MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society For Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

ANTIPROLIFERATIVE EFFECT OF ASPIRIN AND DICLOFENAC ON MDA-MD-231 TRIPLE-NEGATIVE BREAST CANCER CELLS

Yow Hui-Yin[1,2]*, Tang Yin-Quan[1,3] and Lee Jyy-Shiuan[2]

[1]*Centre for Drug Discovery and Molecular Pharmacology, Faculty of Health and Medical Sciences, Taylor's University, 47500 Subang Jaya, Selangor, Malaysia*
[2]*School of Pharmacy, Faculty of Health and Medical Sciences, Taylor's University, 47500 Subang Jaya, Malaysia*
[3]*School of Biosciences, Faculty of Health and Medical Sciences, Taylor's University, 47500 Subang Jaya, Malaysia*

*Corresponding Author: HuiYin.Yow@taylors.edu.my

SHORT COMMUNICATION

History
Received: 13 December 2021
Accepted: 31 May 2022

Keywords:

Aspirin; Diclofenac; Breast Cancer; Anticancer; Cytotoxicity

Abstract

The role of nonsteroidal anti-inflammatory drugs in triple-negative breast cancer remains unclear. This study aimed to investigate the antiproliferative effect of aspirin and diclofenac on MDA-MB-231 cells using MTT assay. Aspirin (1 – 100 mM) inhibited cell proliferation at dose- and time-dependent manner. At 24 and 48 h treatment, 1 – 100 mM of aspirin exhibited significant antiproliferative effect ($p < 0.05$). Only ≥ 5 mM aspirin demonstrated significant effect at 72 h ($p < 0.05$). Similar trend was found with diclofenac (0.01 – 1 mM). This study highlights the potential of aspirin and diclofenac in the management of triple-negative breast cancer.

INTRODUCTION

Cancer has been one of the prominent causes of mortality globally over the years. In 2020, almost 10 million cancer-related deaths and 19 million new cancer cases were reported [1]. Breast cancer is ranked as the most diagnosed cancer with an estimated 2.3 million new cases in 2020 [1]. Although triple-negative breast cancer (TNBC) accounts for approximately 20% of breast cancer cases, it is known to be the most invasive with high metastatic potential and recurrence rate [2]. Among breast cancers, TNBC is the most difficult-to-treat cancer that imposes challenges to patients and clinicians due to its poor prognosis and only a few effective treatments. This is because TNBC lacks estrogen receptor, progesterone receptor, and HER2/neu oncogene expression, which are the targets for hormonal or HER2 treatment [2]. Currently, chemotherapy remains the main approach to treat TNBC due to its non-selective properties. Nevertheless, the treatment outcomes are disappointing due to poor disease prognosis, intra-tumoral heterogeneity and drug resistance issues in treating TNBC [2]. Therefore, this urges the need for new drug development or approach for the treatment of TNBC.

Drug repurposing approach has been utilised in drug research to identify a new indication of currently available medicines and provides a promising effect to cancer patients [3]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed for their analgesic and antipyretic effects. They are known to have an important role in inflammation due to their ability to inhibit the cyclooxygenase enzyme and prostaglandin-endoperoxide synthase [4]. In view of carcinogenesis is closely associated with chronic inflammation, targeting an inflammation has become one of the approaches for cancer therapy. As such, NSAIDs have gained great interest in researchers for their role in cancer prevention and treatment.

Over past decades, studies have shown that long-term NSAIDs intake (particularly aspirin) is associated with reducing the risk of some types of cancer [5-8]. The evidence is more compelling for colorectal cancer, with a decrease in risk for other cancers, including breast, lung and prostate cancer [9]. Their role in cancer is thought to be associated

with their ability in inhibiting cyclooxygenase enzymes, which catalyse the formation of prostaglandins, the potent mediators that trigger chronic inflammation involved in carcinogenesis [4]. However, these studies are predominantly focused on colorectal cancer cell line and aspirin, while the studies on other NSAIDs is scarce. Diclofenac is also reported with an ability in reducing cancer cell growth in human ovarian adenocarcinoma and colon cancer cell lines [10,11]. Hence, this study investigated on the cytotoxic effect of aspirin and diclofenac on triple negative breast cancer cells, MDA-MB-231 cells.

MATERIALS AND METHODS

Materials

Aspirin and 5-Fluorouracil (5-FU) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diclofenac sodium was obtained from Supelco (Bellefonte, PA, USA). Dulbecco's modified eagle's medium (DMEM), penicillin-streptomycin and trypsin-EDTA solution with phenol red were purchased from Nacalai Tesque (Kyoto, Japan). 10% Fetal bovine serum (FBS) was purchased from PAA Laboratories GmbH (Paching, Austria). MTT [3-(4,5-dimethyl- 2-thiazoyl)- 2,5- diphenyl- 2- tetrazolium bromide] was purchased from PhytoTechnology Labs (Lenexa, KS, USA). Dimethylsulfoxide (DMSO) was purchased from Friedemann Schmidt (Frankfurt, Germany).

Cell Culture and Drug Treatment

MDA-MB-231 cells (American Type and Culture Collection, Manassas, VA, USA) were cultured in DMEM containing 10% FBS, 1% penicillin-streptomycin in 5% CO_2 at 37℃ incubator. After cell confluency achieved approximately 80%, the cells were detached by adding 1 ml of 0.25% Trypsin-EDTA solution with 1 min of incubation and transferred to the 96-well plates. The cells were seeded with 3 x10^4 cells per 100 uL of DMEM complete culture medium in each well, followed by incubation at 37℃ with humidified atmosphere of 5% CO_2 for 24 hours to ensure good cell attachment.

Aspirin and diclofenac sodium were dissolved in DMSO and achieved a final concentration of ≤ 1% DMSO after dilution with phosphate buffered saline (PBS) in each well. The cells were treated with aspirin (1, 2.5, 5, 10, 25, 50, 100 mM) and diclofenac (0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1 mM) in triplicates for three independent experiments (i.e. 9 samples per replicates). The concentration selection for both drugs were determined from the literatures [10,12]. Positive control cells were treated with 0.03 mM 5-FU [13], whereas negative control cells were treated with 0.1% DMSO. The cells were incubated in 5% CO2 at 37℃ for 24, 48 and 72 h after the drug treatment.

Determination of Cytotoxicity

Determination of antiproliferative activity was performed by MTT assay, which is defined by forming blue formazan (with an absorbance maximum at 570 nm) through mitochondrial dehydrogenase found in normal cells. The intensity of purple is directly proportional to the number of viable cells. Dead cells lose their ability to convert MTT into insoluble formazan, thus colour formation serves as a useful marker of the viable cells only [14]. After each treatment duration (i.e., after 24 h, 48 h and 72 h), 20 uL of prepared MTT solution (5mg/mL in PBS) was added to the wells in a dark room, then incubated for 4 hours at 37℃. After incubation, excess MTT solution was aspirated from each well and added with 100 uL of 100% DMSO to dissolve the precipitate of formazan crystal. Next, the mixture was mixed by optimal shaker for 15 minutes. The absorbance was measured at a wavelength of 570 nm and a reference wavelength at 630 nm by a microplate reader (EpochTM, BioTek Instruments Inc, Netherlands). The MTT assay was performed in three independent experiments.

The IC_{50} for both NSAIDs, which is the concentration of drug that inhibits 50% of cell growth compared to the control group) was determined from the curve on percentage of cell viability plotted against concentration of aspirin and diclofenac. Cell viability of each group was determined from the formula below [6]:

$$\text{Cytotoxicity} = \left(1 - \frac{mean\ absorbance\ of\ toxicant}{mean\ absorbance\ of\ negative\ control}\right) \times 100\%$$

$$\text{Percentage of cell viability} = 100 - \text{Cytotoxicity } \%$$

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM) and analysed using the JMP Statistical Discovery software (SAS Institute, Cary, NC, USA). One-way analysis of variance (ANOVA) test with Tukey post-hoc test was used to determine the difference between control and treatment groups. The p value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSIONS

Different concentrations of aspirin and diclofenac demonstrated significant antiproliferative effect on MDA-MB-231 cells in dose-and time-dependent manner (Figure 1 and 2). At 24 h treatment, 1 – 100 mM of aspirin exhibited significantly high antiproliferative effect compared to the negative control ($p < 0.05$). Aspirin demonstrated comparable antiproliferative effect to 5-FU and significant greater cytotoxicity than 5-FU at higher concentration, i.e., 50 and 100 mM ($p = 0.0044$ and 0.0053, respectively). Similarly, 1 – 100 mM of aspirin exhibited significant

antiproliferative effect compared to negative control following 48 h of incubation ($p < 0.05$), yet aspirin at 10, 25, 50 and 100 mM had significantly greater cytotoxicity than 5-FU ($p = 0.0091$, 0.0002, 0.0001 and 0.0002, respectively). However, after 72 h of incubation, only higher concentrations of aspirin (≥ 5 mM) were statistically distinguished from negative control. The antiproliferative effect of aspirin on MDA-MB-231 cells is consistent with study conducted by Amaral et al. that reported that aspirin reduced cell viability at 10 mM over 72 h of treatment, which is lower than the concentration of current study [15]. This discrepancy could be related to the choice of vehicle for aspirin. DMSO was used in this study for aspirin dilution, whereas ethanol was selected by Amaral et al. [15]. Interestingly, this study also revealed that the higher the concentration and longer duration of treatment on MDA-MB-231 cells, the more notable the inhibitory effect of aspirin was.

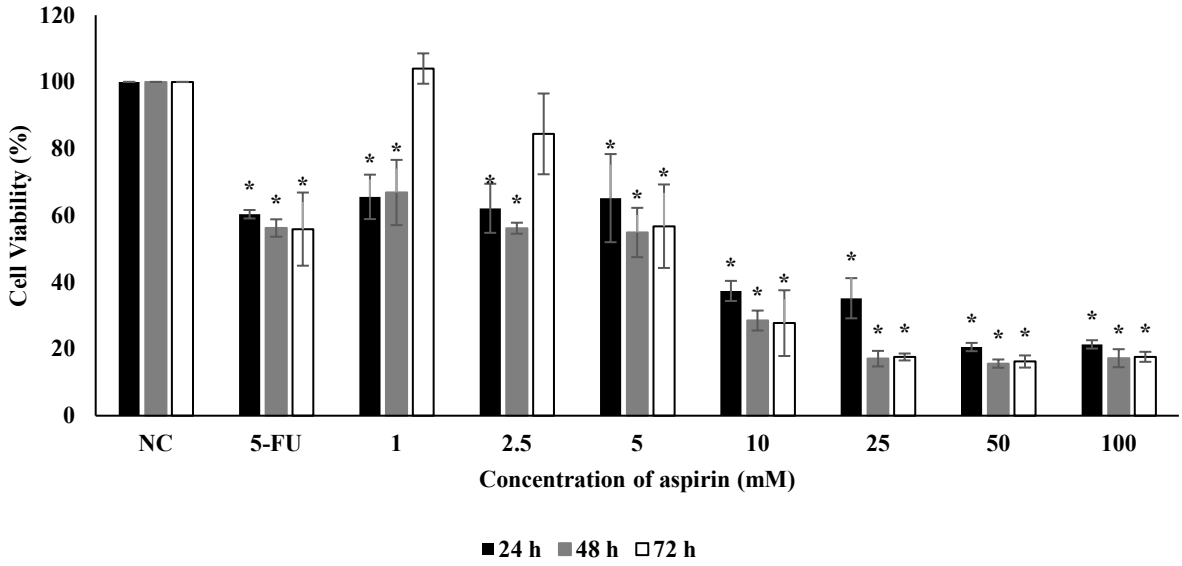

Figure 1: Antiproliferative effect of aspirin on MDA-MB-231 cells. Data were expressed as means ± SEM of three replicates in three independent experiments. NC: negative control; 5-FU: 5-fluorouracil at 0.03 mM; *One-way analysis of variance (ANOVA) with Tukey post-hoc test; significance level at $p < 0.05$.

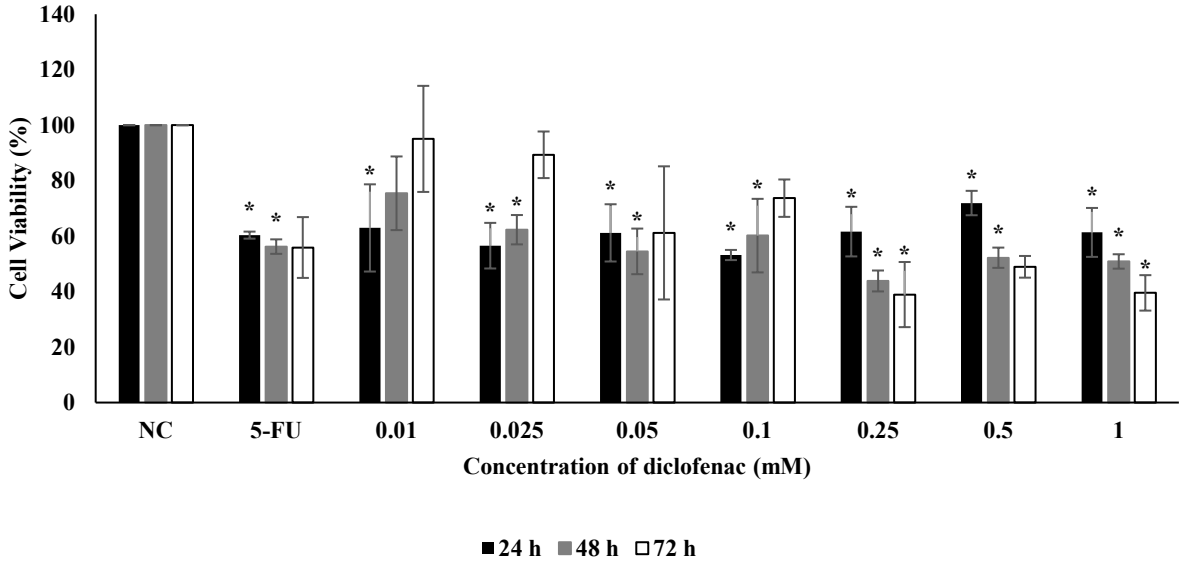

Figure 2: Antiproliferative effect of diclofenac on MDA-MB-231 cells. Data were expressed as means ± SEM of three replicates in three independent experiments. NC: negative control; 5-FU: 5-fluorouracil at 0.03 mM; *One-way analysis of variance (ANOVA) with Tukey post-hoc test; significance level at $p < 0.05$.

The mechanisms by which the aspirin exert the antiproliferative effects against MDA-MB-231 cells might be due to various pathways in addition to its anti-inflammatory activity, including induction of apoptosis, inhibition on tumor growth, induction of reprogramming factors of mesenchymal to epithelial transition and inhibition on synthesis of osteoclast cytokines [16,17]. Nevertheless, a previous study reported that treatment of 1 mM aspirin exerted an apoptosis effect through COX-independent mechanisms by upregulating DNA mismatch repair (MMR) protein expressions in human colon cancer cells [18]. A recent study reported that aspirin suppressed the cell proliferation in hepatocellular carcinoma by upregulating the levels of miRNA-137 and inhibiting the epidermal growth factor pathway [19]. The exact mechanism of aspirin in inhibiting the cell growths of MDA-MD-231 cells remains unclear and warrants further investigations.

In term of IC_{50}, aspirin exhibited 50% of antiproliferative activity at concentration of 6.53 ± 2.18 mM, 5.85 ± 1.22 mM and 6.62 ± 1.87 mM, respectively on MDA-MB-231 cells (Table 1). These IC_{50} values are far higher than the IC_{50} reported for aspirin in various cancer cells, i.e., 0.94 mM in HCT116 cells, 1.28 mM in SW620 cells, 1.867 in Ht-29 cells, 0.98 in MCF-7 cells [5,11,20]. Nevertheless, the IC_{50} values of aspirin in MDA-MB-231 are congruent with the previous study in SW742 cell line, at 5.597 mM following 48 h treatment [6]. This finding suggests that MDA-MB-231 cell line tended to be less sensitive to cytotoxicity of aspirin compared to HCT116, SW620, Ht-29, MCF-7 cell lines and displayed a comparable sensitivity to SW742 cell line.

Table 1. Cytotoxicity of aspirin and diclofenac on MDA-MB-231 cells after 24, 48 and 72 h

Drug	IC_{50}* (mM)		
	24 h	48 h	72 h
Aspirin	6.53 ± 2.18	5.85 ± 1.22	6.62 ± 1.87
Diclofenac	0.06 ± 0.04	0.11 ± 0.07	0.33 ± 0.08

*IC_{50}: the concentration of drug that inhibits 50% of cell growth. Each data point represents the mean of three independent experiments ± SEM

Correspondingly, diclofenac reduced the cell viability significantly compared to untreated cells at different concentrations (0.01 – 1 mM; $p < 0.05$) and shown comparative cytotoxicity effect as 5-FU following 24 h of treatment. At 48 h, diclofenac at 0.01 mM had not statistically different from negative control while significant inhibition on cell growth was observed at other concentrations (0.025 – 1 mM; $p < 0.05$). Lower concentrations of diclofenac (0.01 – 0.1 mM) shown no difference from negative control following 72 h of treatment, yet the antiproliferative effect was significant for other concentrations (0.25 and 1 mM; $p < 0.05$). This effect is parallel with the previous study that diclofenac inhibited MDA-MB-231 cell proliferation at ≥ 0.2 mM [21], in which the effect of diclofenac was not investigated at any concentration less than 0.2 mM. This study also found that the higher the concentration and longer time of treatment time of diclofenac on MDA-MB-231 cells, the inhibitory was more notable. This parallel finding is reported by Yang et al. [21]. The antiproliferative effect of diclofenac was revealed to be associated with impairment of glucose metabolism in MDA-MB-231 cells by downregulating GLUT1 and c-Myc expression and decreased the hexokinase activity [21].

To the best of our knowledge, this is the novel study to report on IC_{50} of diclofenac on MDA-MB-231 cells at 24, 48 and 72 h, which were 0.06 ± 0.04 mM, 0.11 ± 0.07 mM and 0.33 ± 0.08 mM, respectively (Table 1). These IC_{50} values are higher than those reported previously for DLD-1 (0.037 mM), but comparable to Ht-29 (0.055 mM) and SW480 (0.170 mM) [11].

CONCLUSIONS

In conclusion, the results of this study strongly suggest that aspirin and diclofenac showed their antiproliferative effect on MDA-MB-231 breast cancer cell line at dose- and time-dependent manner. This study highlights the potential of aspirin and diclofenac in the management of triple-negative breast cancer. Experimental investigations are required to further establish the role and the underlying mechanisms for aspirin and diclofenac as anti-proliferative agents in MDA-MB-231 breast cancer cells.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Malaysia Ministry of Higher Education (MOHE) for the funding support under the Fundamental Research Grant Scheme (FRGS/1/2019/SKK09/TAYLOR/03/1).

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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