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Unveiling the Effects of Cisplatin and Diallyl Disulfide on MDA-MB-231 Breast Cancer Cells

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Abstract

Cancer is one of the most aggressive diseases and is the primary cause of mortality around the world. This can be prevented by combining anti-cancer drugs to reduce the resistance to monotherapy and thereby reduce the toxic effects. Cytotoxic anticancer drugs can potentially elicit cancer cell death by apoptosis or necrosis. Our present study aimed to investigate the combined cytotoxic potential of cisplatin (CDDP) and diallyl disulfide (DADS) on MDA-MB-231 breast cancer cell lines. The clonogenic assay was also performed to assess the effects of the drug on the proliferation of breast cancer cells. The results showed that CDDP/DADS (CDDP and DADS) markedly inhibited cell proliferation and significantly reduced the colony formation potential, migration, and invasion abilities of MDA-MB-231 cells. The apoptosis assay confirmed that cell death was through an apoptotic pathway. Cell cycle analysis results indicated that the combination effect of the drugs resulted in arresting cells in the G2/M phase of the cell cycle. Further, the haemolytic assay revealed that the CDDP/DADS is nontoxic to RBCs. In conclusion, combining these two drugs inhibits the oncogenic properties of TNBC cells, including their growth, survival, migration, and invasiveness.

Keywords: Cancer, wound healing, colony formation, apoptosis, cell cycle, Haemolysis.

Introduction

Cancer is a very complex disease, which is highly heterogeneous, with a multi-step process involving various molecular events underlying the initiation and progression of tumours. According to the GLOBOCAN Statistics 2022, breast cancer (11.5%) is the second most common disease after lung cancer (12.4%). Breast cancer is the second most frequent type of cancer in the world and the fifth most common cause of death from cancer (1). Although it is still the most frequent cause of cancer death in women in underdeveloped regions, in more developed countries, it is the second most frequent cause of cancer death in women (2).

Breast cancer mainly affects women than men; however, this is a rare condition, accounting for less than 1% of cases. Approximately 2.29 million new breast cancer cases were diagnosed in women in 2022, which represents 23.8% of all cancers and over 15.4% of global breast cancer deaths (https://gco.iarc.who.int) (3). It follows, therefore, that numerous people across the world have breast cancer, something which has made it to be regarded as a significant public health

problem due to its extensive research focus. Of all breast cancer-related issues, triple-negative breast cancer (TNBC) is the most aggressive form. Triple-negative breast cancers (TNBCs) are distinguished by the lack of receptors for progesterone and estrogen and the absence of HER2 overexpression (4). These tumours are a distinct subtype of breast cancer, having a poor prognosis. To overcome this problem, it is crucial to understand how complicated the disease is and how it behaves to achieve effective treatment and management measures for patients suffering from the disease.

The cells in the tumour, stromal tumour microenvironment (TME), and extracellular matrix (ECM) significantly impact how the tumour behaves inside the body. The ECM provides structure to cells outside of them, while TME is known for low pH outside of cells with high hypoxia (5). These factors influence the dormant phenotypes of tumour cells, bringing about drug resistance and poor prognosis among cancer patients. Tumours are biologically similar to chronically unhealing wounds, which show continuous inflammation that leads to tumorigenesis, growth and metastasis. Other cells, such as those attracted to the tumour stromal microenvironment, contribute significantly towards cancer growth, metastasis and therapy failure (6). Thus, cancer therapy combines two or more treatments targeting different pathways that stimulate carcinogenesis or facilitate cellular support. Treatment of various types of cancer often entails mono-therapy; however, this traditional approach is frequently less efficacious than combination approaches. Traditional mono-therapeutic techniques are not selective for actively dividing cells but instead, result in killing all non-cancerous ones as well as unhealthy tissues. Chemotherapy may be harmful to a patient, causing numerous side effects and dangers, too (7).

Cisplatin (CDDP) demonstrates significant antitumor activity across various cancers by forming cross-linked adducts with DNA, distorting its structure, and hindering cell division (8). Diallyl disulfide (DADS), found in garlic, exhibits promising anticancer properties by inhibiting the growth of various types of cancer cells and decreasing carcinogen-induced cancers in experimental animals (9). These two compounds differ significantly in terms of their mechanisms of action. Cisplatin damages DNA, while diallyl disulfide has multiple mechanisms of action, including activation of detoxifying enzymes, suppression of DNA adduct formation, antioxidant effects, regulation of cell cycle arrest, induction of apoptosis and differentiation histone modification, and inhibition of angiogenesis and invasion. Both compounds have been studied for their potential role in cancer therapy. Our present study aimed to investigate the combined cytotoxic effects of CDDP and DADS (CDDP/DADS) on MDA-MB-231 breast cancer cell lines.

Our findings, which delve into the effects on migration, apoptosis, cell cycle, and toxicity, hold significant potential to contribute to developing more effective and less toxic breast cancer treatments. This underscores the relevance and impact of our research, inspiring further exploration and advancement in the field.

Materials and Methods

Materials

Cisplatin (CDDP), diallyl disulfide (DADS), and triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). In addition, Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, antibiotics, Crystal violet, and phosphate-buffered saline (PBS) were procured from HI Media Laboratories (Kelton, PA, USA). The apoptosis and cell cycle kits were purchased from Merck (Darmstadt, Germany). All chemicals and solvents used were of analytical grade.

Cell Culture

The MDA-MB-231cell line, a widely used and well-characterized breast cancer cell line known for its triple-negative breast cancer

(TNBC) phenotype, was obtained from the National Centre for Cell Science (NCCS), Pune.

Determination of IC_{50} concentration by MTT assay

All CDDP/DADS treatments for the below experiments were made with the IC_{50} of the drugs, which was determined using the MTT assay (10).

Clonogenic Assay

The clonogenic assay was performed to assess the effects of the drug on the proliferation of breast cancer cells. MDA-MB-231 cells (200 cells/well) were cultured in six-well tissue culture plates for 16 h. Then, drugs were added to the respective wells according to the IC_{50} concentrations (15.78 µM) described in our previous report (11), and the plates were re-incubated for 5–10 days. The media was changed every alternate day, and the plates were observed for colony (clusters of 20 or more cells) formation under an inverted microscope (Nikon, ECLIPSE Ti2, Tokyo, Japan). The colonies were then stained with 0.5% crystal violet solution and scored (12). The data has been represented as % survival derived from the following equation.

% survival = (Number of colonies after treatment/ Number of tumour cells seeded) X100.

Apoptosis assay

The MDA-MB-231 breast cancer cells (1×10^6 cells/well) were seeded and incubated till the cells were attached to the surface. After incubation, cells were treated with IC_{50} concentration of CDDP/DADS (15.78 µM) and allowed to incubate for 24 h. They were then harvested and washed twice with ice-cold PBS. Subsequently, the cells were labelled with Annexin V and the Dead Cell assay kit according to the manufacturer's instructions and incubated for 20 mins in the dark (13,14). This assay is based on the detection of phosphatidylserine (PS) on the apoptotic cell surface using fluorescently labelled Annexin V. The samples were determined by the Muse Cell Analyzer (Millipore, USA) and analysed by software provided by Merck Millipore (15).

Cell cycle analysis

MDA-MB-231 breast cancer cells (1×10^6 cells/well) were seeded in 6-well plates and incubated till the cells were attached to the surface. After incubation, cells were treated with IC_{50} concentration of CDDP/DADS (15.78 µM) and allowed to incubate for 24 h. The cells were detached, pipetted, centrifuged at 500 × g at 4°C for 5 min, and washed once with 1 × Phosphate-buffered saline (PBS). The cells were fixed with 1 ml (70%) of ice-cold ethanol and incubated at 4°C overnight. The ethanol-fixed cells were washed with PBS, and 100 µL of Muse cell cycle reagent was added. The tubes were incubated for 30 min at dark and analysed on a Muse flow cytometer (Millipore, USA). DNA content and cell cycle distribution were analysed using the software provided by Merck Millipore (16).

Haemolysis activity

The haemolytic efficiency of the CDDP/DADS was assessed by following the protocol by Hu *et al*. (2013), with slight modifications (17). A fresh blood sample (5 ml) was collected in heparinised tubes and centrifuged at 2000 rpm for 5 mins. After discarding plasma, the red blood cells were washed three times with phosphate buffer saline. The 2% solution of red blood cells was prepared in phosphate buffer saline for the analysis. 500 µL of the red blood cell solution was added to a micro-centrifuge tube containing CDDP/DADS (IC_{50} concentration) to study the haemolytic activity (18). Phosphate buffer saline was taken as a negative control, and Triton X-100 (10% v/v) as a positive control. The samples were incubated at 37ºC for 3-4 h on the shaker, following the centrifugation at 2000 rpm for 5 mins, and the release haemoglobin content was scanned at 540 nm. The following equation is used to calculate the haemolysis (%):

% of haemolysis = (absorbance of test

compound (CDDP/DADS)-absorbance of PBS)/ (absorbance of Triton X-100)*100

Statistical analysis

Data was expressed as the mean ± standard deviation, and a t-test was performed to identify the significance between groups. The * $p \leq 0.05$ is significant, ** $p \leq 0.01$ is most significant, and 'ns' represents not significant ($p > 0.05$).

Results and Discussions

Clonogenic Assay

The colony formation or clonogenic assay is a widely used method to evaluate cell survival and proliferation under *in vitro* conditions (19). This assay helps estimate cell death before or after treatment with adjuvant therapy or cytotoxic drugs. Studies have shown that CDDP and DADS, as a single therapy modality or combined with other chemotherapeutic drugs, have demonstrated anticancer effects on various human cancer cells (20).

After treating MDA-MB-231 cells with CDDP/DADS for 12 h and 24 h, the control group formed distinct colonies compared to the treatment group. The CDDP/DADS group inhibited colony formation with a 70% reduction in clonogenic ability, and the colonies were much smaller than those formed by the control (Fig. 1).

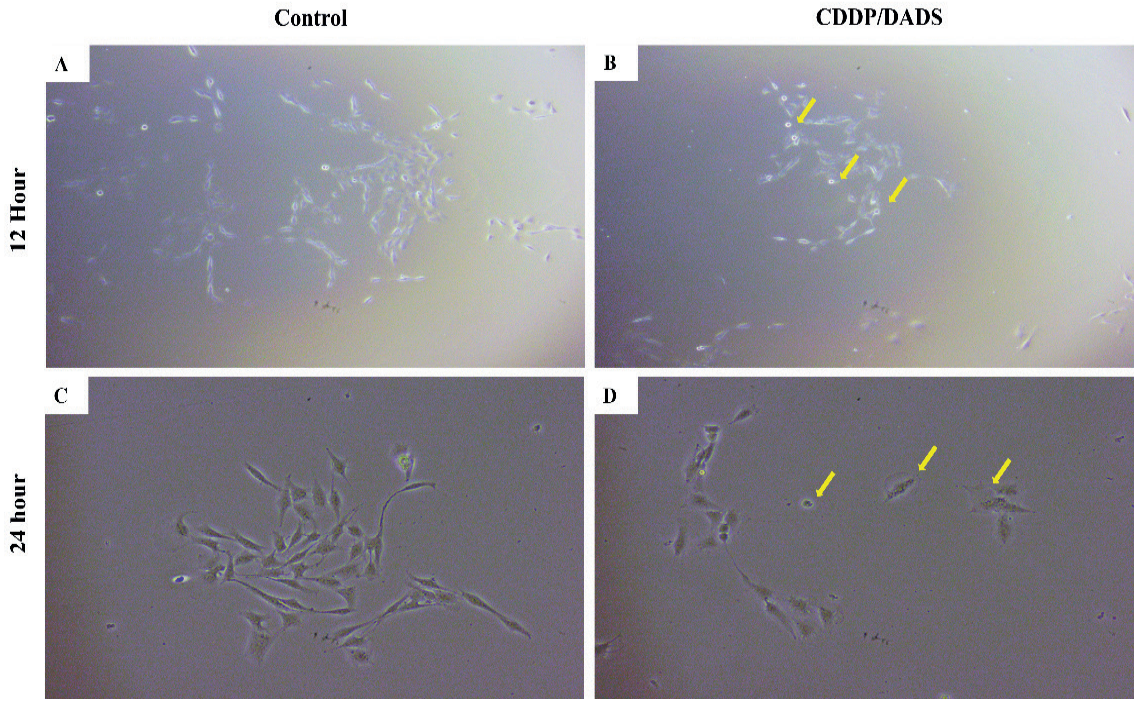

Figure 1: Comparative observation of colony formation in MDA-MB-231 cells subjected to CDDP/DADS treatment with control cells at 12 h and 24 h. The yellow arrow marks denote the apoptosis-undergoing cells in the CDDP/DADS-treated group.

The long-term effects of combination treatment with CDDP/DADS were assessed by clonogenic assay. We found that colony formation in MDA-MB 231 breast cancer cells treated with the combination of these drugs for 24 h was suppressed to 30% (Fig. 2) (21,22).

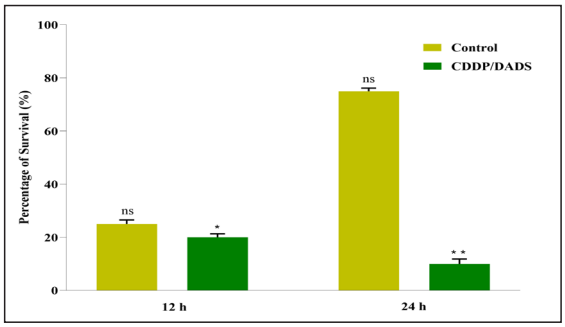

Figure 2: The percentage of MDA-MB-231 cells that survived after treatment with CDDP/DADS compared to the control at 12 and 24 h.

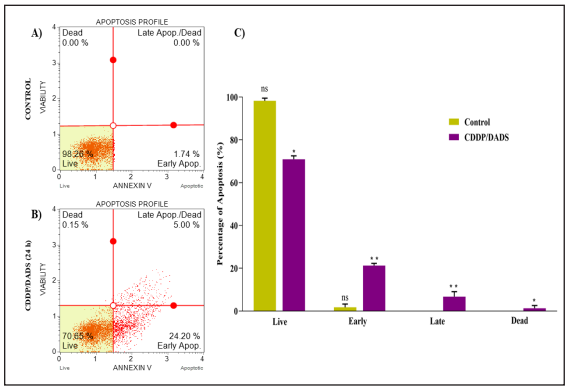

Figure 3: Apoptotic activity of CDDP/DADS on MDA-MB-231 cells. (A) Control cells. (B) CDDP/DADS treated cells. (C) Graph delineating the percentage of apoptosis for each quadrant (live, early, late, and dead).

Cell death (apoptosis)

Cancer cells are potentially killed by necrosis or apoptosis with cytotoxic antitumor drugs. Cell death was identified by analysing the cell trajectories on the dot-plot diagrams of Annexin-V-FITC/PI-stained treated cells. The plots are divided into four quadrants: live cells in the lower left quadrant, early apoptotic cells in the lower right, late-apoptosis cells in the upper right, and dead cells in the upper left (23).

To determine whether the cytotoxic effect of CDDP/DADS was associated with apoptosis, annexin V-FITC/PI double staining was used to determine the number of apoptotic cells by flow cytometry analysis. Phosphatidylserine (PS) is a crucial biomarker of early apoptosis and is translocated to the extracellular domain from the cytosolic portion of the membrane, identified by annexin V-FITC staining. As apoptosis proceeds further, the cell membrane is destroyed, and the PI that goes into the nucleus eventually stains the DNA (24). After treatment, the highest early apoptotic population was 24.20%, with the populations of viable cells at 70.97% in the CDDP/DADS group compared to the control, which had 98.26% live cells (Fig. 3).

Cell cycle analysis

The importance of chemotherapies functioning as cell-cycle modulators has increased due to the recent confirmation of the concomitant involvement of apoptosis and cell-cycle inhibition. Flow cytometry analysis was performed to analyse the various cell cycle checkpoints. We have evaluated the effect of CDDP/DADS on the DNA content of MDA-MB-231 cells by cell cycle phase distribution (G0, G1, S, G2, and M). The analysed data suggested that cells exposed to CDDP/DADS significantly increased the accumulation of DNA contents up to 40% in the G2/M phase. The treatment decreased G0/G1 and S phase cells (Fig. 4). It has been observed that cisplatin arrests cells at sub-G1, S, and G2 phases (25), and diallyl disulfide arrests cells at the G2/M phase. This statement also indicates the accumulation of the DNA contents up to 31% in the G0/G1 phase. The cell cycle analysis results indicate that the combination effect of CDDP/DADS resulted in arresting cells in the G2/M phase of the cell cycle.

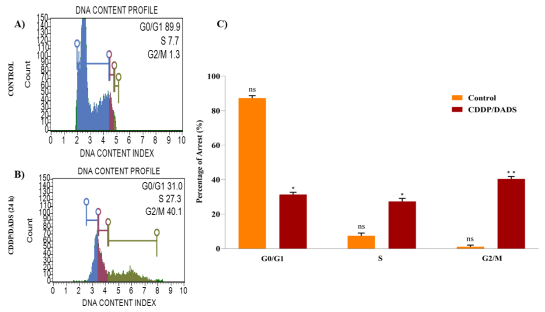

Figure 4: Analysis of cell cycle arrest of CDDP/DADS in MDA-MB-231 cells. (A) Control cells. (B) CDDP/DADS treated cells. (C) Graph presenting the percentage of cell cycle arrest for each phase (G0/G1, S, and G2/M).

Haemolytic Analysis

Haemolysis assay is a commonly used test to evaluate the potential toxicity of compounds on red blood cells. The assay examined the released haemoglobin from the lysis of red blood cells. The haemolytic activity of CDDP/DADS is shown in Figure 5. The results revealed that control and phosphate buffer saline (negative control) showed 0.0% haemolysis. Meanwhile, triton X-100 (positive control) showed 70.60% haemolysis. Moreover, the haemolysis caused by CDDP/DADS at 3% for 12 h and 8% for 24 h was negligible compared to Triton X-100. According to the regulatory guidelines provided by ISO-10993-4: 2107, if the compound causes less haemolysis (below 10% for humans), it is considered a non-haemolytic compound and safe for *in vivo* application. (26,27).

The results (Fig. 5) show that CDDP/DADS in IC_{50} concentration do not show haemolysis activity. According to the percentage of haemolysis activity, a lower concentration of CDDP/DADS was found to be harmless and biocompatible. Thus, the CDDP/DADS combination could be considered non-haemolytic and safe for further applications. The CDDP/DADS combination can be an excellent anticancer agent in preventing breast cancer growth and proliferation.

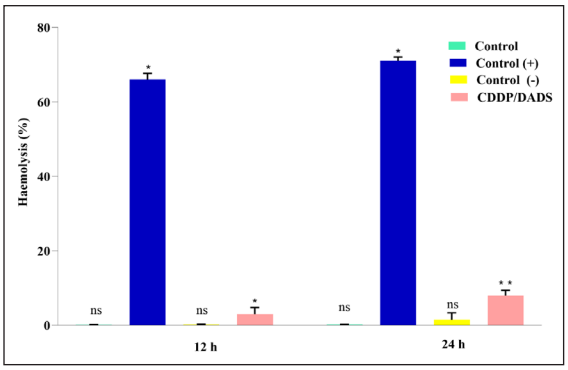

Figure 5: Haemolytic activity of CDDP/DADS at 12h and 24 h. Control is free RBCs, Positive control (control +) is Triton X-100, and Negative control (control -) is PBS.

Conclusions

The combination of CDDP/DADS inhibits various oncogenic properties of TNBC cells, including their growth, survival, migration, and invasiveness. CDDP/DADS can be an excellent anticancer agent in preventing breast cancer growth and proliferation. Additionally, CDDP/DADS induces apoptosis in cancer cells and arrests them in the G2/M phase of the cell cycle. The haemolysis assay showed that CDDP/DADS is non-haemolytic, making them a good choice for various cancer therapies.

Conflict of interest

The authors declare no conflict of interest.

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