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Effects of acetylsalicylic acid and salicylic acid on the growth of HT3 cervical cancer cell line

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Abstract
Cervical cancer is one the commonest type of cancers affecting women worldwide with high mortality rate. Human Papilloma Virus (HPV) is the common risk factor for about 99% of cervical cancer infection worldwide. HPV causes cervical cancer through the up-regulation of COX. COX-1 and COX-2 which is present in small amount in every cell. It plays a key role in regulation of cervical cancer neoplasia. Cell viability was determined using cell titre blue by doubling dilution from 1,000,000 to 31,250 cells per ml on HT3. Dose response for Acetylsalicylic Acid ASA and SA was carried out at different concentrations from 0-20mM concentrations and incubated at different time intervals 24, 48 and 72hrs incubation. A 10mM concentration of ASA and Salisalic Acid (SA) was used to determine the caspase activity using caspaseglo on the cell line for the period of 0-24hrs incubations. Western blot was carried out using active anti- caspase3 antibody for caspase3 proteins. The number of cells was found to increase as the absorbance increases, but the relationship breaks up above 600000 cells per ml. Effects of drugs on the viability of HT3 indicated that SA has more effect by inhibiting the viability of HT3 from 10-20mM concentrations at 48 and 72hrs incubations. SA shows more differential effect on caspase with more increase in caspase activity from 8-16hrs incubations. Western blot shows no expression of protein for caspase3, using β actin as a housekeeping gene. This study indicates that SA has more effect on HT3 cervical cancer cell line. This finding might be as a result of differences in the chemical properties of the drugs.

1. Introduction

Cervical cancer is one of the commonest type of cancers affecting women under 35 in UK and the second most common cancer-affecting women in the world with high mortality rate (1). It is estimated that about 530,000 of women in 2008 were diagnosed with cervical cancer in the world (almost one in ten (9%) of women) is diagnosed with cervical cancer(2). In less developed countries the incidence of cervical cancer is high particularly due to lack of awareness, poor government support, suboptimal infrastructure and absence of life saving vaccination. Cervical cancer is among the few cancers that are easily identified and treated at precursor stage (3, 4). There are two different states in cervical cancer, cervical intraepithelial neoplasia (CIN) as well as invasive cervical cancer.

Human papillomavirus (HPV) is the high risk causative agent of cervical cancer infection. HPV 16 and 18 are the types of viruses associated with almost 99% of cervical

cancers worldwide (5, 6). High- risk HPV oncoproteins increase cervical carcinogenesis through the enhancement of unusual functioning of the genes and proteins that usually control cell homeostasis. It is by this process that the viral oncoproteins succeeds in the upregulation of cyclooxygenase (COX)-dependent pathways (7). The main mechanism however is through the inactivation of p53 and retinoblastoma. Cyclooxygenase are a very important enzyme that initiates the first process of prostaglandin synthesis and as well play a vital role in cell growth, immunity and inflammation.

The age at which women starts sexual intercourse is also an important risk factor of having cervical cancer because at that time the cervix is still developing (8). The chances of contacting HPV and having cervical cancer in women who started sexual intercourse between ages of 15 years is double that of those started at the age of 20 years (8). This may have greater implications for cervical cancer occurrence.

There are other cofactors that facilitate the development of cervical cancers; smoking (9), contraceptive drugs use (10, 11) as well as *Chlamydia* disease (12, 13). The tendency that women of less socio-economic standard have greater chances of having cervical cancer may be as a result of rampant smoking and involvement in high risk sexual relationship in their teenage years (14).

Screening has brought about a reduction in the incidence and mortality of cervical cancer in most developed countries. However, this is not the case in developing countries, particularly among women in rural communities, uneducated, and older women (8). Health education, the use of condoms for sexual intercourse (particularly in young women), sticking to one partner, reduction in the incidence multiple sex partners and safer sex planning have been suggested as possible technique to reduce the prevalence and spread of HPV (15, 16).

Cyclooxygenases enzymes are of two types, COX-1 and COX-2. COX-1 is present in small amount in every cell and participates in COX-2 and prostaglandin regulation in cervical cancer neoplasia. In most normal tissues, the expression of COX-2 is usually undetectable, less expressed or weakly present in the cervix unless during pregnancy, labour, perturbation as well as some stages of menstrual cycle (17). However, it is over-expressed in considerable amount in cervical cancers at every significant phases of growth, including precancerous lesions stage (18, 19).

In developmental processes of cervical cancer, COX enzymes possibly promote cell growth, enhance chronic inflammation as well as carcinogen production, promote the production of blood vessel (angiogenesis) and tumour invasiveness, possibly inhibit immune recognition and programmed cell death (apoptosis) (7, 20, 21, 22,).

Pharmacological inhibition of COX-1 or COX-2 decreased tumourigenesis (23). This combine with the fact that over expression of COX-2 in these tumours is related with poor prognosis (24), makes COX-2 a very important tool for both therapeutic target and chemoprevention of cervical neoplasia.

1.1. Aspirin (Acetylsalicylic Acid) as COX Inhibitor

Acetylsalicylic acid (ASA) is one of the commonest medication taken by many people worldwide and has been used for the treatments of many diseases and conditions for more than 100 years. Aspirin was initially recognized as an analgesic, anti-pyretic as well as anti-inflammatory drug. For more than 25 years, many studies have shown that application of aspirin significantly decreases the rate of occurrence of epithelial cell cancers, more especially colorectal cancer (20, 25, 26, 27). Application of aspirin for long duration possibly decreases the incidence of many cancers, such as bladder, gastric as well as breast cancer, by 40-50% (28).

The molecular processes associated with aspirin's actions on cancer have been well identified. Some factors like concentration, duration, stage as well as type of cancer have revealed that actions of aspirin on cancerous cells are very complex and associated with various signalling pathways.

ASA as a COX inhibitor, the physiological actions of aspirin are related specifically to its ability to inhibit COX-2 enzymes as shown in figure 1 below (22-28). The inhibition of COX-2 in cancer cells result to a decrease in production of prostaglandin, which eventually result to decrease cell growth, initiate apoptosis as well as increased immune surveillance as shown in figure 1 (29). ASA varies from other non-steroidal anti-inflammatory drugs (NSAIDs) in its characteristic of inhibiting irreversibly both COX-1 and COX-2, but with much regard to COX-1 (20). This shows that COX-1 is also over-expressed in cervical cancer cells; there is possibility that effect of aspirin is by dual-COX inhibition.

ASA can acetylate proteins associated with cancer; effect of COX as well as prostaglandins cannot exclusively be as a result of chemo- preventive effects of aspirin, as similar effects have been observed in COX deficient cell model as well as prostaglandin deficient animal model studies (20). Aspirin comprises of an acetyl and salicylate group and both groups were identified to have a clear variation of molecular targets. Aspirin's acetylation characteristics are considered to be the justification why it varies from other NSAIDs.

Evidences from some current studies have revealed that aspirin has the capacity to bind and modulate multiple cellular proteins in cancer cells by acetylation of lysine and serine residues (30). For example, effect of aspirin as a COX inhibitor is as a result of attachment of its acetyl group with serine residues on cyclooxygenases; result in an irreversible inhibition of function shown in figure 1 (31). At physiological concentration of aspirin, aspirin has the ability to acetylate many cellular components relevant to cervical cancer, like the tumour suppressor gene p53, responsible for regulation of over growth or cell death (32).

Apoptosis is programmed cell death as a result of cell stress, cell damage, inflammation or mutations. Caspases, which are the major initiator and regulator of apoptosis, can respond to extracellular inducers like tumour necrosis factor (TNF) or Fas

ligand, or to intracellular inducers like mitogen activated kinases. More importantly, this process has to be related with tumour suppressor gene p53 as well as mitochondria. Cervical cancer cells are recognized to inhibit apoptosis by the inhibition of different kind of pro-apoptotic proteins like p53 as well up regulation of anti-apoptotic proteins (29, 33).

Some studies have shown that aspirin can influence and promote caspase-dependent apoptosis in many cancer cells, usually by up regulation of p53 (34). Previous studyrevealed that aspirin modulates calpain gene expression, resulting to activation of caspase-3 (35). Similarly, inhibition of COX by aspirin results to aggregation of ceramide, which as well promote and induce caspase-dependent apoptosis via cellular stress signalling (20).

Cell death occurs by apoptosis, an extrinsic apoptotic pathway occurs by tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor, which is a cell membrane-bound protein that initiates tumour-specific apoptosis. Activation of the TRAIL receptor pathway is the selective mechanism of eradicating cancerous cells completely, but there are some situations in which most cancers are known to be resistant to TRAIL therapy. But with multiple therapies that is combination of TNF and aspirin treatment has shown an improved capacity to bring caspase-induced cell death in cancer cell rather than TRAIL therapy alone (36). Aspirin also decreases specific amount of protein surviving and sensitises cells to TNF/TRAIL-induced cell death (37).

Figure 1 show that aspirin can inhibits angiogenesis; this is one of the most important aspect in tumour development and growth (that is the ability to produce new blood vessels around them in order to supply nutrients and oxygen for the growth and survival of tumour cells). COX has been shown to control angiogenesis in colon cancer through promoting endothelial activity (38). It has been shown that there is possibility for aspirin to inhibit vascular remodelling as well to inhibit pro-angiogenic factors like metalloproteinases (MMPs) or vascular endothelial growth factor (VEGF) (39). In cervical cancer especially cervical neoplasia, COX-1expression plays a very important function in improving as well as maintaining angiogenesis (40). Aspirin being an effective inhibitor of COX-1, there is possibility that aspirin inhibits angiogenesis by COX-1 dependent pathways in cervical cancer as it shown in figure 1.

NSAIDs, especially ASA has characteristics of anti-tumour effect, as seen in epidemiological studies on colorectal cancer (25, 41). The chemopreventive as well as anti-proliferative ability of ASA in cell cultures and cancer cells was observed to be as a result of induction of apoptosis (42, 43). There are different molecular processes involved in apoptotic effect of ASA recognized, which involve p38 MAP kinase (Mitogen-activated protein kinases) activation in human fibroblasts (44), effect on human telomerase reverse transcriptase in colorectal cancer cells (45), caspases activation (46, 47), down regulation of the anti-apoptotic Bcl-2 protein (48, 49), it also cause alteration of the signalling pathway of the transcription factor NF-κB in human gastric cells (50). ASA brings about tumour apoptotic cell death through promoting the onset of the mitochondrial permeability transition (51, 52).

1.2. Salicylic Acid as COX Inhibitor

Salicylic acid (SA) acts through COX-independent pathways, SA plays an important function in COX-2 inhibition than acetylation as shown in figure 1. Acetylation of serine on COX-2 possibly does not result in inactivation (20). Previous study revealed that SA might possibly be a weak competitor for COX with its target arachidonic acid (53). SA is a weak inhibitor of COX directly, so far is still seen to decrease amount of COX metabolites, the prostaglandins (54). There is also possibility that SA modulates COX indirectly through nuclear transcription factors, which bring the equality between cell life and cell death as shown in figure 1. Inhibition of nuclear factor kappa B (NFκB) transcriptional activation is possibly to be the major target of SA, as NFκB plays an important function in controlling COX expression (55, 56). There is also possibility SA to degrade NFκB's inhibitory co-factor genes in cervical cancer models (57, 58). The process by which SA succeeded in this process is thought to be independent of COX (59). Other nuclear transcription factors related to COX in cervical cancers possibly as a result of pharmacological action of aspirin, like AP-1 and PPAR receptor (17, 54, 60).

Mitochondria play an important role in cell death by apoptosis as an execution of apoptosis through action of its Bcl_2 proteins as well as the release of cytochrome- C(61). ASA and SA are capable of altering these proteins. For example, it has revealed that SA can down-regulate the anti-apoptotic Bcl_2 family group MCL-1 (62). There is also possibility that ASA can initiate apoptosis through mitochondrial cytochrome-C release by inhibition of the ubiquitin proteasome pathway, which can result to stopping protein signalling of the key transcription factors (63). Mitochondrial calcium uptake inhibition is additional process whereby ASA brings about cell death as shown in figure 1 below (64).

NSAID like ASA and SA are found to be inhibitor of COX enzymes as shown in figure 1, these drugs has been in application for anti-oxidative, anti-microbial as well as anti-inflammatory (65, 66, 67, 68, 69, 70). Some researchers have shown that ASA and SA could be applied as anti-tumour drugs in many cancer cell lines (71, 72, 73, 74). NSAIDs, such as ASA and SA has been applied as chemo- preventive agents of cancers to initiate apoptosis, it also decreases the possibility of tumour formation and growth in different organs, like colorectal cancer (42), lung cancer (75), as well as stomach cancer (76). ASA initiates apoptotic cell death through mitochondrial permeability transition and bring about death of the cell by TRAIL (52, 77).

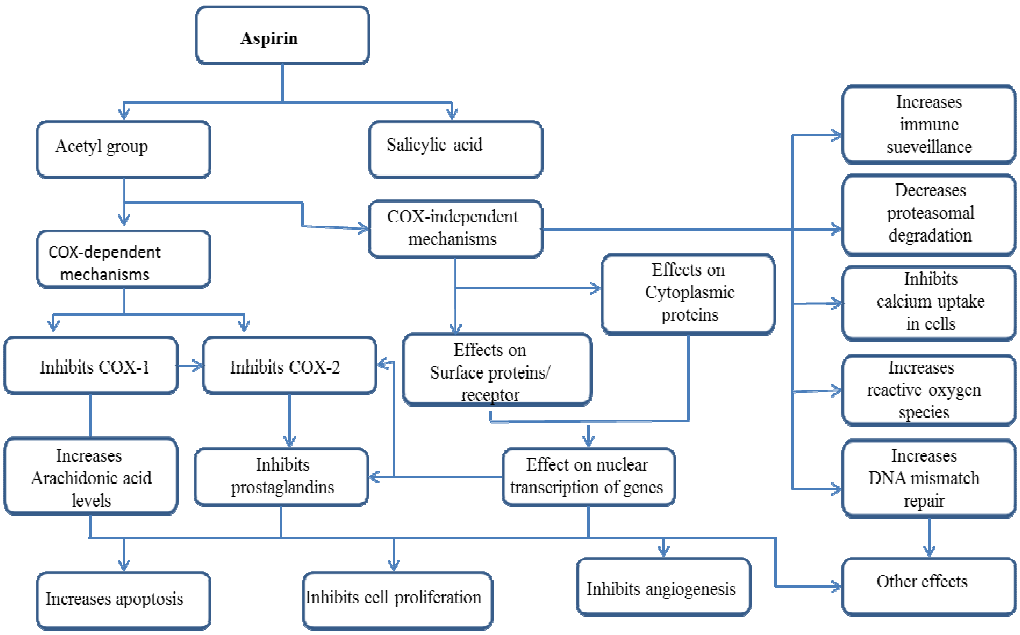

Figure 1. *Molecular Pathway to show multiple anticancer effects of aspirin in epithelial cancer*

Evidence from literatures and previous researches suggests that ASA and SA are very important weapons against growth and development of cervical neoplasia and carcinoma in situ, due to its multi action mechanisms in which it initiates its effect. Many studies suggest that ASA inhibits COX enzymes, prostaglandins, cell proliferation as well as angiogenesis on cervical cancer cells. It also inhibits tumour invasiveness and encourages immune surveillance. These processes are only achieved through COX-dependent and COX-independent pathways, using multi-action characteristics of its acetyl group and its salicylate group. In carrying out this present research on effect of aspirin on cervical cancer growth, we have taken into consideration many factors; the concentration of the drugs and route of administration (it administered orally there is possibility most of its effects would not be seen because of clearance and excretion limitations). Another factor considered was the fact that epithelial tumour cells might not behave in the same way as other epithelial cancer cells. Most studies on effect of aspirin on cancer cells conducted on colorectal cancer may show some differences when compared to results observed with cervical cancer as a result of variation in mutations and genes associated with these tumours. Other factors to be considered are the adverse side effect on application of aspirin, like gastrointestinal bleeding, ulceration, hypersensitivity reactions (78), renal damage and macular degenerative disease (79).

Cervical cancer is among the cancers that affect woman worldwide with high mortality rate. Although screening and treatment are being implemented, the mortality rates are becoming high. The purpose of this present research is to evaluate the effect of Aspirin (acetylsalicylic acid) and salicylic acid on cervical cancer cell lines in vitro as well as to investigate the mechanism by which these drugs kill cervical cancer cells. ASA and AS was used as a drugs for this research because both drugs are COX inhibitor in cancer cells and COX over expressed in cervical cancer.

2. Material and Methods

2.1. Determination of Cell Viability of Adherent Cells Using Celltitre Blue™

This experiment was carried out in order to determine the viability of the cell line HT3 before assessing the viability with the drugs. HT3 cervical cancer cell line was grown to 80% maximum cell as recommended by the provider (American Collection of Cell Cultures). Briefly, the cells were washed with Dulbecco's PBS once, the cells were trypsinized using Cardiff Metropolitan University (CMU) tissue culture guidelines, and re-suspended in 3 ml of fresh supplement media. Cells were counted using haemocytometer and re-suspended to a density of 1 million cells per ml. Doubling dilutions was performed of the cell numbers of 1,000,000, 500,000, 250,000, 125,000, 62,500, 31,250 and 0 cell per ml as media only (negative control). The microplates were incubated for overnight. The media from the wells were aspirated out and a fresh media containing 20 µL Celltitre Blue per 100 µL media was added in to each well and incubated in the dark for 1 hour. The microplates were inserted into the Tecan M200 Infinite multi detection reader and the fluorescence of each well was detected using i-control software (excitation of 560 nm and emission of 590 nm set on optimal gain)

2.2. Dose Response for Acetylsalicylic Acid and Salicylic Acid Using Cell Titre BlueTM

HT3 cervical cancer cell line was processed as mentioned earlier. 100 µL at a density of 200000 cells per ml of cells were pipetted into 96 well plate. The plate was incubated for

overnight. The media in the plate was aspirated out, 100 µL of different concentrations of ASA (acetylsalicylic acid), and SA (Salicylic acid) from 0, 1, 5, 7.5, 10, 15 and 20mM was pipetted in to each well, one row left without the drugs media only as control and incubated for 24 hours. The media was aspirated again from the wells; it was washed once with 300 µL DPBS (Dulbecco's Phosphate-Buffered Saline). Prepared pre-warmed media containing 20 µL Celltitre Blue per 100 µL media was added in to the wells. The plate was incubated for 1 hour in dark. The microplate was inserted into the Tecan M200 Infinite multi detection reader and the fluorescence of each well was detected using i-control software (excitation of 560 nm and emission of 590 nm set on optimal gain). The experiment was repeated for viability after 48 hours incubation as well as after 72 hours incubation, and the fluorescence of each was measured using the Tecan M200 Infinite multi detection reader.

2.3. Determination of Caspase Activity in Adherent Cells Using Caspase 3/7 glo™

HT3 cervical cancer cell line was processed as mentioned earlier. 50 µL at a density of 100000 cells per ml of HT3 cells were pipetted into 96 wells plate. The plate was incubated for overnight. The media in the plate was aspirated out and 10 mM of ASA, 10mM of SA solution were prepared using phenol red, 50 µL of ASA solution was pipetted in to three rows of HT3 plate, 50 µL of SA solution in to another three rows of HT3 plate, one row was left without the drugs, media only as control, the plate was incubated for 24 hours. Caspaseglo reagent from refrigerator was thawed beforehand to equilibrate to room temperature. One vial of caspaseglo buffer was mixed with one vial of caspaseglo lyophilised substrate. 50 µL of mixed caspaseglo was added in to each well of the plate, it was wrapped with aluminium foil to protect it from light (Caspaseglo is light sensitive), and the plate was incubated at room temperature for 1 hour. The plate was inserted into the Tecan M200 Infinite multi detection reader and the luminescence of each well was determined using i-control software. Caspase activity was measured through the following incubation period 0, 2, 4, 6, 8, 16, 20 and 24 hours respectively.

2.4. Western Blotting Detection of Active Anti Caspase 3

Method: 20µL of the samples HT3 with the drugs were pipetted in to the wells of electrophoretic gel, about 12 samples together with a ladder, it was filled up with buffer solution and an electric current passed through for 50 minutes in dark room. The gel was removed; iBlot machine was used to blot membrane paper for 5 minutes. 5% non-fat dried milk in TBST (Tris- Buffered Saline with Tween) was used as blocking buffer to block the antibody on blotting paper; the blotting paper was placed on shaking machine for 1 hour. The blotting paper was washed with buffer TBST solution 3 times 5 minutes interval each on shaking machine. A primary antibody Active anti caspase (abcam NO: 32042) 1:600 dilution, 25 µL in 15 ml (5% TBST) was applied and incubated at 4^0C on shaking machine overnight. The blotting paper was washed with buffer TBST solution 3 times 5 minutes interval each on shaking machine. A secondary antibody goat FAB2 (abcam NO: ab6013) 1:5000 dilution, 3 µL in 15 ml (5% blocking buffer) was added and incubated for 1 hour at room temperature. The blotting paper was washed again with buffer TBST solution 3 times 5 minutes interval each on shaking machine. The membrane paper was incubated in West Pico Dura Luminol substrate (2.5 ml luminol and 2.5 ml peroxide substrate) for 5 minutes at room temperature, excess solution was blotted using tissue paper, air bubble was removed using roller. The membrane was placed on protected membrane in a film cassette with the protein side facing up, it was exposed to KODAK X-Ray film for 1 minutes. The film was developed using 20% (v/v) developer and fixing agent (100 ml in 400 ml deionised water), various bands were produced on the film, all the processes for developing carried out in dark room.

The result from western blot analysis did not show any expression of proteins from the samples. The above processes were repeated using β-actin as primary antibody (Housekeeping gene) in order to confirm the western blot analysis was done correctly. The membrane paper was washed in buffer TBST solution 3 times 5 minutes interval each on shaking machine in order to remove active anti caspase antibody and apply β-actin antibody as housekeeping gene, because no expression shown from the results of western blot for active anti caspase, the process was repeated on the membrane paper, but in this case β-actin was used as primary antibody. It was exposed for KODAK X-Ray film for 1 second.

3. Results

3.1. HT3 Cervical Cancer Cells Viability and Growth

Figure 2 below shows the graph from the result (absorbance) of HT3 cervical cancer cell line using cell titre blue. Tecan M200 Infinite multi detection reader was used in detecting fluorescence of the cells using i-control software (excitation of 560 nm and emission of 590 nm set on optimal gain). A doubling dilution was carried out from 1000000 cells per ml to 7800 cells per ml for HT3 cervical cancer cell line, from figure 2 below shows that the absorbance increases as the number of cells increases. But the relationship breaks up from 500000 cells per ml and above with the decrease in absorbance. The relationship between cells density and absorbance on cervical cancer cell line shown as the mean ± SEM (n=3).

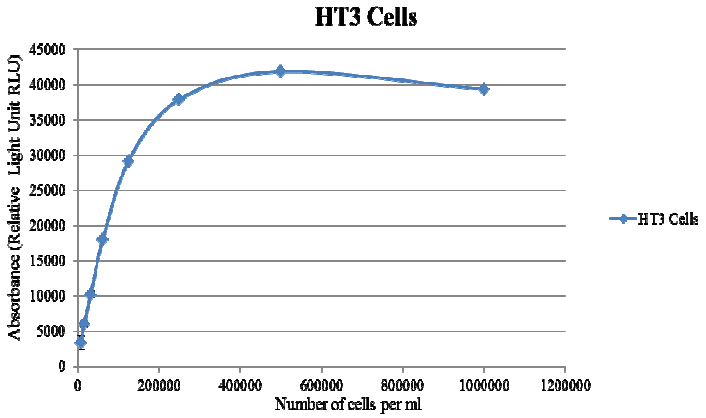

Figure 2. *Relationship between cell density and absorbance on HT3 cells using cell titre blue*

3.2. Dose Response for Acetylsalicylic Acid and Salicylic Acid on HT3 Cervical Cancer Cell Line

Figure 3 shows the graph from the results (absorbance) of the effects of ASA and SA on viability of HT3 cervical cancer cell line using cell titre blue. Tecan M200 Infinite multi detection reader was used in detecting fluorescence of the cells using i-control software (excitation of 560 nm and emission of 590 nm set on optimal gain). Figure 3 shows the effectiveness of ASA on viability of HT3 cervical cancer cells line, from the concentration of 0mM to 20mM for 24, 48 and 72hrs incubation. From 10, 15 and 20mM shows effectiveness at 48 and 72hrs incubations, while 24hrs shows effectiveness only in 20mM concentration. 10000 cells per ml were chosen, because in the relationship between cells density and absorbance results, there is much increase in absorbance that started above 10000 cells per ml. At 48 and 72hrs incubations it shows more effectiveness in cells viability. VC- Vehicle control, this serves as negative control for the experiment and it did not shows any effect of inhibition on the viability of HT3 cells line after 24, 48 and 72hrs incubations. The effects of cell viability on HT3 cervical cancer cell line with ASA shown as the mean ± SEM (n=3).

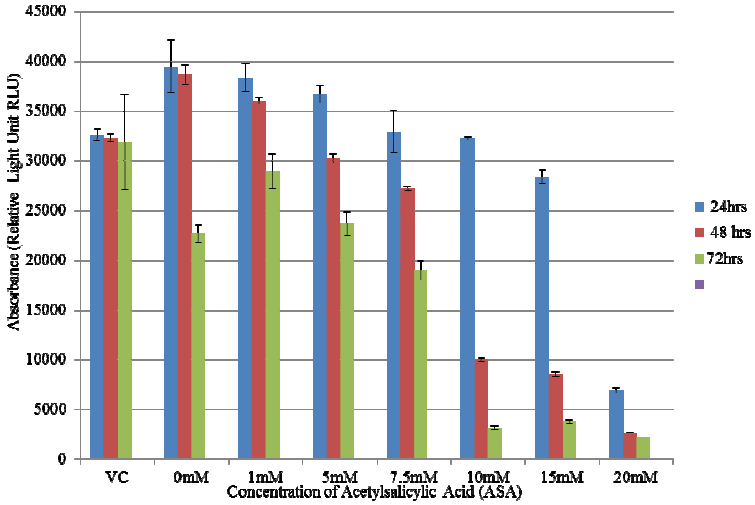

Figure 3. *Effects of ASA on viability of HT3 cervical cancer cell lines*

From figure 4 below, shows the effectiveness of SA on viability of HT3 cervical cancer cells line from the concentration of 10mM to 20mM for only 48 and 72hrs incubations, with more effectiveness in 72hrs incubation at concentration of 15mM. 10000 cells per ml were chosen because in the relationship between cells density and absorbance results, there is much increase in absorbance that started above 10000 cells per ml. VC- Vehicle control, this serves as negative control for the experiment and it did not shows any effect of inhibition on viability of HT3 cells line after 24, 48 and 72hrs incubations. The effects on cells viability on HT3 cervical cancer cell line with SA shown as the mean ± SEM (n=3).

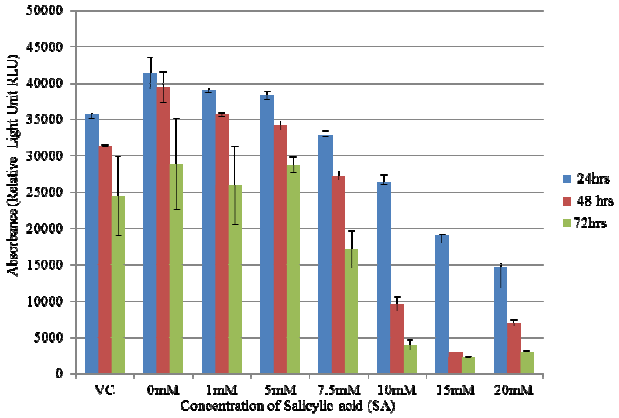

Figure 4. *Effects of SA on viability of HT3 cervical cancer cell lines*

3.3. Caspase Activity on HT3 Cervical Cancer Cell Line

The results from figure 5 show the graph of the absorbance of differential effects of ASA and SA on caspase activity on HT3 cervical cancer cell line using caspaseglo. Tecan M200 Infinite multi detection reader was used in detecting fluorescence of the cells using i-control software (excitation of 560 nm and emission of 590 nm set on optimal gain). The graph shows differential effects of ASA and SA on caspase activity on HT3 cervical cancer cells line; from the figure it shows the increase in caspase activities in both drugs from 0hr to 8hrs. But from 8hrs to 16hrs it shows much caspase activities in both drugs, and activities falls after 8hrs to 24hrs. Differential effects of ASA and SA on caspase activity on HT3 cervical cancer cells line shown as the mean ± SEM (n=3).

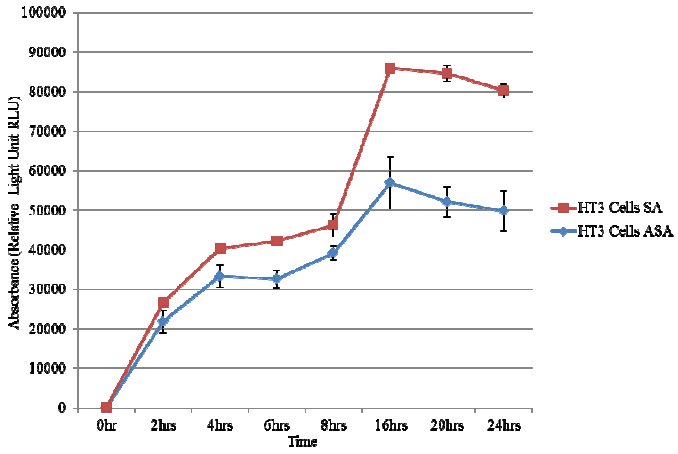

Figure 5. *Differential effect of ASA and SA on caspace activity on HT3 cervical cancer cell lines*

3.4. Western Blot Analysis for Active Anti Caspase 3

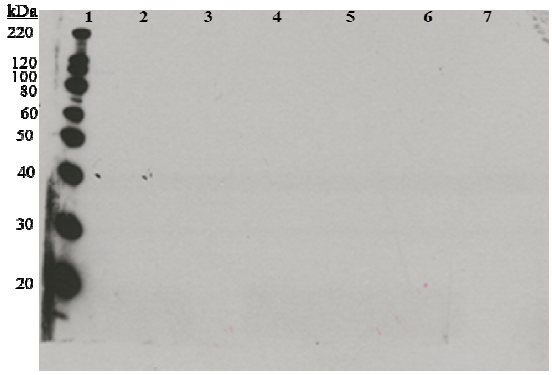

Figure 6. *Electrophoretogram illustrating migration of active anti caspase after 1 minute exposure*

Figure 6 shows the results of western blot analysis of the samples using active anti caspase 3 antibody for the expression of proteins in the samples. From the results no any expression shown by active anti caspase 3.β actin as housekeeping gene was used in order to confirm western blot by active anti caspase 3 was done correctly, also to confirm the presence of protein in the samples. A ladder was used as a molecular weight size marker with a known concentration and size, as a standard that are used to identify the approximate size, base pair, different bands produced by the samples on the gel. The ladder shows different bands of molecular weight ranging from 20 to 220kDa. Active anti caspase 3 expected to show a band around 17kDa, but no any expression shown from this result.

Result from figure 7 shows the expression of protein for β-actin as a housekeeping gene at molecular weight of 43kDa after 1-second exposure of the film; lane 8, 9 and 10 shows

more expression of protein than others, while lane 3 shows very small expression of protein. A ladder was used as a molecular weight size marker with a known concentration and size, as a standard that are used to identify the approximate size, base pair, different bands produced by the samples on the gel. The ladder shows different bands of molecular weight ranging from 20 to 220kDa. From this result it shows western blot analysis was done correctly, it also shows the presence of protein for active anti caspase 3 in the samples, it might be as result of small amount of sample used in western blot analysis or the protein is in small amount that is why it did not show any expression.

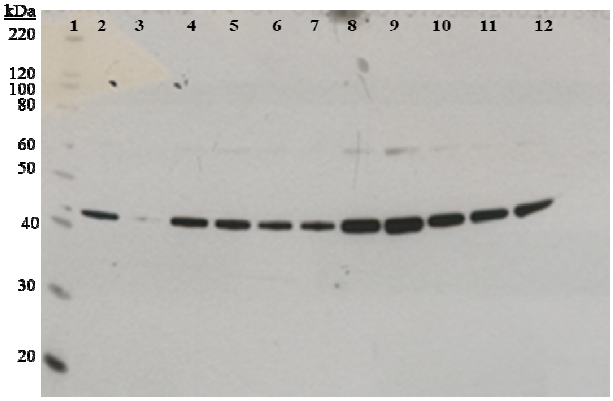

Figure 6. *Electrophoretogram illustrating the migration of β-actin after 1 second exposure*

4. Discussion

In this study the relationship between cells density and absorbance on HT3 cervical cancer cell line using cell titre blue by doubling dilution from 1000000 to 7800 was determined. We observed that the absorbance increases as the number of cells increases from 0 cells per ml to 1000000 cells per ml. The absorbance increases as the number of cells increases from 0 cells per ml to 500000 cells per ml, after 500000 cells per ml the relationship between cells density and absorbance breaks up down to 1000000 cells per ml, the absorbance decreases after 500000 cells per ml. This could be as result of HT3 being HPV negative. Cervical cancer cells that are HPV-16 or -18 positive are known to express small amount of p53 transcript as a result of E6-activated ubiquitin-dependent protease digestion (80). It was also reported that there is possibility for cervical cancer with smaller HPV copy number to be more susceptible to growth inhibition by Adeno-associated virus (81).

In this present study the effects of ASA on viability of HT3 cells from 0mM to 20mM concentrations incubated for 24hrs, 48hrs and 72hrs was determined. The result showed no inhibition of viability from 0mM to 15mM after 24hrs incubation on HT3 cells line, but at 20mM, it inhibits the viability of the cells after 24hrs. After 48hrs incubation from 0mM to 7.5mM, ASA shows no inhibition of viability of the cells on the cells line also, but from 10mM to 20mM, it shows the inhibition of viability of the cells with much more inhibition on 20mM after 48hrs. After 72hrs incubation with ASA from 0mM to 7.5mM, there is no inhibition of viability of the cells by ASA, but from 10mM to 20mM it inhibits the viability of the cells of HT3 cervical cancer cells line. From this result, it shows that ASA inhibits the viability of HT3 cervical cancer cells line more from 10mM to 20mM concentration after 72hrs incubation. This shows that a 0 to 7.5mM concentration was not enough to inhibit the viability of HT3 cells at 24hrs incubation. Evidence shows that ASA inhibits COX-2 pathway when used in high doses for long period (82), also COX-2 is over-expressed in considerable amount in cervical cancers at every significant phases of growth, including precancerous lesions (18, 19).

Figure 4 shows the effect of SA on HT3 cervical cancer cells line 0 to 20mM concentrations incubated for 24hrs, 48hrs and 72hrs intervals. The result shows no inhibition of viability on HT3 cells from 0 to 20mM concentrations of drugs after 24hrs incubation. There is also no viability inhibition on the cells from 0 to 7.5mM after 48hrs incubation, but it shows an inhibition of viability at the concentration of 10 to 20mM with much more inhibition at 15mM after 48hrs. After 72hrs incubation, there is no viability inhibition by the drugs at the concentration from 0 to 7.5mM, but the drugs inhibit the viability of HT3 cells at the concentration from 10 to 20mM with much more inhibition at the concentration of 15mM after 72hrs. From these results it shows that SA inhibits the viability of HT3 cervical cancer cells line at the concentrations from 10mM to 20mM after 72hrs incubation. This seems to prove the evidence that SA can modulates COX indirectly through nuclear transcription factors, brings about inhibition of cell viability and result to cell death. A nuclear transcription factor may possibly to be a major target of SA, as nuclear transcription factor play a role in controlling COX expression (55, 56). From the chemical properties of SA, it shows that SA is soluble in water due to its hydroxyl group and thus can penetrates cells very well and initiate its effects.

In this study the differential effect of SA and ASA on caspase activities on HT3 cervical cancer cells line seeded with 10mM concentration of the drugs with caspaseglo after 0 to 24hrs incubations was determined. The results from figure 5 shows differences in caspase activity from 0 to 16hrs incubations in cells seeded with SA, in which the caspase activity increases from 0 to 16hrs, and the caspase activity decreases from 16 to 24hrs incubations. Caspase activity also increases from 0 to 16hrs incubations in cells seeded with ASA; the caspase activity also decreases from 16 to 24hrs incubations. This result shows that caspase activities are higher from 16 to 20hrs incubations; SA shows much higher caspase activity than ASA. The result of HT3 with ASA shows an increase in caspase activity from 0 to 16hrs incubations with much increase in caspase activity from 8 to 16hrs incubations, but the caspase activity decreases after 16hrs incubation. HT3 with SA shows an increase in caspase activity from 0 to 20hrs incubations with much increase in caspase activity from 8 to 16hrs incubations, but the caspase activity decreases after 20hrs incubation. From these findings

it shows that the drugs and cell line induces caspase activity at 16hrs incubation. From these findings also, SA induces more caspase activity than ASA, this is in support as a result of the differences in chemical properties as well as chemical functions of the two drugs (83), (82).

Previously, non-steroidal anti-inflammatory drugs like ASA and SA were used as an anti-inflammatory and analgesic drugs (67, 69, 84, 70). Findings from this new research and has shown that these drugs can be potentially used to reduce the risk of many cancers in clinical observations (65, 66, 68, 74). It was also identified that these drugs can as well induce apoptosis in various types of cancers (71, 73). Another study also shows that ASA could be used as an anti-tumour drug in many cancer cell lines (71, 73). In this present study, non-steroidal anti-inflammatory drugs ASA and SA were used on cervical cancer cell line HT3. Evidence from this study has shown that these drugs inhibits the growth of cervical cancer cell lines as well induces apoptosis through caspase 3 activation.

Previous studies show that ASA and SA have anti-neoplastic properties on colorectal cancer (25, 41). The anti-tumour effect of ASA has been linked to its effect on apoptosis (42, 43). Mechanisms involved in apoptotic effect of ASA observed in human fibroblast include p38 MAP kinase activation (44), inhibition of human telomerase transcriptase in colon cancer cells (45) and activation of caspases (46, 47). In this current study, we have shown that ASA and SA inhibit the viability of cervical cancer cell line HT3, it also shows a differential effect of the drugs on the cells and increase in caspase activity by the drugs on the cell line. ASA and SA have been used as chemo- preventive medication to induce apoptosis and decrease risk of tumour growth in some organs like colon (42), lung (75) and stomach (76). ASA initiates apoptotic cell death through mitochondrial permeability transition (52, 77), Studies was carried out also on the effect of SA on colorectal cancer, and it shows the possibility of decreasing precancerous adenomas through blocking COX-2 (85, 86, 87, 88). In this current study ASA and SA were used to determine the effectiveness on the viability of cervical cancer using HT3 cell line, the result shows inhibition of cell lines viability, it also shows a differential effect on caspase activity using caspase3glo which brings about apoptosis and cells death.

Some studies also show that ASA has the capacity to attach and modulate multiple cellular proteins in cancer cells through acetylation of lysine and serine residues (30). ASA as COX inhibitor, its effect is as a result of attachment of its acetyl group with serine residues on cyclooxygenase, bring about an irreversible inhibition of function in cancer cells (31). At physiological concentration of ASA, it has the ability to acetylate various components related to cervical cancer, like tumour suppressor gene p53, responsible for regulation of cell growth and bring about cell death (32), this is in support of the results of this study that shows at certain concentrations 10 to 20mM of the drugs ASA and SA, inhibits the viability of HT3 cervical cancer cell line.

Apoptosis is a programmed cell death due to cell stress, cell damage, inflammation or mutations. Caspases regulate and brings about apoptosis that will result to cell death, can also respond to extracellular inducers and intracellular inducers. Caspases are also related to tumour suppressor gene p53 and mitochondria that brings about apoptosis and cell death. Cervical cancer cells are known to inhibit apoptosis through inhibition of pro-apoptotic proteins and up regulation of anti-apoptotic proteins (29, 33). Previous studies also show that ASA can influence and increase caspase-dependent apoptosis in many cancer cells through regulation of p53 (34). Evidences also showed that ASA modulates calpain gene expression and brings about activation of caspase-3 (35). However, inhibition of COX by ASA results to initiate caspase-dependent apoptosis through cellular stress signalling (89). This previous studies corroborate with our study in which we used caspaseglo to show the inhibition of cells viability, and from the results of this study it showed a differential effects of the drugs on caspase activity and inhibition of the viability of the cervical cancer cell line. A study also showed that cell death occurs through apoptosis, extrinsic apoptotic pathway that occurred by tumour necrosis factor-related apoptosis-inducing ligand receptor, which brings about tumour apoptosis. Through combination with ASA and tumour necrosis factor for treatment of cancer has shown an improved capacity to promote caspase-induced cell death in cancer (36). This study also corroborate with our current study in which a caspaseglo was used in combination with ASA and SA on cervical cancer cell line, and the result showed a differential effects of the drugs on caspase activity and showed the inhibition of cervical cancer cell viability.

From this current study the result shows that ASA and SA inhibit the viability of cervical cancer cell lines. It is reported that ASA being an effective inhibitor of COX-1 has the ability to inhibit angiogenesis by COX-1 depended pathways in cervical cancer (40). In this current study also, it was found out that SA inhibits the viability of cervical cancer cell lines more than the ASA. It is reported from the previous studies that SA may possibly be a weak competitor for COX inhibition (53). SA is a weak inhibitor of COX directly, but still seen to decrease amount of COX metabolites (54). It is also reported from the previous studies that SA can degrade NFκB's inhibitory co-factor genes in cervical cancer (57, 58). According to the results obtained from this study, it shows that caspase activity occurred in HT3 cervical cancer cell line with ASA and SA, which results to initiates apoptosis and cell death. It is reported that mitochondria is the key factor for cell death by apoptosis as execution of apoptosis by action of Bcl2 proteins and release of cytochrome C (61). It is reported again SA and ASA are capable of altering these proteins and brings about release of cytochrome C and initiate apoptosis and cell death (62). There is also possibility that ASA initiate apoptosis by the release of mitochondrial cytochrome C (63), mitochondrial calcium uptake inhibition is additional way by which ASA brings about cell death (64).

Results from this study showed that SA induces greater caspase activity than ASA, these might be as a result of differences in chemical properties between AS and ASA, and

might lead to the differences in their actions, SA has a chemical structure C_6H_4 (OH) COOH while ASA has $C_9H_8O_4$ in which hydroxyl group in SA was replaced with acetyl group in ASA (83), (82), the hydroxyl group in SA attaches with serine residue in COX enzymes and inhibit the action of COX, while the acetyl group in ASA attaches with serine residue in COX and inhibits the action of COX. The hydroxyl group in SA is soluble in water; it will make the bond very strong; also it can easily penetrate the cell and initiate its functions more than ASA. While the acetyl group in ASA is slightly insoluble in water, the bond will not be strong as well it cannot penetrate the cell easily and initiate its function. Also SA has been shown to activate adenosine monophosphate-activated protein kinase (AMPK), and suggest being the reason for its anti-tumour effects more than ASA (90). In this study, ASA and SA were used to find their effect on cervical cancer cell line, because these drugs was shown to inhibit COX in cancer cells, and COX over-expressed in cervical cancer. These drugs also were shown to induce apoptosis in cancer cells. In the development of cervical cancer, COX has the ability to promote the growth, chronic inflammation, carcinogen production, angiogenesis, and tumour invasiveness, inhibit immune recognition and apoptosis (7, 20, 21, 22); these drugs ASA and SA can block COX and stop all these processes.

Western blotting analysis is a technique mostly used for the determination and analysis of proteins according to their ability to bind to certain antibody. Western blot has been in used in science for detection and analysis of proteins. This is a simple technique; require simple equipment and inexpensive reagents, rapid technique. The specificity of the antibody-antigen reaction makes the target protein to be determined in the midst of complex protein mixture (91). The results of western blot of active anti-caspase in figure 6 of this study shows no any expression of proteins, which is contrary from the literature in which an active anti-caspase shows an expression around 17kDa large unit and 12kDa small subunits (92). But using β-actin as a house keeping gene it shows the expression of the proteins with different bands of the samples around 43kDa shown in figure 7. This shows that the western blot analysis for active anti caspase was done correctly, it might be as a result of using small amount of sample in an electrophoretic gel, or protein for active anti caspase 3 was not enough in the sample to show the expression.

5. Conclusions

In conclusion, this study examined the effect of ASA and SA on the viability of HT3 cervical cancer cell line. Findings from this study indicates that ASA and SA at 10mM to 20mM concentrations inhibits the viability of HT3 cervical cancer cell line after 48 and 72hrs incubations. It also shows a differential increase in caspase activities by the medication on the cell line from 8 to 16hrs incubations. This shows that ASA and SA can induce apoptosis and bring about cell death on cervical cancer, and these drugs can be good drugs for the treatment of cervical cancer.

Future Study

Based on the results of this study, we recommend that future studies may be needed to increase the number of concentrations of the drugs Acetylsalicylic acid and Salicylic acid from 0 to 30mM, because in this study we used 0 to 20mM concentration, and the inhibition of the drugs started showing from 10mM concentration. In caspase activity assay, we suggest that the incubation period be from 8 to 16hrs with 1 or 2hrs interval. In this present study, we observed more increase in caspase activity from 8 to 16hrs. Further studies are required to identify how Salicylic acid and Acetylsalicylic acid possibly induce the translocation of Bax to the mitochondria in cervical cancer, and whether this is the major mechanism for Salicylic acid and Acetylsalicylic acid induced apoptosis in cervical cancer. Also studies are needed to determine the role of mitochondria and cytochrome C release in Acetylsalicylic acid and Salicylic acid induced apoptosis in cervical cancer. For Western Blot analysis, we suggest an increase in the amount of sample loaded on the wells of electrophoretic gel. We hypothesize that it might be enough to show the expression of active anti-caspase protein in the samples.

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