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Alicyclic diamide derivatives as potential anticancer agents

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Abstract: The present study was carried out in the attempt to design a new class of potential anticancer agents comprising sixteen compounds (**10-25**) sharing the alicyclic diamide as a core. Their biological potential towards breast cancer was tested by using MTT assay on breast cancer (MCF-7)and non-tumorigenic breast (MCF-10-2A)cell lines.Screening results show that the anti-proliferative effects of compounds (**13, 19** and **20**) correlate with the lipophilicity and selectivity of compounds. Interestingly, the active compounds distinguished from others with a quite high selectivity value that is comparable to 5-FU. Therefore, new small molecules containing alicyclic diamide fragments will have a significant contribution to the development of new era anticancer drug candidates for breast cancer.

Keywords: Phenyl alicyclic diamides; breast cancer; anti-proliferative; anti-cancer. ©2020 ACG Publications. All right reserved.

1. Introduction

Cancer is one of the leading causes of human mortality worldwide, with an estimated 14 million new cancer cases projected for 2030.[1] Among the cancer types, breast cancer is the most common cancer for females and its incidence tends to increase year by year. Currently, the backbone of therapy for breast cancer is mainly chemotherapy, however its toxicity in normal cells and acquired tumour resistance to the drug used is considered as the main barriers. Therefore, there is still an urgent need for the development of more effective and less toxic breast cancer therapeutic agents.[2]

In the cancer researches, inflammatory processes have been interested in their inclusion as one of the hallmarks of cancer.[3] Pro-inflammatory cytokines produced in inflammatory processes have caused to influence cell proliferation and cell survival, angiogenesis, metastasis leading to tumour growth, tumour cell migration and progression.[4] Among these, tumour necrosis factor-alpha (TNF-α) is a protein that has key roles in immune cell activation, inflammation and cognitive function in the brain.[5] Recent advances have shown that TNF-α has also been investigated to play a key role in many different types of cancer. For example, thalidomide, known as an immunomodulatory drug is effective for the treatment of certain kinds of cancers, such as colon cancers, prostate cancers, renal cell carcinoma and advanced breast cancer, probably because of its TNF-α production-inhibiting activity and antiangiogenic activity.[6,7] In brief, it has been observed that immunomodulatory molecules can be effective in many biological applications such as cancer and especially breast cancer.[8]

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In our previous study, a series of novel alicyclic oxalamide derivatives were synthesized and their immunomodulatory potentials were tested *in vitro* on the macrophage cells. Based on our previous results, these molecules have differential effects on the production of the TNF-α and IL6 pro-inflammatory cytokines by the lipopolysaccharide-stimulated macrophages.[9] Their differential effects on the production of the TNF-α and IL6 open new venues for their medicinal applications such as an anticancer agent. Accordingly, in here, we have carried out *in vitro* tests of the immunomodulatory molecules we have previously synthesized in the breast cancer cell line.

The modification of known drugs represents a traditional and effective approach in drug design.[10] For example, antimicrobial studies of compounds made by oxalamide modification of the sulfamethoxazole compound have been shown to give better results than reference drugs isoniazid and sulfamethaxazole.[11] In the literature, several functionalities like amides, diamides, oxalamides, semicarbazones, and phthalimido-glutarimide have proved to enhance the anticancer activity of many synthetic molecules. For example, the oxalamide derivative molecule **1** has anti-cancer effect on RNA polymerase II inhibitor,[12]the semicarbazone derivative molecule **2** procaspase-3 kinase inhibitor (EC_{50}: 0.25 µM) and anti-proliferative for breast cancer cell line MDA-10-2A (0.88 µM),[13] the diamide derivative molecule **3** has anti-proliferative effect on breast cancer cell line MCF-7 (IC_{50}: 4.37 µM),[14] the phthalimido-glutarimide derived thalidomide molecule **4** has anticancer effect on many cancer varieties include breast cancer.[15] In addition, it was found that molecule **5**, which contains morpholine, pyrrolidine and piperidine groups depending on the oxalamide structure, has anti-proliferative effects on breast cancer cell lines, MCF-7 and MDA-MB-231, up to 20 µM concentrations[16] (Figure 1).

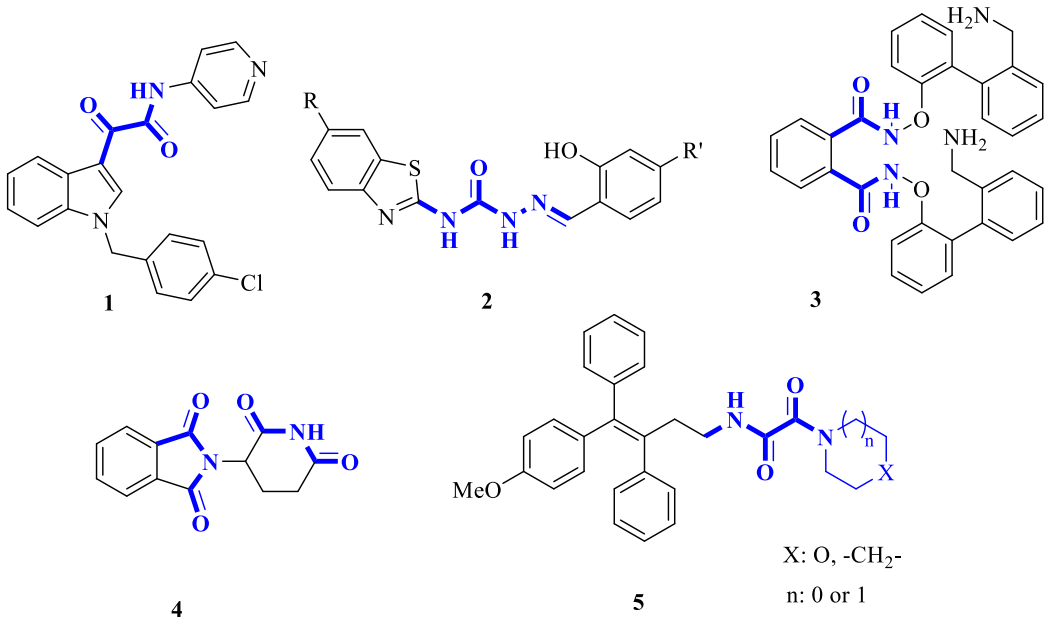
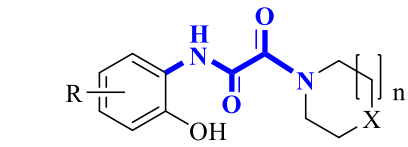


Figure 1. Structures of some compounds with diamide functional group having anticancer properties

Based on the molecular similarity of these molecules with diamide structures and remarkable anti-proliferation results against MCF-7 breast cancer cell line, in this study, we investigated the anti-proliferative effect of molecules in a series of alicyclic diamide derivative structures (Figure 2), in which we previously examined the immunomodulatory effects. Their anti-proliferative effects were tested against estrogen positive (ER+) breast cancer MCF-7 and non-tumorigenic MCF-10-2A human breast cells *in vitro.*



R: H, CH_3, Cl, NO_2

n:0 or 1

X: $-CH_2-$, $-CH-CH_3$, -O-

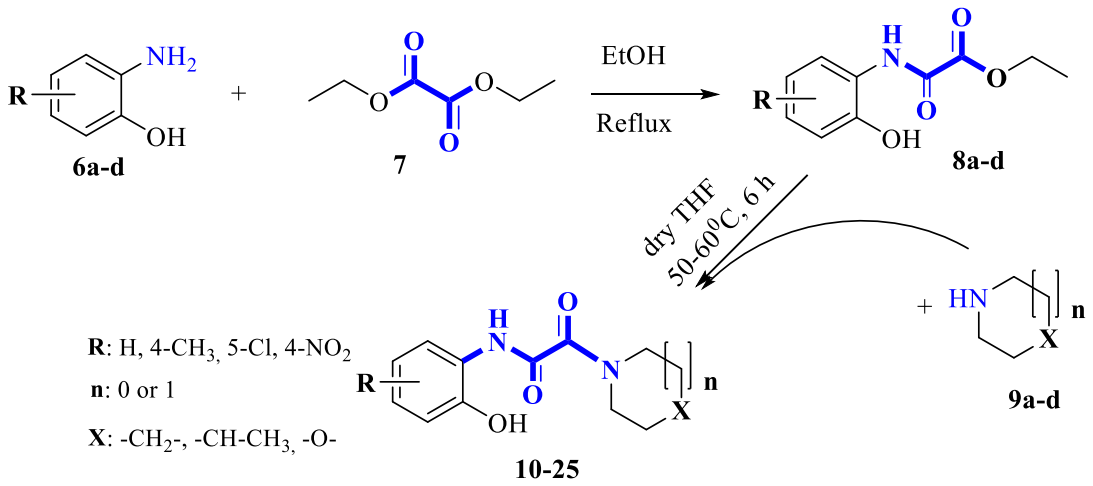
Figure 2. Target molecule structures for anticancer agent

2. Experimental

2.1 Chemistry

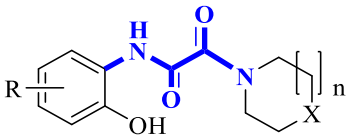
2. 1. 1. General Synthesis Procedure

The synthetic strategy for the preparation of the target compounds is depicted in Scheme 1. To a stirred solution of 2-aminophenol derivatives **6a-d** in ethanol, excess diethyl oxalate **7** was added at room temperature. The reaction mixture was stirred at reflux for overnight. The reaction mixture was cooled to room temperature, solid particles filtered and the intermediates **8a-d** obtained. The intermediates **8a-d** were used after crystallization in ethanol to reach final products. Compounds **8a-d** were reacted with corresponding alicyclic amines **9a-d** in dry THF. After completion of the reaction (confirmed by TLC), the mixture extracted with ethyl acetate. The combined organic layer was dried over sodium sulfate and concentrated using rota-evaporator. Crude residues were purified using column chromatography using n-hexane:ethylacetate, (5:1) as an eluent to get pure compounds **10-25**. All synthesis procedures and results are detailed in our previous study.[9]



Scheme 1. General synthesis procedure

Table 1. The synthesized alicyclic diamide derivatives



Compounds	R	X	n
10	H	-CH_2-	0
11	H	-CH_2-	1
12	H	-CH-CH_3	1
13	H	O	1
14	5-Cl	-CH_2-	0
15	5-Cl	-CH_2-	1
16	5-Cl	-CH-CH_3	1
17	5-Cl	O	1
18	4-CH_3	-CH_2-	0
19	4-CH_3	-CH_2-	1
20	4-CH_3	-CH-CH_3	1
21	4-CH_3	O	1
22	4-NO_2	-CH_2-	0
23	4-NO_2	-CH_2-	1
24	4-NO_2	-CH-CH_3	1
25	4-NO_2	O	1

2.2. Biochemistry

2.2.1. Cell Lines and Cell Culture

Estrogen positive (ER+) breast cancer MCF-7 (ATCC® HTB-22™) was used as *in vitro* cancer models. Non-tumorigenic MCF-10-2A (ATCC® CRL-10781™) human breast cells were used as corresponding normal breast cells. MCF-7 and MCF-10-2A cells were kindly provided by MEITAM from Mersin University, Turkey. MCF-7 cells were cultured in DMEM-high glucose medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin; MCF-10-2A cells were cultured in DMEM containing 5% horse serum, 20 ng/mL epidermal growth factor,100 ng/mL cholera toxin,0.01 mg/mL bovine insulin,500 ng/mL hydrocortisone and maintained in a humidified incubator with 5% CO_2 at 37 °C.

2.2.2. Preparation of Stock Solutions for Compounds and 5-Fluorouracil

5 mg of each compound was dissolved in appropriate amounts of dimethyl sulfoxide (DMSO) to have 10 mM stock solutions. Then, stock solutions were diluted and aliquoted to 100 µM intermediate stocks and stored at -80 °C. Commercially purchased 5-Fluorouracil (MedChemExpress, NJ, USA; 5-FU) was dissolved in DMSO to have a 20 mM stock solution following the manufacturer's protocol. 5-FU stock solution was diluted and aliquoted to 100 µM and stored at -80 °C until use. Final concentration of DMSO in the cell culture medium after addition of the compounds was calculated to be less than 1%.

2.3. Cytotoxicity evaluations

2.3.1. MTT assay

Cell viability was evaluated by using MTT assay. This assay is based on the ability of viable cells to metabolize yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial succinate dehydrogenase and spectrophotometric measurement of the product at 570 nm.

Briefly, cells were seeded at a density of $1x10^5$ per well in 96-well plates; subsequently, after overnight incubation, they were treated with various concentrations (0.15μM, 0.20μM, and 0.30 μM) of compounds. Cells were put back to 37°C 5% CO_2 incubator for 24 h, 48 h,and 72 h incubation. The untreated or DMSO treated well was considered as a negative control, and all samples were prepared in triplicates.

After 24h, 48h, and 72h of incubation, 10 μL of MTT reagent was added into each well and samples were further incubated for 4 h at 37°C, 5% CO_2. As a last step, 100μL of SDS based ROCHE Detergent reagent was added into each well. Cytotoxic effects were monitored by measuring the absorbance values of each well at 570 nm.

3. Results and Discussion

To evaluate the selectivity of the compounds, their toxicity was tested against normal cells using non-tumorigenic MCF-10-2A human breast cells. The selectivity of the compounds was calculated as their IC_{50} values for normal cells divided by their IC_{50} values for the specific cancer cell MCF-7, according to Equation 1. (Eq. 1.)

$$\text{Selectivity index} = \frac{IC_{50} \text{ value of compound against normal cell line}}{IC_{50} \text{ value of compound against cancer cell line}} \quad \text{Eq. 1}$$

Table 2. *In vitro* nontoxic concentrations against normal cells and cancer cell lines, cLogP,and the selectivity index of the compounds.

Compounds	IC_{50} (μM)*		Selectivity index**	cLogP***
	MCF-10-2A	MCF-7		
10	213.6	160.6	1.33	1.113
11	286.2	87.1	**3.29**	1.452
12	454.1	264.5	1.71	1.978
13	288.0	138.8	2.07	0.251
14	272.3	401.8	0.67	1.735
15	241.1	180.1	1.34	2.075
16	105.0	138.1	0.76	2.601
17	135.9	107.0	1.27	0.873
18	360.8	247.5	1.46	1.550
19	263.3	169.0	1.56	1.889
20	242.7	43.5	**5.88**	2.415
21	239.3	83.7	**2.86**	0.688
22	258.0	169.5	1.52	1.007
23	197.9	151.2	1.31	1.347
24	182.4	132.2	1.37	1.872
25	186.4	154.5	1.20	0.145
5-FU	540.0	93.2	5.79	-0.787

MCF-10-2A: Non-tumorigenic human breast cells, **MCF-7**: Estrogen positive (ER+) breast cancer cells
*IC_{50} values were determined after 24 h incubation.
**Selectivity index of the compounds: the ratio for each compound of its IC50 value for normal cells (MCF-10-2A) to its IC_{50} value for the cancer cell MCF-7.
***cLogP value of the compounds **10-25**(calculated from ChemBioDrawUltra 2015)

As a result of the anti-proliferative effects of the synthesized compounds (Table 2), compound **20** appears to have the greatest effect on the MCF-7 cell line. This result indicates that compound **20** has similar activity to the standard drug 5-FU. It also appears to have no toxic effect on the normal cell line MCF-10-2A. The selectivity index study shows that the molecule shows the most selectivity for the MCF-7 cell line. Similar result was found in Compound **21.**

In the structure-effect relationship research, it can be said that the activity increases with the electron donating methyl substitution of the phenyl group. Among the synthesized molecules, **10-13** group with unsubstituted phenyl ring is more active than those carrying back Cl (Compounds **14-17**) and NO_2 group (Compounds **22-25**). The most active molecule in this series was found to be compound **11**. Molecules with electron attracting nitro group show average activity, while the presence of halogen has reduced the activity considerably.

It appears that the anti-proliferative activity increases with the increased lipophilicity in 4-methyl substituted phenyl compounds **18,19**, and **20**. In compound **20**, the electron donor methyl substituent being in the 4 position and containing 4-methyl piperidine substitute provided more effective results than the standard drug 5-FU. However, as seen in compound **21**, replacing the 4-methyl group with morpholine caused the activity to decrease significantly with decreasing lipophilicity. This confirms the result that the anti-proliferative effect increases with lipophilicity. It follows that the phenyl ring will increase the activity by increasing the lipophilicity of the alkyl group as the electron donor group and by finding a more lipophilic group in the alicyclic position.

With the alicyclic system being 4-methyl piperidine in the series carrying the methyl in the phenyl ring compound **20**, which is the most active among the molecules, the increase in lipophilicity has been strong in both activity and selectivity. However, increased lipophilicity for other molecules did not significantly affect activity and selectivity. Based on these results, in the future, it is planned by substituting aliphatic or electron donating groups for the phenyl group and replacing the alicyclic group with different functional groups to design new molecules with stronger anti-proliferative activity.

4. Conclusion

In this study, we aimed to introduce a number of alicyclic diamide-based compounds that may have potential therapeutic effects against breast tumor cells. All the synthesized sixteen compounds (**10-25**) were screened for the *in vitro* anticancer activities, using MCF-7 (human breast adenocarcinoma) and MCF-10-2A (non-tumorigenic human breast cells). The results of anti-proliferative screening suggest that all of compounds can be highly potent since they were biologically active even at low concentration ranges. Among them compounds **11, 20,** and **21** have more potent anti-proliferative effect on breast cancer cells (MCF-7).Furthermore, compound **20** showed the highest inhibition in human MCF-7 cell line and no-toxic effect on non-tumorigenic human breast cells even at low concentrations. It is crucial to adjust their dosage properly to prevent unwanted side effects during their possible applications in the future. This compound has the potential to develop as a new class of anticancer agents. Therefore, we are currently evaluating their mechanism of action at molecular level in breast cancer cells.

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