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Biological evaluation of donor-acceptor aminonaphthoquinones as antitumor agents

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ABSTRACT

Several members of the phenylamino-1,4-naphthoquinone series were prepared in order to investigate structure–activity relationships (SAR) and to explore the antitumor effects associated with this scaffold. The cytotoxic effects of the aminoquinones (EC_{50}) against a panel of cancer cell lines (MCF7, DU145 and T24 cells) and healthy fibroblasts (BALB/3T3) were assessed *in vitro* using the MTT reduction assay 48 h after drug exposure. SAR analysis of the aminonaphthoquinone series showed that insertion of a chlorine atom in the acceptor quinone nucleus and/or insertion of a methyl group at the nitrogen atom of the donor phenylamino group induced significant changes in cytotoxic activity. Quinones *7* and *9*, which exhibited the highest selective indexes (5.73 and 6.29, respectively), were further characterized using the following assays: Colony formation, caspase-3 activity, and ATP content. The results showed that aminoquinone *7* strongly influenced ATP levels and impaired the proliferative capacity of T24 cells without activating caspase-3.

1. Introduction

Normal cells fit their environment perfectly and respond to external signals via tightly regulated pathways that either trigger or repress growth. Cancer arises when a cell escapes the normal brakes placed on its growth and begins to divide in an uncontrolled fashion. Tumorigenesis appears to be a multistep mechanism reflecting the genetic alterations that progressively drive a normal tissue to malignancy [1,2]. Interestingly, this evolutionary process seems to be related to the acquisition of six main alterations in cell physiology: Self-sufficiency in growth signals, insensitivity to antigrowth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis, and evasion of apoptosis [3]. The exploration of new ways to induce cancer cell death, including the reactivation of apoptosis, is therefore a major goal of chemo- and radiotherapy procedures today.

In this context, it has been shown that oxidative stress induced by menadione (2-methyl-1,4-naphthoquinone) leads to cell death by either necrosis or apoptosis [4,5]. Working with murine hepatoma cells, we recently reported that inactivated 2-euryfuryl-3-nitro-1,4-benzoquinone could undergo an activation process by a redox mechanism causing necrosis-like cell death, while 2-euryfurylbenzoquinone, which is less capable of bioreductive activation, seems to induce apoptosis [6]. The redox cycling ability of 2-euryfuryl-1,4-naphthoquinone and its 5- and 5,8-hydroxyderivatives was further studied in the presence or absence of a biologically relevant reducing agent like ascorbate. We found that the electron donor effect of the *peri*-hydroxyl substituents on euryfurylnaphthoquinones and the hydrogen bond between the *peri*-hydroxy and quinone carbonyl groups influenced the electron-acceptor capability of the quinone nucleus, thus modifying electron transfer from ascorbate to the electroactive quinone nucleus [7]. Furthermore, we recently reported that the cytotoxicity of furyl-1,4-quinones is dependent on the nature of the substituent linked to the quinone electroactive nucleus and, therefore, apparently associated with their LUMO energies and their hydrophobic properties [8].

1,4-Naphthoquinones, possessing an amino or a substituted amino group in the 2-position, have been a subject of study for many years because of their use in a variety of medical and biological applications, including as antituberculars, antimalarials,

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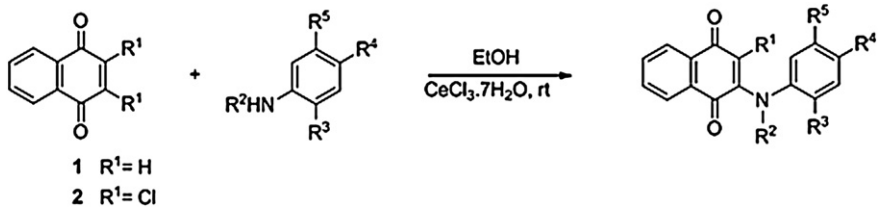

Fig. 1. Phenylaminonaphtoquinones prepared by amination of quinones 1 and 2.

antibacterials, antitumor agents, larvicides and molluscicides, herbicides, and fungicides [9–13]. The presence of the nitrogen atom allows modulation of the substituent's effects on the electronic properties of the quinone system, as well as modification of the geometry of the neutral molecules and of their reduction intermediates. Since no data have been reported regarding the influence of the donor-acceptor properties of phenylaminonaphthoquinones on cytotoxic activity in normal and cancer cells, we synthesized a variety of phenylaminonaphthoquinones to evaluate their cytotoxic properties against a panel of four cell lines, including non-tumor 3T3 BALB fibroblasts and three human-derived tumor cell lines, namely DU145 (prostate), T24 (bladder) and MCF7 (breast).

2. Chemistry

Substitution at both the acceptor quinone nucleus and the donor phenylamino group were designed to study the reactivity of 1,4-naphtoquinone derivatives. To this end, the synthesis of the required phenylaminonaphthoquinone series was achieved by amination of 1,4-naphthoquinone *1* and 2,3-dichloro-1,4-naphthoquinone *2* with a variety of aryl- and arylalkylamines, using $CeCl_3.7H_2O$ as the Lewis acid catalyst (Fig. 1 and Table 1). The structures of the new members were established on the basis of their spectral properties (IR, ^1H NMR and ^{13}C NMR) and microanalytical data.

3. Pharmacology

To investigate the reactivity of our quinoid compounds (3–14), we tested them on a panel of four cell lines including non-tumor BALB/3T3 fibroblasts and three cancer cell lines derived from different human tumors, namely DU145 (prostate), T24 (bladder) and MCF7 (breast). We first calculated the EC_{50} values of the quinones, i.e. the concentration that decreases cell viability by 50%, for each cell line using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) reduction assay. We then determined the safety index of each compound by comparing the EC_{50} measured in cancer cells versus that obtained in normal fibroblasts. As a result of this screening, selected quinones were chosen for further study. The potential anticancer properties of these compounds were characterized by various assays: cell proliferation (clonogenic assay), caspase-3 activation (DEVDase activity), and the intracellular content of ATP.

Table 1
Phenylaminonaphthoquinones prepared by amination of quinones 1 and 2.

Product	R^1	R^2	R^3	R^4	R^5	Yield (%)
3	H	H	H	H	H	95
4	Cl	H	H	H	H	97
5	H	H	Me	H	H	87
6	Cl	H	Me	H	H	93
7	H	H	H	OH	H	92
8	Cl	H	H	OH	H	94
9	H	H	H	OMe	H	84
10	Cl	H	H	OMe	H	89
11	H	H	OMe	H	OMe	85
12	Cl	H	OMe	H	OMe	93
13	H	Me	H	H	H	78
14	Cl	Me	H	H	H	88

4. Results and discussion

4.1. Cytotoxicity of quinones on normal fibroblasts and cancer cells

Table 2 shows the EC_{50} values, expressed as μg/mL, calculated for the selected quinones *3* to *14*. These values were calculated from the effects of the quinones on MTT reduction in three cancer cell lines and 3T3 fibroblasts as a function of their concentration during 48 h of incubation. In general, quinone activity was quite heterogeneous: For example, DU145 cells seemed to be more resistant while T24 and MCF7 cells and healthy fibroblasts exhibited variable sensitivity. Quinone *10* had no cytotoxic effects on cancer cells, while quinones *3*, *8*, *12* and *14* had no selectivity, affecting cancer and non-tumor cells in a similar manner. Another group of quinones (*4*, *5*, *6*, *11*, and *13*) showed little activity, irrespective of the cell line tested. The most active quinones were *7* and *9*. As shown in Table 3, these two quinones had a similar profile with a high safety index (5.73 and 6.29, respectively), low hydrophobicity (logP of 1.16 and 1.43, respectively), and low redox potential (−775 and −856 mV, respectively). Because we were interested in obtaining information on the mechanisms underlying cytotoxicity, aminoquinones *7* and *9* were, therefore, selected for further experiments.

4.2. Effects of quinones 7 and 9 on T24 cancer cells

With regard to the potential impairment of cell proliferation induced by quinones, their effects (at concentrations ranging from

Table 2
EC50 values[a] of phenylaminonaphthoquinones3–14.

Product	Cell lines[b]			
	DU145 Cancerous	MCF7 Cancerous	T24 Cancerous	BALB/3T3 Fibroblasts
3	4	2.6	1.2	0.2
4	66.8	6.3	14.8	9.1
5	7.7	0.8	7.7	6.2
6	25.8	4.9	9.3	3.2
7	0.9	0.8	2.3	6
8	1.9	2.8	0.6	0.6
9	7.6	3.7	8.1	35.7
10	> 1 mg/ml	> 1 mg/ml	437.5	13.71
11	35.2	1.2	10.9	11.5
12	6	4.6	2.4	0.7
13	20.9	7.8	8.4	43.3
14	6.7	1.2	6	0.8

[a] Values are expressed as μg/ml.
[b] Cells (10 000 cells/well) were incubated for 48 h with or without the compounds at varying concentrations. They were then washed twice with warm PBS and incubated with MTT (0.5 mg/ml) for 2 h at 37 °C. Blue formazan crystals were solubilized by DMSO and the colored solution was read at 550 nm.

Table 3
Index of efficacy and safety and physico-chemical properties of the quinones.

Product	DU145C/T[a]	MCF7 C/T	T24C/T	Mean C/T	logP	−E1/2 (mV)
3	0.05	0.08	0.16	0.09	1.55	770
4	0.14	1.44	0.61	0.73	1.59	618
5	0.81	7.80	0.81	3.14	2.04	727
6	0.12	0.65	0.34	0.37	2.08	640
7	6.49	8.05	2.65	5.73	1.16	775
8	0.31	0.21	0.91	0.41	1.20	690
9	4.70	9.76	4.42	6.29	1.43	856
10	n.d.[b]	n.d.	n.d.	n.d.	1.46	658
11	0.33	9.82	1.05	3.73	1.30	767
12	0.11	0.14	0.28	0.18	1.34	614
13	2.07	5.55	5.18	4.27	2.34	760
14	0.12	0.67	0.13	0.31	2.38	503

[a] C/T = EC50 values fibroblasts/EC50 values tumor cells.
[b] n.d. = not determined.

1 to 100 μg/mL) were tested on T24 cells using the clonogenic assay (Fig. 2). T24 cells were chosen over other cancer cells because of the facilities available to manipulate these cells in proliferation assays. It should be noted that the EC_{50} values obtained in T24 cells after 48 h of incubation with quinones *7* and *9*, were 2.3 and 8.1 μg/mL, respectively (Table 2). As expected from these EC_{50} values, quinone *7* was more active than quinone *9* and, at a dose of 10 μg/mL, quinone *7* almost completely inhibited T24 cell proliferation whereas quinone *9* showed a strong inhibitory effect (75%). Fig. 3 shows the effects of quinones *7* and *9* on ATP levels in T24 cells after 6 h of incubation. Both quinones provoked a dose-dependent decrease in ATP, achieving almost total depletion when they were used at the highest doses of 100 μg/mL.

Although quinones mainly impair the capacity of T24 cells to proliferate, we explored whether a process of cell death also takes place when cells are incubated in the presence of quinones *7* and *9*. Table 4 shows the effects of the two quinones, tested at the same range of concentrations (1–100 μg/mL), on caspase-3 activation as

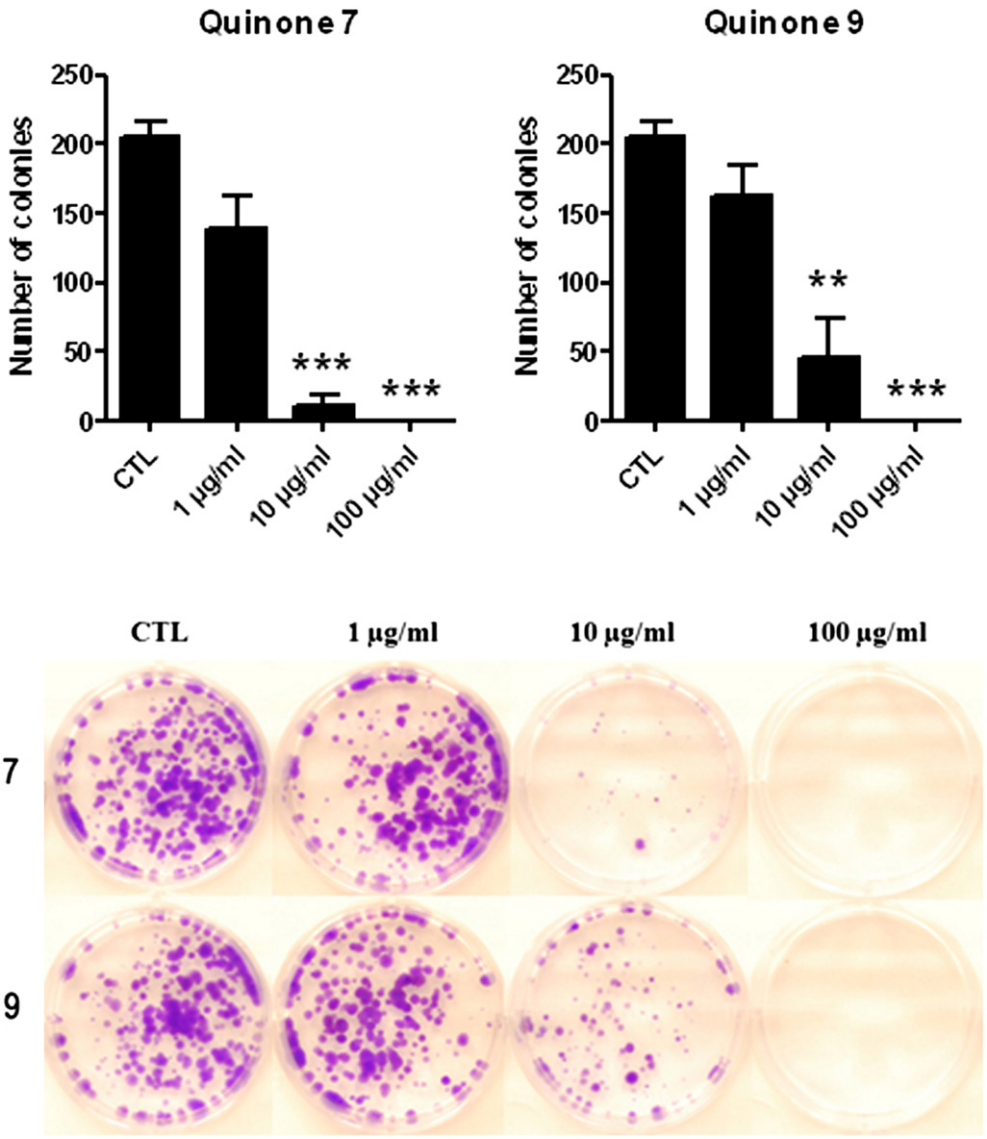

Fig. 2. Effects of quinones 7 and 9 on the proliferative capacity of T24 cells. T24 cells (500) were seeded in six-well plates at a single-cell density. Cells were allowed to adhere overnight, then treated with quinones for 24 h, washed with warm PBS, given fresh medium, and allowed to grow for 10 days. Clonogenic survival was determined by staining colonies using crystal violet. Results are shown as histograms and as colony forming units. (**) $p < 0.01$, (***) $p < 0.001$ compared to control conditions.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

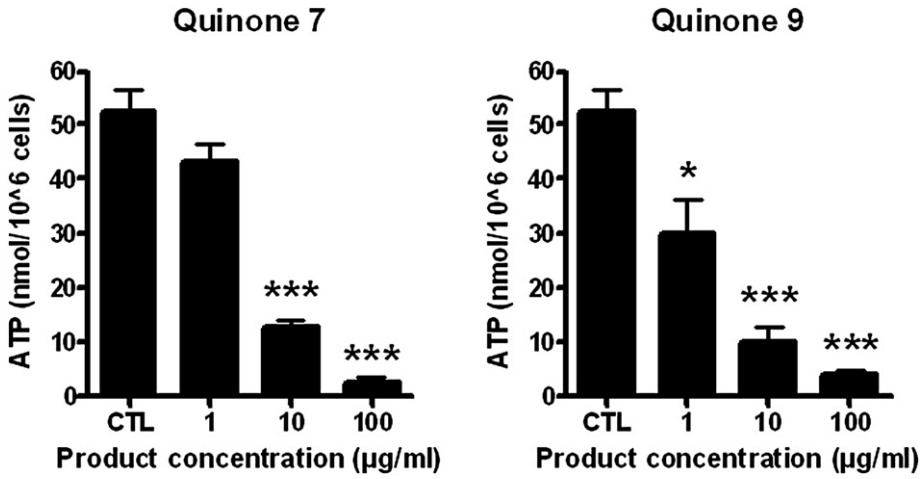

Fig. 3. Effect of quinones 7 and 9 on intracellular ATP content. T24 cells were incubated for 6 h with or without compounds 7 and 9. ATP content was determined using the ATPlite assay (Perkin Elmer, Waltham, MA, USA) according to the procedures described by the suppliers, and the results are expressed as nmol ATP/10^6 cells. (*) $p < 0.05$, (***) $p < 0.001$ compared to control conditions.

measured by DEVDase activity. No caspase-3 activation was detected in T24 cells incubated with the quinones. The relevance of this lack of activation is reinforced by the observation that sanguinarine, a flavonoid which is a well-known inducer of apoptosis via caspase-3 activation [14], induced a 100-fold increase in DEVDase activity in T24 cells. It should be noted that immunoblots against poly(ADP-ribose) polymerase (PARP) protein, a well-known substrate of caspase-3, as well as the lack of effect shown by a pan-caspase inhibitor, namely Q-VD-OPh, suggest that caspase-3 is not activated in T24 cells when incubated with either quinone *7* or quinone *9* (data not shown).

Quinone moieties are present in many drugs, such as anthracyclines, daunorubicin, doxorubicin, mitomycin, mitoxantrones and saintopin, which are used clinically in anticancer therapy. The cytotoxic effects of quinones are mainly due to the formation of reactive oxygen species (ROS) or to covalent binding to macromolecules through arylation reactions [15]. Although, in contrast to ROS, the role of arylation in quinone cytotoxicity is not fully understood, a recent quantitative SAR study performed in different cancer cell lines showed that the cytotoxic activities of 1,4-naphthoquinones depend largely on their hydrophobicity [16]. Analysis of the data in Table 3 clearly indicates that the insertion of a chlorine atom into the quinone nucleus, as occurred in quinones *4*, *6*, *8*, *10*, *12* and *14*, induced an increase in redox capability (low half-wave potential values) compared to the corresponding non-halogenated precursors *3*, *5*, *7*, *9*, *11* and *13*. Insertion of chlorine had no significant effect on lipophilicity. The SAR analysis, which focused on this substitution effect, showed that chlorine insertion in quinones *3*, *5* and *9*, creating compounds *4*, *6* and *10*, respectively, was associated with a marked decrease in cytotoxicity. In contrast, insertion of a chlorine atom into quinones *11* and *13*, creating compounds *12* and *14*, respectively, resulted in an increase in cytotoxic activity. Taken together, these results indicate that there is no parallel correlation of lipophilicity and half-wave potential with cytotoxic activity.

A particular behavior is observed by comparing the activities of compound *7* and its chlorine analog *8*, which were the most cytotoxic members of the aminoquinone series against the four cell lines tested. Indeed, by calculating a mean EC_{50} from the individual EC_{50} values obtained for each cancer cell line, a similar mean value was obtained: 1.75 and 1.3 µg/mL for *7* and *8*, but these compounds differed markedly in terms of their safety index. As shown in Table 3, chlorine insertion reduced the safety index from 5.73 to 0.41, indicating a loss of selectivity of the chlorine analog *8* towards cancer cells. It should be noted that compound *7*, the most promising antitumor member of the aminoquinone series, had the lowest electron capability according to its half-wave potential value and the highest hydrophilicity among the members of the series (lowest lipophilicity). Therefore, the polarity and hydrogen bond capacity of the hydroxyl group in compounds *7* and *8*, together with the electron capability and lipophilicity properties could facilitate interactions with the biological targets involved in anticancer activity.

The SAR analysis also revealed that insertion of a methyl group at the nitrogen atom of the phenylaminoquinones induced a decrease in the cytotoxic activity of compound *13* compared to compound *3*. In contrast, insertion of a methyl group at the nitrogen atom induced an increase in cytotoxic activity of compound *14* compared to compound *4*. These data suggest that the stereoelectronic interaction between the donor and acceptor fragments, through the nitrogen atom, seems to be crucial for the cytotoxic activity of the phenylaminonaphthoquinone chromophore.

Among the quinones tested in this series, compounds *7* and *9* appeared to be the most active and had the best safety index. Indeed, both compounds inhibited the proliferation of T24 cells as shown by the clonogenic assay (Fig. 2). When considering which type of cell death these compounds might induce, it should be noted that no activation of caspase-3, a classical hallmark of apoptosis [17], was observed. The lack of caspase-3 activation may also be explained by the marked decrease in ATP, which is required for apoptosome formation [18].

Table 4
DEVDase activity.

Product	Concentration (µg/ml)	DEVDase at 6h (U/mg of protein)	DEVDase at 24h (U/mg of protein)
None (CTL)		6.6 ± 1.6	5.9 ± 0.6
7	1	6 ± 0.9	5.1 ± 0.7
7	10	2.7 ± 1.3	4.9 ± 0.8
7	100	3 ± 1.5	4 ± 2.2
9	1	5.9 ± 1.4	5 ± 0.4
9	10	5 ± 1.4	4.5 ± 0.2
9	100	3.4 ± 1.6	3.4 ± 0.6

Positive control consisted of sanguinarine-treated cells (5 µM, 4 h) and resulted in a DEVDase activity of 639.2 ± 43.5 U/mg of protein.

5. Conclusion

In conclusion, we have shown that the cytotoxicity of phenylaminonaphthoquinones depends upon the nature of the donor phenyl and the acceptor quinone groups. The two selected quinones (*7* and *9)* markedly influenced cellular metabolism and impaired cell proliferation. They appeared relatively safe when assessed on healthy fibroblasts. Given the high incidence of undesirable adverse effects induced by the majority of currently available anticancer drugs and considering the selective cytotoxic effects of quinones *7* and *9*, these compounds appear to have promising and interesting potential anticancer activities.

6. Experimental protocols

6.1. Chemistry

6.1.1. General remarks

All reagents were commercially available reagent grades and were used without further purification. Melting points were determined on a Stuart Scientific SMP3 apparatus and are uncorrected. ^{1}H NMR spectra were recorded in deuterochloroform ($CDCl_3$) on a Bruker AM-200 instrument. ^{13}C NMR spectra were obtained in $CDCl_3$ at 50 MHz. Chemical shifts are expressed in ppm downfield relative to tetramethylsilane (TMS, δ scale), and the coupling constants (J) are reported in Hertz. The elemental analyses were performed in a Fison SA, model EA-1108 apparatus. Silica gel Merck 60 (70–230 mesh) was used for preparative column chromatography and thin layer chromatography (TLC) aluminum foil 60F254 for analytical TLC.

6.1.2. General procedure for the synthesis of phenylaminonaphthoquinones

A suspension of quinone *1* (500 mg, 3.16 mmol) or *2* (500 mg, 2.20 mmol), the required amine (2 equiv.), $CeCl_3.7H_2O$ (5% mmol in respect to *1* or *2*), and ethanol (15 mL) was left at room temperature with stirring until completion of the reaction as indicated by TLC. The reaction mixture was evaporated under reduced pressure and the residue was column chromatographed (1:1:2 AcOEt/CH_2Cl_2/light petroleum) to yield the corresponding aminoquinone (Table 1). Aminonaphthoquinones *3*, *4*, *5*, *6*, *7*, *8*, *9*, *10*, *13* and *14* were identified by comparing their spectral properties (Melting point, IR, ^{1}H NMR and ^{13}C NMR) to those reported for these compounds in the literature [19–22].

6.1.2.1. 2-[(2,5-Dimethoxyphenyl)amino] −1,4-naphthoquinone (11). Prepared from quinone *1* and 2,5-dimethoxyaniline (24 h, 85%): Red crystal, mp 128.2 °C-128.5 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.14 (t, J = 3.8 Hz, 1H), 8.11 (t, J = 3.8 Hz, 1H), 8.0 (bs, 1H, NHPh), 7.78 (dd, J = 1.6 Hz, 1H), 7.69 (dd, J = 1.6 Hz, 1H), 6.72 (s, 1H), 6.54 (s, 1H), 6.26 (d, J = 3.0 Hz, 1H), 6.22 (d, J = 3.0 Hz, 1H), 3.88 (s, Me, 3H), 3.73 (s, Me, 3H). ^{13}C NMR (50 MHZ, $CDCl_3$): δ 183.9, 181.9, 153.7, 143.6, 141.8, 134.8, 133.2, 132.3, 130.5, 127.6, 126.5, 126.1, 111.7, 111.3, 109.0, 102.1, 56.2, 55.9. Anal. Calcd. for $C_{18}H_{15}NO_4$: C, 69.89; H, 4.89; N, 4.53. Found: C, 69.88; H, 4.92; N, 4.55.

6.1.2.2. 2-Chloro-3-[(2,5-dimethoxyphenyl)amino] -1,4-naphthoquinone (12). Prepared from quinone *2* and 2,5-dimethoxyaniline (24 h, 93%): Dark purple crystal, mp 146.7 °C-146.9 °C; ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.22 (d, J = 3.8 Hz, 1H), 8.14 (d, J = 3.8 Hz, 1H), 7.80 (t, J = 3.7 Hz, 1H), 7.72 (t, J = 3.7 Hz, 1H), 7.65 (s, 1H), 6.86 (d, J = 4.4 Hz, 1H), 6.75 (m, 1H), 6.6 (s, 1H, NHPh), 3.85 (s, Me, 3H), 3.81 (s, Me, 3H). ^{13}C NMR (50 MHZ, $CDCl_3$): δ 180.4, 177.4, 152.9, 146.9, 141.7, 134.9, 132.9, 132.6, 130.1, 127.1, 127.0, 126.9, 115.1, 111.4, 111.3, 111.0, 56.1, 55.9. Anal. Calcd. for $C_{18}H_{14}ClNO_4$: C, 62.89; H, 4.10; N, 4.07. Found: C, 62.88; H, 4.12; N, 4.02.

6.1.3. Electrochemical results

The redox potentials of compounds *3-14* were measured by cyclic voltammetry in acetonitrile as a solvent at room temperature, using a platinum electrode and 0.1 M tetraethylamonium tetrafluoroborate as the supporting electrolyte. The voltammograms were run in the potential range 0.0–2.0 V versus non-aqueous Ag/Ag+.

6.2. Pharmacology

6.2.1. Cell lines

Human cancer cell lines (T24, DU145, MCF7) were cultured in high-glucose Dulbecco's modified Eagle medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). Balb/3T3 cells (normal mouse fibroblasts) were cultured in the same medium, except that the fetal calf serum was replaced by 10% newborn calf serum. All cultures were kept at 37 °C in 95% air/5% CO_2 at 100% humidity. Phosphate-buffered saline (PBS) was purchased from Gibco. Cells were incubated at the indicated times at 37 °C with or without quinones at various concentrations.

6.2.2. Cellular assays

The cytotoxicity of the quinones was assessed by following the reduction of MTT to formazan blue [23]. Briefly, cells were seeded into 96-well plates at a density of 10 000 cells/well for 24 h and then incubated for 48 h with or without the compounds. Cells were then washed twice with warm PBS and incubated with MTT (0.5 mg/mL) for 2 h at 37 °C. Blue formazan crystals were solubilized by adding 100 μl DMSO/well, and the colored solution was subsequently read at 550 nm. Results are expressed as % of MTT reduction compared to untreated control conditions. ATP content was determined after 6 h of incubation using the ATPlite assay from Perkin Elmer (Waltham, MA, USA) according to the procedures described by the suppliers, and the results are expressed as nmol ATP/mg of protein. The amount of protein was determined by the method of Lowry using BSA as a reference [24]. Caspase-3 activity was monitored after 6 or 24 h incubation. Briefly, after incubation with the test compounds, cells were washed twice with PBS, lysed and centrifuged. Cell supernatants were then incubated with a specific peptide substrate, Asp-Glu-Val-Asp-AFC (DEVD-AFC). Substrate cleavage was determined kinetically at room temperature using a Victor X2 spectrophotometer (380 nm excitation, 500 nm emission) (Perkin Elmer, Waltham, MA, USA). The results are expressed as Units/mg protein, as originally described by Nicholson et al. [17]. Clonogenic assays were performed by seeding cells (500) in six-well plates at a single-cell density. Cells were allowed to adhere overnight, then treated with quinones for 24 h, washed with warm PBS, given fresh medium, and allowed to grow for 10 days. Clonogenic survival was determined by staining colonies using crystal violet.

6.3. Statistical analysis

Data were analyzed using a one-way analysis of variance (ANOVA) followed by Bonferroni test for significant differences between means. For statistical comparison of results at a given time point, data were analyzed using a Student's t test. The level of significance was set at $p < 0.05$.

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