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Dimerization enhances cytotoxicity and tumor/non-tumor cell selectivity of juglone

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Abstract: The study investigates the biological activity of spaced juglone dimers derived from the reaction of juglone with dicarboxylic acid dichlorides of varying chain lengths. It builds upon the observation that dimeric structures can exhibit enhanced biological activity compared to their monomeric counterparts. The cytotoxic effects of the synthesized compounds were assessed against a range of human cancer cell lines and non-malignant fibroblasts. Results indicate that the cytotoxicity varied depending on the length of the spacer, with specific dimers showing significantly improved activity. Furthermore, these compounds demonstrated a higher selectivity towards cancer cell lines than non-malignant fibroblasts, suggesting potential for targeted anticancer therapy.

Keywords: Juglone; Cytotoxicity; dimerization.

1. Introduction

Over several decades, plant-derived secondary natural metabolites have been extensively investigated and used in the chemotherapy of diseases, especially cancer. Juglone (**1**, Scheme 1), 5-hydroxy-naphthalene-1,4-dione occurs naturally in plants of the *Juglandeceae* family, for example, in Black walnuts (*Juglans nigra*, but also in *J. manshurica*, and *J. regia*), and also in Lemongrass (*J. cinerea*). Juglone is toxic to many plants; consequently, it has been used as an herbicide 1 and as a dye (juglone is also known as C.I. Natural Brown 7, C.I. 75500). Since its first isolation by Hartwich in 1887 [2] from walnuts, followed by the first synthesis [3] in the same year, many publications dealt with this compound, and several reviews have also been published focusing on its versatile biological activities [4-18].

Furthermore, extracts of these plants have already been used for many years in Traditional Chinese Medicine to treat - among other diseases – cancer [19]. Later, it was assumed that juglone's cytotoxicity is mediated by ROS production and apoptosis in a mitochondria-dependent pathway [20-22]. Furthermore, juglone is cytotoxic against a wide range of human cancer cell lines [23-30], and it also holds some anti-angiogenic activity on K-ras mutated MIA Paca-2 pancreatic cells [22]. However, the cytotoxicity of parent juglone proved low, and some derivatizations [31] have taken place to increase cytotoxicity, for example, by preparing a [Pd(Jug)(phen)]Cl complex or by substitution of the phenol [32]. Juglone's molecular biological modes of action and its derivatives in cancer therapy have been reviewed recently [17].

In order to improve the biological activity of compounds, their dimerization has been suggested - based on the observation that many biological targets dimerize upon an initial interaction with a drug. Consequently, dimers often hold significantly higher bioactivity than their monomeric precursors [33-36]. Several reviews have been published summarizing successful examples of this concept [37-39].

2. Results and Discussion

As a result of these reports in the literature, we decided to prepare a small proof-of-concept study of several juglone dimers and investigate their cytotoxic activity by employing a panel of selected human tumor cell lines. Naphthoquinones of a rather complex structure have already been isolated from various natural sources. However, as outlined in this study, 33 instead of "simple" dimers have never been prepared before; they are first-in-class.

For their synthesis, dicarboxylic acids were converted in situ into the corresponding acid chlorides that were allowed to react with juglone to furnish **2-5**.

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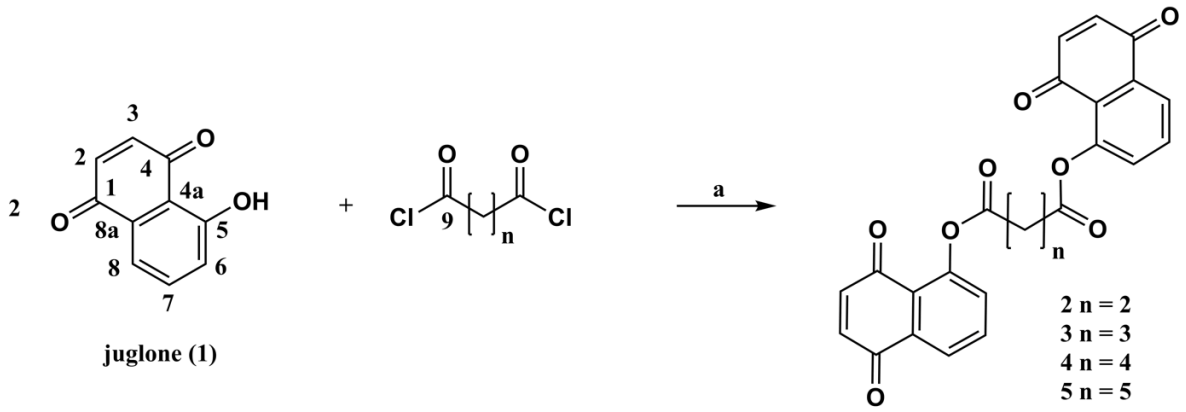

Scheme 1. Synthesis of juglone dimers **2**-**5** from juglone (**1**); a) DCM, TEA, DMAP (cat.), 0 °C → 21 °C, 1 h; yields: **2** 69%, **3** 59%, **4** 69%, **5** 88%

The characterization of these compounds proved easy due to the symmetry of the molecules: As exemplified for **2**, in the ^{1}H NMR spectrum, the CH groups of the quinone were detected at δ = 6.95 ppm and 6.86 ppm each as a doublet holding a vicinal coupling constant ^{3}J = 10.3 Hz; in the ^{13}C NMR spectrum the carbonyls were detected at δ = 184.2 and 183.6 ppm, respectively; the ester at δ = 170.6 ppm. A quasi-molecular ion *m/z* = 430.3 corresponds to $[M+H]^{+}$. Fig. 1 shows a comparative depiction of the ^{13}C NMR spectra of compounds **2**–**5**.

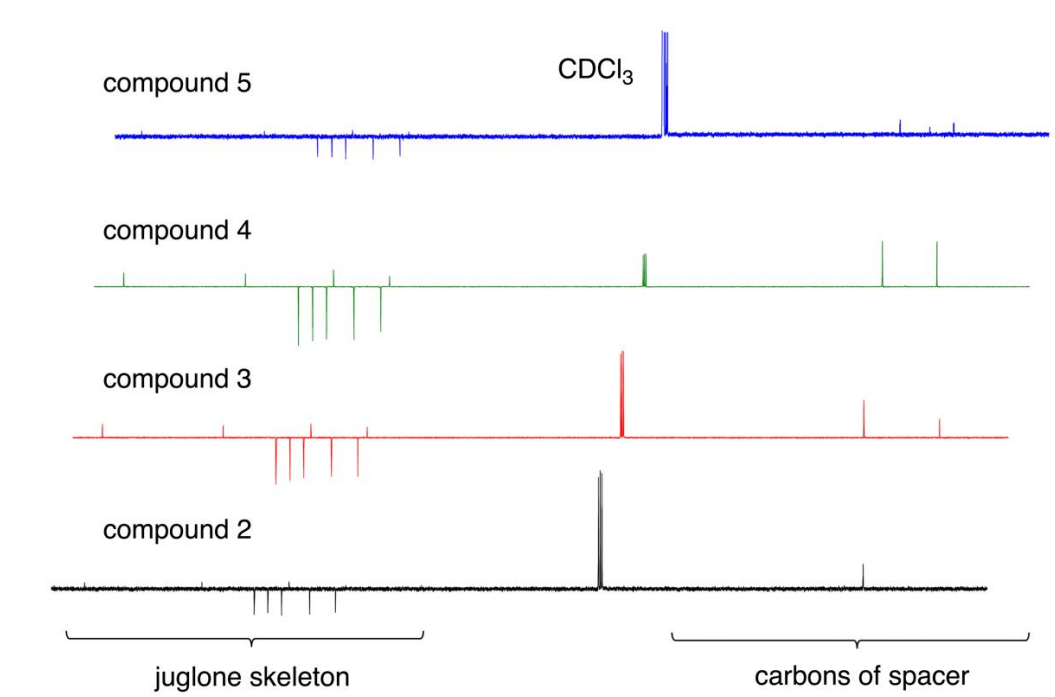

Figure 1. Comparative depiction of the ^{13}C NMR spectra for compounds **2**–**5** (APT-NMR, 126 MHz, $CDCl_3$)

To assess their cytotoxic activity, sulforhodamine assays (SRB) were performed using a panel of human tumor cell lines. The results from these assays are compiled in Table 1. Five human tumor cell lines and a non-malignant cell line (NIH 3T3, murine fibroblasts) have been employed. Doxorubicin has been used as a positive standard. As a result, the lowest IC_{50} values were observed for **3** and **4**; a shorter length of the spacer (as in **2**) or an elongated spacer (as in **5**) resulted in a slightly diminished cytotoxicity. As compared to parent juglone, cytotoxicity improved for the dimeric compounds two to three-fold; furthermore, selectivity (defined as the ratio between the IC_{50} of non-malignant NIH 3T3 cells and the IC_{50} value of the tumor cell lines) more than doubled. (cf. Table 2).

Table 1. Results from the cytotoxicity assays (SRB; incubation for 48 h); IC_{50} values in µM (each value represents the mean value of three independent experiments each performed in triplicate; confidence interval CI = 95%); used human tumor cell lines: A375 (melanoma), HT29 (colorectal carcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), FaDu (squamous hypopharyngeal carcinoma) and NIH 3T3 (murine fibroblasts, non-malignant). Doxorubicin (**DX**) has been used as a positive standard; n.d., not determined.

Compound	A375	HT29	MCF-7	A2780	FaDu	NIH 3T3
Juglone	9.0 ± 0.8	17.1 ± 0.6	12.1 ± 1.1	9.3 ± 0.6	13.0 ± 1.2	11.1 ± 0.9
2	4.2 ± 0.5	15.1 ± 0.4	5.0 ± 0.7	4.0 ± 0.3	7.3 ± 0.7	7.5 ± 0.8
3	3.2 ± 0.4	11.1 ± 0.9	3.1 ± 0.4	3.4 ± 0.4	5.3 ± 0.2	7.0 ± 0.7
4	3.0 ± 0.3	9.3 ± 0.7	3.0 ± 0.5	3.1 ± 0.3	5.0 ± 0.4	7.3 ± 0.5
5	3.5 ± 0.4	10.6 ± 0.6	4.7 ± 0.6	4.5 ± 0.6	6.2 ± 0.5	7.7 ± 0.4
DX	n.d.	0.9 ± 0.01	1.1 ± 0.3	0.01 ± 0.006	n.d.	0.4±0.07

Table 2. Malignant cell to non-tumor cell selectivity [calculated by IC_{50} (NIH 3T3) / IC_{50} (tumor cell line)].

Compound	**A375**	**HT29**	**MCF-7**	**A2780**	**FaDu**
Juglone	1.23	0.65	0.92	1.19	0.85
2	1.79	0.50	1.50	1.88	1.03
3	2.19	0.63	2.26	2.06	1.32
4	2.43	0.78	2.43	2.35	1.46
5	2.20	0.73	1.64	1.71	1.24

Some extra screening experiments (Annexin V/FITC/PI staining) were performed to get a deeper insight into the mode of action of these compounds. This staining is a valuable property exclusive to apoptotic cells — neurotic cells do not exhibit it. Phosphatidylserine is translocated from inside the membrane to the outside during apoptosis, along with cell shrinkage, chromatin condensation, and the creation of apoptotic bodies. The cell membrane undergoes a fast shift in phospholipid symmetry, a process that initially retains the cell membrane's barrier function. When calcium is present, a cellular protein called annexin V can attach itself to the displaced phosphatidylserine. Four categories were identified from the flow cytometric data: living cells, early and late apoptotic cells, and necrotic cells. The control group consisted of untreated cells. The results from **4** (twice IC_{50} concentration, incubation for 48h) and A2780 cells are depicted in Fig. 2.

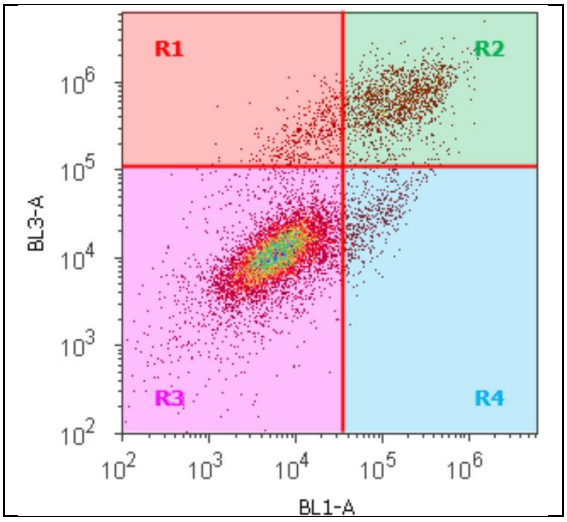

Figure 2. Representative Annexin V/FITC/PI assay plot as determined by flow cytometry: R1: necrosis, R2: sec. necrotic/late-stage necrotic; R3 vital, and R4 apoptotic. A: control; B: A2780 cells incubated with 2 x IC_{50} concentration of **4** for 24h

3. Conclusion

The concept that dimeric structures sometimes exhibit higher biological activity than their monomeric precursors has been observed in numerous natural products. Extending this concept, a first small series of spaced juglone dimers was obtained by reacting the parent juglone with dicarboxylic acid dichlorides of different chain lengths. The resulting compounds were tested for cytotoxic activity against a panel of human cancer cell lines. The results showed that depending on the size of the spacer, a significant improvement in cytotoxicity was observed. In addition, a higher selectivity of the cytotoxic effect between cancer cell lines (A375, HT29, MCF-7, A2780, FaDu) and non-malignant fibroblasts (NIH 3T3) was observed. These results provide an excellent basis for further modifications and future research.

4. Experimental

Equipment and details of the bioassays have been described in previous communications [40-44]. Juglone was purchased from local vendors and used as received.

General procedure for dimerization (GP)

To a solution of juglone (0.40 g, 2.3 mmol, 1 eq) in dry DCM (5 mL), the respective dicarboxylic acid chloride (0.5 eq) at 0°C, TEA (2 eq.) and catalytic amounts of DMAP were added. The reaction solution was allowed to warm to 21°C, and stirring was continued for another hour. The reaction mixture was washed with dil. aq. hydrochloric acid (1 M, 10 mL), and the aq. phase was re-extracted with DCM (3 x 50 mL). The combined organic phases were dried ($MgSO_4$), the solvent was evaporated under diminished pressure, and the residue was subjected to chromatography (silica gel, ethyl acetate/ hexanes, 1:7) to yield the dimeric compounds **2-5**.

Bis(5,8-dioxo-5,8-dihydronaphthalen-1-yl) butanedioate (2)

Yield: 69%; orange colored solid; m.p. 138-141°C; R_f = 0.30 (SiO_2, toluene/ethyl acetate/formic acid/heptane, 80:26:5:1);
IR (KBr): ν = 2935*br*, 1751*s*, 1660*s*, 1589*m*, 1395*w*, 1332*m*, 1294*m*, 1281*m*, 1267*s*,1230*m*, 1135*m*, 1098*m*, 1081*m* cm^{-1};
UV/Vis (MeOH): λ_{max} (log ε) = 250 (4.50), 345 (3.72) nm;
^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.06 (*dd*, *J* = 7.8, 1.3 Hz, 2H, 8-H + 8'-H), 7.77 (*t*, *J* = 7.9 Hz, 2H, 7-H + 7'-H), 7.42 (*dd*, *J* = 8.1, 1.3 Hz, 2H, 6-H + 6'-H), 6.95 (*d*, *J* = 10.3 Hz, 2H, 3-H + 3'-H), 6.86 (*d*, *J* = 10.3 Hz, 2H, 2-H + 2'-H), 3.28 (*s*, 4H, 10-H + 10'-H) ppm;
^{13}C NMR (126 MHz, $CDCl_3$): δ = 184.2 (*C*=O, C-4 + C-4'), 183.6 (*C*=O, C-1 + C-1'), 170.6 (*C*=O, C-9 + C-9'), 149.3 (C-5 + C-5'), 139.8 (C-2 + C-2'), 137.3 (C-3 + C-3'), 134.8 (C-7 + C-7'), 133.5 (C-8a + C-8a'), 129.7 (C-6 + C-6'), 124.9 (C-8 + C-8'), 125.1 (C-4a + C-4a'), 29.2 (C-10 + C-10') ppm;
MS (ESI, MeOH): *m/z* = 430.3 (20%, $[M+H]^+$), 453.0 (90%, $[M+Na]^+$), 882.7 (35%, $[2M+Na]^+$);
analysis calcd for $C_{24}H_{14}O_8$ (430.37): C 66.98, H 3.28; found: C 66.70, H 3.51.

Bis(5,8-dioxo-5,8-dihydronaphthalen-1-yl) pentanedioate (3)

Yield: 59%; orange-colored solid; m.p. 158-160°C; R_f = 0.3 (SiO_2, toluene/ethyl acetate/formic acid/heptane, 80:26:5:1);
IR (KBr): ν = 2936*br*, 1750*s*, 1661*s*, 1591*m*, 1393*w*, 1332*m*, 1293*m*, 1282*m*, 1266*s*,1231*m*, 1135*m*, 1099*m*, 1082*m* cm^{-1};
UV/Vis (MeOH): λ_{max} (log ε) = 250 (4.50), 345 (3.72) nm;
^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.05 (*dd*, *J* = 7.8, 1.3 Hz, 2H, 8-H + 8'-H), 7.77 (*t*, *J* = 7.9 Hz, 2H, 7-H + 7'-H), 7.42 (*dd*, *J* = 8.1, 1.3 Hz, 2H, 6-H + 6'-H), 6.94 (*d*, *J* = 10.4 Hz, 2H, 3-H + 3'-H), 6.85 (*d*, *J* = 10.3 Hz, 2H, 2-H + 2'-H), 2.98 (*t*, *J* = 7.3 Hz, 4H, 10-H + 10'H), 2.33 (*p*, *J* = 7.3 Hz, 2H, 11-H) ppm;
^{13}C NMR (126 MHz, $CDCl_3$): δ = 184.1 (*C*=O, C-4 + C-4'), 183.6 (*C*=O, C-1 + C-1'), 171.4 (*C*=O, C-9 + C-9'), 149.4 (C-5 + C-5'), 139.9 (C-2 + C-2'), 137.3 (C-3 + C-3'), 134.8 (C-7 + C-7'), 133.5 (C-8a + C-8a'), 129.8 (C-6 + C-6'), 125.0 (C-8 + C-8'), 123.3 (C-4a + C-4a'), 33.0 (C-10 + C-10'), 19.3 (C-11) ppm;
MS (ESI, MeOH): *m/z* = 481.1 (100%, $[M+Na]^+$), 938.7 (65%, $[2M+Na]^+$), 954.7 (10%, $[2M+K]^+$);
analysis calcd for $C_{25}H_{16}O_8$ (444.40): C 67.57, H 3.63; found: C 67.39, H 3.80.

Bis(5,8-dioxo-5,8-dihydronaphthalen-1-yl) hexanedioate (4)

Yield: 69%; orange-colored solid; 173-175°C; R_f = 0.3 (SiO_2, toluene/ethyl acetate/formic acid/heptane, 80:26:5:1);
IR (KBr): ν = 2955*br*, 1763*s*, 1664*s*, 1611*w*, 1592*m*, 1463*w*, 1412*w*, 1378*m*, 1330*m*, 1290*m*, 1263*m*, 1233*s*, 1123*s*,1099*s*, 1080*s* cm^{-1};
UV/Vis (MeOH): λ_{max} (log ε) = 250 (4.56), 345 (3.75) nm;
^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.05 (*dd*, *J* = 7.8, 1.3 Hz, 1H, 8-H + 8'-H), 7.76 (*t*, *J* = 7.9 Hz, 1H, 7-H + 7'-H), 7.40 (*dd*, *J* = 8.1, 1.3 Hz, 1H, 6H + 6'-H), 6.94 (*d*, *J* = 10.3 Hz, 1H, 3-H + 3'-H), 6.85 (*d*, *J* = 10.3 Hz, 1H, 2-H + 2'-H), 2.88 – 2.83 (*m*, 4H, 10-H + 10'H), 2.04 – 2.00 (*m*, 4H, 1H + 11'-H) ppm;
^{13}C NMR (126 MHz, $CDCl_3$): δ = 184.2 (*C*=O, C-4 + C-4'), 183.6 (*C*=O, C-1 + C-1'), 171.6 (*C*=O, C-9 + C-9'), 149.5 (C5 + C5'), 139.9 (C-2 + C-2'), 137.3 (C-3 + C-3'), 134.7 (C-7 + C-7'), 133.5 (C-8a + C-8a'), 129.8 (C-6 + C-6'), 124.9 (C-8 + C-8'), 123.3 (C-4a + C-4a'), 33.8 (C-10 + C-10'), 23.8 (C-11 + C-11') ppm;
MS (ESI, MeOH): *m/z* = 467.1 (100%, $[M+Na]^+$), 910.8 (80%, $[2M+Na]^+$), 926.8 (10%, $[2M+K]^+$);
analysis calcd for $C_{26}H_{18}O_8$ (458.42): C 68.12, H 3.96; found: C 67.85, H 4.19.

Bis(5,8-dioxo-5,8-dihydronaphthalen-1-yl) heptanedioate (5)

Yield: 88%; orange-colored solid; 137-140°C; R_f = 0.3 (SiO_2, toluene/ethyl acetate/formic acid/heptane, 80:26:5:1);
IR (KBr): ν = 2941*br*, 1763*m*, 1663*s*, 1612*w*, 1591*m*, 1460*w*, 1443*w*, 1356*w*, 1330*m*, 1288*m*, 1267*m*, 1234*m*, 1219*m*, 1122*s*, 1080*s* cm^{-1};
UV/Vis (MeOH): λ_{max} (log ε) = 250 (4.52), 346 (3.70) nm;
^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.05 (*dd*, *J* = 7.8, 1.3 Hz, 1H, 8-H + 8'-H), 7.76 (*t*, *J* = 7.9 Hz, 1H, 7-H + 7'-H), 7.39 (*dd*, *J* = 8.1, 1.3 Hz, 1H, H-6 + H-6'), 6.94 (*d*, *J* = 10.3 Hz, 1H, H-3 + H-3'), 6.85 (*d*, *J* = 10.3 Hz, 1H, H-2 + H-2'), 2.80 (*t*, *J* = 7.5 Hz, 4H, H-10 + H-10'), 1.93 (*p*, *J* = 7.6 Hz, 2H, H-12), 1.72 – 1.61 (*m*, 4H, H-11 + H-11') ppm;
^{13}C NMR (126 MHz, $CDCl_3$): δ = 184.2 (*C*=O, C-4 + C-4'), 183.6 (*C*=O, C-1 + C-1'), 171.8 (*C*=O, C-9 + C-9'), 149.5 (C5 + C5'), 139.9 (C-2 + C-2'), 137.2 (C-3 + C-3'), 134.7 (C-7 + C-7'), 133.5 (C-8a + C-8a'), 129.8 (C-6 + C-6'), 124.9 (C-8 + C-8'), 123.3 (C-4a + C-4a'), 33.9 (C-10 + C-10'), 28.5 (C-12), 24.1 (C-11 + C-11') ppm;
MS (ESI, MeOH): *m/z* = 495.1 (100%, $[M+Na]^+$), 966.8 (50%, $[2M+Na]^+$);
analysis calcd for $C_{27}H_{20}O_8$ (472.45): C 68.64, H 4.27; found: C 68.36, H 4.63.

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