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Research Article

Discovery of Antiproliferative Activity of Novel Bisurea Derivatives Induces Apoptosis in MDA-MB-231 Breast Cancer Cells

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ABSTRACT

Breast cancer is the most common type of cancer in women. The prognosis is bleak for triple-negative breast cancer due to the rapid metastasis and ineffective therapeutic options. Finding and developing new medications to treat breast cancer immediately is of the utmost importance. Herein, we investigated the potential mechanisms of bisurea derivatives of p-xylylene diamine cytotoxic activity against breast cancer cells. MTT and microscopic tests were used to measure cell death and cell growth, respectively. It was found out what effects bisurea derivatives of p-xylylene diamine have on caspases. Annexin V/PI staining and cell Analyzer observation of nuclear pieces were used to find cells that had gone into apoptosis. The compound 3c suppressed cell proliferation in the MDA-MB-231 breast cancer cell lines but did not affect normal mammary cells. Additionally, the caspase activities were induced in a higher range in the MDA-MB-231 breast cancer cell lines. Treatment with bisurea derivatives of p-xylylene diamine increased the number of apoptotic cells and led to nuclear pyknosis, fragmentation, and apoptotic body formation in breast cancer cells. The results conclude taken together. Our results suggest that bisurea derivatives of p-xylylene diamine induce apoptosis by activating caspases in MDA-MB-231 breast cancer cells. Moreover, bisurea derivatives of p-xylylene diamine may be an effective strategy for the treatment of breast cancer.

Keywords: Apoptosis, Bisurea derivatives, Breast cancer, Caspases, Cytotoxicity.

INTRODUCTION

Among cancers affecting women, breast cancer (BC) accounts for a quarter of all cases. One of the most pressing public health issues on a worldwide scale, BC affects around two million women and accounts for over 620,000 fatalities per year, making it the top cancer killer in this demographic.[1,2] A woman's risk of breast cancer increases with age, the number of pregnancies she has, her ethnicity, her genetic makeup, and her use of oral contraceptives.[3,4] One-way cells destroy themselves is by a process called apoptosis, which is also known as programmed cell death. One of the main goals of cancer treatment is overcoming the cancer's inherent resistance to cell death, which is present in all tumors regardless of their origin or kind.[5] The intrinsic pathway and the extrinsic pathway also called the death receptor pathway, are the two main mechanisms in which cell death can take place. According to Nair *et al*.[6] DNA damage, cellular stress, cellular senescence, and increased ROS production are examples of intracellular signals that can launch an intrinsic pathway. In contrast, numerous extracellular signals can initiate an extrinsic pathway. Genetic damage, cell cycle halt, endoplasmic reticulum stress, and elevated reactive oxygen species production often constitute an inherent pathway in cancer cells that are induced to die by both natural and manmade treatments.[7,8] The literature also confirms that malignant cells undergo apoptosis when tiny molecules convert the dormant zymogen procaspase three into caspase 3.[9] According to Kapinova *et al*,[7] several in vitro studies have shown that both natural and manufactured medicines like can induce cell death in cancer cells through caspase-mediated mechanisms.

The management of cancer relies heavily on modern approaches in oncology that target breast cancer, such as innovations in detection, therapy, and prevention. Tong *et al*.[10] found that as our understanding of BC's biological heterogeneity grows, better personalized medicine therapy approaches are born. New medications with targeted cancer suppressive effects have emerged as a result of significant advancements in BC treatment over the previous several decades. The mortality rates for breast cancer remain high despite the availability of numerous therapies such as surgery, radiation, and chemotherapy. One reason for that is the emergence of resistance of cancer cells to anticancer drugs.[11] Consequently, innovative approaches to treating breast cancer are critically needed.

The development of novel anticancer medications should rely heavily on natural ingredients, which are a rich source of both current and potential pharmaceuticals.[12] Half of all new medications have come from natural sources, either directly or indirectly, since 1981.[13] Natural compounds accounted for almost two-thirds of the anticancer medications. A few examples of these are epothilone,[14] podophyllotoxin,[15] and paclitaxel.[16] Adding urea and thiourea fragments to the structure increased the structural diversity of urea and thiourea derivatives, which in turn improved the anticancer therapeutic candidates. As a result, designed and synthesized a series of bisurea derivatives of p-xylylene diamine and studied their biological activities against tumor cell proliferation.

MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Penicillin and Streptomycin were purchased from Gibco (St W Ste, US). Propidium iodide, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was procured from Sigma Aldrich (St Louis, MO, United States). Tris-EDTA, THF, Urea, and p-xylylene diamine were procured from Sigma-Aldrich, Bangalore, India. Enzyme Linked Immunosorbent Assay (ELISA) kits (Texas, US) and other chemicals used were of analytical grade and purchased from standard manufacturers in India.

Synthesis of bisurea derivatives of p-xylylene diamine

A single-step process was used to synthesize a series of new bisurea derivatives 3(a-e). At 10 to 40°C, *p*-xylylene diamine was treated with various isocyanates 2(a-e) in THF in the presence of Et_3 N to form 3 (a-e). TLC was used to monitor the progress of the reaction at various time intervals, and the crude products obtained after removing the solvent was purified by column chromatography on silica gel with ethyl acetate and hexane (2:3) as step-grade mixtures as eluents, the characterization of compounds (not presented) and evaluate the anticancer activity.

In-vitro cell culture experiments

Breast cancer cell lines, namely MDA-MB-231, were obtained from the National Centre for Cell Science, Pune, India. They were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10 μg/mL streptomycin and penicillin, incubated at 37°C in an incubator with 5% CO_2.

MTT assay for cytotoxicity

The cytotoxic effect of novel synthesized compounds was determined by 3-[4,5-dimethylthiazole-2-yl]2,5-di-phenyl-tetrazoliumbromide (MTT) assay. Briefly, 100 μL of MDA-MB-231 cells were aliquoted and placed into two separate 96-well plates at a density of 1.5 x 10^4 cells/well and leaves to attain 70 to 80% of cell confluence for 24 hours. Then, the cells were treated with novel synthesized compounds (10 μM/mL) and incubated for 24 hours. After the treatment, 0.8 mg/mL of MTT solution was added to each well of the cells. After 4 hours incubation, the supernatant was removed, following 100 μL of DMSO was added into each well to dissolve the formazan crystals. The plates were incubated on a shaker and the absorption was read at 570 nm using a microplate reader (BioRad). All experiments were performed in triplicate. The relative cytotoxicity was compared using untreated control cells as the baseline.[17]

Cell viability by Microscopic assay

The MDA-MB-231 cells were seeded in a T-75 flask until reach 70 to 80% cell confluence, then 1.5 x 10^4 MDA-MB-231 cells were cultured in each well of a 6-well plate containing DMEM media with 10% FBS, 1% antibiotic. The cells were treated with novel synthesized compounds (10 μM/mL) to each well and incubated for 24 hours. The viable cells were observed under the fluorescent microscope.

Caspases assays

Caspase-3 and caspase-9 are key biomarkers for apoptosis. In vitro activities of caspase-3 and Caspase-9 were measured using a colorimetric assay kit (Genscript, NJ, USA). As per the manufacturer's instruction, MDA-MB-231 cells incubated in DMEM medium containing 0.2% FBS were treated with different concentrations of thiourea derivatives. The cells were then lysed to allow for the detection of chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA. The absorbance was measured at a wavelength of 405 nm.

Measurement of cell apoptosis

Cell apoptosis was measured using the Muse Cell Analyzer (Luminex, USA). In brief, MDA-MB-231 and MCF-7 cells were seeded in separate 6-well plates at a density of 1.5 x 10^4 cells/well and after achieving 70 to 80% of cell confluence, the cells were exposed to ursolic acid (10 and 20 μg/mL) for 24 hours, then collected and centrifuged at 3000 rpm for 4 minutes, and then washed with TE buffer before being incubated for 30 minutes in the dark with 5 μL of annexin-V reagent and propidium iodide (PI). Apoptosis was measured as a percentage of fluorescence using a cell analyzer.[17]

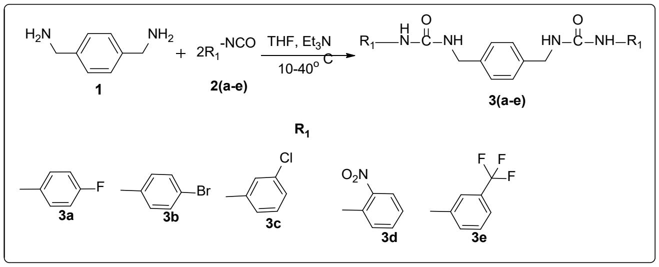
Statistical analysis

All the experiments were carried out in triplicates. Data were loaded to Microsoft Excel, Prism 8.0 and a one-way ANOVA program was used to analyze the data. All the data were presented as mean ± SD. Statistically, the significance between control and treated groups was indicated with different superscripts.

RESULTS AND DISCUSSION

Chemistry

By reacting with various isocyanates 2(a-e) in the presence of triethylamine in THF, we were able to make a series of bisurea derivatives of p-xylylene diamine (1). Scheme 1 summarizes the synthesis of several bisurea derivatives. To get pure compounds in excellent yields, the obtained final compounds 3(a-e) were washed with a small amount of methanol. Column chromatography was used to further purify the chemicals. The bisurea derivatives of synthesized title compounds 3(a-e) such as 1,1'-(1,4-Phenylenebis(methylene))bis(3-(4-fluorophenyl)urea) (3a), 1,1'-(1,4-Phenylene bis(methylene))bis(3-(4-bromophenyl)urea) (3b), 1,1'-(1,4-Phenyl enebis(methylene))bis(3-(3-chlorophenyl)urea) (3c), 1,1'-(1,4-Phe nylenebis(methylene))bis(3-(2-nitrophenyl)urea) (3d) and1,1'-(1,4-Phenylenebis(methylene))bis(3-(3-(trifluoromethyl)phenyl)urea) (3e) were summarized in Scheme 1.



Scheme 1: Synthesis of bisurea derivatives of *p*-xylylene diamine 3(a-e)

Cytotoxicity by MTT assay

The cytotoxic efficacy of 5-bromo-pyrimidine derivatives against HCT116, A549, K562, and U937 cell lines has been observed *in-vitro* and thiourea derivatives exhibited comparable Bcr/Abl antiproliferation action with an IC_{50} value of 0.012 mg/mL and high cytotoxicity against K562 cells.[18] The cytotoxicity of novel synthesized compounds against MDA-MB-231 breast cancer cell lines at different dosages (5, 10, 15, and 20 M/mL) during 24 hours treatment was tested using the MTT assay. The results are presented in Table 1. On MDA-MB-231 cells, all newly synthesized compounds exhibited moderate to good cytotoxicity. In comparison to the reference medication Doxorubicin (IC_{50} values of 16.60 μM/mL), the compounds 3c and 3d demonstrated strong cytotoxicity with IC_{50} values of 20.73, and 21.58 μM/mL, and IC_{50} of doxorubicin treated cells showed 18.48 μM/mL, respectively (Table 1). The lowest IC_{50} values for cytotoxicity show the greatest antiproliferation efficacy against breast cancer cells.

Cell viability

The fluorescent microscopic image observation supported the cytotoxicity assay; the novel synthesized compound treated MDA-MB-231 cells demonstrated moderate to well reduced cell viability, with compounds 3c and 3d exhibiting the lowest cell viability, as shown in Fig. 1. The lowest cell viability shows the greatest anticancer activity.

Caspase-3 and Caspase-9 Assay

Caspases are aspartate-directed proteases that rely on cysteine. Caspases are typically constitutively produced and activated by fixed proteolytic cleavage from their inactive zymogen form, which is called procaspases.[19] Programmed cell death (apoptosis) resulting from proapoptotic cascades is primarily mediated by two kinds of caspases, caspase-9 and caspase-3.[20] Caspases-3 are essential for the apoptotic processes of cell death, chromatin condensation, and DNA fragmentation. Caspases 9 and 10 are also active at the apoptosome during intrinsically initiated apoptosis.[21] Both apoptosis and necrosis are mechanisms by which anticancer drugs can cause cell death. In order to analyze apoptotic events in MDA-MB-231 cells, the microtiter plate reader test was utilized. Table 2 displays the results of treating MDA-MB-231 cells with bisurea derivatives of p-xylylene diamine. After 24 hours of treatment with 10 mM of five synthetic chemicals, caspase-3 and caspase-9 activity was measured in MDA-MB-231 cells. The 3c and 3d compounds significantly raised the amounts of caspase-3 and caspase-9 when compared to doxorubicin.

Cell Apoptosis

Programmed cell death (PCD) includes but is not limited to apoptosis, autophagy-induced cell death, necroptosis, ferroptosis and pyroptosis, all of which regulate various physiological and pathological processes. Diaryl urea-containing sulfonylureas have attracted the attention of researchers with their antitumor properties.[22] The cell analyzer was

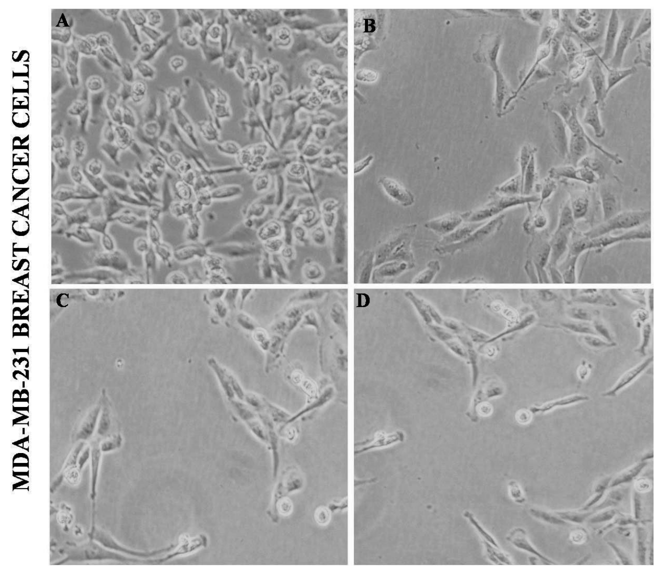


Fig. 1: Cell Viability by Microscopic assay The novel synthesized compound significantly showed toxic effects on MDA-MB-231 breast cancer cells and their microscopic evaluation (A) Control cells (untreated/DMSO), (B) 3c compound treated cells, (C) 3d compound treated cells and (D) Doxorubicin (standard drug) treated cells.

Table 1: Effect of synthesized novel compounds on the percentage of inhibition of MDA-MB-231 cell viability by MTT assay

Compounds	*% of inhibition of MDA-MB-231cells*			
	5 μM/mL	*10 μM/mL*	*15 μM/mL*	*20 μM/mL*
3a	6.06 ± 0.84	10.87 ± 1.14	19.64 ± 1.69	29.53 ± 2.21
3b	5.56 ± 0.49	8.74 ± 0.88	16.34 ± 1.26	25.36 ± 2.05
3c	12.11 ± 1.02	19.93 ± 1.78	29.56 ± 2.44	48.22 ± 2.94
3d	11.29 ± 0.97	18.51 ± 1.39	27.13 ± 2.19	46.33 ± 2.39
3e	10.41 ± 1.03	15.11 ± 1.22	22.19 ± 2.15	34.53 ± 2.03
Doxorubicin	13.49 ± 1.16	21.58 ± 1.86	32.15 ± 2.77	54.11 ± 3.02

Data were expressed as means ± SD (standard deviation)

w 2: Caspase-3 and 9 concentrations on bisurea derivatives of p-xylylene diamine treatment with MDA-MB-231 cells after 24 hours in comparison with doxorubicin

Compounds	*Caspase-3 (ng/mL)*	*Caspase-9 (ng/mL)*
3a	162	6.52
3b	184	8.44
3c	244	10.63
3d	223	10.18
3e	195	9.64
Doxorubicin	249	10.81

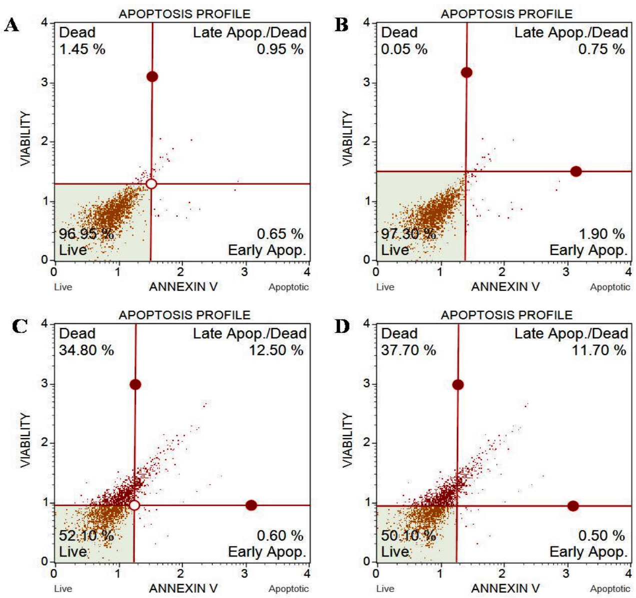


Fig. 2: Cell apoptosis assay by cell analyzer: (A) Control (untreated cells), (B) Vehicle control (DMSO-treated cells), (C) 3c compound treated MDA-MB-231 cells and (D) doxorubicin compound treated MDA-MB-231 cells and all cells were stained by Annexin-V and PI to detected apoptosis

used to measure the apoptosis profile and cell death using annexin-V and propidium iodide (PI). In the annexin-V and PI experiment, cells treated with 20 μM/mL of 3a-e novel synthesized compounds for 24 hours had significant alterations (p <0.05) in live, early, late, and necrotic states. When the apoptotic profile was measured, live cells percentage was detected as 96.95 and 97.30%, respectively in control and DMSO-treated MDA-MB-231 cells. In contrast, the live cell percent in 3c, and doxorubicin-treated MDA-MB-231 cells were 52.10 and 50.10%, respectively. Coinciding with this, the apoptosis profile increased from 34.80 and 37.80%, respectively, which was shown in Fig. 2. Thus, our results demonstrate that 3c treatment had significantly decreased the live cells percent and close to doxorubicin treated cells and apoptotic profile was nearer to doxorubicin treated cells.

CONCLUSION

In summary, a class of new compounds of bisurea derivatives of p-xylylene diamine of 3(a-e)) was designed and synthesized. The compound 3c exhibits potential antiproliferative activity with increased apoptotic protein caspases. Apoptosis results have demonstrated that the compound 3c was selected as a representative example of induced MDA-MB-231 breast cancer cell apoptosis, in addition to its activation of caspase-3 and caspase-9, which might mediated. Our study results show 1,1'-(1,4-Phenylenebis(methylene)) bis(3-(3-chlorophenyl)urea) (3c) could be suggesting that designed and ideal for proposing as selective novel anticancer agents.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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