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Novel 2,3-disubstituted 1,4-naphthoquinone derivatives and their metal complexes – Synthesis and *in vitro* cytotoxic effect against mouse fibrosarcoma L929 cells

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ABSTRACT

Quinones have various pharmacological properties including antibacterial, antifungal, antiviral, anti-inflammatory, antipyretic and anticancer activity. Two novel 2,3-disubstituted 1,4-naphthoquinones and their metal complexes were synthesised, characterized and tested. The cytotoxic potential of the novel 2,3-disubstituted 1,4-naphthoquinones and their metal complexes was studied against L929 murine fibroblasts cells grown *in vitro*. The treatment resulted in a concentration-dependent cytotoxicity as indicated by MTT assay.

1. Introduction

Research in the field of cancer therapy has led to the development of many modern medicines and therapeutics with promising anticancer activity. Among the many natural and synthetic compounds explored for anticancer potential, compounds with quinone containing moieties form a major part. Quinones are widely distributed compounds in nature and they are reported to exhibit diverse pharmacological properties including anticancer activity [1,2]. One of the mechanisms by which quinones could induce cytotoxicity was induction of oxidative stress [3]. According to this proposed mechanism, quinones are first reduced to hydroquinones or semiquinone radicals by cellular reductases at the expense of NADPH. Then, both hydroquinones and semiquinone radicals undergo rapid autoxidation with the regeneration of the parent quinones and the concomitant formation of superoxide [3]. These quinones with the ability to induce oxidative stress are responsible for initiation of tissue damage selectively in tumour cells and this seems to be a promising approach for targeting cancer cells [4].

1,4-naphthoquinone pharmacophore is known to impart pronounced biological effects in 1,4-naphthoquinone derivatives, leading to antitumour [5,6], antiproliferative [7], antimycobacterial [8], antiplatelet, anti-inflammatory, antiallergic [9], antimalarial [10] and antileishmanial activities [11]. The incorporation of sulphur atom in 1,4-naphthoquinone derivatives has led to antifungal, antibacterial, antiviral, and anticancer activities [12–16].

It has been reported that menadione (an analogue of 1,4-naphthoquinone) could induce reactive oxygen species (ROS), which mediate DNA damage in many human cultured cells, suggesting that this activity might be important for its therapeutic effects of naphthoquinone derivatives [17,18].

The literature offers data related to the complex compounds formed by transition metals and ligands having conjugated double bonds [19–21]. The special interest about these complexes is due, among others, to the fact that the complex compounds readily participate in reversible electron-transfer reactions. Literature mentions the important biologic-active, antimalaric, antiviral and antitumoural properties of this type of ligands; the same properties are shown by the complexes that these ligands form with metal ions, which act in the biological structures as essential microelements [19–21]. The importance of these compounds can be exemplified by

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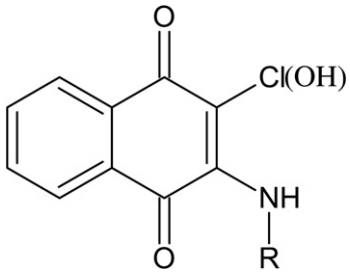


Fig. 1. 3-alkyl- or 3-alkylamino-substituted derivatives of 2-hydroxy-1,4-naphthoquinone or 2-chloro-1,4-naphthoquinones.

the interesting biological activities associated with many 3-alkyl- or 3-alkylamino-substituted derivatives of 2-hydroxy-1,4-naphthoquinone or 2-chloro-1,4-naphthoquinones (Fig. 1) [22].

Further need for a study of compounds of this type is illustrated by the observation that the ortho-amino quinoid unit is present in many antitumoural antibiotics such as actinomycins, mitomycin C, porfiromycin and streptonigrin [23].

In this study, in order to continue the existing investigations on 2,3-disubstituted 1,4-naphthoquinones [24], we introduced substituents groups containing N, S or O atoms, knowing the fact that in many natural structures, such as proteins or aminoacids, these groups are responsible for various biological activities. Moreover, the potential biological activity of the new synthesized compounds can be cumulated due to the 1,4-naphthoquinone properties. Also, because there are few literatures [25–27] regarding the complexes of the general [M-N2S2] type, especially metal complexes with naphthoquinonic ligands, we hope that the data provided by this paper will contribute to enrich the knowledge in this insufficiently studied field.

Cell lines are useful models for doing research since they provide large amounts of consistent cells for prolonged use and because most cellular characteristics are maintained, reliable experimental data can be compared among research reports, in which the same cell lines are used. L929 is a cell line popular in many experiment aspects such as material biocompatibility testing [28–30], drug cytotoxicity testing [31,32] and cell biology studies [33–35].

In order to develop new antitumoural drugs with less secondary effects, the final purpose of this study was to evaluate the potential biological activity of the novel synthesized 2,3-disubstituted 1,4-naphthoquinone compounds in terms of cytotoxicity by MTT assay [36], using L929 mouse fibroblasts.

2. Methods

2.1. Chemicals

The reagents and the solvents for the synthesis were used without further purification. 2-amino-3-chloro-1,4-naphthoquinone and 2,3-dichloro-1,4-naphthoquinone were purchased from Fluka (Steinheim, Germany), chloroacetic acid from Merck (Darmstadt, Germany), ethanol, methanol, dilituric acid, anhydrous nickel chloride and hexahydrate chloroplatinic acid from Sigma–Aldrich (Steinheim, Germany), metallic sodium from Aldrich (Steinheim, Germany), thiourea, NaOH and glacial acetic acid from UTCHIM (Ramnicu Valcea, ROMANIA) and Water Chromasolv from Riedel-de Haën (Seelze, Germany).

2.2. Instruments

Melting points (m.p.) of the ligands and metal complexes were determined by open capillary method using Sunsim electric melting point apparatus and were uncorrected. Elemental analysis of C, H, N, S and O was performed with a Thermo Finnigan Flash 2000 Automatic Elemental Analyzer. The metal content was determined using an Analytic Jena NOVAA 300 Absorption Atomic Spectrophotometer. The IR spectra were recorded in the region 4000–400 cm^{-1} on a FT-IR MAGNA 750 NICOLET in anhydrous KBr pellets. A Jasco V-670 UV–Vis spectrophotometer was used to perform the electronic spectra.

2.3. In vitro cytotoxicity assay

In order to determine the cytotoxicity of the compounds, the *in vitro* cytotoxicity MTT assay was performed using L929 mouse fibroblasts (kindly provided by Dr. Ronald Doyle, University of Louisville School of Medicine, Louisville, KY, USA).

The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) (Sigma–Aldrich, Inc. St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (BIOCHROM AG, Berlin, Germany) and 100 U/mL penicillin-streptomycin (Lonza, Verviers, Belgium) and incubated at 37 °C in a humidified atmosphere with 5% CO_2, in flat-bottom 96-well tissue culture plates (starting concentration: 6 × 105 cells/mL, 100 μL/well).

Test compounds were dissolved in dimethylsulphoxide (DMSO) (Merck, Darmstadt, Germany) at a final concentration of 1% in culture medium.

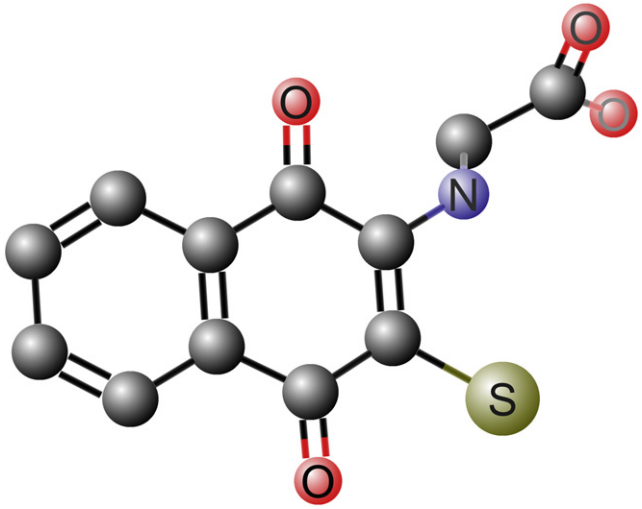


Fig. 2. 2-acetamino-3-mercapto-1,4-naphthoquinone.

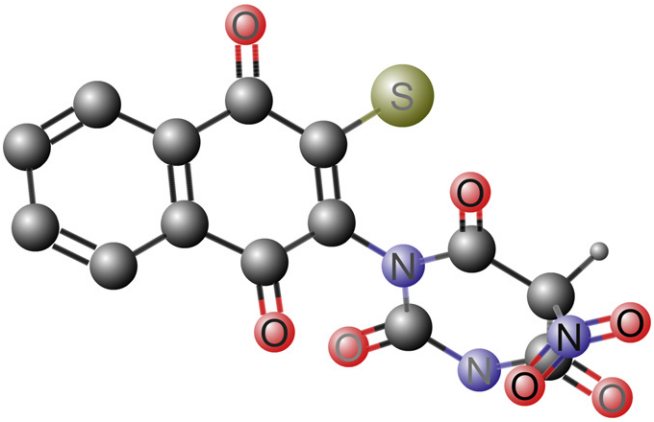
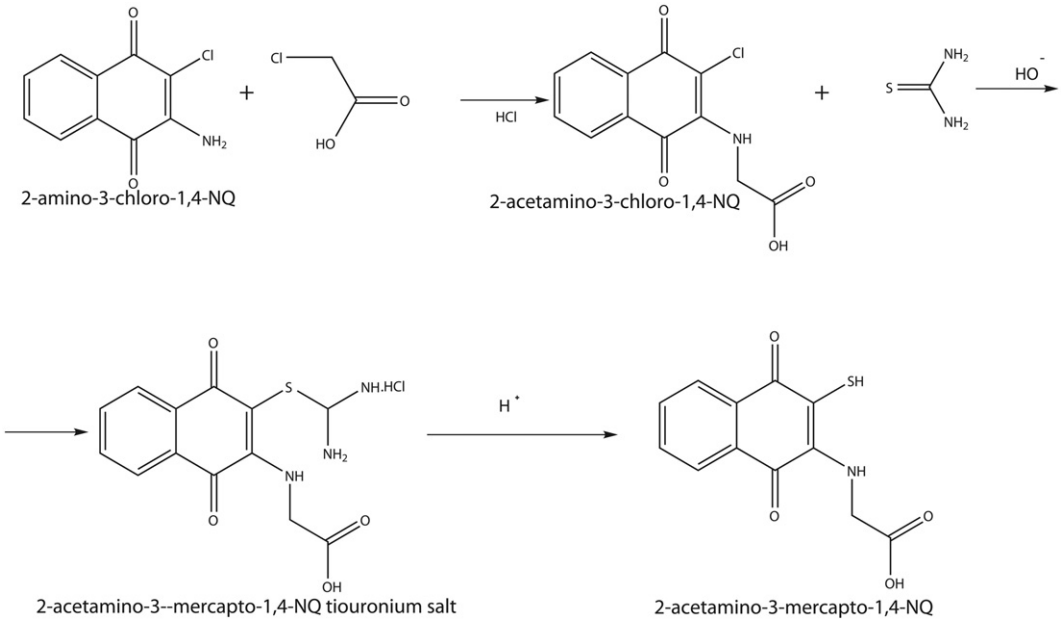


Fig. 3. 2-mercapto-3(5-nitrobarbituro)-1,4-naphthoquinone.



Scheme 1. Synthesis of AMNQ.

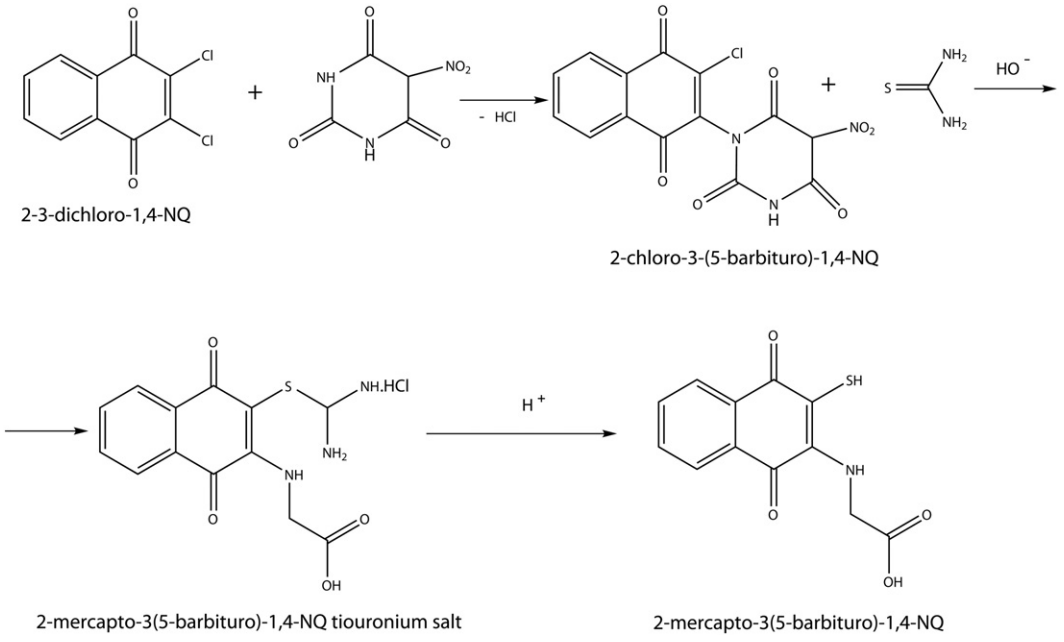
Ten binary dilutions were made by serial dilution in culture media from a starting concentration of 0.200 mg/mL. After incubation, cells were exposed to serial dilutions test samples for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. Viability of the cells was measured by MTT assay (MTT: (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) purchased from Sigma–Aldrich Corp., St. Louis, MO, USA) [36]. MTT solution was added to cell cultures in final concentration of 0.5 mg/mL and cells were incubated for 3 h.

Thereafter, 100 μL of lysis solution (20% sodium dodecyl sulphate in 50% *N*,*N*-dimethylformamide, 0.4% acetic acid, and 0.04 N HCl) was added and the conversion of MTT to formazan by metabolically viable cells was measured by a Tecan Sunrise microplate reader (Tecan Group Ltd., Mannedorf, Switzerland) at 540 nm. Each dilution was tested in two replicates. The 50% inhibition concentration (IC50) was determined by curve fitting using "ic50 package" in *R* [37,38].

3. Results and discussion

3.1. Synthesis

The new naphthoquinonic ligands containing $X = S$, $Y = N$ as donor atoms were: 2-acetamino-3-mercapto-1,4-naphthoquinone (AMNQ) (Fig. 2) and 2-mercapto-3(5-nitrobarbituro)-1,4-naphthoquinone (MNBNQ) (Fig. 3). These ligands were synthesized according the Schemes 1 And 2.



Scheme 2. Synthesis of MNBNQ.

Table 1
Preparation details and elemental analyses for AMNQ and [Pt(AMNQ)$_2$].

Compound	Mol wt calcd	mp (°C)	tPSA[a] / clogP[b]	Appearance	Yield %	Element	Calc./found%
AMNQ	263.27	140	83.47	Brown	93	C	54.75/54.74
						H	3.42/3.42
			1.3462			N	5.32/5.31
						O	24.34/24.35
						S	12.17/12.18
[Pt(AMNQ)$_2$]	719.60	170	149.36	Red-brown microcrystalline	95	C	40.06/40.05
						H	2.24/2.26
			5.9268			N	3.89/3.87
						O	17.79/17.77
						S	8.91/8.89
						Pt	27.11/27.16

[a] tPSA – molecular polar surface area.
[b] clogP – octanol/water partition coefficient.

Table 2
Preparation details and elemental analyses for MNBNQ and [Ni(MNBNQ)$_2$].

Compound	Mol wt calcd	mp (°C)	tPSA[a] / clogP[b]	Appearance	Yield %	Element	Calc./found%
MNBNQ	361.29	142	153.43	Purple-brown	94	C	46.54/46.52
			–			H	1.95/1.97
			1.2946			N	11.63/11.60
						O	31.00/31.02
						S	8.88/8.89
[Ni(MNBNQ)$_2$]	779.25	154	286.40	Dark brown microcrystalline	96	C	43.16/43.14
						H	1.55/1.57
			1.9334			N	10.78/10.77
						O	28.74/28.72
						S	8.23/8.24
						Ni	7.54/7.56

Values calculated with ChemBioDraw, v. 11.0.01, Cambridge Soft.
[a] tPSA – molecular polar surface area.
[b] clogP – octanol/water partition coefficient.

Table 3
Infrared absorption frequencies (cm^{-1}) for AMNQ and [Pt(AMNQ)$_2$].

Compound	ν_{N-H}	ν_{N-Pt}	ν_{SH}	$\nu_{C=O}$	δ_{NH}	ν_{C-N}	ν_{C-H}	ν_{C-S}
AMNQ	3346.09	–	2231.18	1673.15	1590.83	1280.23	841.95	594.56
	m		s	m	s	m	w	s
[Pt(AMNQ)$_2$]	3423.11	3867.15	2360.71	1604.51	–	1275.12	841.30	–
	w	s	s	s		m	w	

w – weak; m – medium; s – strong.

Synthesis of AMNQ: 2-amino-3-chloro-1,4-naphthoquinone (0.053 mol) and chloroacetic acid (0.074 mol) in absolute ethanol (100 mL) were stirred and refluxed at 120 °C for 3 h. The reaction mixture was then cooled and after filtration the intermediary, 2-amino-3-chloro-1,4-naphthoquinone was formed. This compound (0.022 mol) was then stirred and refluxed in absolute ethanol (80 mL) with thiourea (0.422 mol) at 120 °C for 2 h. The reaction mixture volume was then half reduced by distillation under vacuum, 100 mL pure water and 10 g (0.4 mol) NaOH were added to the solution and the mixture was refluxed another 2 h. In order to obtain the final product, AMNQ, 1 N acetic acid solution was added to the reaction mixture. The product was then filtered, washed with pure water and dried. The purification of AMNQ was achieved by consecutive dissolution in 1 N NaOH solution and precipitation in 1 N acetic acid solution.

Synthesis of MNBNQ: 2,3-dichloro-1,4-naphthoquinone (0.048 mol) and dilituric acid (0.049 mol) in absolute ethanol (100 mL) were stirred and refluxed at 120 °C for 3.5 h. The reaction mixture was then cooled and after filtration the red intermediary, 2-dilituro-3-chloro-1,4-naphthoquinone was formed. This compound

Table 4
nfrared absorption frequencies (cm^{-1}) for MNBNQ and [Ni(MNBNQ)$_2$].

Compound	ν_{N-H}[a]	ν_{N-Ni}	ν_{S-H}	$\nu_{C=O}$[a]	$\nu_{C=O}$	ν_{NO2}[a]	$\nu_{C=C}$	ν_{C-N}[a]	ν_{C-N}	ν_{C-S}
MNBNQ	3417.24	–	2854.48	1836.77	1673.37	1569.57	1524.5	1386.05	1285.63	697.43
	m		w	w	s	m	w	w	s	w
[Ni(MNBNQ)$_2$]	3415.33	3742.10	–	1836.02	1687.20 1642.39	1549.85	1524.64	1390.52	–	693.13
	i	m		s	w	m	w	w		w

w – weak; m – medium; s – strong.
[a] N cycle from the ligand and the complex.

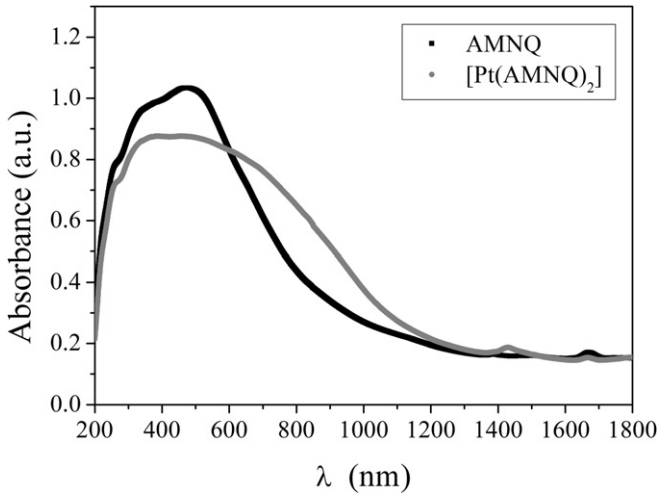


Fig. 4. Electronic spectra of AMNQ and [Pt(AMNQ)$_2$].

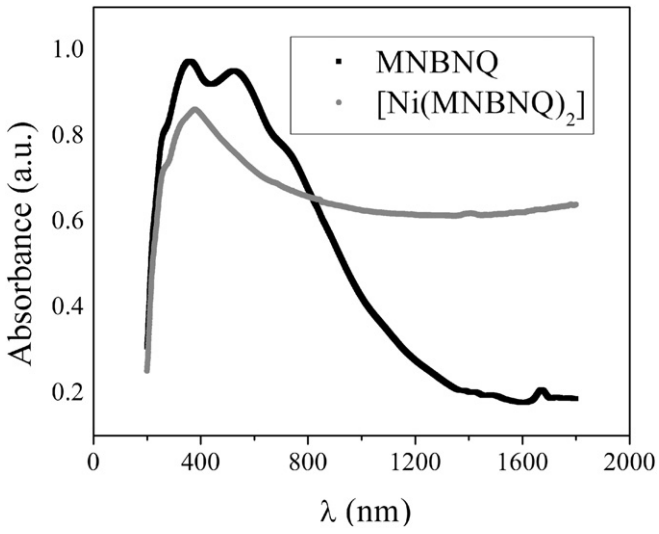


Fig. 5. Electronic spectra of MNBNQ and [Ni(MNBNQ)$_2$].

(0.022 mol) was then stirred and refluxed in absolute ethanol (80 mL) with thiourea (0.44 mol) at 120 °C for 1.5 h. The reaction mixture volume was then half reduced by distillation under vacuum, 100 mL pure water and 10 (0.4 mol) g NaOH were added to the solution and the mixture was refluxed another 2 h. In order to obtain the final product, MNBNQ, 1 N acetic acid solution was added to the reaction mixture. The product was then filtered, washed with pure water and dried. The purification of AMNQ was achieved by consecutive dissolution in 1 N NaOH solution and precipitation in 1 N acetic acid solution.

The transitional metals complexes of Pt (II) and Ni (II) with naphthoquinonic ligands were prepared by the procedure described by Jensen and Nielsen [39]. The complexes are microcrystalline powders, with melting points higher than that of the pure ligand (Tables 1 and 2). They are air-stable, insoluble in ordinary organic solvents (such as chloroform, acetone, ether, ethanol, or carbon tetrachloride), sparingly soluble in dichlorethane and dioxane and soluble in dimethylformamide (DMF) or dimethylsulphoxide (DMSO).

3.2. Spectra interpretation

In order to explain the structures of the metal complexes, we must admit that AMNQ and MNBNQ act as bidentate ligands. The assumption has been confirmed by the infrared spectra for the free ligands and complex compounds. These results are showed in Tables 3 and 4.

The absorption bands due to vibrations groups not involved in the coordination appear in the infrared spectra of the free ligand and of the complex in the same spectral regions, with unchanged or at most slightly modified intensities, because of the electromeric effects due to the coordination. The peaks assignment was made according to Pretsch et al. [40]. The characteristic bands of the N–H, C–N, C–S and S–H groups, which appear in the IR spectra of the ligand as intense or medium intense bands in the complex spectra the bands are modified in intensity and appear at modified frequencies or they cannot be detected, proving the involvement of these groups in the coordination. [Pt(AMNQ)$_2$] and [Ni(MNBNQ)$_2$] are also characterized by the appearance of new bands at 3867.15 cm^{-1} and 3742.10 cm^{-1}, respectively, which can be assigned to ν(M–N) frequencies. So, IR spectra indicate the participation of both sulphur and nitrogen atoms in coordination to the metal.

In order to establish the coordination geometry of the new complexes, a spectral analyses in the visible and UV range was performed. The bands observed in the electronic absorption spectra of the studied complexes were assigned according to Gray and Ballhausen [41] and Vanquickenborne [42]. The absorption bands that appear in the UV range are considered to be the band for the ligands.

Fig. 4 presents the electronic spectra for AMNQ and [Pt(AMNQ)$_2$]. The absorption spectrum of AMNQ presents a band

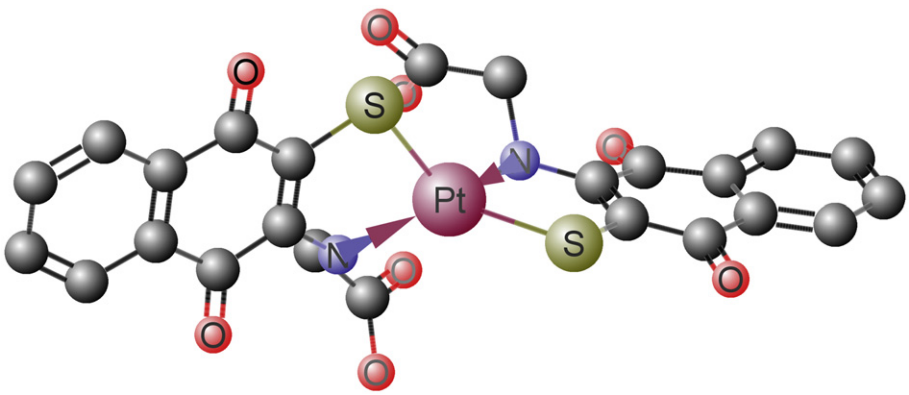


Fig. 6. Configuration of [Pt(AMNQ)$_2$].

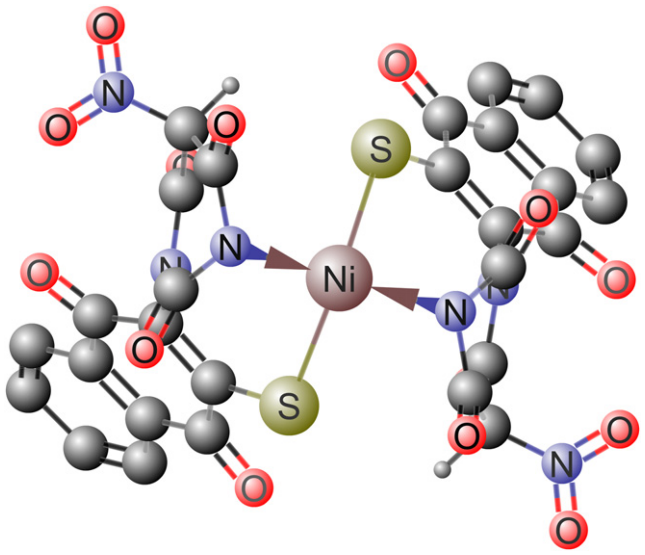


Fig. 7. Configuration of [Ni(MNBNQ)$_2$].

with a maximum at 410 nm, which is characteristic to a $\pi \rightarrow \pi^*$ transition of the aromatic moiety. The electronic spectrum of the complex consists in a broad absorption band in the region 200–800 nm (most probably this broad band is an overlap of bands: the band characteristic for the free ligand, the charge transfer band, and d–d transitions characteristic for Pt (II) (d^8) ion). The majority of compounds of Pt (II) are four-coordinated with a square-planar stereochemistry. According to the literature data [41], electronic spectrum of a compound containing Pt (II) shows three d–d spin allowed transitions (d^8 ion, ground state $^1A_{1g}$). These ones allow d–d transitions corresponding to the transitions from the three lower d levels to the empty d_{x2-y2} orbital. The ground state is $^1A_{1g}$ and the excited states, corresponding to these transitions are $^1A_{2g}$, $^1B_{1g}$ and ^1E_g. These three bands are observed in the region 470–525 nm, 405–448 nm and 335–394 nm and are attributed to $^1A_{1g} \rightarrow {}^1A_{2g}$, $^1A_{1g} \rightarrow {}^1B_{1g}$ and to $^1A_{1g} \rightarrow {}^1E_g$ transitions.

Fig. 5 presents the electronic spectra for MNBNQ and [Ni(MNBNQ)$_2$]. The electronic spectrum of MNBNQ presents a band centred at ~395 nm that is attributed to a $\pi \rightarrow \pi^*$ transition. The [Ni(MNBNQ)$_2$] complex presents a band at 375 nm. Most of the Ni (II) complexes present a strong absorption in the 15 000–25 000 cm^{-1} (666–400 nm) region and another one in the 23 000–30 000 cm^{-1} (435–333 nm) interval. The square-planar Ni (II) complexes differ from the octahedral or tetrahedral complexes because no electronic transitions occur below 10 000 cm^{-1}. This is a consequence of the large field splitting in a square complex; the energy separation between the d_{x2-y2} orbital and the next lowest orbital is invariably greater than 10 000 cm^{-1}.

The transitions assignments prove the involvement of the C–S group to coordination. This conclusion is sustained also by the absorption maxima shift in the complexes, for the $\pi \rightarrow \pi^*$ transition, according to a hypsochromic effect.

The structures of the complexes were explained using the molecular orbital approach. The results of the spectral interpretation confirmed the proposed structures for the new synthesized ligands and also the square-planar configuration for the transitional metal complexes. These complexes are the [ML_2] type, where M = Pt (II) or Ni(II) and L = AMNQ or MNBNQ (Figs. 6 and 7). In order to explain this composition, we must admit that AMNQ and MNBNQ act as bidentate ligands.

3.3. Evaluation of cytotoxicity

To explore the *in vitro* antitumor activity of novel naphthoquinones derivatives, cultures of mouse fibrosarcoma L929 cells were treated with the two ligands (AMNQ and MNBNQ) and metal complex [Pt(AMNQ)$_2$] for 24 h, and cell viability was determined by MTT assay (Fig. 8). Each determination represents the average means of two replicates. The metal complex of MNBNQ with Ni^{2+} could not be tested due to low solubility. Both novel synthesized naphthoquinonic ligands were cytotoxic for L929 fibrosarcoma cell line. Samples at high concentration interfere with detection, resulting in an artificial increase in signal (due to absorbance overlap), although no sign of reduced MTT crystals were visible at microscopic examination. The IC50 differed between these two

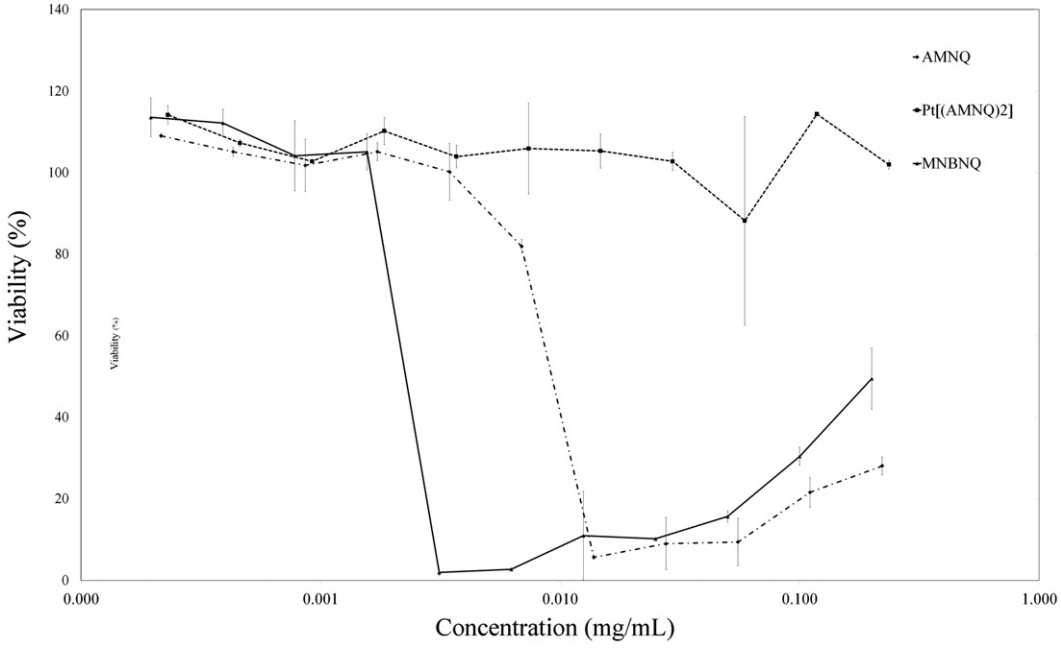


Fig. 8. Cells viability determined by MTT assay.

ligands: 0.0088 mg/mL (95% CI: 0.0074–0.0104) for AMNQ and 0.0022 mg/mL (95% CI: 0.0018–0.0027) for MNBNQ, respectively.

Similar results were reported by Srinivas et al. [18] with a structurally related naphthoquinone, plumbagin, in human cervical cancer cell line, ME-180.

It has been reported that 1,2- and 1,4-naphthoquinones fused with furan or pyran ring are important groups for cytotoxicity to cancer cell lines, with 1,2-naphthoquinones having better activity [43].

In these cases, the cytotoxic effect of 1,4-naphthoquinones-induced cell death was attributed to the induction of apoptosis. Our data do not allow for a conclusion regarding the mechanisms by which the new synthesized compounds AMNQ and MNBNQ exerted their cytotoxic effect on L929 and further studies are needed to elucidate if cell apoptosis or necrosis occurred. Results of a QSAR study have shown that the cytotoxic activities of 1,4-naphthoquinones depend largely on their hydrophobicity [44].

Also, it has been showed the steric interactions of the substituents at either position-2 or position-6 might be also important for cytotoxic effect [45].

4. Conclusions

In conclusion, we have synthesized two novel 2,3-disubstituted 1,4-naphthoquinones and their transitional metal complexes. Correlation between elementary analysis and physical–chemical determinations suggests that Pt and Ni complexes are $[ML_2]$ type. The coordination is assured by the two sulphur atoms from the "mercapto" group and the two nitrogen atoms. ANMQ and NBMNQ are cytotoxic, but, unfortunately, after complexation with transitional metal ions this property disappears. In order to correlate the noticed cytotoxic activity with chemical structure for both ligands and Pt complex, further physical and chemical studies are mandatory.

Acknowledgements

We are grateful to University from Craiova for allowing us using its instruments and laboratories. Authors want to bring a post-mortem tribute to Professor PhD Viorica Muresan, who shared her knowledge in coordination chemistry and spectra interpretation.

Appendix

Marvin was used for drawing, displaying and characterizing chemical structures, substructures and reactions, Marvin 5.4.1.1, 2011. ChemAxon (http://www.chemaxon.com).

Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jorganchem.2011.10.036.

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