

An in vitro comparative study with furyl-1,4-quinones endowed with anticancer activities

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Summary We describe the biological activity of some furylbenzo- and naphthoquinones (furylquinones) on hepatocarcinoma cells and healthy rat liver slices. The effects of furylquinones on cancer cells (Transplantable Liver Tumor, TLT) were assessed by measuring cell death (membrane cell lysis); intracellular contents of ATP and GSH and the activity of caspase-3 were used to determine the type of cell death. Most of the furylquinones tested (at a concentration of 25 µg/ml) induced caspase-independent cell death but compound **4** had no cytotoxic effects. The levels of

both ATP and GSH were severely affected by quinones **1**, **2** and **5**, while no effect was observed with compound **4**. These cytotoxic properties of quinones are associated with physico-chemical properties as shown by the LUMO energies and lipophilicity. Interestingly, no cytotoxic effects of furylquinones were detected when the in vitro model of precision-cut liver slices (PCLS) was used. Indeed, although CYP2E1 activity was slightly affected, ATP and GSH levels as well as protein synthesis were not modified by furylquinones. Paracetamol, a well-known hepatotoxicant, reduced these parameters by more than 80% compared to control conditions. Taking into account the considerable incidence of adverse-effects induced by most current anti-cancer drugs, the selective cytotoxicity shown by compounds **1**, **2** and **5**, in particular that of **1**, represents a safety factor that encourages the further development of these quinones as new drugs in cancer therapy.

In memoriam to Dr. Henry S. Taper who died on 24/04/2009

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Introduction

Cancer is the second leading cause of death in the world. In Europe, the number of persons dying of cancer was estimated to be 1,700,000 in 2006 [1], while 1,479,350 new cancer cases and 562,340 deaths from cancer were projected to occur in the United States in 2009 [2]. From a biochemical point of view, cancer cells have some remarkable features, including angiogenesis, invasive capacity and resistance to apoptosis induction [3], high rates of glycolysis [4, 5], and low antioxidant enzyme activity [5, 6]. Apoptosis reactivation procedures and the exploration of

new and alternative ways to induce cancer cell death are currently the major goals of chemo- and radio-therapy procedures.

Since cancer cells usually exhibit low antioxidant enzyme activity [5, 6], this raises the possibility that they could be killed by oxidative stress. A large body of evidence supports the idea that oxidative stress induced by menadione (2-methyl-1,4-naphthoquinone) leads to cell death by either necrosis or apoptosis [7–9]. In agreement with this, we recently studied the cytotoxicity of sesquiterpene quinones, namely 2-eryfuryl- and 2-eryfuryl-3-nitro-1,4-benzoquinone, on Transplantable Liver Tumor (TLT) cells, a murine hepatoma cell line [10]. The results revealed that inactivated 2-eryfuryl-3-nitro-1,4-benzoquinone could undergo an activation process by a redox mechanism causing necrosis-like cell death, whereas 2-eryfurylbenzoquinone, which would be less able to perform bioreductive activation, seemed to induce apoptosis [10]. We subsequently focused our study on 2-eryfuryl-1,4-naphthoquinone and its 5- and 5,8-hydroxy derivatives. We postulated that the electron donor effect of the *peri*-hydroxyl substituents on eryfurylnaphthoquinones and the hydrogen bond between the *peri*-hydroxy and quinone carbonyl groups influence the electron-acceptor capability of the quinone nucleus and thus modify electron transfer from ascorbate to the electroactive quinone nucleus [11].

In continuation of these studies, we report here the results obtained with several bioactive furylquinone compounds on tumor and non-tumor targets. Indeed, the ultimate issue for every compound expected to possess antitumor activity is its clinical relevance (i.e., efficacy and safety). To this end, two different *in vitro* methods were employed: a murine hepatoma cell line, namely TLT cells, and precision-cut rat liver slices (PCLS). The first model (TLT cells) has been widely used to assess both *in vivo* and *in vitro* cytotoxic anticancer effects [12–14], facilitating the investigation of possible mechanisms involved in cancer cell death. The second method, PCLS, is a suitable model for studying the toxic effects of xenobiotics on healthy tissues at both molecular and cell biology levels [15–18]. The end points for assessing the biological reactivity of furylquinones in TLT cells were LDH leakage (index of cell survival), the intracellular content of reduced glutathione (GSH) and ATP (indexes of cell metabolism which are more sensitive to LDH leakage), and caspase-3 activity (type of cell death). To assess the reactivity of furylquinones towards a non-transformed tissue (PCLS), a simultaneous evaluation of cellular markers associated with energetic status (ATP content) and oxidative stress (GSH levels) together with xenobiotic detoxication capacity (chlorzoxazone hydroxylation by CYP2E1) and tightly regulated hepatic function, the biosynthesis of proteins, were investigated. In addition, we compared the results

obtained with furylquinones to those obtained with paracetamol, which was used as the reference compound because its liver toxicity has been attributed to the formation of a quinoneimine reactive metabolite, namely N-acetyl-p-benzoquinoneimine [19].

Materials and methods

Chemical synthesis

The dimethylaminohydrazonefurylquinones **1–3** employed in this study were prepared by oxidative coupling reactions of 2-furaldehyde *N,N*-dimethylhydrazone with 1,4-naphthoquinone, 2-chloro-1,4-naphthoquinone and 2-methoxy-1,4-benzoquinone in acetic acid according to a previously reported procedure [17]. The aldehydes **4–6** were obtained by acid-induced hydrolysis of compounds **1–3** under mild conditions (Fig. 1).

Cell culture conditions

Murine hepatocarcinoma cells, namely TLT, were cultured in DMEM/F12 (Dulbecco's modified eagle medium, Gibco) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and gentamicin (50 µg/ml). The cultures were maintained at a density of $1-2 \times 10^5$ cells per ml. The medium was changed at 48–72 h intervals. All cultures were kept at 37°C in a 95% air/5% CO₂ atmosphere with 100% humidity. TLT cells were incubated at the indicated times at 37°C in the presence or absence of furylquinones at different concentrations.

Cellular assays

Cellular viability was estimated by measuring the activity of lactate dehydrogenase (LDH), according to the procedure of Wroblewsky and Ladue [21], in the culture medium and in the cell pellet obtained after centrifugation. Results are expressed as the ratio of released activity to total activity. ATP content was determined using the Roche ATP Bioluminescence Assay Kit CLS II (Mannheim, Germany) according to procedures described by the suppliers, and the results are expressed as nmol ATP/mg protein. The protein content was determined using the method of Lowry et al. [22] with bovine serum albumin (BSA) as reference. The GSH content was determined in clean-supernatants using a modified Cohn and Lyle method, after the formation of a fluorescent complex with o-phthalaldehyde (oPT) and measurement at 345 nm excitation and 420 nm emission [23]. The results are expressed as nmol GSH/ mg protein. Caspase-3 activity was monitored by cleavage of a specific peptide substrate, Asp-Glu-Val-Asp-AFC (DEVD-AFC)

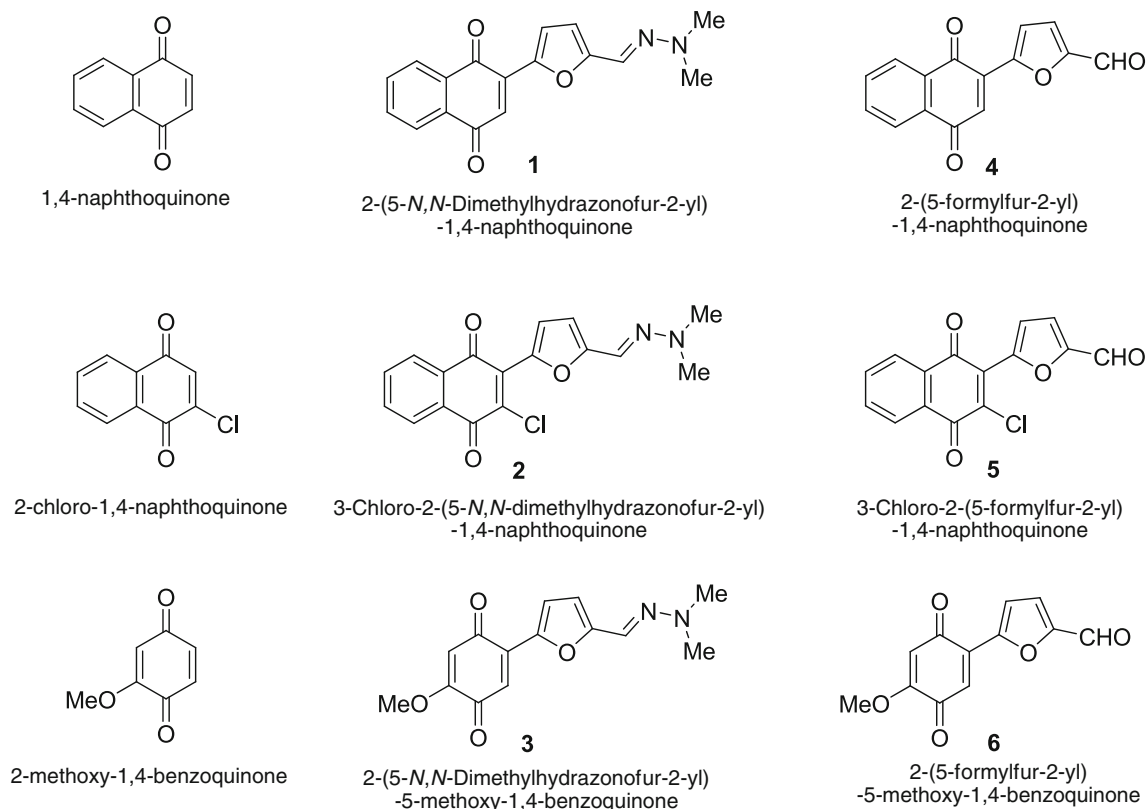


Fig. 1 Furylquinones 1-6 prepared from 1,4-quinones

according to the procedure outlined in the instructions for the “FluorAce apopain assay” kit (Biorad). The results are expressed as Units/mg protein.

Preparation and culture conditions of PCLS

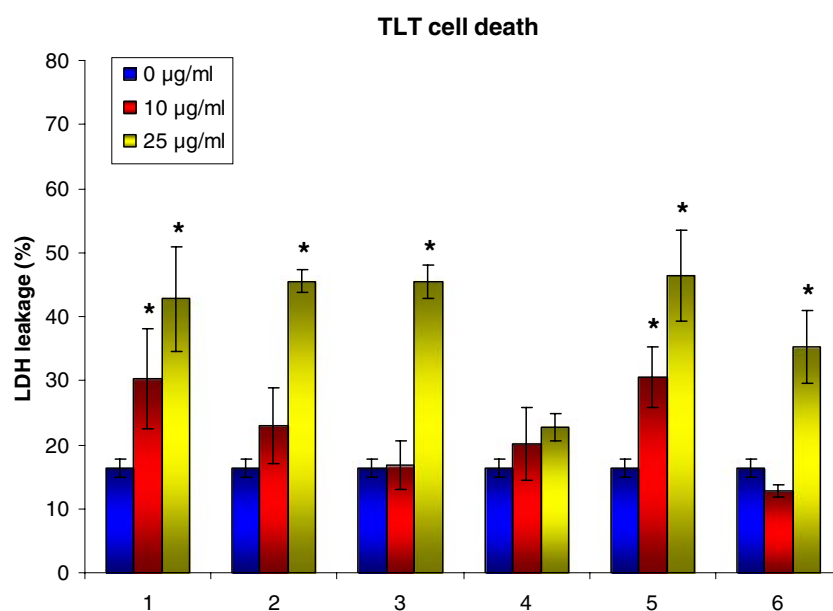
PCLS were prepared from male Wistar rats (3 months old) purchased from Harlan (the Netherlands). Rats were housed in individual cages in a temperature- and light- controlled room (12 h dark/light cycle). They received a standard diet (A03 UAR, France) and water ad libitum. Animal care and experiments were performed according to the Biosafety and Ethical rules in application in Belgium as adopted by the Bioethical Committee of the Université Catholique de Louvain. After one week of acclimatization, rats were weighed and killed under pentobarbital anesthesia (60 mg/kg i.p.). The liver was removed and PCLS (250-300 μm thickness) were prepared using the Krumdieck tissue slicer according to procedures previously described [16]. The PCLS were kept for 30 min at 4°C in Williams’ medium E (WME) containing 10% fetal calf serum (FCS), glutamine (2 mM) and insulin (100 nM). After preincubation, the PCLS were transferred to vials containing WME (one slice in 2 ml) supplemented with glutamine (2.4 mM), insulin (100 nM), and 50 $\mu\text{g}/\text{ml}$ gentamicin sulfate. The PCLS were incubated in a shaking water-bath (100 cycles/min) at

37°C under a continuous flow of O_2/CO_2 , (95%/5%) for 24 h. All chemicals were ACS reagent grade.

PCLS assays

Protein synthesis was estimated by measuring the incorporation of [^{14}C] Leu (specific activity 94 $\mu\text{Ci}/\text{mmol}$, Amersham; 0.8 mM unlabelled Leu) into the pelleted material obtained by perchloric acid precipitation as described by Seglen [24]. Results are expressed as dpm/mg protein. The rate of chlorzoxazone metabolism (index of CYP2E1 activity) was quantified by HPLC following the formation of its 6-hydroxy-derivative as previously reported by Wauthier et al [25]. After 24-hours’ incubation in the presence or absence of chemicals (quinones and paracetamol), PCLS were washed with PBS and further incubated for 20 min at 37°C in medium supplemented with 600 μM chlorzoxazone. The incubation medium was recovered and the reaction was stopped by the addition of 200 μl ZnSO_4 (15% W/V). Chlorzoxazolone was added as an internal standard and, after centrifugation, the supernatant analyzed by HPLC – UV at 287 nm. The stationary phase consisted of a C18 column; the mobile phase (ammonium acetate 10 mM (pH 4.25) – acetonitrile [81:29]) was delivered at a flow rate of 1.5 ml/min. Results are expressed as μg of 6-hydroxy-chlorzoxazone/mg

Fig. 2 Effects of furylquinones on TLT cell survival. TLT cells were incubated for 24 h at 37°C in the presence of furylquinones at different concentrations as indicated in the figure. Aliquots of cell suspensions were taken and LDH leakage, as a marker of cell death, was measured as indicated in the “Materials and Methods” section. The results represent the mean values \pm S.E.M. of at least three different experiments. * $p < 0.05$ compared to control values



protein. Chlorzoxazone and chlorzoxazolone were purchased from Sigma Chemicals (St. Louis, MO, USA). 6-Hydroxychlorzoxazone was purchased from Ultrafine Chemicals (Manchester, UK). Solvents used were of HPLC grade and all other chemicals were of the purest quality available.

Statistics

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

Results

Antitumor activity of furylquinones on hepatocarcinoma cells

The effect of the selected furylquinones **1-6** on TLT cell survival as a function of their concentration during 24 h of incubation is shown in Fig. 2. Quinones **1** and **5** (10 µg/ml) showed a significant increase in cell death; indeed, values of LDH leakage of about 30% were observed in treated cells compared to 16% in untreated control cells. At a higher concentration (25 µg/ml), all the furylquinones were cytotoxic demonstrating more than 35% LDH leakage, except quinone **4** which did not influence the survival of TLT cells to any significant extent at either concentration, as shown by LDH leakage values that remained fairly stable at around 20–22%.

Since some furylquinones already induced membrane cell lysis at 10 µg/ml, the dose was decreased to 5 µg/ml to

determine the nature of cell death. Table 1 shows the effects of furylquinones on caspase-3 as measured as DEVDase activity. Caspase-3 was not activated in cells incubated with quinones. Conversely, a decrease in caspase-3 activity of about 30–40% was observed in cells incubated with quinones **1**, **2** and **5**, the same compounds that induced membrane cell lysis. The relevance of this lack of activation is noted by the 8.2-fold increase observed when cells were

Table 1 Effects of furylquinones on ATP and GSH contents and on caspase-3 activity in TLT cells

	ATP (nmol/mg protein)	GSH	Caspase-3 activity (Units/mg protein)
Control	4.78 \pm 0.56	9.70 \pm 0.55	13.45 \pm 1.3
Sanguinarine (10 µM)	nt	nt	110.15 \pm 6.4*
1	1.44 \pm 0.41*	6.17 \pm 0.89*	7.30 \pm 0.2*
2	0.86 \pm 0.33*	5.45 \pm 0.96*	9.40 \pm 1.8*
3	2.85 \pm 0.67*	7.04 \pm 0.97*	14.20 \pm 5.2
4	5.53 \pm 1.21	9.26 \pm 1.69	17.50 \pm 5.3
5	3.62 \pm 0.89	5.37 \pm 0.87*	7.40 \pm 1.4*
6	2.92 \pm 0.89*	6.86 \pm 0.66*	13.20 \pm 1.7

TLT cells were incubated for 24 h at 37°C in the presence of furylquinones at 5 µg/ml. Sanguinarine (10 µM) was used as the reference compound. Aliquots of cell suspensions were taken and different markers were measured as indicated in the “Materials and Methods” section. The markers were caspase-3 activity as a marker of apoptosis; ATP content as a marker of energetic status; and GSH content as a marker of oxidative stress. The results represent the mean values \pm S.E.M. of at least three different experiments. (nt = not tested)

* $p < 0.05$ compared to control values

incubated with sanguinarine (10 μ M), a flavonoid that is a well known inducer of apoptosis via caspase-3 activation [9]. It should be noted that at this concentration (5 μ g/ml) none of the furylquinones affected the LDH leakage of TLT cells. Interestingly, the same profile was observed for both ATP and GSH contents, two end-points which are more sensitive than LDH leakage. When considering the loss of ATP compared to control conditions, the subgroups of quinones had different effects. A first group, which caused a severe decrease in the intracellular ATP content, is represented by the dimethylhydrazonofuryl-bearing naphthoquinones **1** and **2** which decreased ATP by 70% and 82%, respectively. A second group, composed of the benzoquinones **3** and **6**, decreased ATP by nearly 40%. Finally, within the formylfuryl-bearing naphthoquinone group, a minor effect on ATP was observed: **5** decreased ATP by only 32%, while **4** had no effect on ATP content. For the second selected metabolic marker, namely the level of GSH, the situation was rather different to that observed for ATP and more closely resembled the effect observed on membrane cell lysis (Fig. 2). Indeed, almost all the quinone compounds decreased the level of GSH by approximately 30-40%. In agreement with the previous results, only the aldehyde-bearing naphthoquinone **4** had no effect on GSH.

Biological activity of furylquinones on precision-cut liver slices

To evaluate the toxicity and safety of furylquinones we utilized PCLS, an in vitro model reflecting the biological activity of non-transformed cells. Indeed, PCLS were employed because they preserve a higher level of tissue organization, close to the one normally found in the intact organ, and thus are a better reflection of the in vivo situation. In addition, they preserve cell heterogeneity, including non-parenchymal cells such as Kupffer and endothelial cells, and due to the maintenance of cell-cell and cell-matrix interactions, there is preservation of a differentiated state. The effects obtained by incubating PCLS with the quinone compounds on the four parameters selected, were compared to those caused by paracetamol, a well-known hepatotoxicant (Table 2). Indeed, compared to control conditions, paracetamol decreased the content of ATP by 95%, induced an 80% depletion in GSH, inhibited protein synthesis by more than 85%, and decreased CYP2E1 activity by 88%. None of the quinones significantly influenced ATP or GSH levels. Surprisingly, although not statistically significant, when slices were incubated in the presence of the most cytotoxic quinones **1** and **5**, a slight increase, ranging from 20% to 38%, was observed in the levels of both ATP and GSH.

Two additional parameters of major hepatic functions, namely the ability to synthesize proteins and the capacity to

Table 2 Effects of furylquinones on ATP and GSH contents and on protein synthesis and CYP2E1 activity in PCLS

	ATP (nmol/mg protein)	GSH	Protein synthesis (dpm/mg protein)	CYP2E1 (activity)
Control	4.77 \pm 0.55	7.40 \pm 0.51	1624 \pm 175	0.48 \pm 0.05
Paracetamol	0.22 \pm 0.08*	1.46 \pm 0.18*	228 \pm 30*	0.06 \pm 0.02*
1	6.20 \pm 1.54	10.27 \pm 2.64	1794 \pm 311	0.45 \pm 0.04
2	5.30 \pm 0.64	6.87 \pm 1.34	1826 \pm 144	0.32 \pm 0.03*
3	4.20 \pm 0.92	8.80 \pm 2.33	1173 \pm 175*	0.33 \pm 0.07*
4	4.68 \pm 0.59	7.52 \pm 1.51	1433 \pm 234	0.39 \pm 0.11
5	5.72 \pm 1.08	10.09 \pm 1.88	1148 \pm 213*	0.27 \pm 0.09*
6	4.88 \pm 0.30	9.54 \pm 1.09	1751 \pm 107	0.28 \pm 0.02*

PCLS were incubated for 24 h at 37°C with 25 μ g/ml of each furylquinone and 5 mM of paracetamol. The contents of both ATP and GSH were measured in perchloric acid-deproteinized fraction obtained after PCLS homogenization by Ultraturax. Protein synthesis was measured as follows: after 24 h incubation in the presence of quinone compounds and paracetamol, PCLS were further incubated for 2 h in the presence of radiolabeled leucine. Afterwards, PCLS were rinsed twice with saline and homogenized by Ultraturax. Radioactivity was measured in the protein pellet obtained after perchloric acid precipitation. CYP2E1 activity was estimated following the formation of 6-hydroxychlorzoxazone. Briefly, after 24 h incubation in the presence of furylquinones and paracetamol, PCLS were further incubated for 20 min in the presence of chlorzoxazone. At the end of the incubation, aliquots of incubation medium were analyzed by HPLC. All these parameters were measured as indicated in the "Materials and Methods" section. The results represent the mean values \pm S.E.M. of at least three different experiments

* p <0.05 compared to control values

detoxify xenobiotics, were assessed in PCLS. Compared to paracetamol, the quinone compounds were rather ineffective for both these measures. For protein synthesis, a slight but marginal inhibitory effect of about 30% was observed for compounds **3** and **5**. Nevertheless, almost all the quinone compounds decreased CYP2E1 activity by about 30% to 40%, with the exception of **4** and **1**.

Physico-chemical analysis

There are several studies on 1,4-quinone derivatives which demonstrate that the cytotoxic activities of 1,4-quinones depend on redox capability and lipophilicity. On this basis, we calculated the LUMO energies and CLog P of the tested compounds **1-6** in order to gain insight into the influence of these parameters on the cytotoxic effects of these agents on cancer and normal cells (Table 3). Calculations of the LUMO energies of quinones **1-6** were performed using the semiempirical PM3 method [26] and lipophilicity was estimated using the CSChem 3D software. The values of the E_{LUMO} energies calculated for the 1,4-naphthoquinones **1**, **2**, **4** and **5** are displayed within the range 1.4321-1.7240 eV. Accord-

Table 3 LUMO energies^a and CLogP^b values of furylquinones 1-6

Furylquinone	-E _{LUMO} (eV)	CLogP	Furylquinone	-E _{LUMO} (eV)	CLogP
1	1.4321	1.56	4	1.6386	1.34
2	1.5233	2.29	5	1.7240	2.06
3	1.7115	-0.64	6	1.7330	-0.86

^a Calculated by the semiempirical PM3 method. ^b Determined by the CSChem 3D software

ing to these data, these quinones showed a similar electron capacity to accept one electron but naphthoquinone **4** did not demonstrate a cytotoxic effect towards TLT cells. This fact could be attributed to a marked effect of the lipophilicity which, in the case of compound **4**, was lower than that of analogues **1**, **2** and **5**. Consequently, on the basis of this explanation and taking into account that the E_{LUMO} energies of the 1,4-benzoquinones **3** and **6** were quite similar, the smaller cytotoxic effect of **6** as compared to **3** may be explained by the lower lipophilicity of the former compound.

Discussion

Compared to healthy cells, cancer cells usually have high levels of reactive oxygen species (ROS), which stimulate cell proliferation and promote genetic instability [27, 28]. This difference is due to oncogenic stimulation and the high metabolic rates of cancer cells, rendering them particularly vulnerable to any treatment affecting redox homeostasis. It has recently been reported that inhibition of antioxidant defenses leads to accumulation of ROS, affecting mostly cancer cells [30] and most probably due to an impairment of antioxidant enzymes during carcinogenesis [5, 6, 29]. In support of this hypothesis, tumors that exhibit low antioxidant enzyme levels respond better to radiotherapy than tumors with greater enzyme levels [31, 32]. Techniques leading to increased ROS formation should, therefore, reinforce the efficacy of anticancer treatments. Indeed, we have recently reported that euryfuryl benzo and naphthoquinones in the absence or presence of vitamin C, can alter the survival of hepatocarcinoma cells [10, 11]. The results of the current study indicate that, in addition to the ability to induce ROS formation, other physicochemical properties may also influence the activity of quinones. For instance, among naphthoquinone derivatives, those which exhibited the highest lipophilicity (CLogP: 1.56; 2.29 and 2.06), namely compounds **1**, **2**, and **5**, were the most toxic against TLT cells. The benzoquinone derivatives **3** and **6**, which had the lowest CLogP values, were less active and showed the same profile of biological activity, except for the slight inhibitory effect of compound **3** on protein synthesis.

The antitumor activity of quinones **1** and **2** against TLT cells (hepatocarcinoma), is in agreement with our previous work using other cancer cell lines, namely MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), and SF-268 (CNS cancer), in which cytotoxic activities were demonstrated at concentrations of about 10 μ M [20]. Although immortalized cell lines have several limitations and drawbacks (e.g., disruption of cell-to-cell contacts, loss of normal polarity and the architecture of the whole tissue, etc), they enable molecular studies to be performed that explore mechanisms and metabolic pathways underlying a particular effect by a given xenobiotic. Thus, the calculations of $IC_{50\%}$ for a new chemical entity may be related to its potential therapeutic application. To reinforce this approach, rather than to merely record the extent of membrane cell lysis, we preferred to include additional end-points that are more sensitive to LDH leakage, dye exclusion, and MTT reduction. These endpoints also provide some indications regarding the mechanism and the nature of cell death. For example, while compound **1** shows antitumor effects in different cancer cell lines, it was rather inactive towards rat liver slices, indicating a high degree of safety. The measurement of a number of functional parameters, namely two standard end-points (ATP and GSH content) and two major hepatic functions (protein synthesis and the ability to metabolize xenobiotics), demonstrated that the biochemical integrity of the liver slices was well preserved during the 24 h of incubation in the presence of quinone compounds. In contrast, in the presence of paracetamol, a well-known hepatotoxic agent, all these functional parameters were strongly depressed. It should be noted that almost all the quinones induced a slight inhibition of CYP2E1-mediated activity, with the exception of compounds **1** and **4**. We do not have a satisfactory explanation for this effect, but because CYP2E1 can convert molecular oxygen into ROS [33, 34], we can hypothesize that quinone **1** would be able to block this ROS formation, thus reducing the oxygen radical-mediated cytotoxicity.

In conclusion, we have demonstrated that the cytotoxicity of furyl-1,4-quinones is dependent on the nature of the substituent linked to the quinone electroactive nucleus. The biological effect is apparently associated with the LUMO energies and the hydrophobic properties. The latter parameter seems to have a major influence, since compound **4** was totally inactive among the naphthoquinone derivatives, and the two benzoquinone derivatives with the lowest CLogP values, namely compounds **3** and **6**, were less active than compounds **1**, **2** and **5**. Quinones induce a necrotic-like cell death strongly affecting TLT cellular metabolism because cancer cells are sensitive to an oxidant insult and strongly dependent on ATP for their survival. Nevertheless, quinones appear relatively safe when they are challenged in

a healthy in vitro model such as PCLS, because healthy cells have antioxidant defenses against oxidative stress. Given the high incidence of undesirable adverse-effects induced by the majority of currently used anticancer drugs, and considering the selective, cytotoxic effects of quinone 1, these results reinforce our aim of developing new chemicals to be used as promising and new anticancer drugs.

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