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ABSTRACT

This work was aimed to the development of a set of new naphtoquinone derivatives that can act against glioma. The compounds were tested in order to find out their ability to inhibit the growth of glioma cells, and the results of these assays were correlated with electrochemical analysis and NMR-based reoxidation kinetic studies, suggesting that a redox mechanism underlies and may explain the observed biological behavior. In addition to a full description of the synthetic pathways, electrochemistry, NMR and single crystal X-ray diffraction data are provided.

1. Introduction

Gliomas are tumors most frequently affecting the central nervous system (CNS). According to the gliomas classification provided by the World Health Organization [1], the most malignant grade is glioblastoma (Grade IV, GB). The current therapeutic approach that FDA approved consists in neurosurgical resection, followed by chemotherapy with temozolomide (TMZ) in combination with radiotherapy, though only 26% of treated patients reached 2 years of survival [2]. Despite all the recent advances in neurosurgery [3] and chemotherapy [4], glioblastoma presents highly invasive pattern and recurs in 90% of cases [5]. Many attempts to define the specific biomolecular characteristics of these complex and variable tumors have been carried out during the last decades [6], though no promising discoveries successfully migrated from the bench to the clinic [7]. At present, investigation on modulation of specific targets and consequent effects on glioma cells is one of the most concrete approaches towards a better understanding of the mechanism involved in gliomas malignancy. Alternative therapeutic approaches based on novel compounds and/or discovery of interesting biological activities of known compounds are therefore necessary.

Recently, indeed, the interest towards natural derivatives developed and naphthoquinones, among the others, continuously captivated biological interest. In this connection, the applications of both natural (as in the case of 3-hydroxy-5-methoxy-2-methylbenzoquinone and heliquinone, from Sterculiaceae family) [8] and semi-synthetic derivatives have been widely investigated. For instance it has been reported that water-soluble naphthoquinone derivatives – *i.e.* their conjugated with carbohydrates – show cytotoxic activity in JB6 P^{+} Cl41 cells [9], while other 1,4-naphthoquinones have been synthesized and evaluated for many other purpouses, such as their trypanocidal activity [10].

Juglone is a natural compound deriving from the Juglandaceae family, particularly *Juglans nigra*, whose toxic and growth-stunting effects are well known [11]. While Juglone was reported to induce generic oxidative stress in both healthy cells and cancer cell lines [12], we previously highlited the cytotoxic effect of its derivative 1 in inducing apoptotic cell death on human glioma cell lines [13] as a result of a screening on our in-house database. Starting from the discovery of this promising lead, the synthesis of novel naphthoquinone derivatives stems out with the aim of describing and

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comparing structure–activity relationship (SAR) of Juglone and the obtained compounds by evaluating their effects on glioma cell death, allowing the enlightenment of the mechanism of action and, eventually, the optimization of the lead.

2. Results and discussion

2.1. Chemistry

Juglone (JUG) was obtained from radical oxidation of 1,5-dihydroxynaphthalene in the presence of CuCl according to a literature procedure and to the indication previously reported by Zonta et al. [14–16]. Starting from Juglone a series of derivatives containing various functional groups and structural variations was prepared to test for the existence of a structure–activity relationship. A selection of the synthesized compounds is summarized in Fig. 1 (6, 14, and 16 were known compounds). A first set of naphthoquinone derivatives was synthesized starting from Juglone *via* a modified *Micheal's addition.* Unfortunately, these reactions gave poly-oxidized species that were difficult to eliminate. Then the reaction was tried under inert nitrogen atmosphere. The reactions showed moderate to good yields and the raw products had to be purified by flash chromatography while recrystallization from *n*-hexane gave satisfactory results only for few derivatives. Hetero- and homo-nuclear 2D NMR studies proved that substitution occurred at position 3 (see Supporting Information). Another class of derivatives containing sulfur (6, 8) was prepared in a similar manner by direct addition of the appropriate nucleophilic thiophenol precursor to the position 3 of Juglone, but ethanol was used as solvent instead of acetic acid. Preparation of a 3-bromo Juglone precursor (7) *via* chemoselective bromination of Juglone and subsequent reaction of the bromo-juglone with amines gave few more derivatives (9, 10) that, with the sulfur derivative, showed different redox properties potentially useful to establish a SAR. NMR studies proved that substitution occurred at position 3. More in detail, 7 was used as the model compound and deeply investigated through 2D homo- and heteronuclear 2D NMR experiments. Correlation Spectroscopy (CoSY), Heteronuclear Single Quantum Coherence (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) spectra (see Supporting Information) confirmed the reactivity of the 3- position of the naphthoquinone scaffold.

Derivatives 6, 7 and 8 were also unambiguously identified through single crystal X-ray diffraction analysis (see Fig. 2 for 7 and 8, whereas for 6, already reported in the literature [17], see Supporting Information). In these structures, C–O(H) bond distances are within 1.339(2) – 1.356(6) Å, slightly shorter than those commonly observed for phenolic hydroxyls [1.362(15) Å] [18]. The C=O bond lengths, comparing with the average value reported for benzoquinones [1.222(13) Å] [18], show a bimodal distribution where those observed for the C=O group proximal to the hydroxyl are longer [1.223(5) – 1.227(2) Å] than those of the distal C=O group [1.206(5) – 1.218(2)Å]. In all structures the hydroxyl group is intramolecularly H-bonded to the proximal C=O group to form a six-membered pseudo-cycle. Such features are common among juglone-related compounds [17,19].

O-Acetylated derivatives (3, 5) and 7-methyl-8-chloro naphthoquinones derivatives (14, 15) were also synthesized to explore the influence of these groups on structure–activity relationships.

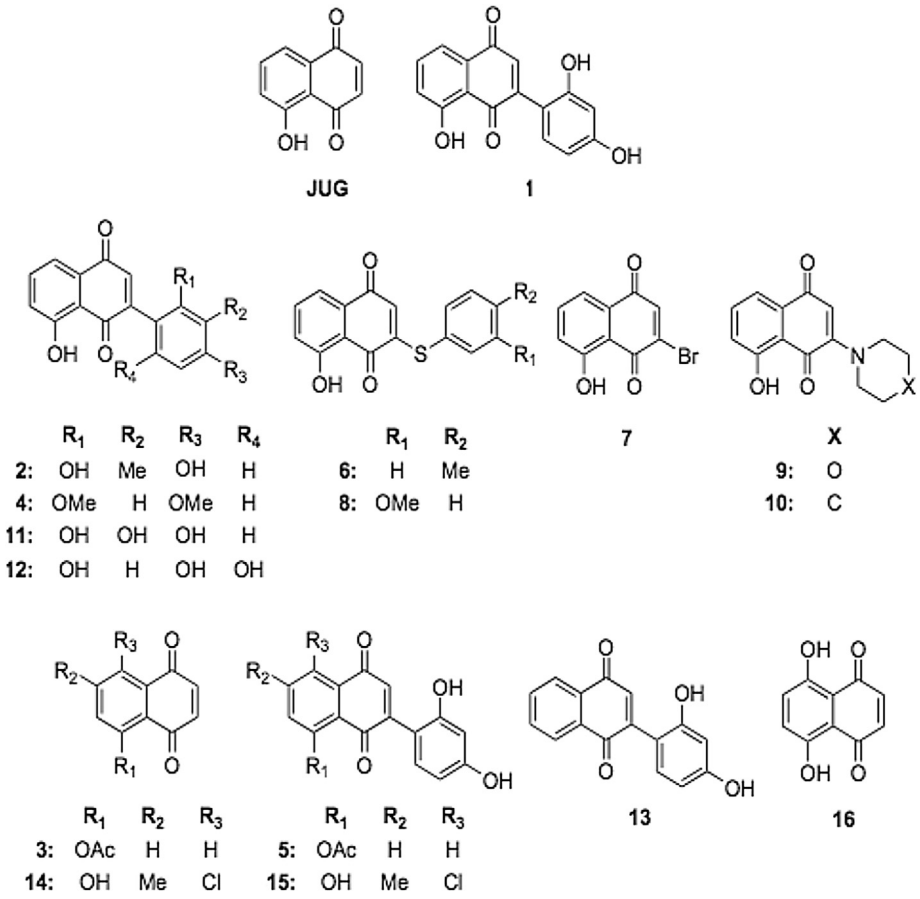


Fig. 1. Juglone and its derivatives synthesized in this work.

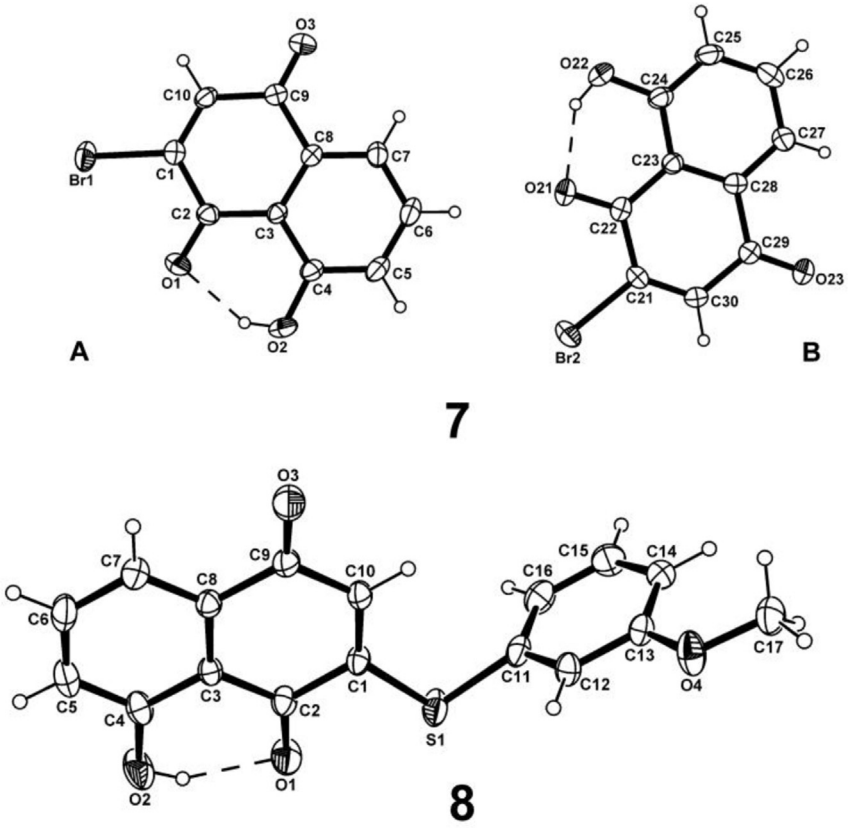


Fig. 2. X-ray diffraction structures of Juglone derivatives 7 and 8. The two crystallographically independent molecules in the structure of 7 are indicated with **A** and **B**, respectively. Displacement ellipsoids are drawn at the 30% probability level. Intramolecular H-bonds are represented by dashed lines.

2.2. Cytotoxicity

Synthesized compounds were tested at different concentrations (50 μM, 5 μM, 0.5 μM or 0.05 μM) using GLI36 human glioma cell line. Cells were incubated with the compounds (1–16), Juglone (JUG), Temozolomide (TMZ) and Paclitaxel (PTX) for 24 or 72 h. After treatment cells were tested for cell viability with MTT assay and cell death (apoptosis and necrosis) with morphological analysis (see Supporting Information).

Cell cultures treated with 1 for 24 and 72 h showed a significant decrease in cell viability in comparison with JUG, TMZ, PTX and most of the other tested compounds (see Supporting Information).

Unraveling the status of the treated cells with morphological analysis, apoptosis and necrosis were evaluated in all the conditions tested. Data showed a strongly significant increase in percentage of apoptotic cells and a decrease of necrosis level in comparison with reference compounds (JUG, TMZ and PTX) and the other synthesized compounds (Fig. 2). Analysis carried out at 72 h provided similar results (see Supporting Information).

According to what previous research works suggest [20], we postulated, as working hypothesis, that the preliminary results of biological assays could be explained by a redox-based mechanism promoted by the examined compounds (see Fig. 3).

In this connection we synthesized 1,4,5-trihydroxy naphthalenes as reduction products of selected naphthoquinones of a particular biological interest (JUG and 1): compounds JUG-R and 1-R were prepared *via* reduction of Juglone and 1, respectively, with $Na_2S_2O_4$ (Fig. 4).

This reaction involving 1 was found to produce the quite unstable and easily reoxidable 1-R, as indicated by color changes and confirmed by an NMR-based kinetic study (see Supporting Information), where the signals in the proton spectra of JUG-R and 1-R were compared to those of JUG and 1, respectively. In general, a shift towards lower δ was observed for the reduced derivatives, especially for what concerning the singlet attributed to the proton in the 2- position. In order to prevent reoxidation of the reduced compounds to the naphthoquinone form, NMR spectra for characterization and biological assays were carried out in the presence of $Na_2S_2O_4$.

The synthesized compounds were subsequently used for cytoxicity assays. Glioma cells were incubated with JUG, JUG-R, JUG + $Na_2S_2O_4$, JUG-R + $Na_2S_2O_4$, 1 + $Na_2S_2O_4$, 1-R + $Na_2S_2O_4$ and $Na_2S_2O_4$ as control, at different concentrations (50 μM, 5 μM, 0.5 μM or 0.05 μM). After 24 h apoptosis and necrosis were evaluated by morphological analysis. MTT test could be otherwise affected by the presence of $Na_2S_2O_4$.

The cytotoxic effect (apoptosis and necrosis) induced by JUG and 1 was not influenced by the presence of $Na_2S_2O_4$ while the reduced trihydroxy naphthalene derivatives JUG-R and 1-R showed a strong decrease in cytotoxicity in comparison with the original hydroxynaphthoquinones (Fig. 5). The significantly lower level of apoptosis and necrosis induced in human glioma cells by the reduced derivatives (JUG-R and 1-R) could represent a first important indication supporting the hypothesis that a redox mechanism is involved. This is hypothesized to be related to a marked difference in their reoxidation tendency showing that 1-R is more promptly reconverted to 1 as compared to JUG-R to JUG.

The potential capability of the compounds of reaching and interacting with the target cells in a more complex system was investigated by evaluating *in silico* the logP values, ranging from −0.5 to 2.7, in line with the one calculated for TMZ. Nevertheless the major limitation of this study is related to this point: the

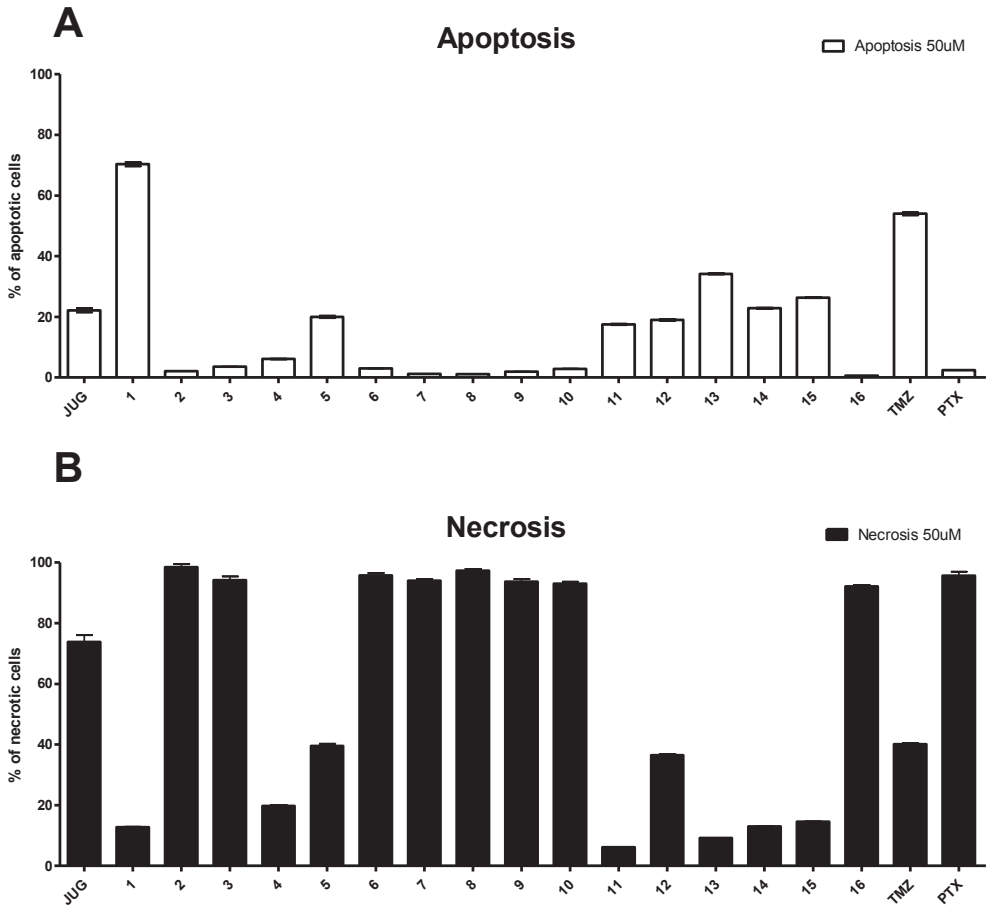


Fig. 3. Morphological assays. Histograms (Mean ± SEM) representing the percentage of cell death. Apoptosis (A) or necrosis (B) were determined with morphological analysis, in GLI36 treated for 24 h with JUG, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, TMZ and PTX. The panels refer to the 50 μM concentration tested (for all the concentration tested please refer to Supporting Information – Biological Assays). All the apoptosis effects reported significantly differ from TMZ; and all the necrosis effects reported differ significantly from TMZ with the exception of 5. t-test: $P < 0.05$.

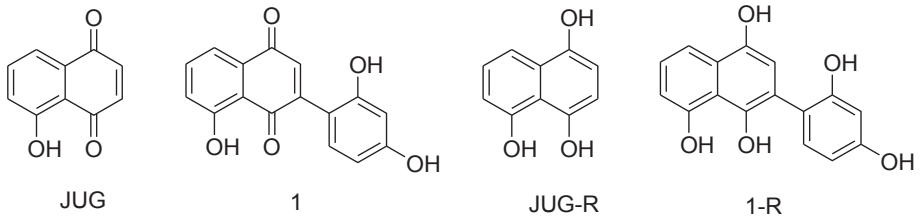
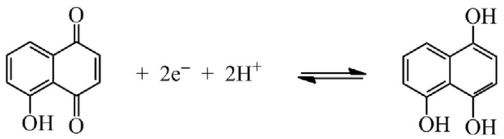


Fig. 4. Juglone, 1 and their reduced derivatives.

biological evaluation was carried out at *in vitro* level. Further insights in complex systems are needed to assess this issue.

2.3. Electrochemistry

To account for the different biological activity of the reduced and non-reduced naphthoquinone derivatives cyclic voltammetry (CV) analysis of naphthoquinones was performed to better understand if and how much the redox activity may be involved in inducing cell death. Since most of the investigated compounds are sparingly soluble in water, all voltammetric investigations were carried out in aqueous solutions containing 20% (v/v) CH_3CN and the data for the derivatives reaching an adequate concentration are repoted below. The redox chemistry of quinones is well documented in the literature and it is known that both the voltammetric pattern and the redox potential are strongly influenced by pH [21–23]. Therefore all experiments were conducted in buffered solutions, using 0.1 M phosphate buffer at pH 7; the bath temperature was set at 25 °C. Examples of cyclic voltammograms are reported in Fig. 6 for juglone and some other representative naphthoquinone derivatives. It is well known that reduction of quinones in protic media occurs by a $2e^-$, $2H^+$ reaction leading to the formation of the corresponding hydroquinones. The redox reaction in the case of juglone is



(1)

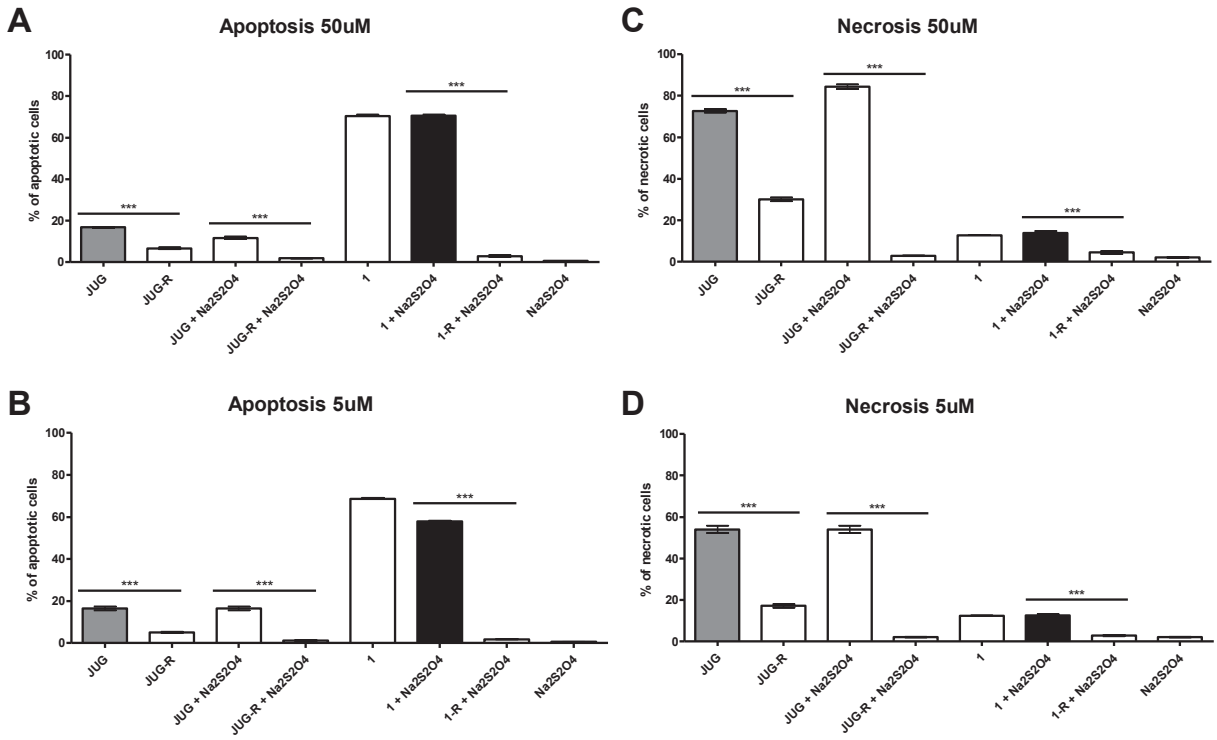


Fig. 5. Morphological assays after 24 h of incubation with reduced compounds. Histograms (Mean ± SEM) represent the percentage of cell death. Apoptosis (A,B) or necrosis (C,D) were determined with morphological analysis, in GLI36 treated for 24 h with JUG, JUG-R, JUG + $Na_2S_2O_4$, JUG-R + $Na_2S_2O_4$, 1, 1 + $Na_2S_2O_4$, 1-R + $Na_2S_2O_4$ and $Na_2S_2O_4$. The panels refer to the concentration tested: 50 μM (A,C), 5 μM (B,D). t-test: *p < 0.05, **p < 0.01, ***p < 0.001.

A single peak couple similar to those shown in Fig. 6 was observed for all investigated compounds and therefore the voltammetric pattern was assigned to the quinone/hydroquinone redox couple according to the redox reaction of Eq. (1). Table 1 summarizes the cathodic and anodic peak potentials, E_{pc} and E_{pa} respectively, as well as the peak separation, $\Delta E_p = E_{pa} - E_{pc}$, and half-wave potential $E_{1/2}$, measured for each naphthoquinone at the scan rate $v = 0.05\ Vs^{-1}$. The separation between the cathodic and anodic peaks is significantly greater than 30 mV, predicted for a reversible $2e^-$ process and increases with increasing scan rate, indicating that these compounds undergo quasi-reversible electron transfer processes [24]. The standard reduction potential, E^o, can be measured from cyclic voltammetry by assuming it to be approximately equal to the half-wave potential:

$$E^o \approx E_{1/2} = \frac{E_{pa} + E_{pc}}{2} \quad (2)$$

Values of $E_{1/2}$ are included in Table 1 (last column). As can be observed, although an extensive series of naphthoquinones bearing both electron withdrawing and electron donating groups was chosen for this study, the measured redox potentials lie in a rather narrow range. In fact, if 9 is excluded, the maximum difference between $E_{1/2}$ values for the whole series is only 65 mV, indicating that the ability of these molecules to act as oxidizing agents is little

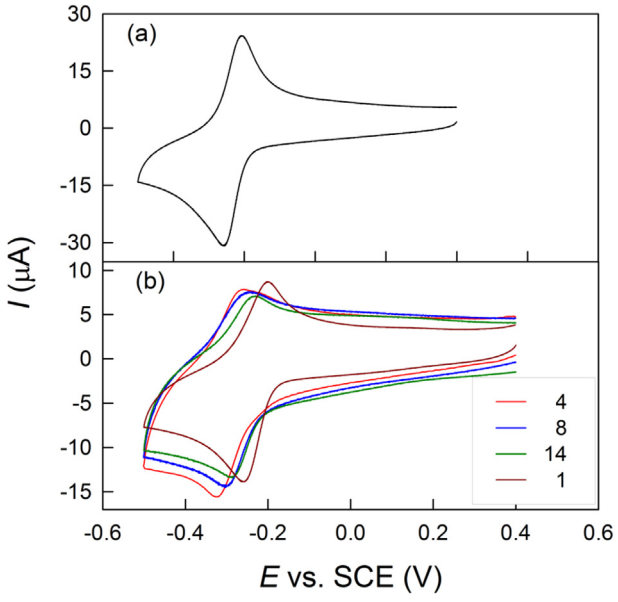


Fig. 6. Cyclic voltammetry of (a) 1.0 mM juglone and (b) 0.5 mM of 4, 8, 14 and 1 in phosphate buffer pH 7/CH_3CN mixture (80:20 v/v) recorded on a GC electrode at $v = 0.1\ Vs^{-1}$.

Table 1
Redox data of various naphthoquinones in phosphate buffer pH 7/CH_3CN mixture (80:20 v/v) measured at $v = 0.05\ V^{-1}$ at 25 °C.

Quinone	E_{pc} (V)	E_{pa} (V)	ΔE_p (mV)	$E_{1/2}$ (V)
JUG	−0.254	−0.208	46	−0.231
1	−0.256	−0.199	57	−0.228
2	−0.385	−0.200	185	−0.293
4	−0.313	−0.263	50	−0.288
5	−0.291	−0.197	94	−0.244
8	−0.299	−0.247	52	−0.273
9	−0.413	−0.355	58	−0.384
11	−0.292	−0.177	115	−0.235
14	−0.282	−0.231	51	−0.257
15	−0.347	−0.195	152	−0.271

affected by molecular structure. Therefore if the biological activity of the substrates is strongly related to their redox power as oxidizing agents, the activity is not expected to vary significantly throughout the whole series of naphthoquinones. This is in part true, but as we shall discuss in the next section there are important exceptions, underscoring the importance of structural factors and/or presence of functional groups not affecting $E_{1/2}$.

Fig. 7 enlightens a definite linear correlation between the half-wave potential and the citotoxicty expressed in terms of apoptosis. According to the reported data, the fit to the straight correlation line is better at 5 μM concentration of naphtoquinones but compounds show a similar behavior also at different concentrations. On the other hand Fig. 8 shows a scattered correlation pattern between the $E_{1/2}$ values and the cell necrosis data.

3. Conclusions

A set of naphthoquinone derivatives have been synthesized and tested for their ability to inhibit the growth of glioma cells. Preliminary data [13] on the peculiar properties of 2-(2′,4′-dihydroxyphenyl)-8-hydroxy-1,4-naphthoquinone (1) as strong cytotoxic agent for glioma cells were confirmed within this small focused library of 1,4-naphthoquinone derivatives. The results obtained from biological studies were found to be in good agreement with preliminary kinetic data, supported by NMR results, and strongly suggests that a redox mechanism underlies. In addition to the redox pathway, which has often been reported in connection with the biochemical properties of hydroxynaphthoquinone derivatives [25,26], kinetic aspect of the reaction was demonstrated to be strongly involved, since only 1-R showed fast reoxidation to 1. In conclusion, besides the similar behavior described by the electrochemical results we assume, as working hypothesis, that the reaction kinetic is influenced by the effects of the substituents, leading to different reoxidation tendency and, hence, different degrees of cytotoxicity in human glioma and normal cells.

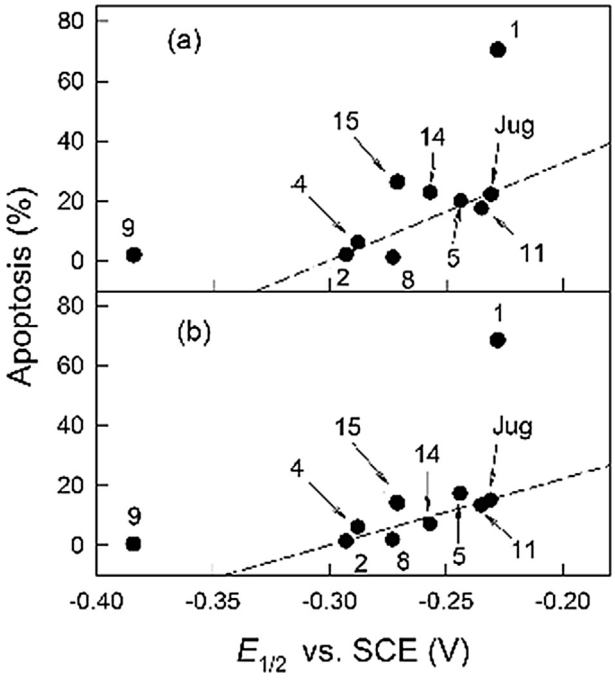


Fig. 7. Percentage of cell death by apoptosis versus $E_{1/2}$ of naphthoquinones, measured in phosphate buffer pH 7/CH_3CN mixture (80:20 v/v). Apoptosis determined with morphological analysis, in GLI36 treated for 24 h at quinone concentrations of (a) 50 μM or (b) 5 μM.

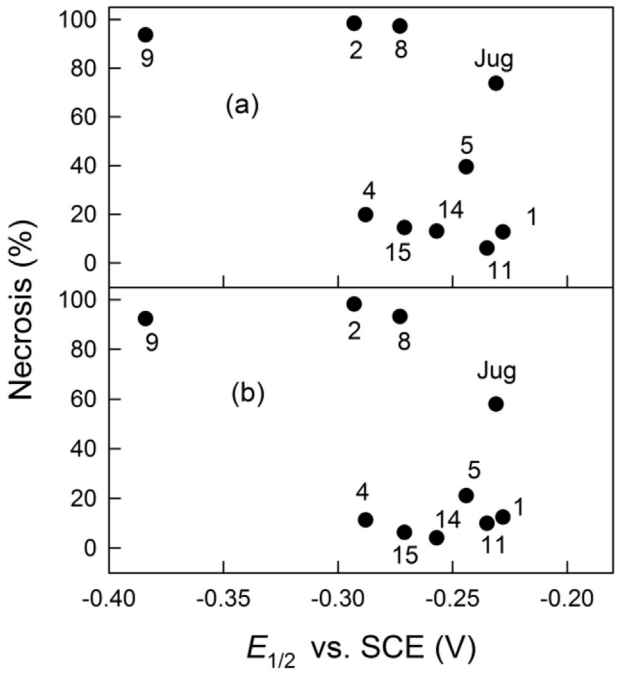


Fig. 8. Percentage of cell death by necrosis versus $E_{1/2}$ of naphthquinones, measured in phosphate buffer pH 7/CH_3CN mixture (80:20 v/v). Apoptosis determined with morphological analysis, in GLI36 treated for 24 h at quinone concentrations of (a) 50 μM or (b) 5 μM.

4. Experimental section

4.1. Chemistry

4.1.1. Materials and instruments

Commercially available chemicals were purchased from Aldrich, and used as received, unless otherwise stated. All air-sensitive manipulations were conducted according to Schlenk line techniques, using dry nitrogen and glassware. If required, solvents were dried prior to use. For work-up and chromatographic purification, commercial grade solvents were used; chromatographic separations were carried out using silica gel 60 (230–400 mesh, Grace Davisil). In addition, semi-preparative and preparative purification of the derivatives were carried out on Isolera One, an automated flash chromatography system provided by Biotage (Upsala, Sweden); the chromatography was carried out using disposable cartridges made of silica gel as stationary phase and bench solvents as mobile phase.

1H and 13C{1H} NMR spectra were recorded on a Bruker Avance III 400 MHz and a Bruker AMX 300 MHz spectrometers. All spectra were recorded at room temperature, the solvent for each spectrum is given in parentheses. Chemical shifts are reported in ppm and are relative to TMS internally referenced to the residual solvent peak. The multiplicity of signals is reported as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), broad (br) or a combination of any of these.

High resolution mass-spectra were recorded on a ESI-TOF Mariner from Perseptive Biosystem (Stratford, Texas, USA), using electrospray (ES) ionization.

The degree of purity of the compounds synthesised throughout this investigation was assayed by HPLC, using a Varian Pro-Star system equipped with a Biorad 1706 UV-VIS detector (254 nm) and an Agilent C-18 column (5 μm, 4.6 × 150 mm). An appropriate ratio of water (A) and acetonitrile (B) was used as mobile phase with an overall flow rate of 1 mL min^{-1}; the general method for the

analyses is reported here: 0 min (90% A-10% B), 15 min (10% A-90% B), 20 min (10% A-90% B), 21 min (90% A-10% B), 25 min (90% A-10% B).

Cyclic voltammetry was performed on a PC controlled Autolab PGSTAT30 potentiostat (Eco-Chimie, Utrecht, Netherlands), with a positive feedback for ohmic drop compensation. All experiments were carried out at 25 °C in a three-electrode cell with a glassy carbon (GC) working electrode, a Pt counter-electrode and an aqueous saturated calomel reference electrode. All solutions were prepared in a mixture of phosphate buffer (pH 7)/acetonitrile (80:20 v/v) and were carefully deaerated with Ar. The GC electrode was a 3 mm diameter disc embedded in glass, which was fabricated from a GC rod (Tokai, GC-20) and polished to a mirror finish with silicon carbide papers and diamond paste. Before every experiment it was cleaned by polishing with a 0.25 μm diamond paste followed by ultrasonic rinsing in ethanol for about 5 min.

Chemical names and calculated logP values were obtained with ChemDraw Ultra 8.0, CambridgeSoft, USA.

4.1.2. Synthesis of juglone JUG

Juglone was synthesised according to the literature procedure [15,16] and to the indication previously reported by Zonta et al. [14].

4.1.3. General procedure for the synthesis of 3-substituted-5-Hydroxy naphthoquinones

A round bottom flask was charged with 5-hydroxynaphthalene-1,4-dione (juglone, 2 eq) in acetic acid, followed by addition of the appropriate phenol (1 eq) in acetic acid (2 ml) and 0.5 ml of 2 M H_2SO_4. The mixture was stirred at room temperature for 2 h under nitrogen at atmospheric pressure, followed by addition of water (20 ml) and neutralization with 5% sodium bicarbonate. The mixture was extracted with ethyl acetate (3 × 15 ml), and the combined organic layers were dried (Na_2SO_4) and evaporated to dryness. Purification was carried out by recrystallization or by flash chromatography (n-hexane/ethyl acetate = 7:3 v/v) if necessary.

*4.1.3.1. Synthesis of 8-hydroxy-2-(2,4-dihydroxyphenyl)naphthalene-1,4-dione (**1**).* Yield 66%; δ_H (400 MHz, DMSO-d6) 11.93 (1H, s, OH), 9.71 (1H, s, OH), 9.69 (1H, s, OH), 7.8–7.7 (1H, m, ArH), 7.54 (1H, dd J 1.1 Hz, J 6.4 Hz, ArH) 7.37 (1H, dd J 1.1 Hz, J 7.3 Hz, ArH), 7.10 (1H, d J 8.4 Hz, ArH), 6.98 (1H, s, CH), 6.39 (1H, d J 2.3 Hz, ArH), 6.32 (1H, dd J 2.3 Hz, J 8.4 Hz, ArH); δ_C (100 MHz, DMSO-d6) 189.6, 184.6, 160.9, 160.4, 157.2, 147.6, 137.1, 136.3, 132.7, 132.4, 124.3, 118.3, 115.8, 111.7, 107.7, 103.1; HRMS (ESI) $[M + 1]^+$ found 283.0646 $[C_{16}H_{11}O_5]^+$, calcd. 283.0601. HPLC purity 98%.

*4.1.3.2. Synthesis of 8-hydroxy-2-(2,4-dihydroxy-3-methylphenyl) naphthalene-1,4-dione (**2**).* Yield 55%; δ_H (400 MHz, DMSO-d6) 11.91 (1H, s, OH), 9.54 (1H, s, OH), 8.61 (1H, s, OH), 7.80–7.70 (1H, m, ArH), 7.52 (1H, dd J 1.0 Hz J 7.2 Hz, ArH), 7.33 (1H, dd J 1.0 Hz J 7.3 Hz, ArH), 6.85 (1H, s, CH), 6.81 (1H, d J 8.3 Hz, ArH), 6.39 (1H, d J 8.3 Hz, ArH), 1.97 (3H, s, Me); δ_C (100 MHz, DMSO-d6) 184.8, 183.7, 160.9, 158.1, 154.3, 149.1, 137.0, 132.6, 128.5, 124.4, 118.2, 115.9, 113.0, 111.7, 106.9, 106.2, 9.3; HRMS (ESI) $[M + 1]^+$ found 297.0804 $[C_{17}H_{13}O_5]^+$, calcd. 297.0762. HPLC purity 97%.

*4.1.3.3. Synthesis of 8-hydroxy-2-(2,4-dimethoxyphenyl)naphthalene-1,4-dione (**4**).* Yield 20%; δ_H (400 MHz, DMSO-d6) δ 11.95 (1H, s, OH), 7.7–7.6 (1H, m, ArH), 7.48 (1H, d J 7.4 Hz, ArH), 7.20 (1H, d J 7.4 Hz, ArH), 7.16 (1H, d J 8.6 Hz, ArH), 6.83 (1H, s, CH), 6.51 (1H, d J 2.3 Hz, ArH), 6.56 (1H, dd J 8.6 Hz J 2.3 Hz, ArH), 3.82 (3H, s, OMe), 3.73 (3H, s, OMe); δ_C (100 MHz, DMSO-d6) 187.8, 183.3, 160.4, 159.4, 154.4, 145.4, 137.0, 133.4, 129.5, 122.5, 120.0, 116.0, 113.4, 111.7, 107.3, 105.9, 55.2, 45.1; HRMS (ESI) $[M + 1]^+$ found 311.0925 $[C_{18}H_{14}O_5]^+$ calcd.311.1113. HPLC purity 98%.

*4.1.3.4. Synthesis of 8-hydroxy-2-(2,3,4-trihydroxyphenyl)naphthalene-1,4-dione (**11**).* Yield 20%; δ_H (400 MHz, Acetone-d6) 11.97 (1H, s, OH), 9.47 (1H, s, OH), 8.76 (1H, s, OH), 8.49 (1H, s, OH), 7.8–7.7 (1H, m, ArH), 7.55 (1H, d J 6.6 Hz, ArH), 7.37 (1H, d J 8.4 Hz, ArH), 6.94 (1H, s, CH), 6.60 (1H, d J 8.4 Hz, ArH), 6.39 (1H, d J 8.4 Hz, ArH); δ_C (100 MHz, Acetone-d6); 189.9, 184.0, 156.5, 155.8, 149.3, 147.4, 142.5, 136.8, 136.5, 123.7, 121.6, 117.9, 112.8, 107.0, 94.5, 88.4; HRMS (ESI) $[M - 1]^-$ found 297.0803 $[C_{16}H_9O_6]^-$, calcd.297.0800. HPLC purity 94%.

*4.1.3.5. Synthesis of 8-hydroxy-2-(2,4,6-trihydroxyphenyl)naphthalene-1,4-dione (**12**).* Yield 93%; δ_H (400 MHz, Acetone-d6) 11.92 (1H, s, OH), 8.76 (1H, s, OH), 8.57 (1H, s, OH), 8.49 (1H, s, OH), 7.9–7.8 (1H, m, ArH), 7.60 (1H, d J 6.6 Hz, ArH), 7.40 (1H, d J 8.4 Hz, ArH), 6.76 (1H, s, CH), 6.43 (2H, d J 8.4 Hz, 2 × ArH); δ_C (100 MHz, Acetone-d6) 174.3, 173.7, 162.5, 159.4, 159.2, 158.2, 151.8, 132.2, 131.9, 111.0, 102.2, 94.5, 90.4, 79.7; HRMS (ESI) $[M-1]^-$ found 297.0435 $[C_{16}H_9O_6]^-$, calcd.297.0405. HPLC purity 97%.

*4.1.3.6. Synthesis of 2-(p-tolylthio)-8-hydroxynaphthalene-1,4-dione (**6**) [17].* Yield 47%; δ_H (300 MHz, Acetone-d6) 11.66 (1H, s, OH), 7.8–7.7 (1H, m, ArH), 7.6–7.4 (5H, m, ArH), 7.32 (1H, dd J 1.0 Hz J 7.4 Hz, ArH), 5.86 (1H, s, CH), 2.45 (3H, s, Me); δ_C (75 MHz, Acetone-d6) 186.1, 181.0, 161.0, 142.6, 138.6, 136.8, 132.5, 128.3, 124.9, 124.7, 124.6, 124.7, 124.6, 119.2, 21.7; HRMS (ESI) $[M + 1]^+$ found 297.0596 $[C_{17}H_{13}O_3S]^+$ calcd.297.0580. HPLC purity 96%.

*4.1.3.7. Synthesis of 2-(3-methoxyphenylthio)-8-hydroxynaphthalene-1,4-dione (**8**).* Yield 43%; δ_H (300 MHz, Acetone-d6) 11.48 (1H, s, OH), 7.60 (1H, dd J 8.2 Hz J 8.3, ArH), 7.4–7.3 (2H, m, 2 × ArH), 7.15 (1H, dd J 1.0 Hz J 8.3 Hz, ArH), 7.1–7.0 (3H, m, ArH), 5.87 (1H, s, CH), 3.72 (3H, s, Me); δ_C (75 MHz, Acetone-d6) 188.4, 181.7, 162.9, 162.3, 157.0, 138.7, 133.6, 132.7, 130.1, 129.5, 128.9, 124.7, 121.8, 120.1, 117.9, 116.1, 56.4; HRMS (ESI) $[M + 1]^+$ found 313.0588 $[C_{17}H_{13}O_4S]^+$, calcd.313.0531. HPLC purity 94%.

4.1.4. Synthesis of 3-N-substituted juglone derivatives

3-Bromo juglone (7) was synthesised according to the literature [15]. Then, the precursor 7 (1 eq) was dissolved in acetic acid, followed by the addition of the appropriate amine in large excess (7 eq). The work-up followed the procedure previously reported.

*4.1.4.1. Synthesis of 2-bromo-8-hydroxynaphthalene-1,4-dione (**7**).* Yield 59%; δ_H (400 MHz, DMSO-d6) 11.48 (1H, s, OH), 7.78 (1H, dd J 8.3 Hz J 7.8 Hz, ArH), 7.72 (1H, s, CH), 7.58 (1H, dd J 7.8 J 1.1 Hz, ArH), 7.38 (1H, dd J 8.3 J 1.1, ArH); δ_C (100 MHz, DMSO-d6) 182.4, 182.3, 160.9, 141.1, 139.5, 137.5, 132.3, 124.6, 119.4, 114.9; HRMS (ESI) found 250.9301 ($C_{10}H_4O_3Br79$, M79-H -), 252.9370 ($C_{10}H_4O_3Br81$, M81-H -), requires 250.9349 and 252.9330. HPLC purity 95%.

*4.1.4.2. Synthesis of 8-hydroxy-2-morpholinonaphthalene-1,4-dione (**9**).* Yield 39%; δ_H (400 MHz, Acetone-d6) 12.89 (1H, s, OH), 7.7–7.6 (1H, m, ArH), 7.6–7.5 (1H, m, ArH), 7.24 (1H, dd J 1.0 Hz J 7.4 Hz, ArH), 6.00 (1H, s, CH), 3.71 (4H, m, 2 × $O(CH_2)$), 3.59 (4H, m, 2 × $N(CH_2)$); δ_C (100 MHz, Acetone-d6) 190.1, 187.6, 161.6, 153.2, 137.7, 125.1, 124.9, 120.0, 109.8, 109.6, 67.4, 50.4; HRMS (ESI) $[M + 1]^+$ found 260.0912 $[C_{14}H_{14}O_4N]^+$ calcd. 260.0911. HPLC purity 96%.

*4.1.4.3. Synthesis of 8-hydroxy-2-(piperidin-1-yl)naphthalene-1,4-dione (**10**).* Yield 39%; δ_H (400 MHz, Acetone-d6) 13,13 (1H, s, OH), 7.7–7.5 (1H, m, ArH), 7.44 (1H, dd J 6.4 Hz J 1.3 Hz, ArH), 7.26 (1H, dd J 7.4 Hz J 1.3, ArH), 5.97 (1H, s, CH), 3.6–3.5 (4H, m, 2 × CH_2), 2.12 (6H, m, 3 × CH_2); δ_C (100 MHz, Acetone-d6) 189.8, 189.1. 161.9, 156.4, 135.4, 134.6, 124.9, 119.8, 115.8, 108.2, 51.4, 27.8, 25.3; HRMS

(ESI) $[M + 1]^+$ found 258.1123 $[C_{15}H_{16}O_3N]^+$ calcd. 258.1120. HPLC purity 99%.

4.1.5. Synthesis of O-Acetyl juglone derivatives

*4.1.5.1. Synthesis of 1,4-dihydro-1,4-dioxonaphthalen-8-yl acetate (**3**).* A round bottom flask was charged with 5-hydroxynaphthalene-1,4-dione (juglone, 1 eq), acetic anhydride (5 eq) and sodium acetate. The mixture was refluxed at 120 °C and stirred overnight. The mixture was then added to a phosphate buffer (20 mL, pH: 7,4) and extracted with chloroform (3 × 15 ml) and the combined organic layers were dried (Na_2SO_4) and evaporated to dryness. Purification was carried out by recrystallization.

Yield 51%; δ_H (300 MHz, Acetone-d6) 8.06 (1H, dd J 6.6 Hz J 1.3 Hz, ArH), 8.0–7.9 (1H, m, ArH), 7.59 (1H, dd J 6.7 Hz J 1.3 Hz, ArH), 7.07 (1H, d J 10.4 Hz, CH_3), 6.97 (1H, d 10.4 Hz, CH_2), 2.40 (3H, s, CH_3); δ_C (75 MHz, Acetone-d6) 185.2, 184.9, 169.9, 150.9, 141.1, 138.7, 136.3, 134.9, 131.1, 125.6, 124.6, 21.4; HRMS (ESI) found 239.2309 $(C_{12}H_8O_4Na, M + H)^+$ requires 239.0315. HPLC purity 94%.

*4.1.5.2. Synthesis of 1,4-dihydro-2-(2,4-dihydroxyphenyl)-1,4-dioxonaphthalen-8-yl acetate (**5**).* Compound 5 was synthesized according to the general procedure for the synthesis of 3-substituted-5-hydroxy naphthoquinones previously reported, starting from 5-acetoxy-1,4-naphthoquinone and resorcinol.

Yield 17%; δ_H (400 MHz, DMSO-d6) 11.87 (1H, s, OH), 10.21 (1H, s, OH), 7.9–7.8 (1H, m, ArH), 7.59 (1H, dd J 1.0 Hz, J 7.4 Hz, ArH), 7.42 (1H, dd J 1.0 Hz, J 7.4 Hz, ArH), 7.34 (1H, d J 8.5 Hz, ArH), 6.93 (1H, s, CH), 6.81 (1H, dd J 2.3 Hz, J 8.5 Hz, ArH), 6,71 (1H, d J 2.3 Hz, ArH), 2.11 (3H, s, CH_3); δ_C (100 MHz, DMSO-d6) 182.2, 175.4, 169.0, 160.9, 158.7, 154.2, 145.8, 138.8, 135.5, 132.3, 128.4, 127.9, 127.2, 121.9, 112.3, 106.5, 100.3, 20.3; HRMS (ESI) $[M + 1]^+$ found 325.0692 $[C_{18}H_{13}O_6]^+$calcd. 325.0707. HPLC purity 98%.

4.1.6. Synthesis of 5-Hydroxy-7-Methyl-8-Chloro-1,4-naphthoquinones

*4.1.6.1. Synthesis of 5-chloro-8-hydroxy-6-methylnaphthalene-1,4-dione (**14**).* A round bottomed flask was equipped with $AlCl_3$ (28 eq) and NaCl (13 eq) were introduced. The mixture was heated to melt the salts at 180 °C under vigorous stirring and 4-chloro-3-methylphenol (1 eq) and maleic anhydride (4 eq) were added. After 10 min at 180 °C the mixture was poured into ice/water made strongly acid with HCl 12 M, and was stirred at room temperature for half an hour. The precipitate was collected, dried and extracted with n-hexane in a soxhelet. The solvent was evaporated to dryness.

Yield: 67%; %; δ_H (400 MHz, Acetone-d6) 12.61 (1H, s, OH), 7.39 (1H, s, ArH), 7.07 (1H, d J 10.3 Hz, ArH), 7.04 (1H, d J 10.3 Hz, ArH), 2.54 (3H, s, CH_3); δ_C (100 MHz, Acetone-d6) 183.1, 182.2, 160.3, 143.7, 139.1, 138.7, 130.3, 126.6, 122.4, 114.6, 15.5; HRMS (ESI) $[M - 1]^-$ found 220.9937 $[C_{11}H_6O_3Cl35]^-$ calcd. 221.0001; $[M - 1]^-$ found 222.9985 $[C_{11}H_6O_3Cl37]^-$ calcd.222.9999. HPLC purity 93%.

*4.1.6.2. Synthesis of 5-chloro-8-hydroxy-2-(2,4-dihydroxyphenyl)-6-methylnaphthalene-1,4-dione (**15**).* Compound 15 was synthesized according to the general procedure for the synthesis of 3-substituted-5-hydroxy naphthoquinones previously reported, starting from 5-hydroxy-7-methyl-8-chloro-1,4-naphthoquinone and resorcinol.

Yield: 77%; %; δ_H (400 MHz, DMSO-d6) 12.54 (1H, s, OH), 9.75 (1H, s, OH), 9.73 (1H, s, OH), 7.47 (1H, s, ArH), 7.12 (1H, d J 8.4 Hz, ArH), 6.42 (1H, d J 2.3 Hz, ArH), 6.35 (1H, dd J 2.3 Hz J 8.4 Hz, ArH), 2.49, (3H, s, CH_3); δ_C (100 MHz, DMSO-d6) 183.1, 182.2, 160.3, 159.6, 159.2, 143.7, 139.1, 138.7, 130.3, 129.9, 126.6, 122.4, 114.6, 110.4, 108.9, 103.4, 15.4; HRMS (ESI) $[M + 1]^+$ found 331.0372 $[C_{17}H_{10}O_5Cl_{35}]^+$ calcd. 331.0368; (ESI) $[M + 1]^+$ found 333.0407 $[C_{17}H_{10}O_5Cl_{37}]^+$ calcd. 333.0345. HPLC purity 96%.

4.1.7. Synthesis of other naphthoquinones derivatives

*4.1.7.1. Synthesis of 2-(2,4-dihydroxyphenyl)naphthalene-1,4-dione (**13**).* 3-(2,4.diidrossiphenyl)-1,4-naphthoquinone (13) was synthesized following the general procedure reported above for the synthesis of 3-substituted-5-hydroxy naphtoquinone starting from 1,4-naphthoquinone and resorcinol.

Yield: 37%; δ_H (400 MHz, DMSO-d6) 9.70 (2H, sb, 2 × OH), 8.00 (2H, m, ArH), 7.89 (2H, m, ArH), 7.07 (1H, d J 8.4 Hz, ArH), 7.02 (1H, s, CH), 6.39 (1H, d J 2.3 Hz, ArH), 6.32 (1H, dd J 2.3 Hz J 8.4 Hz, ArH)); δ_C (100 MHz, DMSO-d6) 186.6, 183.4, 161.0, 160.2, 147.2, 137.1, 136.9, 134.2, 132.3, 132.0, 124.1, 116.2, 115.2, 111.0, 107.7, 103.2; HRMS found (ESI) 267.0667$C_{16}H_{10}O_4H$ $(M + 1)^+$, requires 267.0579. HPLC purity 98%.

*4.1.8. Naphthazarin naphthalene-1,4-dione (**16**)*

Naphthazarin (16) was used as received from Aldrich and used without any further purification.

4.1.9. General procedure for the synthesis of 1,4,5-trihydroxy naphthalenes

1,4,5-trihydroxy naphthalenes were obtained following a previously reported procedure. A round bottom flask was charged with a solution of $Na_2S_2O_4$ (5 eq) in water (5 mL), followed by the addition of a mixture of diethyl ether (15 mL) and a solution of the naphthoquinone (1 eq) in dichloromethane (3 mL). The mixture was vigorously stirred at room temperature for 15 min and the organic layer was collected and washed with brine. The solvent was evaporated to dryness. In order to prevent reoxidation of the compounds to the naphtoquinone form, NMR spectra and biological assays were carried out in the presence of $Na_2S_2O_4$. Each NMR spectrum was recorded using a mixture of DMSO-d6 (800 μL) and D_2O (200 μL containing 10 mg of $Na_2S_2O_4$) as solvent.

4.1.9.1. Synthesis of naphthalene-1,4,5-triol (JUG-R). Yield 68%; δ_H (400 MHz, DMSO-d6) 10.71 (1H, s, OH), 10.34 (1H, s, OH), 9.36 (1H, s, OH), 7.50 (1H, dd J 8.4 Hz J 1.1 Hz, ArH), 7.22 (1H, dd J 8.4 Hz J 7.8 Hz, ArH), 6.71 (1H, dd J 7.8 Hz J 1.1 Hz, ArH), 6.66 (1H, dd J 8.0 Hz, ArH), 6.54 (1H, d J 8.0, ArH); δ_C (75 MHz, DMSO-d6) 154.4, 146.2, 146.0, 127.4, 126.0, 115.4, 113.7, 109.2, 108.8, 108.3. HPLC purity 95%.

4.1.9.2. Synthesis of 3-(2,4-dihydroxyphenyl)naphthalene-1,4,5-triol (1-R). Yield 59%; δ_H (400 MHz, DMSO-d6) 9.97 (1H, s, OH), 8.14 (1H, s, OH), 7.43 (1H, dd J 8.1 Hz J 1.0 Hz, ArH), 7.19 (1H, dd J 8.1 Hz J 7.6 Hz, ArH), 7.01 (1H, d J 8.8 Hz, ArH), 6.67 (1H, dd J 7.6 Hz J 1.0 Hz, ArH); 6.66 (1H, s; ArH), 6.33 (2H, m, ArH); δ_C (75 MHz, DMSO-d6) 154.7, 154.3, 146.1, 146.0, 142.6127.8, 125.0, 124.7, 118.4, 115.3, 114.1, 112.7, 110.5, 109.9, 108.6, 108.3. HPLC purity 94%.

4.2. Biological assays

4.2.1. Cells

GLI36 Human glioma cell line was established by Dr. Anthony Campagnoni (UCLA, Los Angeles, CA, [27]). Cells were maintained in a monolayer using complete growth medium (CGM): 90% Dulbecco Modified Eagle's Medium (DMEM), 10% Foetal Bovine Serum, 100 I.U./mL penicillin, 10 I.U./mL streptomycin, 10 I.U./mL tetracycline, 25 I.U./mL Plasmocin (InVivogen, Milan, Italy). Cells were incubated at 37 °C in a humidified environment with 95% air and 5% CO_2, up to 80–90% confluence (4–6 days).

4.2.2. MTT assay

Cells were plated in 96-well plates in CGM. After 48 h, addition to CGM of JUG, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, TMZ and

paclitaxel (PTX) was carried out to reach the concentrations of 50 μM, 5 μM, 0.5 μM or 0.05 μM, in 10 μL DMSO. TMZ was used as the reference molecule, since it is used for human glioma treatment. PTX was used as a different anti-tumor agent, not clinically approved for glioma treatment. Control was performed with 10 μL DMSO. After 24 h or 72 h, MTT (2.5 mg/mL in phosphate buffered saline, PBS) was added for additional 3 h and the percentage of cell viability was obtained. Each analysis was performed in 5 replicates and repeated in 3 independent experiments.

4.2.3. Assessment of apoptosis and necrosis by Wright's staining

Cells were cultured in 24-well plates on 9 mm coverslips for 24 h in CGM, followed by incubation for 24 h or 72 h with JUG, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, TMZ and PTX and DMSO as control at different concentrations (50 μM, 5 μM, 0.5 μM or 0.05 μM). Then, cells were washed in PBS, fixed in methanol for 5 min and Wright's stained (250 μL/well) for 5 min. Cell morphology was evaluated under light microscope at 400× magnification. At least 600 cells were counted for each coverslip in 5 different fields. Each experiment was repeated 2 times.

4.2.4. Statistical analysis

Statistical analysis was performed with GraphPad Prism5 (GraphPad Software Inc., San Diego, CA). The percentage of apoptotic and necrotic cells was calculated, t-test was used to estimate the difference in apoptotic and necrotic cells between treated and control cells. Results are expressed as the mean ± SEM, $p < 0.05$ was considered as statistically significant.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. All the authors contributed equally.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.04.039.

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