
Antiproliferative Effect of Aspirin and its Inclusion Complex on Human MCF-7 and K-562 Cancer Cells *In Vitro*

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The effect of aspirin, ibuprofen, and nimesulide the three nonsteroidal anti inflammatory drugs on cell growth of MCF-7 human malignant breast tumour cells was investigated. The results demonstrated that the drugs exerted a preferential cytotoxic effect on actively proliferating cells but to different extents. The inhibitory effect of aspirin i.e. acetyl salicylic acid was around two to four times more pronounced than those produced by the ibuprofen and nimesulide. The IC_{50} obtained with aspirin was 5 μ g/ml whereas those of ibuprofen and nimesulide were 13.5 μ g/ml and 28 μ g/ml respectively. Further the inhibitory effects were dose and time dependent. These effect of acetyl salicylic acid appeared to arise from a clear antiproliferative shift towards a reduced percentage of cells at the S and G₂/M phases, together with an increased percentage of cells at the G₁ phase. Aspirin could be more effective on MCF-7 than K-562 erythroleukemic cells and also as an inclusion compound with the advantage of oral administration and a greater bioavailability of the compound. Using the Chick chorioallantoic membrane model, the aspirin loaded pellets release sufficient acetyl salicylic acid to produce vascular regression and inhibition of angiogenesis.

Numerous epidemiological studies indicate that chronic use of nonsteroidal antiinflammatory drugs (NSAIDs) lowers the mortality rate from colorectal cancer¹. NSAIDs are effective at inducing regression of existing polyps in familial adenomatous polyposis (FAP) patients¹ and reducing the tumour burden in animal models of colorectal cancer². The mechanism of action of NSAIDs is principally due to the inhibition of cyclooxygenase (COX) which inhibits the production of proinflammatory prostaglandins (PGs)³. COX uses arachidonic acid (AA) as a substrate and produces PGs. PGs are lipid mediators whose production is enhanced

in both acute and chronic inflammatory reactions⁴.

COX exists in two isoforms, referred to as COX-1 and COX-2 which differ in their basal expression, tissue localization and induction during inflammation⁵. COX-1 is a constitutive form of the enzyme that is widely expressed in tissues throughout the body⁶. COX-2, a cytokine-inducible form of the enzyme is normally found in very low levels in healthy tissues, but is expressed prominently in inflamed tissues⁶. The pharmacology^{1,7} of COX-1 is also different from that of COX-2, such as that several NSAIDs have been shown to display differential inhibitory activity against COX-2 and COX-1

Enhanced COX-2 expression has been observed in colorectal, gastric, esophageal, pancreatic, liver, breast, bladder, prostate and also lung cancer. These findings

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suggest that COX-2 plays an important role in carcinogenesis¹.

Multiple studies using the mouse model have clearly established antitumour efficacy of NSAIDs, the inhibitors of COX-1 and COX-2. Despite these supportive data, evidence suggests that NSAIDs may also work via COX-independent mechanism. Additionally, NSAIDs have been shown to modulate cell proliferation and cell death in cultured colon cancer cells lacking COX, suggesting that not all of the NSAID effects are based on COX inhibition⁸. Multiple COX-independent mechanisms⁹ have been investigated to date including those involving 15-lipoxygenase, ceramide and inhibition of nuclear factor-KB. Whereas many of these alternative mechanisms may involve signaling pathways related to COX inhibition and prostaglandin biosynthesis, these links have yet to be definitely established.

The aim of the present study was to determine if some of the NSAIDs displayed antitumour properties for breast cancer. Preference was given to aspirin (ASA) since it is locally available and cheaper in price. This study was designed to compare *in vitro* cytotoxic activity of aspirin with other NSAIDs like ibuprofen and nimesulide on human breast cancer MCF-7 cell line. There are hardly any reports describing the activity of NSAIDs on tumours of lymphoid origin. Therefore, we tested the antineoplastic activity of aspirin on K-562 human erythroleukemia.

COX-2 is over expressed in human cancers and is involved in angiogenesis¹⁰ as well as in tumorigenesis. Angiogenesis, the formation of new blood vessels from pre-existing ones, occurs physiologically in the endometrium and pathologically e.g. during tumour growth. We examined the antiangiogenesis induced by ASA by using chorioallantoic membrane (CAM) assay.

MATERIALS AND METHODS

All the NSAIDs were purchased locally. All other reagents were obtained from Sigma (St. Louis, MO). Stock solutions of NSAIDs were prepared with dimethyl sulfoxide (DMSO) and stored in the dark at -20°. Dilution with PBS to the required concentrations was made before each experiment. Control cells received the same concentration of solvent as the cells treated with the test compounds.

Preparation of inclusion complex:

An usual procedure was followed as has been reported earlier¹¹. ASA (0.254 g) and beta cyclodextrin (1.135 g) were taken in distilled water (70 ml) and 25% aqueous ammonia

solution (1.5 ml) was then added for complete solubilization. The solution was frozen in a refrigerator. The solidified material was lyophilized using Labconoco freeze drier at -40° and 50 millibar vacuum. The powder was passed through 60# sieve and stored in a desiccator until further evaluation.

Analysis of ASA in inclusion complex:

An accurately weighed quantity of ASA complex was dissolved in distilled water (50 ml) to obtain a drug concentration of 5 µg/ml. The absorbance was recorded at 253 nm using Milton Roy UV/Vis spectrophotometer 1201 using water as a blank. The solutions were scanned from wavelength 240 to 280 nm. The change in maximum absorption was noted and absorption was compared with the standard curve of ASA obtained earlier.

Tumour cell lines:

The tumour cell lines MCF-7 and K-562 were brought from NCCS, Pune, under ideal storage conditions. Both the tumour cell lines are maintained in Dulbecco's MEM medium supplemented with 10% fetal bovine serum, glutamine (2 mm), penicillin (150 µg/ml), streptomycin (150 µg/ml) as described earlier.

Viability, proliferation and cytotoxicity studies:

Cell growth and survival were assessed by trypan blue staining. 1×10^5 cells/ml were seeded in each 35 mm petridish and allowed to attach overnight. Cells were then treated with various concentrations of NSAIDs and incubated for 3 d. Each assay was performed in triplicate.

Flow cytometric analysis:

The flow cytometric analysis of propidium iodide-stained nuclei was performed as described previously. Briefly, MCF-7 cells were plated at a density of 5×10^6 cells in 35 mm petridishes and treated with various concentrations of ASA and its complex. After 72 h of incubation the cells were trypsinized, washed twice with PBS and fixed in 70% ethanol for 5 h at 4°. The fixed cells were washed twice with PBS, incubated with 1 unit RNase A (Sigma Chemical Co.) for 1 h at 37° and stained with 5 µg/ml propidium iodide (Sigma Chemical Co.) for 1 h at room temperature. The stained cells were analyzed for relative DNA content using a FACScan (Becton Dickinson Labware).

Chorioallantoic membrane (CAM assay):

The CAM angiogenesis assay was performed as described by Lee *et al*¹² with some modifications. Fertilized,

domestic chick eggs were incubated for four days. On day five of incubation, agarose pellets loaded with 2.5 to 10 μg ASA were placed directly on CAM surface. After more

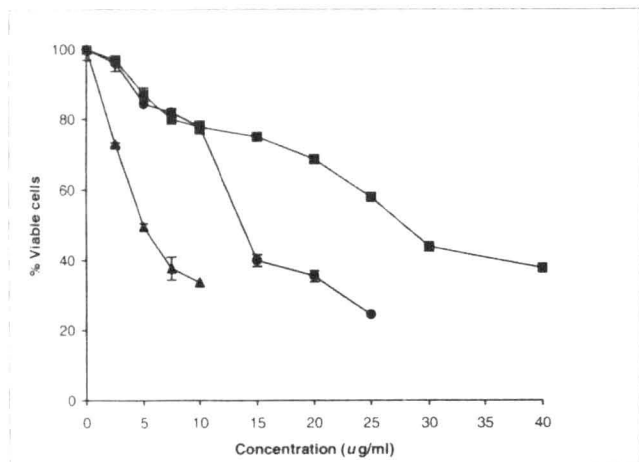


Fig. 1: Dose response effect of NSAIDs on human breast carcinoma cells (MCF-7).

Total number of viable MCF-7 cells was measured by trypan blue exclusion assay. Cells were cultured in the presence of IMDM medium and exposed to various concentrations of ASA (\blacktriangle), ibuprofen (\bullet) or nimesulide (\blacksquare). Values are mean \pm SD. $p < 0.05$ versus control.

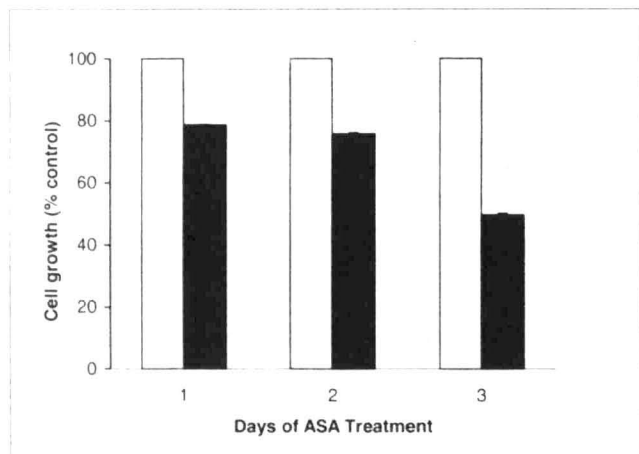


Fig. 2: ASA-mediated growth inhibition of human MCF-7 breast carcinoma cells.

At 25-30% confluency, cultures were fed with fresh medium and treated with either DMSO (\square) or ASA (\blacksquare) at the concentration of 5 $\mu\text{g/ml}$. After 1, 2 and 3 days of ASA treatment both floating and attached cells were collected and cell growth was estimated. Results are the mean \pm SD of 3 separate experiments and are expressed as percent of control.

incubation for two days the vasculature was examined using a stereomicroscope interfaced with a video camera.

RESULTS AND DISCUSSION

A plethora of studies have provided evidence that shows promise indicating NSAIDs are potential anti cancer drugs. In this investigation, we examined the effect of NSAIDs on the growth of the human breast MCF-7 and human erythroleukemic K-562 cells *in vitro*. We demonstrated that ASA, ibuprofen and nimesulide inhibited the growth of MCF-7 cells but their low-doses marginally affect the growth of the cells. Furthermore ASA found to be more potent than the remaining two drugs on the basis of their IC_{50} values. The respective values were 5, 13.5, and 25 $\mu\text{g/ml}$ (fig. 1). ASA shows time and dose dependent effect on MCF-7 cell line (fig. 2).

We first established ASA as a potent cytotoxic molecule on MCF-7 cells, and then we tested its cytotoxic effects on K-562 cell proliferation. We also observed inhibitory effects on K-562 cell growth (fig. 3) with a higher IC_{50} (15 $\mu\text{g/ml}$) which is almost three times the values on MCF-7 cell proliferation. Haematological malignant cells are generally believed to be more sensitive to drug treatment. A higher

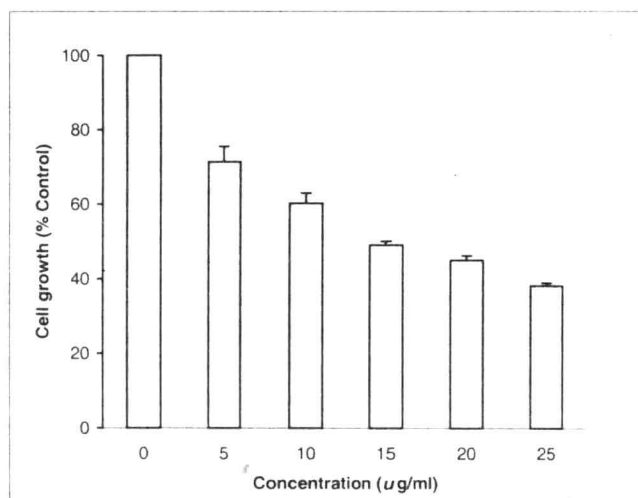


Fig. 3: Dose-dependent effect of ASA on K-562 erythroleukemia cell growth.

After three days of treatment, cells were collected and counted with hemocytometer. Trypan blue dye exclusion was used to determine viable and dead cells. Cell death of K-562 cells is represented as mean of two independent plates; each sample was counted in duplicate. The representative data shown are mean \pm SD, which were reproducible in two independent experiments.

IC₅₀ value is indicative of lower presence of cyclooxygenase enzymes i.e. COX-1 and COX-2. Since there are rare reports on research carried out on haematological malignant tumours, using NSAIDs, K-562 erythroleukemic cell line was incorporated in the present investigation.

Macromolecular drug delivery systems have been developed as one approach to overcome drug resistance and to improve the therapeutic index. Examples include polymeric conjugates of chemotherapeutic agents, these are internalized by endocytosis, resulting in their accumulation in perinuclear lysosomes, thereby rendering drug release from polymer both closer to nuclear targets and less accessible to membrane-linked drug efflux mechanisms than the free drug originally incorporated by diffusion¹³. Cyclodextrin inclusion drug may also have pharmacokinetic advantages over free drug¹⁴. The latter may readily extravagate to normal tissues, whereas the size of the former may restrict such distribution, potentially reducing toxicity. Despite the restriction, the leaky, irregular vasculature of solid tumours may still be readily traversed by these complexes, which, when combined with their greater retention in the tumour interstitium results in superior tumour localization and less toxicity compared with free drug¹⁵. The inclusion complex of ASA shows antiproliferative effect like the parent drug (Table 1) with a higher IC₅₀ with the possibility of slow, sustained and delayed release.

To investigate the mechanism behind the anti proliferative activity, cell cycle analysis was performed to determine the fate of cells after treatment with ASA and its inclusion compound. Flow cytometry was performed along

TABLE 1: EFFECTS OF ASA AND ITS COMPLEX ON HUMAN BREAST CANCER CELLS (MCF-7).

Concentration (mg/ml)	Viability(%)		
	β-Cyclodextrin	ASA	ASA-COMPLEX
0	100	100	100
2.5	100	72.9±3.1	88.0±2.0
5.0	100	49.6±0.5	63.1±1.8
7.5	100	37.7±0.9	56.0±1.0
10.0	98.5	33.7±3.3	50.7±1.2
12.5	-	-	37.1±1.2

Cells were treated with β-cyclodextrin, ASA and inclusion complex at indicated concentrations. Cell number was determined on day 3. Control viability was taken as 100 percent. The control consists of two groups, first PBS and second as 0.1% DMSO treated. Results are the mean ± SD of three separate experiments.

with cell cycle analysis (Table 2). In repeated experiments flow cytometry showed G₁ arrest and loss of cells in G₂/M phase of the cell cycle, whereas treated cells in the remaining phase maintained a cell cycle distribution that was identical to the no treatment group. We failed to detect the induction of programmed tumour cell death (induction of apoptosis).

Angiogenesis, the generation of new blood vessels, is essential for tumour growth, invasion and metastasis. At

TABLE 2: CELL CYCLE PROFILE OF ASA/ASA COMPLEX TREATED MCF-7 CELLS.

Group	Concentration (µg/ml)	Cells(%)		
		G ₁ /G ₀	S	G ₂ /M
Control	—	56.4	34.2	09.3
ASA	5.0	62.5	35.9	01.5
	7.5	61.9	36.3	01.7
	10.0	68.8	31.2	00.0
ASA-COMPLEX	10.0	58.4	38.5	02.9
	20.0	60.5	36.8	02.6

MCF-7 cells were exposed to ASA and its inclusion compound at different concentration at 72 hours. After cell permeabilization and RNase treatment cells were stained with propidium iodide, followed by FACS analysis. Results are representative of atleast three independent experiments.

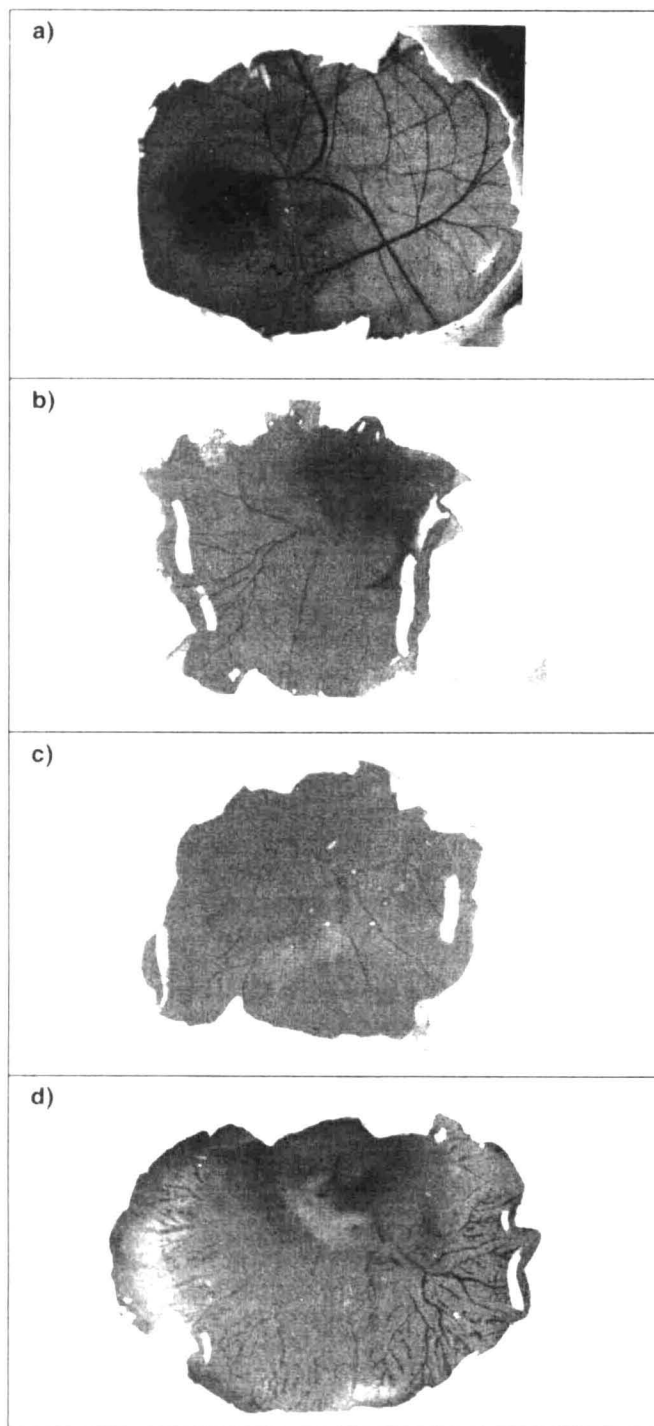


Fig. 4: Antiangiogenic effect of ASA.

Agarose pellets containing 0-10 μg ASA were placed on chorioallantoic membrane (CAM) of 3-day old fertilized eggs. After 48 hours the angiogenic response was evaluated. Control (A), ASA 2.5 μg (B), ASA 5 μg (C), ASA 10 μg (D).

present, a number of strategies are employed for anti-angiogenic therapy^{12,16}. For example, inhibition of endothelial cell proliferation, blocking activity of angiogenic peptides, inhibition of formation of neovessels or stimulation of angiogenesis inhibition are all strategies that may influence tumour growth.

Little is known about the effect of ASA on endothelial cell function and on angiogenesis. This study investigated the effect of ASA on embryonic-induced angiogenesis on an experimental model. Using the Chick chorioallantoic membrane (CAM) model, ASA at 10 μg embedded on agarose pellet significantly inhibited the angiogenic response induced by chick embryo (the reduced number of capillaries as compared to the control was taken as a measure of anti-angiogenic response). The effect was seen at the dose of 5 μg also. However, no response was observed in pellet containing 2.5 μg ASA (fig. 4). This observation indicates that ASA has a strong antiangiogenic activity a property that might contribute to its antineoplastic activity.

In these studies ASA demonstrated significant activity in each of the human tumour cell line. The inclusion complex of ASA was also active on MCF-7 cells. ASA shows promising antiangiogenic activity. We propose that the antiangiogenic property might further contribute to its antineoplastic activity particularly against solid tumours. Taken together, these results suggest that ASA exhibits sufficient properties to merit consideration for further development, alone or in combination with conventional anticancer agents/radiation/hyperthermia.

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