SUPPLEMENTARY MATERIAL

Antiproliferative Activity of Juglone Derivatives on Rat Glioma

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Malignant gliomas are aggressive and life-threatening tumours that still show a poor prognosis: the current therapeutic approach based on surgical resection and chemotherapy combined with radiotherapy does not provide a satisfactory chance of long-term survival to patients. Natural bioactive compounds represent a precious source of molecules with antiproliferative activity, potentially effective also against glioma cells. Among these, juglone is a known allelopathic compound extracted from the eastern black walnut (*Juglans nigra*) which anti-mitotic effect has been extensively described in mammalian cells. We investigated the antiproliferative effect of a synthetic derivative of this natural compound, 2-(2,4-dihydroxyphenyl)-8-hydroxy-1,4-naphthoquinone (DiNAF), in rat glioma cells. We compared this molecule and its effect with the natural reference compound and with newly synthesized derivatives to build a preliminar structure-activity relationship. Biological assays and NMR-based redox experiments confirmed that DiNAF is a promising lead and supported the hypothesis of a redox mechanism underlying its cytotoxic activity.

Keywords: glioma, juglone, TMZ, naphthoquinones

1. Experimental procedures

1.1. Chemistry

Commercially available chemicals were purchased from Aldrich, and used as received, unless otherwise stated. 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 $^{13}\text{C}\{^1\text{H}\}$ 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 and an Agilent C-18 column (5 μm , 4.6 x 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 minutes (90% A-10% B), 15 minutes (10% A-90% B), 20 minutes (10% A-90% B), 21 minutes (90% A-10% B), 25 minutes (90% A-10% B). The purity of all compounds was $\geq 95\%$, unless otherwise stated.

1.1.1. Synthesis of 2-(2,4-dihydroxyphenyl)-8-hydroxy-1,4-naphthoquinone (DiNAF, 1)

Compound 1 was prepared as described by Redaelli *et al.* (Redaelli 2015).

1.1.2. General procedure for the synthesis of substituted naphthoquinones (2-6)

A round-bottom flask was charged with 1.05 mmol of the opportune 1,4-naphthoquinone and 15 mL of glacial acetic acid. 0.68 mmol of the chosen hydroxy benzene, previously dissolved in 3 mL of glacial acetic acid, were added to the mixture followed by 0.5 mL of 2M sulfuric acid. The reaction was allowed to stir under a nitrogen atmosphere (25-50 °C, 5-24 hours depending on the substrate) and followed by TLC using opportune proportions of hexane and ethyl acetate as eluents. To isolate the desired product, 20 mL of water were added and the resulting mixture was extracted with ethyl

acetate. The organic phase was washed with an aqueous solution of sodium bicarbonate, collected and the solvent was evaporated providing an orange-brown solid.

1.1.3. Synthesis of 2-(2,4-dihydroxyphenyl)-1,4-naphthoquinone (2)

The resulting solid was further purified by silica flash chromatography (hexane\ethyl acetate 1:1, R_f 0.38). Yield: 37 %. δ_{H} (400 MHz, DMSO) 6.32 (1H, dd J 8.3 Hz J 1.9 Hz, PhH), 6.41 (1H, d J 1.9 Hz, PhH), 7.09 (1H, d J 8.3 Hz, PhH), 7.11 (1H, s, C=CH), 7.38 (2H, s, PhH), 9.70 (1H, s, OH), 9.73 (1H, s, OH), 12.44 (1H, s, OH), 12.47 (1H, s, OH). δ_{C} (100 MHz, Acetone) 103.6, 109.4, 114.2, 117.5, 117.7, 118.0, 123.6, 123.8, 129.1, 138.3, 145.8, 154.7, 154.9, 159.1, 175.4, 182.2. HMRS (ESI) found 299.0561 ($\text{C}_{16}\text{H}_{11}\text{O}_6$, $[\text{M}+1]^+$), calc. 299.0550. Anal. found C 64.4; H 3.4. Calc. for $\text{C}_{16}\text{H}_{10}\text{O}_6$: C 64.4; H 3.4 %.

1.1.4. Synthesis of 5-hydroxy-2-(2,4-dihydroxyphenyl)-1,4-naphthoquinone (3)

The resulting solid was further purified by silica flash chromatography (hexane\ethyl acetate 7:3, R_f 0.49). Yield: 35 %. δ_{H} (400 MHz, Acetone) 6.34 (1H, dd J 8.2 Hz J 2.1 Hz, PhH), 6.39 (1H, d J 2.1 Hz, PhH), 6.89 (1H, s, C=CH), 7.07 (1H, d J 8.4 Hz, PhH), 7.18 (1H, d J 8.3 Hz, PhH), 7.46 (1H, d J 7.2 Hz, PhH), 7.63 (1H, dd J 8.3 Hz J 7.2 Hz, PhH), 8.54 (2H, s, OH), 11.99 (1H, s, OH). δ_{C} (100 MHz, Acetone) 103.9, 107.9, 113.0, 116.4, 118.7, 124.6, 133.3, 137.6, 137.7, 148.1, 150.1, 157.5, 160.9, 162.3, 184.9, 190.9. HMRS (ESI) found 283.0615 ($\text{C}_{16}\text{H}_{11}\text{O}_5$, $[\text{M}+1]^+$), calc. 283.0601. Anal. found C 68.0; H 3.6. Calc. for $\text{C}_{16}\text{H}_{10}\text{O}_5$: C 68.1; H 3.6 %.

1.1.5. Synthesis of 2-(2,3,4-trihydroxyphenyl)-1,4-naphthoquinone (4)

The resulting solid was further purified by silica flash chromatography (hexane\ethyl acetate 1:1, R_f 0.32). Yield: 19 %. δ_{H} (400 MHz, DMSO) 6.39 (1H, d J 8.2 Hz, PhH), 6.62 (1H, d J 8.2 Hz, PhH), 7.07 (1H, s, C=CH), 7.38 (2H, s PhH), 8.50 (1H, s, OH), 8.75 (1H, s, OH), 9.51 (1H, s, OH), 12.43 (1H, s, OH), 12.48 (1H, s, OH). δ_{C} (100 MHz, Acetone) 103.4, 107.5, 112.7, 130.9, 131.0, 132.9, 133.3, 136.1, 147.0, 157.1, 159.2, 160.6, 161.9, 162.6, 183.0, 184.0. HMRS (ESI) found 315.0482 ($\text{C}_{16}\text{H}_{11}\text{O}_7$, $[\text{M}+1]^+$), calc. 315.0499. Anal. found C 61.1; H 3.2. Calc. for $\text{C}_{16}\text{H}_{10}\text{O}_7$: C 61.1; H 3.2 %.

1.1.6. Synthesis of 2-(2,3,4-trihydroxyphenyl)-1,4-naphthoquinone (5)

The resulting solid was further purified by silica flash chromatography (hexane\ethyl acetate 6:4, R_f 0.40). Yield: 38 %. δ_{H} (400 MHz, DMSO) 6.38 (1H, d J 8.5 Hz, PhH), 6.58 (1H, d J 8.5 Hz, PhH), 6.98 (1H, s, C=CH), 7.88 (2H, m PhH), 8.02 (2H, m PhH), 8.46 (1H, s, OH), 8.70 (1H, s, OH), 9.46 (1H, s, OH). δ_{C} (100 MHz, Acetone) 107.9, 114.7, 122.6, 126.2, 127.3, 132.9, 133.7, 133.8, 134.5,

135.0, 136.4, 145.5, 148.1, 148.2, 184.9, 185.6. HMRS (ESI) found 283.0622 ($C_{16}H_{11}O_5$, $[M+1]^+$), calc. 283.0601. Anal. found C 68.1; H 3.6. Calc. for $C_{16}H_{10}O_5$: C 68.1; H 3.6 %.

1.1.7. Synthesis of 5-nitro-2-(2,4-dihydroxyphenyl)-1,4-naphthoquinone (6)

This compound was synthesized from 1,2,3-trihydroxybenzene and 5-nitro-1,4-naphthoquinone, prepared as follows. A round-bottom flask was charged with 6.30 mmol of 1,4-naphthoquinone and 13 mL of concentrated sulfuric acid. The mixture was cooled in an ice bath to 5 °C and 41.80 mmol of sodium nitrite were slowly added to the reaction under stirring. The mixture was stirred for 25 minutes at 20 °C and for further 5 minutes at 40 °C. The cooled reaction mixture was then poured into crushed ice giving a solid product that was filtered and washed with water. The solid was recrystallized from toluene giving the desired intermediate (yield: 65 %) that was then used in the following reaction step, for which the general procedure was applied.

The resulting solid was further purified by silica flash chromatography (hexane/ethyl acetate 6:4, R_f 0.38). Yield: 27 %. δ_H (400 MHz, DMSO) 6.30 (1H, d J 8.4 Hz, PhH), 6.46 (1H, d J 8.4 Hz, PhH), 7.01 (1H, s, C=CH), 7.96 (1H, dd J 7.9 Hz J 7.7 Hz, PhH), 8.15 (1H, dd J 7.9 Hz J 1.1 Hz, PhH), 8.22 (1H, dd J 7.7 Hz J 1.1 Hz, PhH), 8.51 (1H, s, OH), 8.88 (1H, s, OH), 9.57 (1H, s, OH). δ_C (100 MHz, Acetone) 103.5, 107.4, 112.2, 119.4, 121.4, 124.6, 135.3, 137.6, 137.8, 150.9, 154.1, 159.0, 161.2, 169.3, 186.9, 190.9. HMRS (ESI) found 328.0451 ($C_{16}H_{10}NO_7$, $[M+1]^+$), calc. 328.0452. Anal. found C 58.7; H 2.7; N 4.3. Calc. for $C_{16}H_9NO_7$: C 58.7; H 2.8; N 4.3 %.

1.1.8. Synthesis of juglone (7)

Juglone was prepared as according to a literature procedure (Couladouros 1996) and to the indication of Zonta *et al.* (Zonta 2009).

1.2. Redox kinetic studies

The selected compounds were dissolved in DMSO- d_6 (800 μ L) to a 10 mM concentration and a solution of $Na_2S_2O_4$ (1 equivalent) in 200 μ L of D_2O was added, adapting a procedure we previously reported (Redaelli 2015). 1H NMR spectra were acquired at regular timings and the variations of the integrals of the reacting compound and of the forming reduced species were measured. Experiments were carried out in 3 replicates. Spectra were acquired at 25 °C.

1.3. Biological Assays

F98 rat glioma cells were maintained in monolayer using complete growth medium (CGM) in combination with 90% Dulbecco Modified Eagle's Medium (DMEM), 10% FBS, 100 I.U./mL penicillin, 10 μ g/mL streptomycin, 10 μ g/mL tetracycline, 25 μ g/mL Plasmocin (*InVivogen*, Milan,

Italy). Cells were incubated at 37°C in a humidified environment with 95% air and 5% CO₂, up to 80-90% confluence (4-6 days).

Cytotoxicity assays were performed in two independent tests: morphological analysis and an MTT based cell viability test. The morphological analysis allows a more thorough evaluation of the appearance of the cells compared to automated counts based on single markers, that may miss strange morphology due to definition of sharp cutoffs. Cells were plated on 24-well plates and grown on 9 mm sterile cover glasses. After 48 hours, addition of the following species was carried out whilst maintaining the CGM medium: DiNAF (1) (50 µM, 5 µM, 0.5 µM or 0.05 µM), Juglone (50 µM, 5 µM, 0.5 µM or 0.05 µM), PTX (50 µM, 5 µM, 0.5 µM or 0.05 µM) and TMZ (50 µM, 5 µM, 0.5 µM or 0.05 µM). Cells were incubated for 24 hours, and then fixed with methanol and stained with *Wright's stain*; a total of 2400 cells were counted from each slide. The percentage of apoptotic and necrotic cells was then calculated, following an established procedure (Mucignat-Caretta et al. 2008); *t-test* was used to estimate the amount of apoptotic and necrotic cells (in percentage) between treated cells.

	Morphological Analysis				
	% of apoptotic cells				
	DMSO	DiNAF	Juglone	TMZ	PTX
50uM	3,9±0,17	76,12±1,29	45±0,7	64±0,79	53,4±0,64
5uM	4±0,07	68,56±0,99	30,22±0,64	46,01±0,42	34,71±0,55
0.5uM	3,91±0,05	43,84±0,52	19,25±0,71	36,05±0,91	18,03±0,84
0.05uM	3,78±0,15	14,65±0,53	7,8±0,19	12,33±0,3	7,85±0,41
	Morphological Analysis				
	% of necrotic cells				
	DMSO	DiNAF	Juglone	TMZ	PTX
50uM	3,21±0,17	12,05±0,36	27,65±0,96	21,6±0,25	37,1±0,39
5uM	3,58±0,2	15,32±0,87	26,51±0,33	15,08±0,85	19,03±0,36
0.5uM	3,6±0,22	6,71±0,23	15,99±0,31	6,63±0,23	14,07±1,52
0.05uM	4,15±0,3	4,13±0,24	9,75±0,25	5,61±0,31	8,68±0,2
	MTT assay				
	% of cell viability				
	DMSO	DiNAF	Juglone	TMZ	PTX
50uM	101,69±3,08	12,33±1,33	18,15±1,12	19,33±1,98	21,17±2,11
5uM	102,94±1,47	21,33±1,78	42,11±2,11	36,25±2,46	37,17±1,23
0.5uM	100,82±2,82	36,26±2,21	59,28±2,27	49,11±2,11	61,21±1,36
0.05uM	99,46±1,94	83,11±2,11	87,23±2,33	85,11±2,67	87,13±2,67

Table S1. Biological data of morphological analysis and MTT assay.

Cell viability test was performed on 96-well plates in six repetition, using PTX, TMZ, Juglone, DiNAF (1) and the other synthetic derivatives at the same concentrations tested for the morphological analyses. The data were normalized on control cells and *t-test* was used to estimate the variation in percentage of cell viability between treated cells.

1.4. In-vivo tests

Wistar rats (n=3) were maintained in standard conditions (12:12 hours, lights on at 07:00, temperature $23 \pm 2^{\circ}\text{C}$) in plastic cages (41x25x15), with food and water always available. The procedure was approved by the competent authorities according to EEC directive 86/609/EEC. Rats were anesthetized with a mixture of xylazine (75 mg/kg) and tiletamine/zolazepam (20 mg/kg). DiNAF was dissolved 50 nmol in DMSO 40 μl were injected at the following coordinates: A+1mm, L+1.5mm, D-3.4mm. Animals were examined daily for neurological symptoms for 3, 7 and 10 days. 72 hours after injection one rat was sacrificed, the brain was postfixed in formalin, embedded in paraffin, sectioned and stained with hematoxylin/eosin or Nissl staining.

2. Representative 2D NMR spectra

Figure S1. ¹H NOESY of 1

Figure S2. HMBC of 3

3. Kinetic NMR studies

Figure S3. Figure 3. Reduction kinetic of compounds 1 and 5 towards 1 equivalent of $\text{Na}_2\text{S}_2\text{O}_4$. Data are presented as percentage (Mean $\pm$ SEM, $n=3$) of naphthoquinone form calculated on ^1H NMR integrals. See Supplementary Material for representative spectra.

Figure S4. Comparison of ^1H NMR spectra (DMSO) of juglone before the addition of $\text{Na}_2\text{S}_2\text{O}_4$ (above) and of its reduced form (below), immediately produced after the addition of 1 eq of $\text{Na}_2\text{S}_2\text{O}_4$.

Figure S5. Comparison of the ^1H NMR spectrum (DMSO) of compound 1 before the addition of $\text{Na}_2\text{S}_2\text{O}_4$ (above) and the spectrum recorded after the addition of 1 eq of $\text{Na}_2\text{S}_2\text{O}_4$ (below, $t = 10$ min).

Figure S6. Comparison of the ^1H NMR spectrum (DMSO) of compound 1 before the addition of $\text{Na}_2\text{S}_2\text{O}_4$ (above) and the spectrum recorded after the addition of 1 eq of $\text{Na}_2\text{S}_2\text{O}_4$ (below, $t = 30$ min).

Figure S7. Comparison of the ^1H NMR spectrum (DMSO) of compound 1 before the addition of $\text{Na}_2\text{S}_2\text{O}_4$ (above) and the spectrum recorded after the addition of 1 eq of $\text{Na}_2\text{S}_2\text{O}_4$ (below, $t = 45$ min).

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