# Effect of some aspirin analogues on SW480 Colorectal cancer cell line: Tip of the iceberg

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## Abstract

Colorectal cancer (CRC) is the third most common cancer and second leading cause of death with approximately 1.9 million cases and almost a million deaths worldwide in 2020. It is predicted that the global incidences of CRC will be over 3 million in 2040. Aspirin has been shown to have cytotoxic and immunomodulatory effects on CRC cell lines. However, due to side effects such as gastrointestinal bleeding in older patients, there has always a quest for safer and more effective analogues, leading to the synthesis of aspirin analogues. One of the primary regulators of the mitochondria-mediated pathway to apoptosis is the family known as BCL-2 proteins, which are broadly grouped into pro-apoptotic proteins such as BAX, BAK, BIK, BAD and anti-apoptotic proteins such as BCL-2 and BCL-$X_L$.

Phase contrast images of the SW480 CRC cells treated with aspirin, meta-aspirin, para-aspirin, ortho-thioaspirin, meta-thioaspirin and para-thioaspirin at 0.5 mM for 24 h, 48 h and 72 h were taken at 100X magnification.

Western blot was used to detect BAX and BCL-2 proteins by treating CRC cells with aspirin and its analogues, and analysed by SDS-PAGE and immunoblotting. The blots were then probed with primary antibodies BCL-2 and BAX, after which the HRP bound protein-labelled antibody was visualised using ECL and exposed to CL-XPosure™ Film.

It was observed that the isomers of aspirin (O-ASP), meta-aspirin (M-ASP)

and para-aspirin (P-ASP) significantly increased the density of BAX Ab. O-ASP, M-ASP, P-ASP and ortho-thioaspirin (O-TASP) significantly reduced the density of BCL-2 Ab, thus proposing that the anti-apoptotic effect of this cell line is reversed/reduced by these aspirin analogues.

The results of this study suggest that M-ASP and P-ASP have its antiproliferative effect on the SW480 CRC cell line via driving the pro-apoptotic effect of BAX protein and decreasing the expression of anti-apoptotic of BCL-2 protein, thus, encouraging apoptosis in this CRC cell line.

**Keywords:** Colorectal cancer, aspirin, aspirin analogues, BAX protein, BCL-2 protein, apoptosis

## Introduction

Colorectal cancer (CRC) is the third most common cancer and second leading cause of death with approximately 1.9 million cases and almost a million deaths worldwide in 2020 (Xi and Xu, 2021). It is predicted that the global incidences of CRC will be over 3 million in 2040. These incidences however differ across geographical regions, economic status, life style and more recently, race and ethnicity (Zaki *et al*., 2023). In the United States, CRC is the second most common cause of mortality from cancer (Siegel *et al*., 2020). Despite the fact that prognosis after CRC therapy has greatly improved over the years, the rising number of CRC incidence among younger patients is still a heavy burden financially amongst governments and the patients themselves (Fidler *et al*., 2017; Arnold *et al*., 2017).

Aspirin, which is the ortho-isomer has been shown to have cytotoxic and immunomodulatory effects on colorectal cancer cell lines (Kilari *et al*., 2018; Kadhum *et al*., 2022). Patients that in particular experience increased thromboxane biosynthesis after radical cancer therapy, are most likely to benefit from the use of aspirin (Joharatnam-Hogan *et al*., 2023). The findings in a study by Guo *et al*., 2021 supports the use of aspirin, if initiated at a younger age to reduce the risk of CRC. The regular aspirin that is used medically is the ortho-aspirin (O-ASP), while meta-aspirin (M-ASP) and para-aspirin (P-ASP) are its positional isomers. Ortho-thioaspirin (O-TASP), meta- (M-TASP) and para-thioaspirin (P-TASP) are also synthesised aspirin analogues (Bashir *et al*., 2019).

Several studies have shown the effects of aspirin and its analogues on colorectal cancer, which reiterates their use in chemoprevention. An updated meta-analysis and a CAPP2 (Colorectal Adenoma/carcinoma Prevention Programme) study, both published in 2020 concluded that the regular use of aspirin is associated with reduced risk of colorectal and other digestive tract cancers, including those with a genetic disposition to CRC such as Lynch syndrome and patients with PIK3CA-mutated tumors (Bosetti *et al*., 2020; Burn *et al.*, 2020). This reduced risk is associated with longer duration of aspirin use (Grancher *et al*., 2022). Thus, aspirin is recommended by the United States Preventive Services Task Force (USPSTF) for primary prevention of CRC in all patients aged 50 to 59 with a 10-year risk of cardiovascular events greater than 10% . Aspirin or/and its analogues have also been found to exhibit additive and synergistic effects when used in combination with other medications in CRC cell lines (Voutsadakis *et al*., 2010; Kilari *et al*., 2019; Susan *et al*., 2023).

Due to side effects of aspirin, such as gastrointestinal bleeding in older patients, there has always been a quest for equally cheap but safer and more effective analogues. This has led to the synthesis of different aspirin analogues such as 2-hydroxy benzoate zinc, 4-hydroxy benzoate zinc (Pepper *et al*., 2011), Bis-carboxyphenylsuccinate, Bis-carboxyphenylfumarate, *m*-bromobenzoylsalicylic acid (Claudius *et al*., 2014), ortho-, meta- and para-isomers of NOSH-aspirin (Vannini *et al*., 2015) and those used in this study (Bashir *et al*., 2019). Thus, the interest on the effect and possible mechanism of action for positional isomers of aspirin and thioaspirin on CRC.

Apoptosis is the process of programmed cell death that is involved in many physiological and pathological states (Evan and Vousden, 2001). Thus, dysregulation of this process contributes to many diseases, including cancer. One of the primary regulators of the mitochondria-mediated pathway to apoptosis is the family known as BCL-2 proteins, which are broadly grouped into pro-apoptotic proteins such as BAX, BAK, BIK, BAD and anti-apoptotic proteins such as BCL-2 and BCL-$X_L$ (Kasibhatla and Tseng, 2003). Positional isomerism of different aspirin analogues has been found to reduce colorectal cancer cell growth via inhibition of proliferation and induction of apoptosis (Deb *et al*., 2011; Claudius *et al*., 2014;

Vannini *et al*., 2015). This prompted us to investigate whether our aspirin analogues exhibited their proliferative effect via pro-apoptotic BAX and/or anti-apoptotic BCL-2 proteins.

Another alternative to programmed cell death is necroptosis, a program that combines the features of apoptosis and necrosis. The deregulation of necroptosis is found to cause disease states such as cancer, inflammation and neurodegeneration (Dhuriya and Sharma, 2018).

***Pharmacology of Aspirin***

The modes of action for aspirin as an antiproliferative and chemopreventative agent include

irreversible cyclooxygenase inactivation through non-enzymatic acetylation of a single serine residue, which ultimately leads to the inhibition of prostaglandin biosynthesis (Warner *et al.*, 1999; Patrono *et al.*, 2008; Schror, 2011; Patrignani *et al.*, 2017). Thus, an anti-inflammatory agent. This is the generally accepted pathway. The inhibition of NF-κB activity (Kopp and Ghosh, 1994; Gurpinar *et al.*, 2013), inhibition of the JNK pathway (Schwenger *et al.*, 1997), activation of AMP kinase (AMPK) (Hawley *et al.*, 2012), apoptosis through the Wnt-β-catenin pathway (Deng *et al.*, 2009) providing a link between the Wnt-β-catenin pathway and apoptosis, normalising EGFR expression (Li *et al.*, 2015), perturbation of EGF phosphorylation and signalling (Bashir *et al*., 2019) are also proposed modes of action.

**Materials and methods**

**Aspirin analogues on proliferation of SW480 CRC cell lines:**

Aspirin analogues (Table 1) were synthesised and characterised, which we have previously reported. Aspirin and analogues were prepared in dimethyl sulfoxide (DMSO) at a concentration of 50 mM and then diluted to 25 mM with HEPES (pH8) to adjust the pH. These solutions were then further diluted in Leibovitz's L-15 medium supplemented with 10% (v/v) FBS and 1% (v/v) 10,000 U/ml penicillin-10 mg/ml streptomycin to 0.5 mM final concentrations for studies.

Table 1. List of compounds

| Common name | Chemical name | Source |
|---|---|---|
| Aspirin, ortho-aspirin (O-ASP) | 2-acetoxybenzoic acid (acetylsalicylic acid) | A5376, Sigma® Sigma-Aldrich Company Ltd |
| meta-aspirin (M-ASP) | 3-acetoxybenzoic acid | Bashir *et al*., 2019 |
| para-aspirin (P-ASP) | 4-acetoxybenzoic acid | Bashir *et al*., 2019 |
| ortho-thioaspirin (O-TASP) | 2-acetylthiobenzoic acid | Bashir *et al*., 2019 |
| meta-thioaspirin (M-TASP) | 3- acetylthiobenzoic acid | Bashir *et al*., 2019 |
| para-thioaspirin (P-TASP) | 4-acetylthiobenzoic acid | Bashir *et al*., 2019 |

About 5 X$10^4$ SW480 CRC cells per well were plated in a 6-well tissue culture plate and treated with O-ASP, M-ASP, P-ASP, O-TASP, M-TASP and P-TASP at 0.5 mM for 24 h, 48 h and 72 h. Phase contrast images were taken at 100X magnification.

**Western blot for detection of proteins:** About 5 X$10^4$ SW480 CRC cells per well were plated in a 6-well tissue culture plate and treated with aspirin and some of its analogues at 0.5 mM for 24 h. 150 µl of Laemmli protein buffer was added to the cells before being scraped off and transferred into 1.5 ml microcentrifuge tubes. The cells were then treated at 100°C for 20 min and centrifuged (14,000 rpm) for 5 min. The samples were analysed by SDS-PAGE and immunoblotting. The blots were then probed with primary antibodies BCL-2 (ab32124, Abcam) rabbit mAb at 1:1000 and BAX (sc-493, Santa Cruz Biotechnology) mouse mAb at 1:200. Anti-beta Tubulin Ab (ab15568, Abcam) at 1:2000 was used as loading control overnight on a rocker at 4°C. The blots were then washed in TBST three times for 5 min each and probed with the corresponding HRP-linked secondary antibody diluted in blocking buffer for 1

h on a rocker at RT. The HRP bound protein-labelled antibody was visualised using ECL (GE Healthcare Amersham™ ECL™ Prime Western Blotting Detection Reagent, RPN2232) for 5 min and exposed to CL-XPosure™ Film. ImageJ software v1.53k (National Institutes of Health, USA) was used for densitometric analysis of western blots. Band intensities were quantified (n=3) and BAX and BCL-2 levels were calculated relative to ß-Tubulin, the loading control. Data points represent mean ± standard error of the mean. Statistical analysis was performed using ANOVA (Analysis of variance) followed by Tukey post hoc test.

**Results and discussion**

Due to evidence that aspirin is chemopreventive in colorectal cancer for patients whom its protective benefits outweigh the harms (Drew *et al*., 2016; Kotana and Weiss, 2020; Guo *et al*., 2021), the effect of its analogues, M-ASP, P-ASP, O-TASP, M-TASP and P-TASP were studied on SW480 CRC cell line.

**Effect of aspirin analogues on proliferation of SW480 CRC cell lines:** Aspirin analogues at 0.5 mM reduced the proliferation of SW480 CRC cell line after 24 h, 48 h and 72 h after treatment (Figure 1), which agrees with previous studies (Deb *et al*., 2011). The concentrations of compounds used may seem high in comparison to other chemotherapeutic agents. However, it is common to use concentrations between 1mM and 5mM in *in vitro* studies involving aspirin molecular action (Stark *et al*., 2001; Hawley *et al*., 2012; Bashir *et al*., 2019).

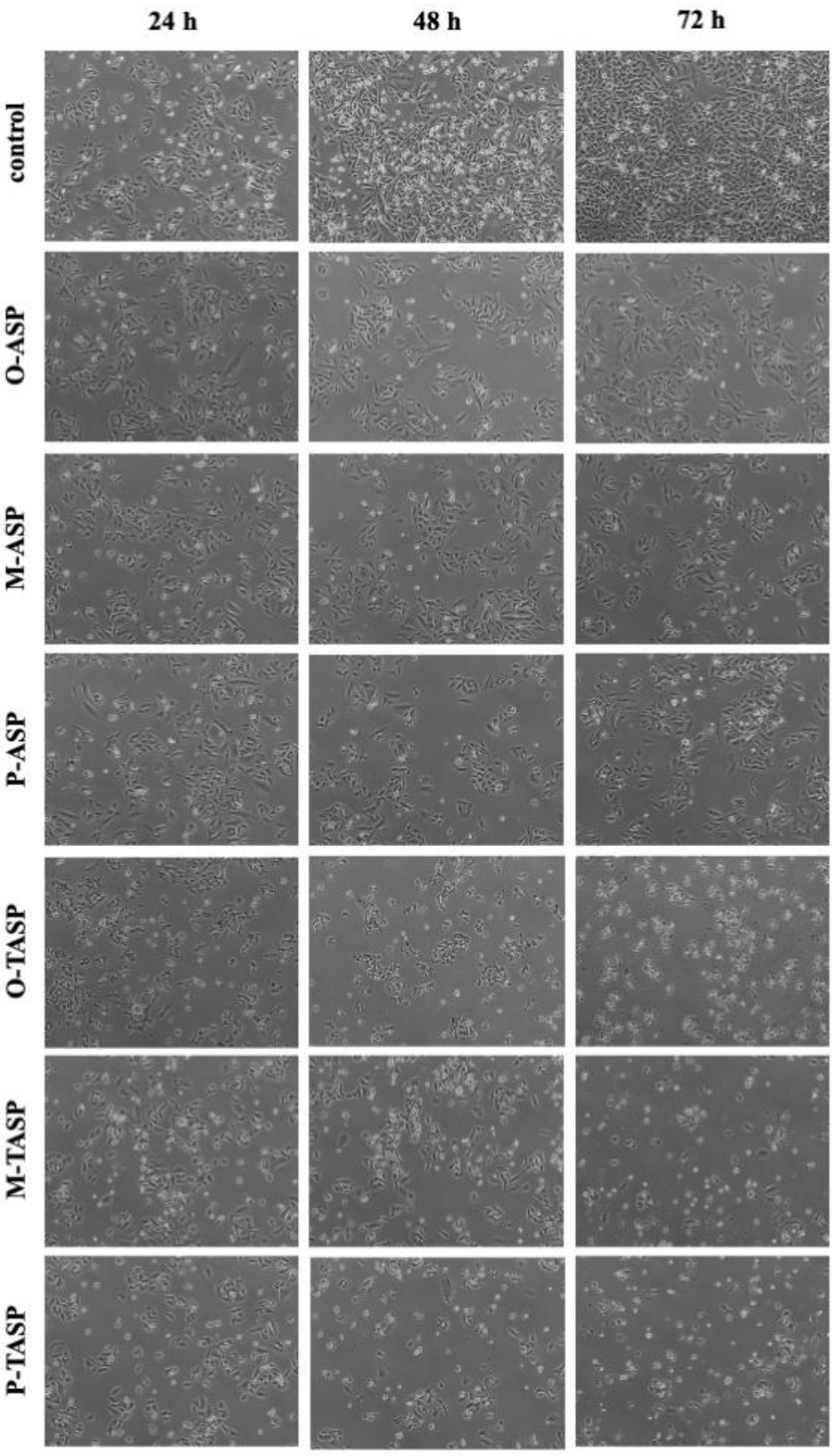



Figure 1. Effect of aspirin analogues on proliferation of SW480 CRC cell line. Phase Contrast

images of SW480 CRC cell line untreated and treated with aspirin analogues (0.5 mM) for 24 h, 48 h and 72 h taken at 100X Magnification.

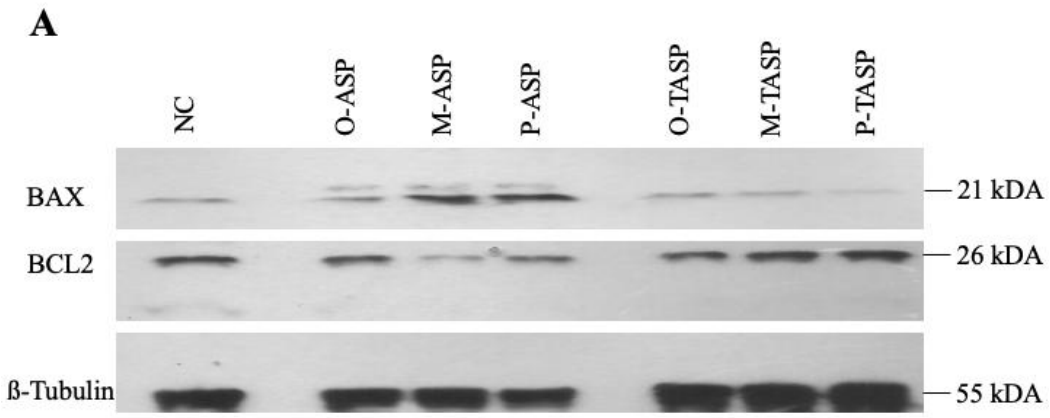
**Effect of aspirin analogues on pro- and anti-apoptotic proteins:**

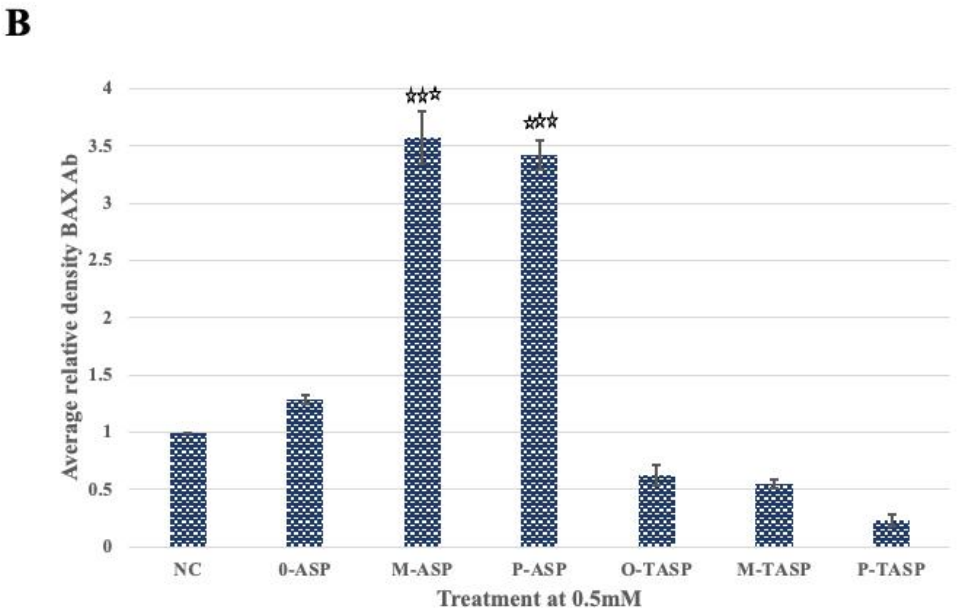
BAX, a pro-apoptotic protein has a low expression in localized tumour, which increases the incidence of CRC. Thus, very important in colorectal carcinogenesis (Pryczynicz *et al*., 2014). Aspirin has previously been reported to cause death in SW480 cells via apoptosis (Stark *et al*., 2001). In this study, it was observed that meta-aspirin (M-ASP) and para-aspirin (P-ASP) significantly increased the density of BAX Ab (Figure 2A, 2B), suggesting that these analogues encourage apoptosis in this cell line. This correlates with previous studies using flow cytometry analysis (Bashir *et al*., 2022).

Destruction of the cell depends on formation of apoptotic bodies that are engulfed by macrophages. BCL-2 proteins, which are also referred to as 'guardians' neutralise pro-apoptotic members thus interfering with apoptosis (Berberich and Hildeman, 2017). In this study, aspirin (O-ASP), meta-aspirin (M-ASP), para-aspirin (P-ASP) and ortho-thioaspirin (O-TASP) significantly reduced the density of BCL-2 Ab (Figure 2A, 2C), thus proposing that the anti-apoptotic effect is reversed/reduced by these aspirin analogues.

The $IC_{50}$ values have been previously reported as 1.8 mM, 3.8 mM, 4.9 mM, 0.2 mM, 0.5mM and 0.8 mM for O-ASP, M-ASP, P-ASP, O-TASP, M-TASP and P-TASP respectively (Bashir *et al*., 2019). Interestingly, concentrations employed for O-ASP, M-ASP and P-ASP were significantly less than their respective $IC_{50}$ values, which are all within a physiologically relevant range of 0.5 and 2 mM.

The ulcerogenic profile of these aspirin analogues have been reported to differ significantly, with an observed decrease in mucosal injury by M-ASP and P-ASP when compared to O-ASP (Kodela *et al*., 2013). Thus, the reduced SW480 CRC cell proliferation, increased density of BAX Ab and reduced density of BCL-2 Ab by M-ASP and P-ASP has an added advantage of a greater safety profile.


A
NC
O-ASP
M-ASP
P-ASP
O-TASP
M-TASP
P-TASP
BAX
BCL2
ß-Tubulin
21 kDA
26 kDA
55 kDA


B
Average relative density BAX Ab
4
3.5
3
2.5
2
1.5
1
0.5
0
NC
0-ASP
M-ASP
P-ASP
O-TASP
M-TASP
P-TASP
Treatment at 0.5mM

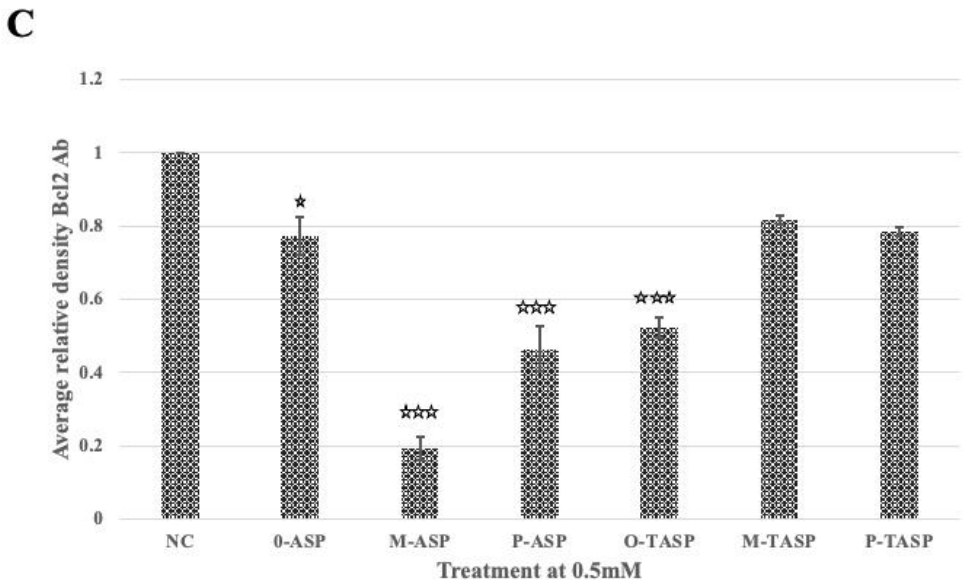

C
Average relative density Bcl2 Ab
1.2
1
0.8
0.6
0.4
0.2
0
NC
0-ASP
M-ASP
P-ASP
O-TASP
M-TASP
P-TASP
Treatment at 0.5mM

Figure 2. Effect of aspirin analogues on BAX and BCL2 protein. SW480 colorectal cancer cells were treated with 0.5mM aspirin analogues (HEPES buffered) for 24 h and (A) probed with BAX Ab and BCL2 Ab (B) histogram presentation for effect of compounds on BAX Ab (C) histogram presentation for effect of compounds on BCL2 Ab. [NC = normal control, O-ASP = ortho-aspirin, M-ASP = meta-aspirin, P-ASP = para-aspirin, O-TASP = ortho-thioaspirin, M-TASP = meta-thioaspirin, P-TASP = para-thioaspirin]

## Conclusion

The results of this study suggest that meta- and para-aspirins have its antiproliferative effect via driving the pro-apoptotic effect of BAX protein and decreasing the expression of anti-apoptotic of BCL-2 protein, thus, encouraging apoptosis in SW480 CRC cell line. An added advantage of production at a low cost will make them perfect adjuvants for the treatment of cancer in developing countries.

## Conflict of interest

There is no conflict of interest. No external financial support was obtained for this study.

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