug resistance and increasing synergistic antitumor efficacy. In this paper, we investigated the anticancer property of aspirin in A549 lung cancer cells. Since the 5-Fluorouracil (5-FU) is a well-established drug against various cancers we used it as a reference to compare our data.

Materials and Methods

Chemical and reagents used

Aspirin (Cat # 027039) and 5- fluorouracil were purchased from Central Drug House (P) Ltd., India, and Fisher Scientific (Cat # A0305173). 5-fluorouracil was dissolved in 1 mM stock of dimethylsulfoxide (DMSO) and stored in tiny aliquots at -20 °C, whereas aspirin was dissolved in 100 mM stock of sterile deionized water. The ultimate dose of DMSO administered to cells was 0.1 percent (v/v). The drugs were quickly diluted in the appropriate medium before usage.

Docking Study

Obtaining protein and ligand structure from databases

Test compounds- Aspirin (PubChem CID-2244) and 5-FU (PubChem CID-3385)

Autodock 4 was used to prepare the ligand for autodockvina by applying torsion and aromaticity criterion on the ligand.

Selection of receptors

The 3D structures of B-cell lymphoma 2 (BCL-2), Bcl-2-associated X protein (BAX), and Caspase-3 were obtained from the protein data bank (PDB, ID: 1R4L)https://www.rcsb.org/ website with PDBID 4BD2, 1G5M, and 1GFW, respectively, for docking of chosen ligands.

The binding sites were analyzed in Discovery Studio 4.0 by receptor cavities option in the define and edit binding site option. Furthermore, the preparation of targeted protein was done with Autodock 4 with added polar hydrogen atoms and Kollman charges for protein optimization.

Molecular Docking

Our major target proteins were docked with aspirin and 5FU in AutoDockVina. The grid coordinates with a dimension of 50 Å × 50 Å × 50 Å set at the binding site of the prepared protein file. Docking results were analyzed by Autodock 4 and visualized by Discovery Studio 4.0 software. Our major target proteins were docked with aspirin and 5FU in AutoDockVina. Molecular docking of our major target proteins with aspirin and 5FU was performed in AutoDockVina.

Conditions for culture and cell lines

The cell line used in this research was Adenocarcinomic human alveolar basal epithelial cells (A549), procured from NCCS, Pune, India. When A549 cells are grown in vitro, they form a monolayer that adheres to the flask. In nature, These cells are squamous in form and are responsible for the diffusion of various chemicals through the alveoli of the lungs, such as water and electrolytes. The A549 cells were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) (cat # AT-189, Himedia) supplemented with an antibiotic-antimycotic cocktail (1 %) and FBS (Fetal bovine serum, 10 %). Cells were incubated at 37 °C, with 5% CO_2 and 95% humid atmosphere inside a CO_2 incubator.

Sub-culturing of A549 cell lines

The cells were sub-cultured after attaining the 80%-90% confluency. The following steps were employed to passage the cells: the exhausted media was discarded out of the flask and cells were rinsed with FBS devoid media. The media used for washing was then discarded. The cells were flooded with 1 ml of trypsin and the flask was put in CO_2incubator at 37 °C for 2-3 min to detach the cells from the adherent surface. After the cells were detached from the flasks, 2 ml medium added to neutralize the activity of EDTA trypsin. The suspension of

the cell was put in centrifugation for 5 minutes at 200xg to obtain the cell pellet. After the supernatant was removed from the centrifuge tubes, the cell pellet was dissolved in 1 ml medium. A suitable volume of cell suspension was aliquoted into a T-25 flask containing a 4 ml new medium that had just been freshly prepared. The flasks were then placed in aCO_2 incubator at 37 °C to allow the cells to develop.

Cell counting by using a hemocytometer

The cell suspension was poured into the hemocytometer using a 20 µl pipette and covered with a coverslip. The cells in the 1 mm square in center and four 1 mm squares in corner of the hemocytometer were counted. At 100x microscope magnification, the circle represents the approximate area covered (10x ocular and 10x objective). Cells that came into contact with the centerline on the upper and left side were tallied, but those that came into contact with the centerline on the lower and right side were not. Formula for the calculation of cell number is

$$\frac{Cells}{ml} = \frac{5n}{5 * 10 * 1000 * dilution}$$

In vitro cytotoxicity determination of aspirin on A549 cells by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay

Mosmann was the first to describe the MTT assay [23] and later modified by Hansen et al [24]is a standard colorimetric assay for measuring the viability of the cells. The cytotoxicity of aspirin against A549 lung cancer cells has been determined at different concentrations (0.16, 0.31, 0.62, 1.25, 2.5, 5, 10 mM). In this study, As a positive control, 5-FU was utilized, and various concentrations (1.6, 3.12, 6.25, 12.5, 25, 50, 100 µM) of this compound were also used. The cells were sown in 96 well plates at a density of 20,000 cells per well and grown overnight under conventional culture conditions. Further, Under normal culture conditions, the cells' culture media was replaced with a medium containing the above-mentioned amounts of both chemicals and incubated for 24 hours. Cells that had not been exposed to any compound were used as a control. Following incubation, each well received 5µl of MTT (5 mg/ml) and was incubated for 3 hours. By the removal of medium, to breakdown the formazan crystals, 200 µl of DMSO was administered. At 570 nm, the absorbance of cells was measured using a plate reader. The cells' survival was calculated using the formula below. The IC50 values of the substances were calculated using the GraphPad Prism software.

Cell viability (%) = [(Mean absorbance of control-Mean absorbance of sample)**/**Mean absorbance of control)]*100

Preparation of MTT dye

To remove the insoluble MTT dye residues, the MTT dye (5 mg, cat no TC191, Himedia) was diluted in 1X-PBS and filtered through a 0.22 µm filter membrane (Sartorius). After that, the solution was kept at 4°C.

Analysis of Apoptosis by Flow cytometry

The cells were stained with propidiumiodide (PI) to detect apoptosis. The cells (5x 104 cells/well) were plated into six-well plates, let it to proliferate, and then given the IC50 dosages of aspirin and 5-FU, as described above.The cells were extracted by trypsinization and pelleted down for flow cytometric analysis. The cells were rinsed twice in cold 1x PBS before putting it back in suspension (resuspended) in 500µl of PBS with RNase (20 µg/ml) and 5 M of PI and allowed to stand in the dark at 37 °C for 30 minutes. A FACS-Calibur flow cytometer was used to examine the prepared samples (Becton Dickinson, San Jose, CA, USA).

To investigate the effect of mRNA gene expression on marker genes, we used Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

qRT-PCR analysis

The same amount of RNA (approximately 1 gm) was reverse-transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis Kit. Then, using TaqMan Universal Master Mix II with UNG, 1µL of diluted cDNA was used as a template for RT-PCR investigations. The PCR was carried out with TaqMan gene assays for Bcl-2, Caspase3, and GAPDH: 10min incubation at 50°C, 2min: Initial denaturation (1 cycle) and then denaturation for 40 cycles; 94°C for 20s, annealing and extension; 60°C for 60s. Chemical dilutions are listed. Primer sequences used are shown in **Table no-3.**

Gene	Primer's (5'-3')
Bcl-2	F-AGGAAGTGAACATTTCGGTGAC R-GCTCAGTTCCAGGACCAGGC
Caspase 3	F-GTGCTACAATGCCCCTGGAT R-GCTGGATGCCGTCTAGAGTC
Gapdh	F-GATTTGGTCGTATTGGGCGC R-AGTGATGGCATGGACTGTGG

Isolation of cellular proteins and detection of protein expression in immunoblots

The cells were collected (treated and control cells), were rinsed in chilled 1X Phosphate Buffer Saline (1X PBS) and RIPA cell lysis and extraction buffer (Amresco-N653) was used to lyse them with 1 mM PMSF (phenylmethylsulfonyl fluoride, HiMedia-RM1592). Cells homogenized at 4°c for 30 min and cell lysate were collected in supernatants by centrifugation at 12,000 for 20 minute.

Bradford protein quantification assay was used to determine the protein concentration. On SDS-polyacrylamide gel electrophoresis, denatured cell lysates were utilised to separate 25µg of proteins, which were then transferred to a PVDF membrane. After blocking with 5% bovine serum albumin (BSA) the PVDF membranes were treated with primary antibodies against Bcl-2 (Abcam-ab32124, 1:1000), Anti-Cleaved Caspase-3 (ab2302,1:1000), and GAPDH overnight at 4 °C (Cell Signaling Technology -14C10, 1:1000). After incubation, PVDF membranes were rinsed in 1X Tris-Bufferd Saline with Tween 20 (1X TBST) and incubated

with HRP-coupled secondary antibodies anti-rabbit (Santa Cruz-sc-2004, 1:5000) for 2 hours at room temperature. Immunoblots were developed with a chemiluminescent substrate (Pierce ECL western blotting substrate, Thermo Scientific-32109) and viewed under Chemidoc system (Image Quant LAS4000, GE). As an internal loading control, GAPDH was used. A protein ladder (3.5–245 kDa, Abcam-ab116029) was used to determine the molecular weight of the protein bands.

Results

In-silico analysis depicts the interaction of 5-Fu and aspirin with Bax, Bcl-2, Caspase-3

Molecular docking analysis of selected target protein with aspirin and 5FU was performed using AutoDockVina. The grid coordinates with a dimension of 50 Å × 50 Å × 50 Å set at the binding site of the prepared protein file. The docking result was analyzed by Autodock 4 and visualized by discovery studio 4.0 software. Results of Aspirin and 5-FU with cancer target protein Bax, Bcl-2, and Caspase-3 were summarized in (table1).

Table 1
Binding Energy of Aspirin and 5FU against selected Target protein

S.NO	Target	Ligands	Binding energy (Kcal/mol)
1	BAX	Aspirin	-5.2
		5-FU	-4.8
2	BCL-2	Aspirin	-5.8
		5-FU	-4.6
3	CASPASE 3	Aspirin	-5.7
		5-FU	-4.4

The docking result displays the aspirin as a more potent drug against the selected target proteins Bax, Bcl-2, and Caspase-3 with binding energy -5.2, -5.8, and -5.7 Kcal/mol. Whereas 5FU binding energy were -4.8, -4.6 and -4.4 find against the selected target protein Bax, Bcl-2, and Caspase-3, respectively. Detailed interaction analysis of best dock result of aspirin-Bcl-2 revealed 5 H-bond interaction with amino acid Asp10, Asn11, Trp195, and His186, while His186 also form Pi-Pi Stacking hydrophobic bond which makes this complex more stable. A detailed information interaction analysis of all docking results is given in (table2). and displayed in (Figure1).

Table 2
Details of interacting amino acid, type of and bond distance

Bax-5fu			
Interacting Amino Acid	Distance	Type of Bond	Sub-type of Bond
A:GLN52:NE2 - :UNK0:F1	3.46657	Hydrogen Bond;Halogen	Conventional Hydrogen Bond;Halogen (Fluorine)
A:GLN52:NE2 - :UNK0:O2	3.15176	Hydrogen Bond	Conventional Hydrogen Bond
UNK0:H11 - A:SER60:O	3.05247	Hydrogen Bond	Conventional Hydrogen Bond
A:GLY29:CA - :UNK0:O2	3.55997	Hydrogen Bond	Carbon Hydrogen Bond
A:PHE30:CA - :UNK0:O3	3.48175	Hydrogen Bond	Carbon Hydrogen Bond
A:ASP33:OD1 - :UNK0	4.57059	Electrostatic	Pi-Anion
A:SER60:CB - :UNK0	3.58568	Hydrophobic	Pi-Sigma
UNK0 - A:LYS64	4.97872	Hydrophobic	Pi-Alkyl
Bax-Aspirin			
A:GLN52:NE2 - :UNK0:O4	3.0668	Hydrogen Bond	Conventional Hydrogen Bond
A:ASP33:OD1 - :UNK0	3.95823	Electrostatic	Pi-Anion
Bcl2-5FU			
A:TYR9:HN - :UNK0:F1	2.45542	Hydrogen Bond;Halogen	Conventional Hydrogen Bond;Halogen (Fluorine)
A:TYR9:HN - :UNK0:O2	2.4502	Hydrogen Bond	Conventional Hydrogen Bond
A:ASN11:HN - :UNK0:F1	2.75481	Hydrogen Bond;Halogen	Conventional Hydrogen Bond;Halogen (Fluorine)
:UNK0:H11 - A:ASN182:OD1	2.6032	Hydrogen Bond	Conventional Hydrogen Bond
A:GLY8:HA1 - :UNK0:O2	2.56308	Hydrogen Bond	Carbon Hydrogen Bond
A:TRP195:HD1 - :UNK0:O2	2.8717	Hydrogen Bond	Carbon Hydrogen Bond
A:HIS186 - :UNK0	5.14647	Hydrophobic	Pi-Pi Stacked
A:TRP195 - :UNK0	4.83499	Hydrophobic	Pi-Pi T-shaped
:UNK0 - A:ILE189	5.14141	Hydrophobic	Pi-Alkyl
Bcl2-Aspirin			
A:ASP10:HN - :UNK0:O4	2.89886	Hydrogen Bond	Conventional Hydrogen Bond
A:ASN11:HN - :UNK0:O4	2.19454	Hydrogen Bond	Conventional Hydrogen Bond

A:TRP195:HE1 - :UNK0:O1	2.64186	Hydrogen Bond	Conventional Hydrogen Bond
A:TRP195:HE1 - :UNK0:O2	2.61809	Hydrogen Bond	Conventional Hydrogen Bond
A:HIS186:HA - :UNK0:O3	2.7727	Hydrogen Bond	Carbon Hydrogen Bond
A:HIS186 - :UNK0	5.84725	Hydrophobic	Pi-Pi Stacked
Caspase-5FU			
A:MET39:N - :UNK0:O3	2.91487	Hydrogen Bond	Conventional Hydrogen Bond
:UNK0:H10 - A:TYR37:O	2.11876	Hydrogen Bond	Conventional Hydrogen Bond
Caspase-Aspirin			
B:ASN208:ND2 - :UNK0:O4	3.04353	Hydrogen Bond	Conventional Hydrogen Bond
B:TRP214:NE1 - :UNK0:O1	2.87386	Hydrogen Bond	Conventional Hydrogen Bond
B:GLN217:NE2 - :UNK0:O3	2.99988	Hydrogen Bond	Conventional Hydrogen Bond
B:PHE247 - :UNK0	4.62511	Hydrophobic	Pi-Pi Stacked
B:TRP214 - :UNK0	5.04057	Hydrophobic	Pi-Pi T-shaped

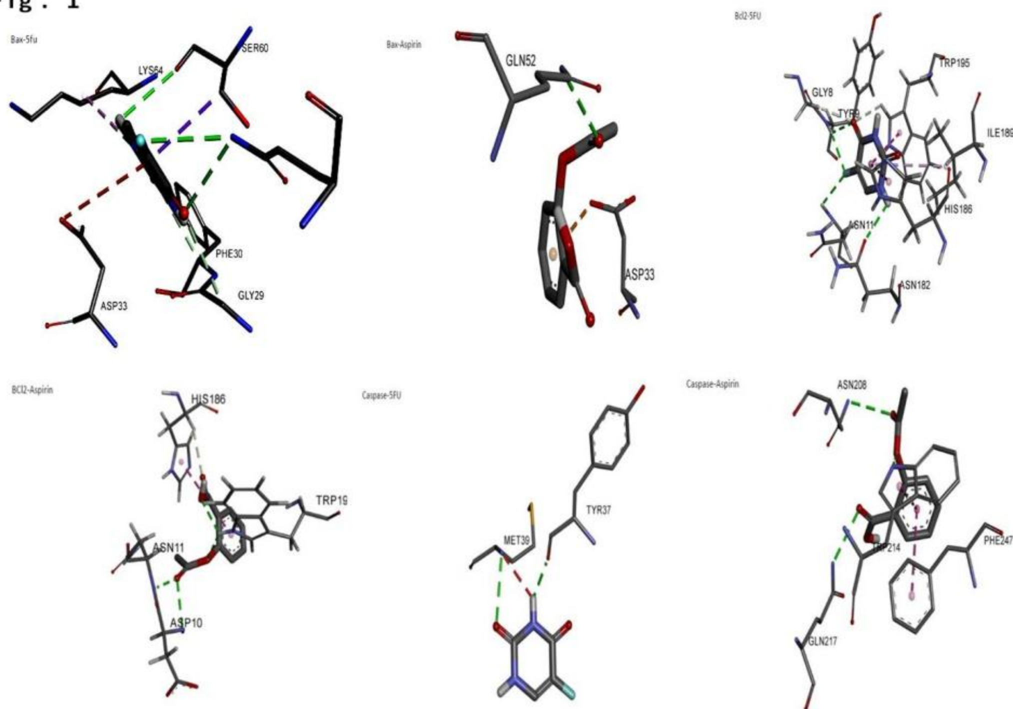

Figure 1: A detailed structural information interaction analysis of docking.

In vitro cytotoxicity determination of aspirin on A549 cells by MTT (3-[4, 5 dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay

Aspirin is said to have consequential anticancer properties against a variety of malignancies, including breast, liver, and colorectal cancers [20, 25, 26, 27]. In this study, we are focused to know that how aspirin show effect on lung cancer cell line A549. We tested a range of aspirin doses (0.078 to 10 mM) and used the MTT assay to assess cell cytotoxicity. The results showed that as the concentration of aspirin was increased, the viability of A549 cells decreased gradually. Though the lowest concentration has no visible effect on cell viability, the concentration of 0.156 mM caused a ~20 % decrease in cell viability. The highest concentration, 10 mM, resulted in a 70% reduction in cell viability. As a positive control, we used 5FU, a well-known medication used to treat a variety of malignancies. As a result, we were able to confirm the efficacy of aspirin in inhibiting the development of A549 cells (Figure2). Since the aspirin could able to inhibit the development of A549 cells, we calculated the IC50 of this compound along with 5FU (positive control) from the MTT assay data. We observed IC50 values of 2.79 mM for aspirin and 23.16 µM for 5FU.

Fig. 2

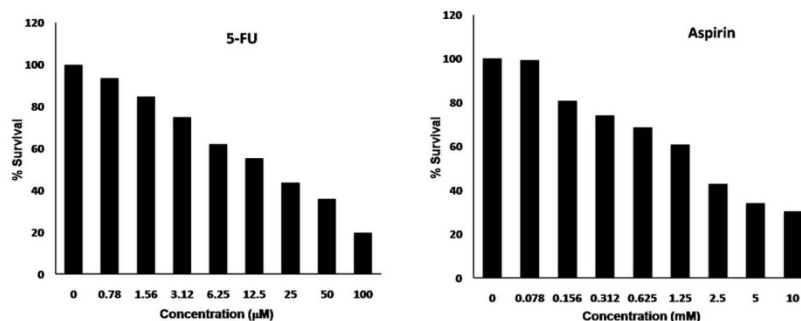

Figure 2: Cytotoxicity determination of aspirin on A549 cells by MTT assay.

Viability of cells by using Aspirin and 5-Fu in A549 cells

The viability of A549 cells was evaluated again by the use of Trypan blue dye exclusion assay at the IC50 values of both drugs. The Trypan blue dye exclusion assay is a simple, fast, and accurate method for determining cell viability. The treatment of cells at IC50 concentration for 24 h resulted in 47 and 35 % decreased viable cells for aspirin and 5FU, respectively. Thus, this experiment confirms that our cytotoxicity experiment was conducted well and IC50 calculation was also successful as at IC50 the aspirin was able to inhibit the growth of ~50% cells. The inhibition efficiency of aspirin was comparable to 5FU, though the concentration was high.

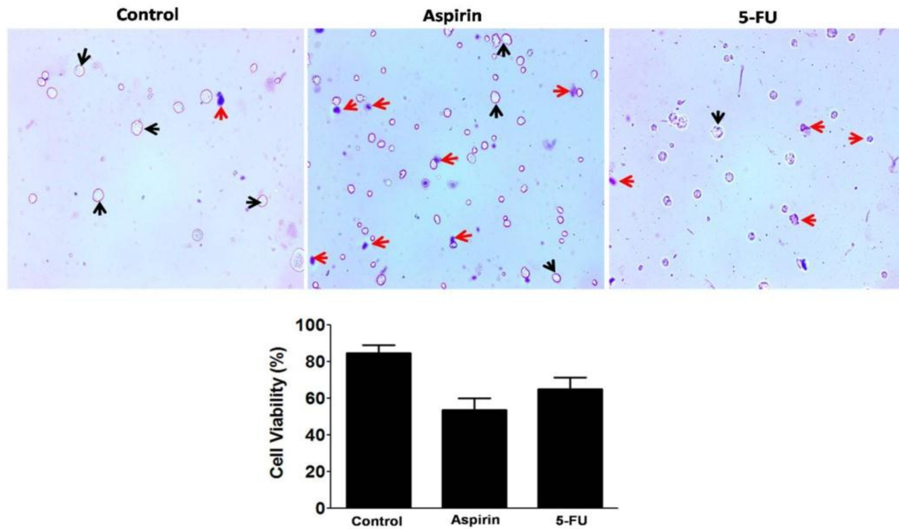

Figure 3: Cell viability was determined using Trypan blue.

Analysis of Apoptosis by Flow cytometry

Further, we sought to know whether aspirin causes cell death by apoptosis, we performed a cell death assay using PI staining by flow cytometry. PI is a tiny fluorescent compound that binds to DNA, however, it is unable to pass through the healthy plasma membrane. Thus it is passively introduced into the dead cells and bind to DNA. Hence during flow cytometric analysis, red fluorescence is obtained in direct proportion to dead cells. In our study, we treated the cells with both the drugs at IC50 concentrations and analyzed them by flow cytometry. The results suggest that the number of dead cells in the treated samples were increased significantly for both the drugs in figure 4.

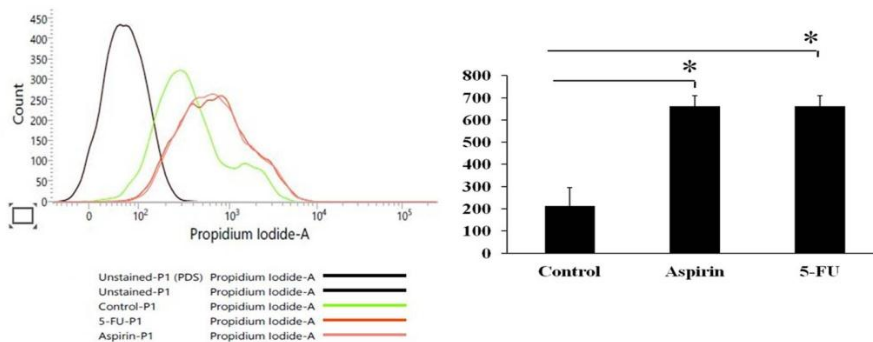

Figure 4: Apoptosis measuring by Flow cytometry analysis.

Aspirin activates the apoptotic marker genes in A549 cells

To check the effect of Aspirin and 5-FU on apoptosis marker genes in A549 cells, mRNA expression was checked by quantitative RT-PCR. The qRTPCR results showed that the mRNA level of BCL2 was increased by (3.53) fold after treatment with Aspirin and 3.0 fold after treatment with 5FU (Figure5A) when compared to control. Similarly, the expression of another apoptotic gene i.e. Caspase3 also increased after treatment with aspirin (2.33 fold) and 5-FU (2.05 fold, Figure5B).

Fig. 5

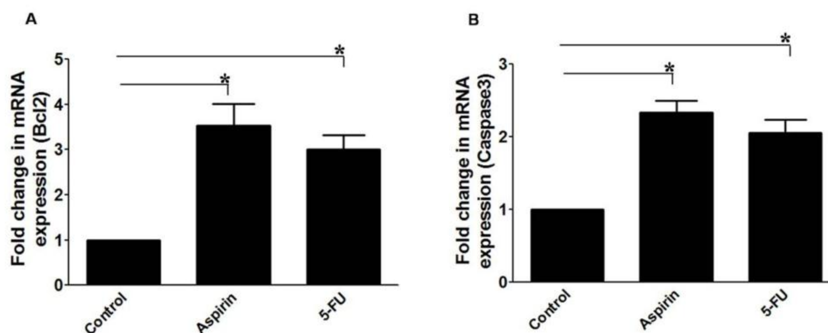

Figure 5: mRNA expression was checked by quantitative RT-PCR.

Effect of Aspirin on apoptotic markers

The results of qRTPCR suggested an increase in mRNA levels of apoptotic genes BCL2 and cleaved Caspase3 after treatment with Aspirin and 5FU. Further, we wanted to know whether the expression of these genes at proteins levels was also increased owing to Aspirin and 5FU treatments. (Figure6) depicts the western blotting data for Bcl2 and cleaved Caspase3 after treatment with both the drugs. The data suggested that exposing the A549 cells with aspirin and 5FU for 24 h enhanced the expression of both the apoptotic proteins, significantly. The findings suggest that aspirin is capable to trigger apoptosis in lung cancer cell lines in a similar way to 5FU.

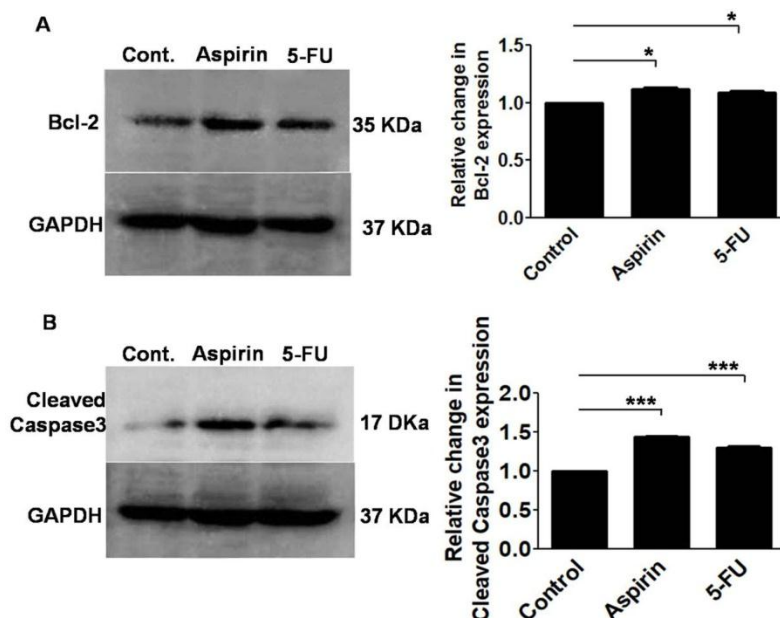

Figure 6: Western blots probed with antibodies of Bcl2 and Cleaved caspase3.

Discussion

In this study, we attempted to determine the effectiveness of aspirin on the apoptosis of lung cancer cell lines. Many of aspirin's medicinal characteristics and mechanisms of action have been discovered since its discovery in 1897. It has anti-platelet characteristics and is used to treat several illnesses such as inflammation, fever, and pain. Cancer is characterised by tumour growth that is practically uncontrollable and the proliferation of abnormal cells. Therapies for cancer are based on the type, location, and stage of the tumour, and typically include a combination of pharmacological techniques.

In-silico analysis proved that both Aspirin and 5-FU have good binding energy with (caspase-3,Bcl-2, and Bax). Results of Aspirin and 5-FU with cancer target protein (caspase-3, Bcl-2, and bax) were summarized in (Table1). The docking result displays the aspirin as a more potent drug against the selected target protein (caspase-3, Bcl-2, and bax) with binding energy -5.2, -5.8, and -5.7 Kcal/mol. Where as 5FU binding energies were -4.8, -4.6, and -4.4 against Bax, Bcl-2, and Caspase-3 respectively. Detailed interaction analysis of best dock result of aspirin-Bcl-2 revealed 5 H-bond interaction with amino acid Asp10, Asn11, Trp195, and His186, while His186 also form Pi-Pi Stacking hydrophobic bonds which makes this complex more stable.

In the present study, We wanted to see how Aspirin affected the A549 lung cancer cell line, A549. We investigated a range of aspirin doses (0.078 to 10 mM) and used the MTT assay to assess cell cytotoxicity. The results show that the concentration of aspirin was increased, the viability of A549 cells decreased

progressively. Though the lowest concentration has no visible effect on cell viability, the concentration of 0.156 mM caused a ~20 % decrease in cell viability. The highest concentration i.e. 10 mM caused about 70 % inhibition of cell viability. We used 5FU, a well-known drug to treat various types of cancers, as a positive control. The findings revealed that when the concentration of aspirin was increased, the viability of A549 cells decreased progressively. The effects of aspirin on several malignant cell lines have also been studied in the past. The effects of a high dose of aspirin (10 mM) on H1299 and A549 lung cancer cells were investigated [22].Several other research groups have used this high dose in in-vitro investigations as well [28]. In a similar work [21] Lung cancer cell lines A549 and H1299 obtained from human, yielded novel results in investigations. In prior research, the capacity of aspirin to stop proteasome activity was suggested but not directly proved. The authors found that Photodynamic therapy (PDT) followed by aspirin prolongs proteasome inhibition, resulting in the death of cells that were resistant to Photodynamic treatment's primary lethal impact (PDT).

When the A549 cells were grown in the presence of aspirin equal to the concentration of IC50, about 50 % of cells were found to be dead. Thus, this experiment confirms that our cytotoxicity experiment was conducted well and IC50 calculation was also successful. The inhibition efficiency of aspirin was comparable to 5FU, though the concentration was high.

A study on the HeLa cell line used changes in morphology of apoptosis and the localization of cell cycle markers to see if aspirin-induced cell growth is linked to apoptosis induction. HeLa TG cells treated with aspirin, showed nuclear condensation, suggesting that aspirin can promote apoptosis in cervical cancer cells. Condensation of chromatin along the blebbing of plasma membrane, followed by fragmentation into tiny, apoptotic entities, were common apoptotic indicators. According to FACS analysis, aspirin boosted the sub-G1 stages of cervical cancer cells. This activity was similar to the susceptibility of colorectal cancer cells to aspirin [29, 30]. Aspirin is an efficient anticancer medication that causes apoptosis in cervical cancer cells, according to these findings.

Apoptosis in different experimental models can be assessed using the flow cytometric assay of propidium-iodide (PI). Propidium iodide (PI) is a tiny fluorescent compound that binds to DNA, however, it is unable to pass through the healthy plasma membrane. Thus it is passively introduced into the dead cells and bind to DNA. Hence during flow cytometric analysis, red fluorescence is obtained in direct proportion to dead cells. In our study, we treated the cells with both the drugs at IC50 concentrations and analyzed them by flow cytometry. The results suggest that revealed both medicines considerably increased the number of dead cells in the treated samples.

The majority of data on the use of aspirin to lower cancer risk comes from observational research, with only a few randomized controlled trials. Furthermore, many trials have focused on cardiovascular disease prevention, which limits our knowledge of the ideal time and dose for cancer risk decrement. It would be challenging to conduct a phase III clinical trial of several aspirin pattern in the general population would be difficult. Before any effects on cancer incidence could be noticed, the project would need volunteers and run for some years.

Furthermore, aspirin is a generic medicine, therefore no pharmaceutical company wants to invest in it. Although evidence from aspirin trials in patients with specific genetic disorders, such as CAPP3, a seven-year study that will evaluate different doses in 3,000 patients with Lynch syndrome, may provide some insight, "I don't think we'll ever have an ideal study of aspirin for cancer prevention," says Andrew Chan, MD, of Massachusetts General Hospital.

Thus, The overall objective of our experiments is to assess the possible function of aspirin in modulating the cross-talk between apoptosis and autophagy by using several substances separately and in combination. The expected outcome of the study may help in obtaining a better insight for treating cancer by targeting proteins.

Conclusion

The expected outcomes of the study will help in increasing the repertoire of anti-cancer drugs with better potency so; That Evidence from a huge number of research suggests that aspirin has an important role in cancer prevention. Aspirin usage leads to a significant decrease in cancer incidence and a huge decrease in cancer death. Aspirin's health benefits appear to be mostly due to its impact against on breast, prostate, and lung malignancies. Although there are worries about aspirin-related bleeding, the majority of cases are mild. Aspirin's potential advantages in terms of cancer prevention and cancer mortality are expected to outweigh bleeding events of equivalent severity. For a medicine developed in the nineteenth century, aspirin might very well become the cancer-prevention drug of the twenty-first century. This research might lead to a new paradigm in cancer therapy through medication repurposing since it would reduce drug resistance, development time, and enhance low-cost medicines to fulfill the rising demand and unmet requirements of cancer patients.

Statistical analysis

Statistical analysis was done by one-way ANOVA through Graph Pad prism 5. Images were quantified using Image J software. Data were presented as mean ± S.D of at least triplicate determinations. $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$.

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