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N . V. Polish, N. G. Marintsova, L. R. Zhurakhivska, A. O. Mylyanych, E. Y. Izmerli
Lviv Polytechnic National University,
Department of Technology Biologically Compounds, Pharmacy and Biotechnology
nataliia.v.polish@lpnu.ua

EVALUATION OF CYTOTOXICITY OF HETEROCYCLIC AMINO-CONTAINING 1,4-NAPHTHOQUINONE DERIVATIVES

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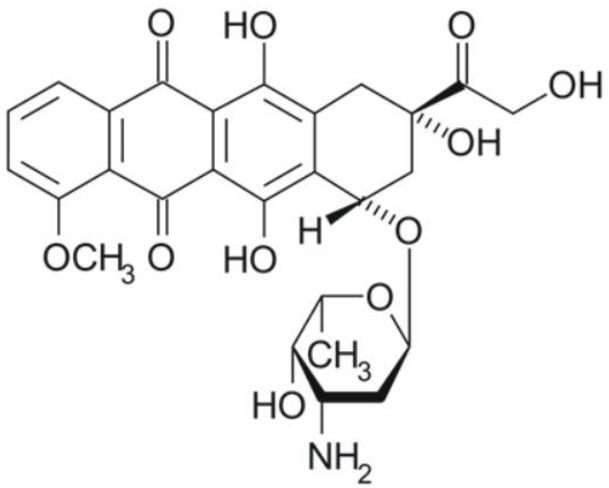
The cytotoxic activity of heterocyclic amino-containing derivatives of 1,4-naphthoquinone *in vitro* on various human breast cancer lines, namely: MDA-MB, MCF-7, SK-BR-3, BT-474 and non-cancerous cell lines (MCF-10A) was investigated. *In silico* prediction of toxicity of the test substances for rats was performed using modern software ProTox-II. It was established that all studied compounds belong to toxicity classes IV and VI and are of low toxicity. It was determined that heterocyclic amino-containing derivatives of 1,4-naphthoquinone can be used as potent anticancer agents with cytotoxic activity against MCF-7 and MDA-MB-231.

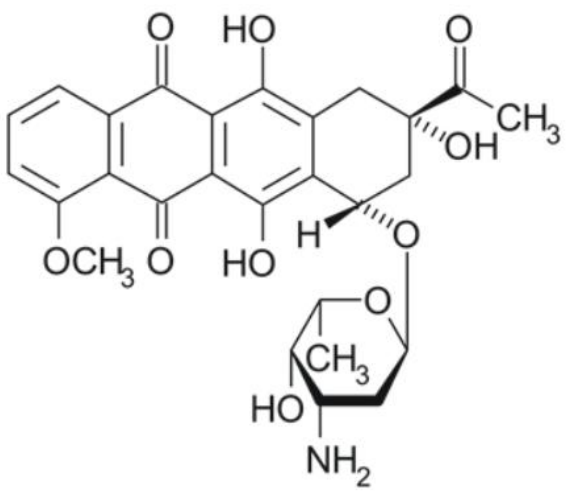
Keywords: heterocyclic amino-containing derivatives of 1,4-naphthoquinone, antitumor activity *in vitro*, ProTox-II.

Introduction

According to the World Health Organisation, cancer is one of the leading causes of death worldwide and claims millions of lives. One of the most common form of cancer among women is breast cancer. More than 1.8 million new cases are recorded annually. This alarming statistic makes today's leading biotechnology and global pharmaceutical companies, which produce the bulk of drugs aimed at combating this disease, develop and improve methods for the synthesis of new low-molecular weight compounds with high biological activity and low toxicity [1–2]. It should be noted that today there are known drugs with pronounced anticancer activity that contain quinoid fragments in their structure, namely daunomycin, doxorubicin and its derivatives epirubicin, idarubicin, pirarubicin, and zirubicin [3–5].



Doxorubicin



Daunomycin

Doxorubicin is a cytotoxic anthracycline antibiotic with high antitumor activity, which is produced by microorganisms *Streptomyces peucetius var. caesius* and is a semi-synthetic daunorubicin derivative [6–8].

Despite its high clinical efficacy, there are significant side effects such as: severe liver dysfunction, cardiotoxicity (progressive heart failure, severe arrhythmia, myocardial infarction), development of resistance, etc. [6–8]. This stimulates the search for alternative quinone structures with high anticancer activity and low toxicity [2]. Particular attention is paid to conformationally compact structures containing various heterocyclic fragments capable of targeted interaction with biological targets. In this context, exofunctionalised 1,4-naphthoquinone derivatives are promising. In contrast to condensed polycyclic anthracycline systems, they are structurally simpler, easier to modify, have increased selectivity and less toxicity [9–14]. The small size of the heterocyclic compounds synthesised by us allows them to target any part of the molecule in the cell, moving freely across the plasma membrane.

The aim of the work was to evaluate the heterocyclic amine-containing 1,4-naphthoquinone derivatives obtained in our previous work [15] as promising anticancer agents. Their *in vitro* cytotoxicity, *in silico* biological potential and the search for a leading compound for further pharmacological screening were determined.

Materials and research methods

Toxicity studies *in silico*

To effectively search for new compounds with biological activity, their careful selection at the synthesis planning stage is necessary. Given the cost of the process and for ethical reasons, we have used *in silico* computer modeling methods to determine the toxicity of new anticancer agents. They allow us to quickly assess the potential toxicity of synthesized compounds before testing them *in vitro* or *in vivo*. This significantly reduces the time and resources spent on preclinical studies and is therefore an integral element in the design of newly synthesized compounds.

In our work, the software ProTox-II was used, which is available at the web link https://tox-new.charite.de/protox_II. With the help of this program, the class of toxicity according to the average lethal dose LD_{50}, hepatotoxicity, cytotoxicity, carcinogenicity, mutagenicity, immunetoxicity of the studied compounds was determined [16, 17].

According to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS), the following toxicity classes are distinguished: values:

- Class I: fatal if swallowed ($LD_{50} \leq 5$)
- Class II: fatal if swallowed ($5 < LD_{50} \leq 50$)
- Class III: toxic if swallowed ($50 < LD_{50} \leq 300$)
- Class IV: harmful if swallowed ($300 < LD_{50} \leq 2000$)
- Class V: may be harmful if swallowed ($2000 < LD_{50} \leq 5000$)
- Class VI: non-toxic ($LD_{50} > 5000$)

Cytotoxicity *in vitro*

In vitro cytotoxicity studies of amine-containing heterocyclic derivatives of 1,4-naphthoquinone were carried out at the Gdansk University of Technology. In vitro cytotoxicity assay was performed using 3-(4,5-dimethyltriazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) on two different types of human breast cancer cell lines (BT474, SKBR-3, MCF7, MDA, MB231) and one non-cancerous cell line (MCF-10A). BT-474, SKBR-3, MCF-7, MDA-MB-231, MCF-10A cells were treated with a 100 μM solution of the test compounds (the corresponding IC_{50} values were determined by the MTT method for compounds 1, 2, 3, 4) during incubation. Cells were fixed with ethanol and stained with propidium iodide.

Briefly, exponentially growing cells were seeded onto the 96-well plate, and after overnight incubation cells were exposed to different drug concentrations and the cell viability was evaluated after 72 h. Cells were exposed to the MTT tetrazolium salt (4 μg/ml) for 2–3 h at 37°C, and the formation of formazan was measured after solubilisation in 100 μl DMSO with ASYS UVM340 microplate reader (Biochrom Ltd.). The concentrations required to inhibit cell growth by 50 % compared to untreated controls were determined from the curves plotting survival as a function of dose by the use of the GraphPad Prism 5program. All values are averages of at least three independent experiments, each done in triplicate [19].

Data were expressed as a percentage reduction in cell viability relative to control cells and expressed as mean ± standard deviation (SD). The mean value of the

control cells was standardized to 100 % of the activity cells.

Statistical analysis

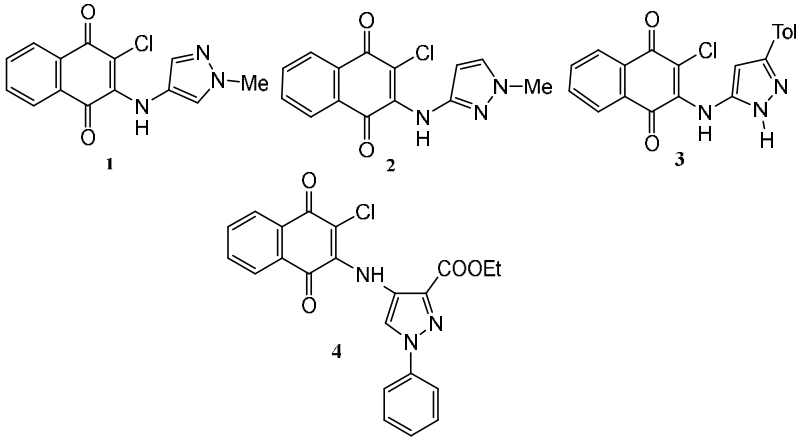
Statistical analysis was performed using Statistica 12 software (StatSoft, Poland). Data are expressed as means ± SD. Statistical differences between samples were evaluated using the Mann-Whitney U test or Student's t-test. Differences were considered significant at *p < 0.05, **p < 0.01, ***p < 0.001 [19].

Results and discussion

This research is a continuation of fundamental research of our department. The synthesis of the studied amine-containing heterocyclic derivatives of 1,4-naphthoquinone was described in previous works [20].

An important indicator for the introduction of new drugs is toxicity, which was determined for the studied compounds **1**–**4** using modern software ProTox-II [16].

The results are presented in Table 1.



where **1** is 2-chloro-3-((1-methyl-1*H*-pyrazol-4-yl)amino)naphthalene-1,4-dione, **2** is 2-chloro-3-((1-methyl-1*H*-pyrazole-3-yl)amino)naphthalene-1,4-dione, **3** – 2-chloro-3-((3-(*p*-tolyl)-1*H*-pyrazol-5-yl)amino) naphthalene-1, 4-dione, **4** – ethyl-4-((3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-yl)amino)-1-phenyl-1*H*-pyrazole-3-carboxylate

Table1

Prediction of the toxicity of substances

Compounds*	Oral toxicity **			Prediction***: active, probability*** of 1				
	toxicity Index	LD_{50}, mg/kg	toxicity prediction, %	hepatotoxicity	carcinogenicity	immunotoxicity	mutagenicity	cytotoxicity
1	4	1260	67.38	0,59 (A<0.7)	0,58 (N<0.7)	0,50 (A<0.7)	0,59 (A<0.7)	0.58 (N<0.7)
2	6	11700	54.26	0.60 (A<0.7)	0.58 (N<0.7)	0.78 (A>=0.7)	0.59 (A<0.7)	0.58 (N<0.7)
3	4	1260	54.26	0.65 (A<0.7)	0.56 (N<0.7)	0.62 (N<0.7)	0.51 (N<0.7)	0.61 (N<0.7)
4	4	800	54.26	0.66 (A<0.7)	0.63 (N<0.7)	0.94 (N>=0.7)	0.53 (N<0.7)	0.63 (N<0.7)
Doxorubicin	3	205	100.00	0.86 (N>=0.7)	0.90 (N>=0.7)	0.99 (A>=0.7)	0.98 (A>=0.7)	0.94 (A>=0.7)
Daunomycin	3	205	100.00	0.89 (N>=0.7)	0.75 (N>=0.7)	0.99 (A>=0.7)	0.99 (A>=0.7)	0.74 (A>=0.7)

*Notes: * ; ** Class I: fatal if swallowed (LD_{50}≤5); Class II: fatal if swallowed (5<LD50≤50); Class III: Toxic if swallowed (50<LD_{50}≤300); Class IV: harmful if swallowed (300<LD_{50}≤2000); Class V: may be harmful if swallowed (2000<LD_{50}≤5000); Class VI: not toxic (LD_{50}>5000). Prediction***: A – Active, N – Inactive.*

As can be seen from Table 1, the compound 2-chloro-3-((1-methyl-1*H*-pyrazol-3-yl)amino) naphthalene-1,4-dione 2 belongs to toxicity class VI and is probably non-toxic (LD_{50}= 11700 mg/kg). The remaining studied compounds 1,3,4 belong to toxicity class IV, which indicates their low toxicity, only in high doses they can be harmful when swallowed ($300 < LD_{50} \leq 2000$). Thus, for comparison, the widely used anticancer drugs doxorubicin and daunomycin belong to class III and are toxic when swallowed ($50 < LD_{50} \leq 300$).

In silico predictions showed that 2-chloro-3-((3-(*p*-tolyl)-1*H*-pyrazol-5-yl) amino) naphthalene-1,4-dione 3 and ethyl-4-((3-chloro-1, 4-dioxo-1,4-dihydronaphthalen-2-yl)amino)-1-phenyl-1*H*-pyrazole-3-carboxylate 4 may cause hepatotoxicity upon regular use, and compounds **1** and **2** may cause hepatotoxicity, immunotoxicity, and mutagenicity. Doxorubicin and daunomycin may cause immunetoxicity, mutagenicity, and cytotoxicity.

Therefore, the results indicate the possibility of further pharmacological research of heterocyclic amino-containing derivatives of 1,4-naphthoquinone.

Cytotoxicity *in vitro*

To determine the efficacy of potential anticancer agents in preclinical studies, the IC_{50} value was estimated. This is a universal indicator by which the activity of test compounds can be compared with each other and with known reference drugs.

The cytotoxic activity of 1,4-naphthoquinone derivatives was evaluated *in vitro* on various human breast cancer lines, namely: MDA-MB-231 (estrogen-negative breast carcinoma cells), MCF-7 (estrogen-positive breast carcinoma receptor cells), SK-BR-3 (breast adenocarcinoma cell lines), BT-474 (estrogen-positive, progesterone-positive epithelial cancer cells) and non-cancerous cell lines (MCF-10A).

As can be seen from the data in Table 2, ethyl 4-((3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-yl)amino)-1-phenyl-1*H*-pyrazole-3-carboxylate (4) is a leader compound and exhibits high cytotoxic activity against epithelial cancer cells BT-474 (IC_{50} = 6.12±0.22 μM), breast adenocarcinoma cell lines SK-BR-3 (IC_{50} = 2.00±0.21), receptor cells of breast carcinoma MCF-7 (IC_{50} = 6.97±0.14), breast carcinoma cells MDA-MB-231 (IC_{50} = 3.83±0.16) and non-cancerous cell lines MCF-10A (IC_{50} = = 3.60±0.10) which indicates its high efficiency. All tested compounds have high cytotoxic activity against MDA-MB-231 and MCF-10A. At the same time, the relatively low cytotoxicity of compounds 1–3 against three cancer cell lines BT-474, SK-BR-3 and MCF-7 indicates the selectivity of their action.

Table 2

IC_{50} [μM]

Compouds	BT-474	SK-BR-3	MCF-7	MDA-MB-231	MCF-10A
1	18.72±0.43	6.73±0.49	11.70±0.88	2.63±0.13	2.04±0.13
2	19.47±0.59	11.62±0.82	13.72±0.91	3.44±0.12	3.22±0.91
3	34.31±3.09	10.9±0.23	27.64±0.93	3.87±0.23	3.97±0.25
4	6.12±0.22	2.00±0.21	6.97±0.14	3.83±0.16	3.60±0.10

Analyzing the obtained results of *in silico* and *in vitro* screening of heterocyclic amino-containing derivatives of 1,4-naphthoquinone, we can conclude that there is a partial correlation between them. Thus, according to the *in silico* method, the studied compounds 1-4 can be attributed to low toxicity (IV-VI class), which is confirmed by *in vitro* studies, in particular, by the high activity of compounds 1-4 against MDA-MB-231 and MCF-10A. This indicates the potential safety and selectivity of the action of heterocyclic amino-containing derivatives of 1,4-naphthoquinone. Ethyl-4-((3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-yl)amino)-1-phenyl-1*H*-pyrazole-3-carboxylate (4) showed the highest cytotoxic activity against all tested cancer and non-cancer cell lines, however, *in silico* screening revealed signs of potential hepatotoxicity, and, therefore, there is a need for further experimental *in vitro* toxicity studies.

Conclusion

The results obtained indicate the feasibility of conducting experimental studies of synthesized heterocyclic amino-containing derivatives of 1,4-

naphthoquinone by in vitro methods for antitumor activity. According to the data of modeling the predicted toxicity of the studied compounds in rats by the ProTox-II method, all the studied compounds belong to IV(**1,3,4**) and VI(**2**) toxicity classes, i.e. to low-toxic and non-toxic substances, respectively.

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Н. В. Поліш, Н. Г. Марінцова, Л. Р. Журахівська, А. О. Миляних, Е. Я. Ізмерлі
Національний університет “Львівська політехніка”,
кафедра технології біологічно активних сполук, фармації та біотехнології

ОЦІНКА ЦИТОТОКСИЧНОСТІ ГЕТЕРОЦИКЛІЧНИХ АМІНОМІСНИХ ПОХІДНИХ 1,4-НАФТОХІНОНУ

Досліджено цитотоксичну активність гетероциклічних аміновмісних похідних 1,4-нафтохінону *in vitro* на різних лініях раку молочної залози людини, а саме: MDA-MB, MCF-7, SK-BR-3, BT-474 та неракових клітинних лініях (MCF-10A). Проведено *in silico* прогнозування токсичності досліджуваних речовин щодо щурів з використанням сучасного програмного забезпечення ProTox-II. Встановлено, що всі протестовані сполуки належать до IV та VI класів токсичності та є малотоксичні. Визначено, що гетероциклічні аміновмісні похідні 1,4-нафтохінону можуть бути використані як потужні протиракові засоби з цитотоксичною дією щодо MCF-7 та MDA-MB-231.

Ключові слова: гетероциклічні аміновмісні похідні 1,4-нафтохінону, протипухлинна активність *in vitro*, ProTox-II.