UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA

DOCTORAL SCHOOL

DOCTORAL THESIS

STUDIES CONCERNING THE SYNTHESIS AND BIOLOGICAL ACTIVITY OF NEW 1,4-NAPHTHOQUINONE DERIVATIVES

ABSTRACT

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Contents

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Introduction

Quinones are a type of widespread aromatic compounds, comprising of substances such as pigments, antibiotics, vitamins and coenzymes. They are found in plants, as secondary metabolites, in fungi, as well as in the kingdom Animalia. Plants containing 1,4-naphthoquinones have been and are used to date in folk medicine all across the world, which is why these compounds have been intensively studied in order to identify their biological actions. Research has revealed some of the most diverse pharmacological effects, including antibacterial, antifungal, antiviral, antiparasitic and antiplatelet actions.

Bacterial resistance to currently used drugs represents the core of a global crisis heralding the end of the antibiotic era. There are more and more pathogens resistant to multiple classes of antibiotics and there seems to be an ascending trend in the rise of strains that are sensitive to an extremely narrow segment of pharmacological agents, which drastically limits the treatment options, especially in immunocompromised patients.

In spite of the development of new antibiotics, the number of drugs available for treating infections remains reduced, which triggers the necessity of developing new, simpler molecules, able to act as antimicrobial agents, on top of having an optimal pharmaco-toxicological profile.

Consequently, naphthoquinone (NQ) compounds have drawn the scientific community's interest, meeting these criteria.

A. Current Stage of Knowledge

NQ compounds can be classified in two large categories: natural and synthetic. Literature studies are focused on the uses and effects of the naturally occurring molecules, as well as on conceiving new optimized structures, built on the 1,4-naphthoquinone scaffold. Last but not least, research based on these compounds is aimed at revealing the mechanisms of action justifying their pharmacological effects.

1. Natural and synthetic biologically active 1,4-naphthoquinone derivatives

Natural NQs are secondary metabolites produced in plants, fungi and animals, playing a wide range of biological roles. NQs are usually orange or brown, representing important natural pigments.

NQ derivatives have been mostly used for their tinctorial properties, but there have also been reported biological activities. Thereby, most studies revolve around their antibacterial, antifungal, antiprotozoal and antitumor effects. Additionally, there have been conducted studies centered on their antiplatelet, anti-inflammatory and antiallergic effects.

Naturally occurring quinones are usually the 1,4-naphthoquinone type, more seldom the 1,2 type, in the form of either monomers or dimers and trimers. They are most commonly found in certain plant families, such as: *Bignoniaceae*, *Avicenniaceae*, *Boraginaceae*, *Droseraceae*, *Ebenaceae*, *Juglandaceae*, *Nepenthaceae*, *Plumbaginaceae.* Moreover, their presence has been confirmed in different fungi genera, such as *Fusarium*, *Marasmius*, *Verticillium*, as well as in algae and lichens.

Most frequently, NQs are found in the yellow or brown pigments of plants. They have also been detected in the defense mechanisms of plants, used by these as antibacterial, antifungal and antimalarial compounds, which is why quinone producing species present with allelopathy.

A special category of NQs is represented by the vitamin K group, which comprises of lipophilic substances having as common structural ground the 2-methyl-1,4-napthoquinone moiety with an aliphatic chain at C3. Vitamins K play a fundamental role in numerous biochemical processes: coagulation (vitamins K are known to influence the activity of coagulation factors II, VII, IX, X), bone metabolism and cellular growth, among others.

As for the synthetic derivatives, the structures described in the literature have in common the 1,4-naphthoquinone ring, condensed with different other rings or endowed with various groups placed on the main ring. The pharmacological effects of the synthetic molecules display the same pharmacological effects of their natural counterparts, with emphasis on the antimicrobial and antitumor actions.

Structures have been designed bearing the NQ ring, endowed with proven actions against bacteria, fungi, protozoa and viruses. As for the antibacterial effect, it seems that NQs tend to be more active against Gram-positive bacteria, having shown little or no effect whatsoever on the Gram-negative ones.

It has been reported the synthesis of NQ compounds capable of inhibiting the phospholipase A_2, as well as the synthesis of derivatives with inhibitory effects on both the formation of the superoxide anion in the neutrophil, as well as on the mast cell degranulation. These properties recommend this type of compounds as antiallergic and anti-inflammatory agents. Moreover, preclinical studies have been conducted, showing the antiplatelet effect of certain NQs in the absence of any coagulating activity.

Another major therapeutic potential displayed by NQs consists of their antitumoral action. Research studies try to exploit this potential, testing different molecules on a variety of cell lines. Some articles cite the investigation of antitumoral effect of NQ compounds substituted at C2, C3, C5 and C8, especially with alkoxy groups.

2. Biochemical mechanisms of action responsible for the activity of 1,4-naphthoquinones

To date, it has not been confirmed a singular hypothesis concerning the means by which NQs act on a cellular and subcellular level, but several partially verified scenarios have been proposed, which could help shed light on why these compounds are endowed with such a polymorphic pharmacological profile.

One of the fundamental hypotheses regarding the mechanism of action of NQs is based on the capacity of inducing oxidative stress, initially noticed in menadione. The efficiency of menadione in various types of cancer is explained by the oxidative stress generated by the redox cycle of the quinone, leading to the formation of reactive oxygen species (ROS), such as hydroxyl radicals, superoxide anions and hydrogen peroxide.

Through the massive production of ROS, the antioxidant capacity of the cellule is exceeded, which eventually leads to cellular death. Quinones can be reduced with one electron, generating semiquinone radicals, or two electrons, turning into hydroquinone.

Another mechanism of action is represented by the arylating capacity of NQs, consisting of the introduction of aromatic structures, such as the NQ ring of menadione, in the molecule of glutathione, forming NQ-glutathione conjugates.

Lastly, the two previously mentioned effects (ROS production, as well as the arylation of thiol moieties of peptides and proteins) do not occur individually, but rather they

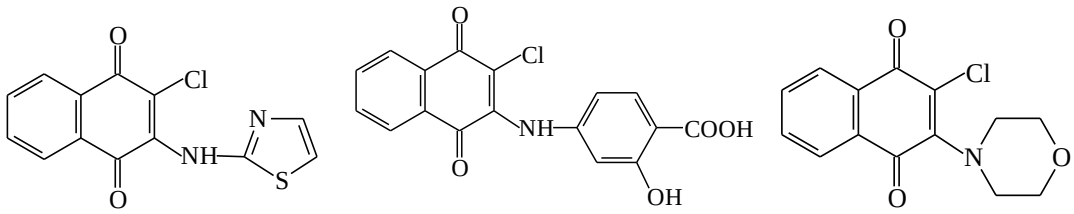
initiate an intracellular signaling cascade, in addition to urging cellular death. Indirectly, NQs are capable of blocking the evolution of the cell cycle from phase S to phase G2, by blocking the replication of cellular DNA. The reason for this is the inhibition of topoisomerases.

All this research validating the hypotheses concerning the mechanisms of action of NQs demonstrates that their cytotoxic activity is manifested through cellular and subcellular phenomena complementing each other, the target cell being therefore attacked on multiple fronts.

B. Personal Contributions

3. Synthesis and physico-chemical characterization of new 2-chloro-3-*N*-substituted 1,4-naphthoquinones

The 2-chloro-3-*N*-substituted 1,4-naphthoquinones were obtained in a one-step reaction of condensation between dichlone (2,3-dichloro-1,4-naphthoquinone) and one of the following amines: 2-aminosulphathiazole, 4-aminosalicylic acid and morpholine. The resulting compounds were as follows: 2-chloro-3-(*N*-thiazole-2-ylamino)-1,4-naphthoquinone (3a), 2-chloro-3-(*N*-[4-(1-carboxy-2-hydroxy-phenyl)amino)-1,4-naphthoquinone (3b), 2-chloro-3-morpholino-1,4- naphthoquinone (3c), as shown below.

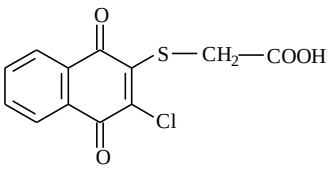


3a **3b** **3c**

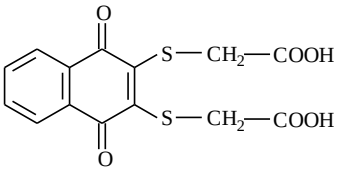
The synthesis lead to the formation of powders with various colors, ranging from red to brown. The reaction yields were of 70-87%. According to the chromatograms, the compounds obtained were pure, due to the presence of a sole peak. The UV-Vis and FTIR spectra have indicated the presence of the characteristic functional groups. Moreover, the validity of the structural formulas of the three compounds was confirmed by the NMR-^{1}H spectra, recorded at 300 MHz, in DMSO-d_6.

4. Synthesis and physico-chemical characterization of new 1,4-naphthoquinone thiol-containing derivatives

The synthesis of these five hetero-1,4-naphthoquinones containing thiol groups substituted with residues of alkanoic acids at 2- or 2,3-positions consists in a condensation reaction between 2,3-dichloro-1,4-naphthoquinone (dichlone), thioacetic acid, 3-thiopropanoic acid and ethyl thioacetate, respectively, heated in the presence of pyridine, at a ratio of 1:1 or 1:2. The resulting compounds were the following: 2-(3-chloro-1,4-dioxo-1,4-dihidronaphthalen-2-yl-thio) acetic acid (1), 2,2'-[(1,4-dioxo-1,4-dihidronaphthalen-2,3-diyl)-dithio] diacetic acid (2), 3-(3-chloro-1,4-dioxo-1,4-dihidronaphthalen-2-yl-thio) propanoic acid (3), 3,3'-[(1,4-dioxo-1,4-dihidronaphthalen-2,3-diyl)-dithio] dipropanoic acid (4), ethyl 2-(3-carboxyethylthio-1,4-dioxo-1,4-dihidronaphthalen-2-ylthio) acetate (5).



1



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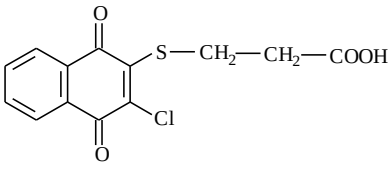

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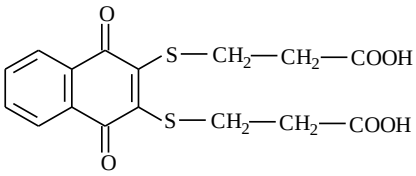

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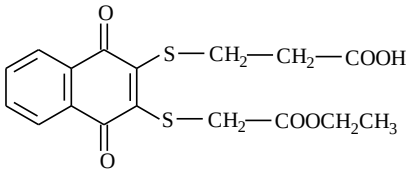
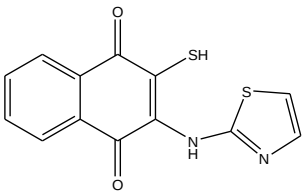


5

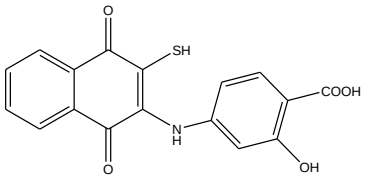
The process lead to the formation of powder with various colorings, varying from yellow to red, with yields of 75-85%. As mentioned for the previous compound series, the presence of a single peak on the chromatograms was proof of the purity of the compounds. The UV-Vis and FTIR spectra indicated the presence of the functional groups of the compounds and the structures were confirmed by the NMR-^{1}H analysis, carried out in the same conditions as mentioned in the previous paragraph.

In the same category we have synthesized three other compounds, with structural formulas shown below. The synthesis of the 2-thio-3-*N*-substituted NQs was possible through a three-step condensation of simpler 2-chloro-3-*N*-substituted NQs with thiourea, sodium hydroxide and acetic acid. The resulting compounds were: 2-thio-3-(*N*-thiazole-2-ylamino)-1,4-naphthoquinone (a), 2-thio-3-(*N*-[4-(1-carboxy-2-hydroxy-

phenyl)amino)-1,4-naphthoquinone (b), 2-(diethylamino)ethyl-4-(1,4-dihydro-2-thio-1,4-dioxonaftalen-3-ylamino) benzoate (c).



a



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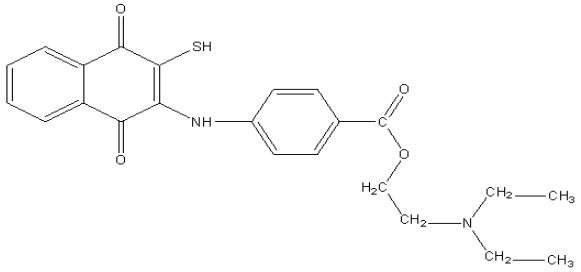
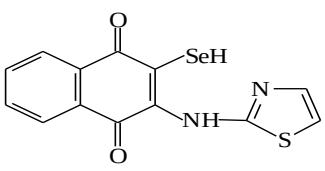


c

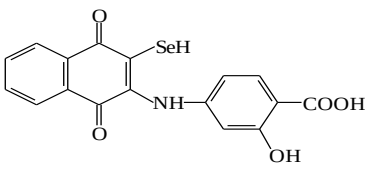
The synthesis process lead to the formation of red or brown powders, with yields revolving around 70%. The invariability of the melting points, in addition to the presence of a unique peak on the chromatograms, indicated that the products were pure. The UV-Vis, FTIR and NMR-^{1}H spectra confirmed, once again, the designed structural formulas.

5. Synthesis and physico-chemical characterization of new 2-hydroseleno-3-*N*-substituted 1,4-naphthoquinones

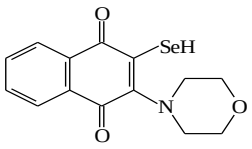
Three novel NQ derivatives were synthesized through a three-step condensation reaction. The process was very similar to the one described for compounds a-c, only this time thiourea was replaced with selenourea. The resulting compounds were 2-hydroseleno--3-(*N*-tiazol-2-ilamino)-1,4-naphthoquinone (6a), 2-hydroseleno-3-(*N*-[4-(1-carboxy-2-hydroxyphenyl)amino)-1,4-naphthoquinone (6b), 2-hydroseleno-3-morpholino-1,4-naphthoquinone (6c). The structural formulas are shown below.



6a



6b



6c

The compounds were of solid nature, in the form of grey or brown powders. The reaction yields were of 70-80%. In terms of physico-chemical analysis, the observations made for the other compounds apply to this series as well.

6. Evaluation of the antimicrobial activity of the newly synthesized 1,4-naphthoquinone derivatives

Firstly, this chapter presents the observations resulted from testing the antibacterial activity of the five thioalkanoic substituted NQs (1-5).

The testing was carried out using the disk diffusion method, according to CLSI recommendations. All compounds were tested on the following pathogen strains: *Staphylococcus aureus* ATCC®25923, *Escherichia coli* ATCC®25922, *Pseudomonas aeruginosa* ATCC®27853, and the antifungal activity was tested on *Candida albicans* ATCC®10231.

Bacterial strains were grown in Mueller Hinton medium. Fungi were grown in Sabouraud Dextrose Agar medium. The results were recorded by measuring the diameter of the zones of complete inhibition, including the diameter of the disk. Disks containing vancomycin were used as antibacterial control. Fluconazole was used as antifungal control, dichlone as structural control and DMSO as blank.

Most of the compounds of this series showed various antibacterial and antifungal activities, some of them exceeding dichlone, used as a structural control. The majority of the compounds showed a superior activity on *S. aureus* compared to dichlone. *S. aureus* has a higher sensibility to compounds 2 and 5, which exerted an antibacterial activity comparable to vancomycin.

The absence of inhibition zones of Gram-negative strains, *E. coli* and *P. aeruginosa*, has proven that the compounds have no antibacterial action in the tested concentration. Regarding the antifungal activity on *C. albicans*, we observed that only compound 5 proved a medium activity comparable to that of fluconazole, while the others were less efficient than dichlone.

Following the determination of the MIC (minimum inhibitory concentration) on *S. aureus*, using the serial dilution method, the results have indicated a uniformity of the MIC values, of 3 µg/ml, for all five compounds. Meanwhile, CMI values have varied on *C. albicans*. The lowest CMI value was found for compound 5, which makes it the most potent derivative in this series, on *C. albicans*.

A similar experiment was conducted for testing the antibacterial and antifungal effect of the chlorinated NQ derivatives (3a, 3b, 3c).

The antibacterial effect of 3a was tested onto *Staphylococcus aureus* ATCC®25923, *Enterococcus faecalis* ATCC®29212, *Escherichia coli* ATCC®25922, *Klebsiella spp.* ATCC®700603. The antifungal effect was tested on *Candida albicans* ATCC®10231. The control substances used were ampicillin, oxacillin, as well as clotrimazole. Compound 3a proved efficient only against Gram-positive strains, although with a lower activity compared to the control antibiotics. The antifungal effect, on the other hand, was similar to clotrimazole.

The antimicrobial activity of compound 3b was evaluated against the same strains as 3a. The two aforementioned derivatives were similar in terms of antibacterial effect, the latter being slightly more effective on *C. albicans.*

3c was tested on *Pseudomonas aeruginosa* ATCC®27853, *Streptococcus pyogenes* ATCC®19615, *Candida albicans* ATCC®10231. Additionally, we tested three strains supplied by the Emergency County Hospital of Craiova: methicillin-resistant *S. aureus* (MRSA), *E. coli* and *C. albicans*. Unlike dichlone, 3c was active against all tested strains, with the exception of *P. aeruginosa*. The strongest activity proved to be against *C. albicans*.

Thiol derivatives a and b were tested onto *Staphylococcus aureus* ATCC®25923, *Enterococcus faecalis* ATCC®29212, *Escherichia coli* ATCC®25922, *Klebsiella spp.* ATCC®700603, as well as on *Candida albicans* ATCC®10231. Both compounds were active solely against Gram-positive strains and *C. albicans*, but with an inferior effect compared to the control substances, the same we used for the chlorinated NQs.

In order to evaluate the antimicrobial activity of the selenoderivatives (6a, 6b, 6c), the following strains were used: *Pseudomonas aeruginosa* ATCC®27853, *Streptococcus pyogenes* ATCC®19615, *C. albicans* ATCC®10231, as well as two of the strains supplied by the Emergency County Hospital of Craiova: *C. albicans* and methicllin-resistant *S. aureus*, respectively. The control substance used for this series was dichlone. All compounds, dichlone included, were inefficient against the *P. aeruginosa* strain, as expected. The most sensitive strain proved to be MRSA, responding to 6b and 6c, the latter having a higher effect than dichlone. However, dichlone proved to be more efficient than compounds 6b and 6c on the indigenous *C.*

albicans, while the standard strain was sensitive to 6b only. On *E. coli*, the only molecule relatively active was dichlone. 6a only exerted an effect on *S. pyogenes*.

7. Evaluation of the anti-proliferative potential of the newly synthesized 1,4-naphthoquinone derivatives

This chapter shows the results of the experiments aimed to determine the effects of some of the novel NQs on cell proliferation of mammary adenocarcinoma and osteosarcoma cell lines.

For the first experiment, human breast epithelial cell line MDA-MB-231 was used, testing thiol derivatives a, b and c. We observed that these three molecules exhibited different effects on the cell line. For instance, compound a had no significant influence on the cell proliferation, compared to control samples, whereas compounds b and c reduced the cell proliferation in a dose-dependent manner. At lower concentrations, the two molecules exerted similar effects, with a slightly better activity for b. However, the effect of compound c increased at higher concentrations. The results show that treatment of cells with NQ-PRO induced dropping of viability to 86.55% for the 1μM concentration, 79.88% for 2.5μM, 25% and 18% for 5μM and 10μM respectively ($p<0.05$), when compared to control. For the compound NQ-PAS, the viability was more influenced at lower concentrations when compared to control (77.14% for 1μM, 72.33% for 2.5μM, 39.12% and 32.5% for 5μM and 10μM, respectively ($p<0.05$).

In order to test the antiproliferative effect of thioalkanoic derivatives 1, 3 and 4, we used cell lines MCF-7 and MG63. MCF-7 is also a breast cancer cell line, isolated from a 69-year-old Caucasian woman, diagnosed with invasive ductal carcinoma. MG-63 is a fibroblast cell line, isolated from the bone of a Caucasian patient with osteosarcoma.

Compounds 1, 3 and 4 were tested along with menadione. The MTT test showed no drop in cell viability for either two cell lines, after 24 h. On the other hand, we noticed different behaviors of the compounds after 48 h. The macroscopic analysis of the resulting assays following the MTT test showed a variation in the intensity of the formazan coloration more significant in MCF-7, compared to MG-63.

On MCF-7 cells, menadione had a profound, relatively dose-independent, antiproliferative effect, the survival rate plummeting significantly compared to the

control, but without relevant differences of the concentrations. Compound 1 only displayed a dose-dependent variability of its effect, on MCF-7. The results indicate that the treatment of the MCF-7 cells with compound 1 did not induce low survival rates at low concentrations. However, there was a drastic survival decrease towards the maximum concentration tested (1000 μM).

With respect to compound 3, the mean value indicated a slight survival rate decrease, however, the standard deviations raises questions over the accuracy of the result. Compound 4 displayed an inconstant behavior towards the survival rate, the cell viability being similar for the limit concentrations of the tested interval.

Conclusions

The purpose of this thesis was to describe the synthesis of novel 1,4-NQ derivatives, as well as the testing of their biological actions (antibacterial, antifungal, antiproliferative).

Specific objectives:

- Synthesis of the designed 1,4-NQ derivatives – three structural categories were developed: 2-chloro-3-*N*-substituted NQs, 2-hydroseleno-3-*N*-substituted NQs, and 1,4-NQ thiol derivatives;
- Physico-chemical characterization of the compounds – purification through recrystallization, purity testing using chromatography, structure confirmation using UV-Vis, FTIR and RMN-^{1}H spectrometry;
- Evaluation of the antibacterial activity using the disk diffusion and serial dilution methods;
- Evaluation of the antifungal activity on *C. albicans* strains;
- Evaluation of the antiproliferative activity of the compounds on two breast cancer cell lines (MCF-7 and MDA-MB-231), as well as on MG-63, an osteosarcoma cell line.

The novel molecules have exerted heterogenous antibacterial activity, being predominantly active against Gram-positive strains. Some of the NQ derivatives also had antifungal activities on both standard and indigenous *C. albicans* strains.

Further research is necessary in order to determine the pharmacological properties *in vivo*, as well as the possibility of using these compounds as antimicrobial agents.

The results obtained from testing the antiproliferative potential encourage follow-up research with the purpose of identifying the underlying mechanisms of action, responsible for the inhibition of the cell proliferation manifested by the tested compounds.

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