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Original article

Part 1: Effect of vitamin C on the biological activity of two euryfurylbenzoquinones on TLT, a murine hepatoma cell line

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Abstract

2-Euryfuryl- and 2-euryfuryl-3-nitro-1,4-benzoquinone **Q2** and **Q3**, prepared *via* oxidative coupling reactions of sesquiterpene euryfuran **1** to 2-nitro-1,4-benzoquinone and 1,4-benzoquinone, were tested for their cytotoxicity towards TLT cells (a murine hepatoma cell line) in the absence and in the presence of vitamin C. Their cytotoxic profile was completely different. In cells incubated with **Q2** (from 1 to 50 μg/ml), cell survival was not modified, both GSH and ATP were depleted to about 50% of control values (at 50 μg/ml); and caspase-3 was activated in a dose-dependent manner. These effects were observed whatever cells were incubated or not in the presence of vitamin C. In the case of **Q3**, the cytotoxicity was rather unrelated to its concentration but the association of vitamin C plus the highest **Q3** concentration (50 μg/ml) results in a strong cell death (more than 60%). At such a concentration, a complete lack of caspase-3 activity was observed, probably due to cell lysis. At lower concentrations of **Q3** (1 and 10 μg/ml), caspase-3 activity was lower than that observed in the absence of vitamin C or even under control conditions. Both GSH and ATP were kept fairly constant as compared to control values but in the presence of vitamin C and **Q3,** at 50 μg/ml, a decrease in their amounts was observed.
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Keywords: Vitamin C; Euryfurylbenzoquinones; Redox cycling; Reactive oxygen species; Cell death

1. Introduction

Cancer, second cause of mortality in the world, is characterized by a deregulation of the cell cycle which results in a progressive loss of the cellular differentiation and a non-controlled cellular growth. Despite the progresses achieved in medicine this last century, cancer is still a leading life-threatening pathology. This situation will not dramatically change in the next years since due to the increase of life-span, human populations are expected to suffer more and more from cancers. Therefore, there is a need for new therapies, especially those that are based on current knowledge of cancer biology as well as by taking advantage on cancer cells phenotype recently described by Hanahan and Weinberg [1]. Indeed, the search on new cellular targets inducing cell death is a critical issue since cancer cells are resistant to an induction of apoptosis (e.g. by bcl-2 over-expression or mutation of p53).

Among several strategies capable of activating cancer cell death, the induction of an oxidative stress represents an interesting approach. Indeed, we and several other laboratories have developed a particular one by exposing selectively cancer cells to an oxidative stress generated during a redox cycling between ascorbate (vitamin C) and menadione [2−10]. Three lines of evidence support this approach: first, due to their lack

Abbreviations: LDH, lactate dehydrogenase; LUMO, lowest unoccupied molecular orbital; Q2, 2-euryfuryl-1,4-benzoquinone; Q3, 2-euryfuryl-3-nitro-1,4-benzoquinone; ROS, reactive oxygen species; TLT, transplantable liver tumor.

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of antioxidant enzymes, cancer cells are more sensitive towards an oxidative stress [11,12]; second, vitamin C is preferentially taken up by cancer cells, due to their over-expression of GLUT transporters [13]; and third, cancer cells show an almost universal glycolytic phenotype, required to survive and, most probably, to invade tissues of their neighborhood [14].

On the other hand, bioreduction of quinones by cellular reductases leads to the formation of free radicals (e.g. semiquinone radical), which causes a cascade of reactions including lipid peroxidation, protein and DNA damage, and, ultimately, cell death [15]. By combining ascorbate and menadione, it may be argued that the semiquinone radical may be quenched by ascorbate to form ascorbyl radical, a stable radical causing little oxidative stress. However, we postulate that such a possibility is unlikely. Indeed, ascorbate (vitamin C) increases the cycle of oxidation–reduction of menadione (vitamin K_3), generates reactive oxygen species (ROS) and leads to a lethal oxidative injury for cancer cells. As outlined in Fig. 1, a redox cycle is initiated by electron transfer from ascorbate to menadione. In agreement with calculations and previsions made by Roginsky et al. [16], we observed a rapid reoxidation of the semiquinone to its quinone form, an enhanced oxygen uptake, and the generation of ROS [5,17]. Moreover, we have recently reported that among these ROS, H_2O_2 appears as the major oxidizing agent formed by redox cycling between ascorbate and menadione, and probably also with other quinone derivatives depending on their half-redox potentials [18]. We took advantage of these features and recently we reported that such an oxidative stress results in an impairment of glycolysis (a critical survival pathway of cancer cells), and leads to cell death and to an in vivo inhibition of cancer cell proliferation [17,19].

Synthetic and natural sesquiterpene quinones represent an important class of biological active molecules. Among these compounds those having an euryfuryl group linked to a benzo- and naphthoquinone moiety are valuable for cytotoxic evaluation studies due to the facile access and variable electron-accepting capacities [20,21]. On the other hand, bioactive quinones such as euryfurylquinones and hydroquinones have been shown to have antiprotozoal activity against *Leishmania amazonensis* [20], antitumor activity [22], inhibition of HIV 1 reverse transcriptase [23] and immunomodulation [24].

The aim of this study was, therefore, to analyse the mechanisms by which potential bioactive substances such as euryfurylbenzoquinones, either alone or in association with vitamin C may affect the survival and the metabolic ability of Transplantable Liver Tumor (TLT) cells. Indeed, we have demonstrated that these cells are deficient in antioxidant enzymes as compared to normal murine hepatocytes [12]. This murine hepatoma-derived cell line has been widely used to assess both in vivo and in vitro anticancer cytotoxic effects [25–27]. Briefly, possible mechanisms involved in such a cell death were investigated. Cell survival was assessed by measuring the % of the cytoplasmic enzyme lactate deshydrogenase (LDH) released into the incubation medium. A hallmark of apoptosis, namely caspase-3 activation, was measured by following the proteolytic cleavage of a synthetic fluorochrome peptide (DEVD-AFC). Finally, additional metabolic end-markers to evaluate the cytotoxicity include both the intracellular contents of ATP and GSH.

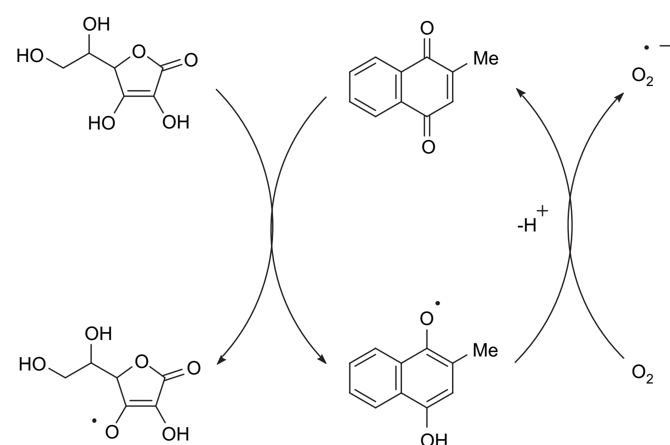
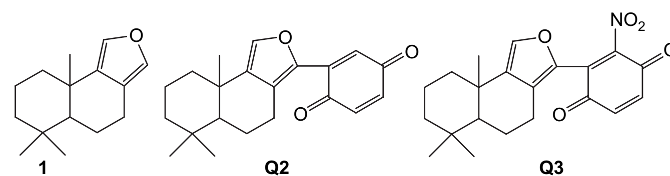


Fig. 1. Ascorbate (vitamin C)–menadione redox cycling. In solution, menadione is non-enzymatically reduced by ascorbate to form dehydroascorbate and the semiquinone free radical. Such a semiquinone radical is rapidly reoxidized to its quinone form by molecular oxygen thus generating superoxide anion radical ($O_2^{\bullet -}$). We hypothesize that hydrogen peroxide is mainly formed due to dismutation of $O_2^{\bullet -}$ either spontaneously or by enzyme catalysis.

2. Chemistry

For the present study, quinones **Q2** and **Q3** were selected as representative members of the euryfurylbenzoquinone series, due to the difference of their redox capacity. In fact, the presence of the electron-withdrawing nitro group on **Q3** should facilitate the electron transfer from ascorbate to quinone moiety compared with the same process on furylquinone **Q2**. Otherwise, it is postulated that **Q3** will act as menadione (as shown in Fig. 1) thus leading to ROS formation. Under such circumstances, a higher cytotoxicity by **Q3** as compared to **Q2** would be expected.



Compounds **Q2** and **Q3** were prepared through oxidative coupling reactions of (+)-euryfuran **1** with 1,4-benzoquinone and 2-nitro-1,4-benzoquinone according to previously published procedures [20,21].

3. Biology

The TLT cells were cultured in DMEM/F12 (Dulbecco's modified eagle medium, Gibco) supplemented with 10% foetal 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/ml. The medium was changed at 48–72 h intervals. All cultures were maintained at 37 °C in a 95% air/5% CO_2 atmosphere with 100% humidity.

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TLT cells were incubated for 24 h at 37 °C in the absence or in the presence of **Q2** and **Q3** at different concentrations (1, 10, and 50 μg/ml). In addition, cells (both untreated and treated with **Q2** or **Q3**) were incubated in the presence of 2 mM vitamin C. Sodium ascorbate (vitamin C) and dimethylsulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were ACS reagent grade.

4. Results

4.1. Biology

Fig. 2 shows the effect of TLT cells on LDH leakage by euryfurylquinone **2** (**Q2**) and **3** (**Q3**), associated or not with vitamin C. In agreement with our hypothesis about electron transfer facility due to the presence of the nitro group, cell death is observed to a large extent when cells were incubated in the presence of **Q3** and vitamin C. Indeed, while **Q2**, at 50 μg/ml, increases the LDH leakage from 20% to 28%, **Q3** induces the same effect already at 1 μg/ml. Nevertheless, when vitamin C was added to both conditions, no change in the % of LDH leakage was observed with **Q2** at 50 μg/ml, but the effect was completely different with **Q3**, since the LDH leakage is reaching more than 60%.

Fig. 3 shows the effects of two euryfurylquinones (**Q2** and **Q3**), either alone or associated to vitamin C, on the intracellular levels of both ATP and GSH. Interestingly, **Q2** induces a decrease in both ATP and GSH levels by about 40% and 60%, in the absence or in the presence of vitamin C, respectively (Fig. 3A). Conversely, when cells were incubated with

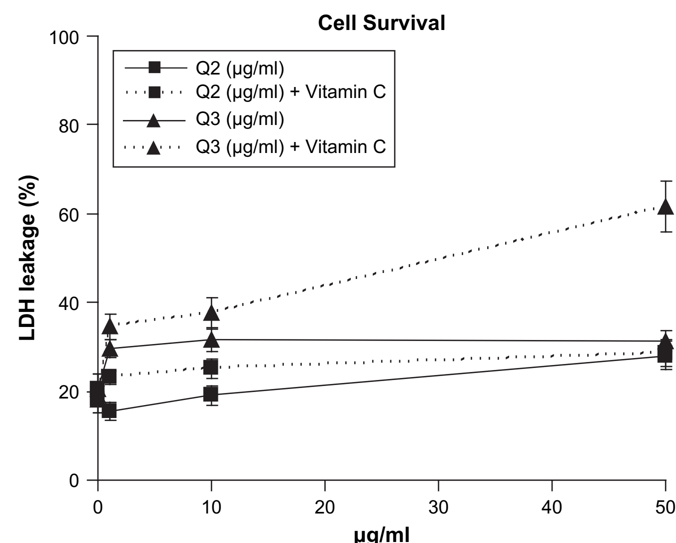

Fig. 2. Effect of compounds **2** and **3,** in the absence or in the presence of vitamin C (VC), on LDH leakage. TLT cells were incubated for 24 h at 37 °C with **Q2** (squares) and **Q3** (triangles) in the absence (solid lines) and in the presence (dashed lines) of 2 mM vitamin C. Aliquots of cell suspension were taken and the activity of LDH was measured as indicated in Section 4. The results are expressed as % of LDH leakage and represent the mean values ± S.E.M. of three different experiments. At the high concentration of 50 μg/ml, the Q3/VC group was significantly different from the three other experimental groups ($p < 0.05$).

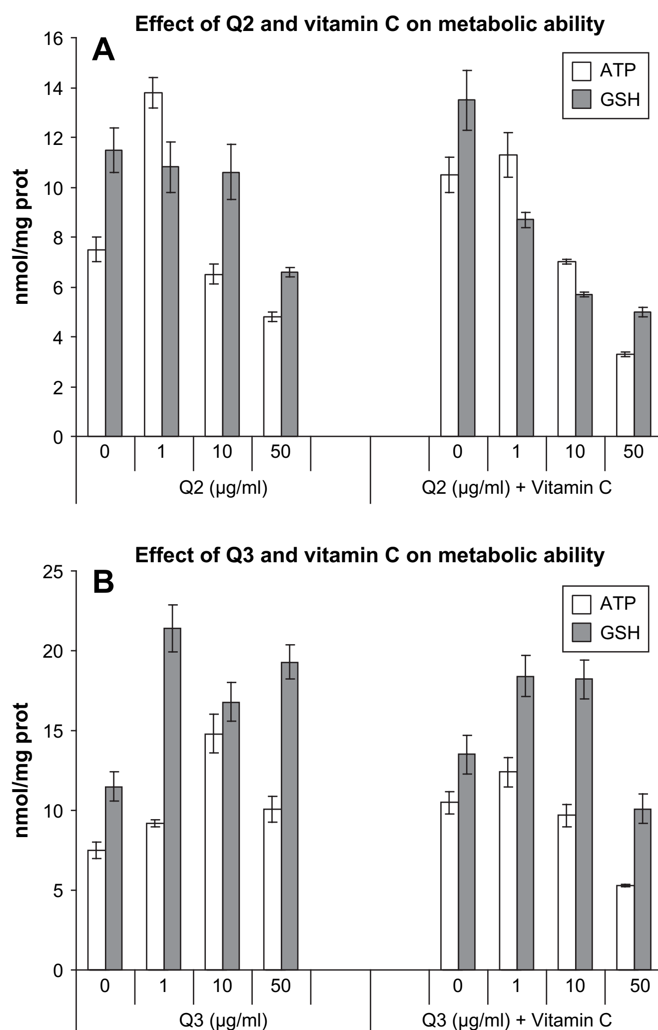

Fig. 3. Influence of vitamin C on metabolic effects induced by **Q2** (A) and **Q3** (B). TLT cells were incubated for 24 h at 37 °C with **Q2** (A) and **Q3** (B) in the absence and in the presence of 2 mM vitamin C (VC). Aliquots of cell suspension were taken and the contents of ATP (open bars) and GSH (closed bars) were measured as indicated in Section 4. The results are expressed as nmol/mg of proteins and represent the mean values ± S.E.M. of three different experiments.

Q3, at low concentrations (1 and 10 μg/ml), higher values as compared to basal condition, of both ATP and GSH were seen regardless of the presence or not of vitamin C (Fig. 3B). Only at the highest concentration (50 μg/ml) and in the presence of vitamin C, **Q3** was able to induce a decrease in the levels of both ATP (by 50%) and GSH (by 25%).

Finally, Table 1 shows the effects of **Q2** and **Q3** on the activity of caspase-3, either in the absence or in the presence of vitamin C. Actually, the increase of enzyme activity by **Q2** (at 50 μg/ml), as compared to basal conditions, was 2.5- and 3.5-times in the absence or in the presence of vitamin C, respectively. Interestingly, **Q3** (at 1 μg/ml) increases more the activity of caspase-3 (31.9 ± 2.8 mU) than **Q2** (21.3 ± 1.9 mU) at the same concentration, but at the highest concentrations it seems that caspase-3 was rather inhibited by **Q3**. This effect was rather confirmed when vitamin C was added to **Q3**-treated cells, since caspase-3 activity was decreased to

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Table 1
Effects of euryfurylbenzoquinones (**Q2**, **Q3**) and vitamin C on caspase-3 activity

		No addition	+Vitamin C (2 mM)
Q2	0 μg/ml	20.1 ± 1.5	16.7 ± 1.2
	1 μg/ml	21.3 ± 1.9	15.1 ± 1.2
	50 μg/ml	37.2 ± 2.5*	25.6 ± 2.1*
	100 μg/ml	51.1 ± 4.3*	57.8 ± 4.5*
Q3	0 μg/ml	20.1 ± 1.5	16.7 ± 1.2
	1 μg/ml	31.9 ± 2.8*	12.8 ± 1.6**
	50 μg/ml	27.4 ± 1.8*	10.7 ± 0.8*,**
	100 μg/ml	22.5 ± 1.7	1.0 ± 0.05*,**

TLT cells were incubated for 24 h at 37 °C with the two euryfurylbenzoquinones (**Q2** and **Q3**) in the absence and in the presence of 2 mM vitamin C. Aliquots of cell suspension were taken and the activity of caspase-3 was measured as indicated in Section 4. The results are expressed as mU and represent the mean values ± S.E.M. of three different experiments.
*$p < 0.05$ as compared to "0 μg/ml" conditions.
**$p < 0.05$ as compared to "no addition" conditions.

values below the basal conditions, and it was completely abolished at 50 μg/ml.

4.2. Chemistry

In order to explain such a difference in the cytotoxicity of these two quinones, we evaluated the electron and Michael acceptor capacity of 3,4-unsubstituted 1,4-benzoquinones **2** and **3** through their LUMO energies [28]. Calculations of the LUMO energies of quinones **Q2** and **Q3** by the semiempirical MM2 method show that quinone **Q3** possesses lower LUMO energy (−1.44739 eV) than quinone **Q2** (−0.681704 eV). Therefore, quinone **Q3** is more reactive to undergo bioreduction and nucleophilic attack processes than **Q2**. These facts could explain the biological differences observed for quinones **Q2** and **Q3**, concerning the necrotic-like cell death induced by **Q3**, and the pathway employed by **Q2** to induce apoptosis. However, an additional non-mitochondrial mechanism of evoking cell death cannot be ruled out [29].

5. Discussion

Our findings reveal that inactivated **Q3** could undergo an activation process by a redox mechanism causing cell death on TLT hepatoma cells, whereas **Q2**, which would be less capable of bioreductive activation, seems to induce apoptosis (as shown by the increased activity of caspase-3), whatever cells were incubated in the absence or the presence of vitamin C. Therefore, the question about the mechanisms conditioning the cell death has been raised. Indeed, by taking in mind the effect of these compounds on both LDH leakage and caspase-3 activity, it is tempting to speculate that **Q3** is killing cells by a necrotic-like cell death, especially when associated with vitamin C, while a putative cell death induced by **Q2** would likely to proceed by apoptosis.

It may be argued that **Q3** (at the lowest concentration of 1 μg/ml) would have a capacity to activate caspase-3, but most probably due to the oxidation of critical cysteinyl groups, it progressively loses this capacity at higher concentrations [30]. To reinforce such a lack of caspase-3 activation, it is tempting to propose that the higher reactivity of **Q3** to undergo a nucleophilic attack may affect the caspase-3 activity. Indeed, active caspase-3 contains five surface-exposed cysteines in addition to the catalytic cysteine, and that allosteric cysteine site is functionally coupled to its active site [31]. Thus, the binding of this compound to the allosteric site of caspase-3 could prevent the peptide binding at this site. This could partially explain the fact that **Q3** (10 and 50 μg/mL) in the presence of vitamin C has a reduced caspase-3 activity.

On the other hand, **Q2** would be less capable of bioreductive activation, or reduction mediated by vitamin C, and have a correspondingly decreased capacity to induce cell death because ROS formation would be less important as compared to **Q3**. It should be noted that although these two quinone-bearing compounds could be involved in a redox cycling process to generate reactive oxygen species (ROS), the presence of an electrophilic quinone double bond should facilitate the Michael addition of cellular nucleophiles to give arylated products that retain the ability to function as redox cycling agents [32]. We would like to hypothesize that such a covalent binding would be then more intense in the mechanism underlying the action of **Q2** as compared to **Q3**, thus explaining the decrease of GSH, and most probably followed by a decrease of ATP (suggesting that glycolysis would be impaired). Conversely, **Q3** would act on TLT cells by mixing both redox properties and covalent binding, at least at the highest concentration. It should be underlined that this hepatoma cell line (TLT) is particularly sensitive to an oxidative injury because they are strongly deficient in antioxidant enzymes as compared to non-transformed murine hepatocytes [12].

Finally, although in normal hepatocytes apoptosis induced by sustained oxidative stress can occur independently of the caspase-3 activation [33], our results in hepatoma cells indicate that **Q3** causes a necrotic-like cell death when associated with vitamin C and/or at high concentrations.

6. Conclusion

We reported in this work a cytotoxic process that takes advantage of tumor metabolism. Indeed, through the generation of an oxidative stress, the combination of one of these quinones with vitamin C induces a necrotic cell death. This mechanism is of interest because cancer cells take up vitamin C, they are expected to be highly sensitive to an oxidant insult and they are dependent on glycolysis (ATP formation) for their survival.

7. Experimental protocols

Cellular viability was estimated by measuring the activity of lactate dehydrogenase (LDH), according to the procedure of Wroblesky and Ladue [34], both in the culture medium and in the cell pellet obtained after centrifugation. The results are expressed as a ratio of released activity to the total activity. ATP content was determined by using the Roche ATP Bioluminescence Assay Kit CLS II (Mannheim, Germany)

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according to procedures described by suppliers, and the results are expressed as nmol ATP/mg proteins. The amount of protein content was determined by the method of Lowry et al. [35] using bovine serum albumin (BSA) as reference. Reduced glutathione (GSH) was measured according to the ortho-phthalaldehyde (oPT) method as reported elsewhere [36] and the results are expressed as nmol GSH/mg proteins. The caspase-3 activity was monitored by cleavage of a specific peptide substrate, Asp-Glu-Val-Asp-AFC (DEVD-AFC) according to the procedure outlined in the instructions for the ‘‘FluorAce apopain assay’’ kit (Biorad). The results are expressed as mU.

Data were analysed 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 analysed using Student's t test. The level of significance was set at $p < 0.05$.

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References

[1] D. Hanahan, R.A. Weinberg, Cell 100 (2000) 57–70.
[2] V. Noto, H.S. Taper, Y.H. Jiang, J. Janssens, J. Bonte, W. De Locker, Cancer 63 (1989) 901–906.
[3] W. De Locker, J. Janssens, J. Bonte, H.S. Taper, Anticancer Res. 13 (1993) 103–106.
[4] H. Sakagami, K. Satoh, Y. Hakeda, M. Kumegawa, Cell Mol. Biol. 46 (2000) 129–143.
[5] J.M. Jamison, J. Gilloteaux, H.S. Taper, J.L. Summers, J. Nutr. 131 (2001) 158–160.
[6] P. Buc Calderon, J. Cadrobbi, C. Marques, N. Hong-Ngoc, J.M. Jamison, J. Gilloteaux, J.L. Summers, H.S. Taper, Curr. Med. Chem. 9 (2002) 2271–2285.
[7] V.E. von Gruenigen, J.M. Jamison, J. Gilloteaux, H.E. Lorimer, M. Summers, R.R. Pollard, C.A. Gwin, J.L. Summers, Anticancer Res. 23 (2003) 3279–3287.
[8] J.M. Jamison, J. Gilloteaux, M.R. Nassiri, N. Venugopal, D.R. Neal, J.L. Summers, Biochem. Pharmacol. 67 (2004) 337–351.
[9] J. Gilloteaux, J.M. Jamison, D.R. Neal, J.L. Summers, Ultrastruct. Pathol. 29 (2005) 221–235.
[10] W. Kassouf, R. Highshaw, G.M. Nelkin, C.P. Dinney, A.M. Kamat, J. Urol. 176 (2006) 1642–1647.
[11] Y. Sun, L.W. Oberley, J.H. Elwell, E. Sierra-Rivera, Int. J. Cancer 44 (1989) 1028–1033.
[12] J. Verrax, J. Cadrobbi, C. Marques, H.S. Taper, Y. Habraken, J. Piette, P. Buc Calderon, Apoptosis 9 (2004) 223–233.
[13] D.B. Agus, J.C. Vera, D.W. Golde, Cancer Res. 59 (1999) 4555–4558.
[14] R.A. Gatenby, E.T. Gawlinski, Cancer Res. 63 (2003) 3847–3854.
[15] H. Kappus, Biochem. Pharmacol. 35 (1986) 1–6.
[16] V.A. Roginsky, T.K. Barsukova, H.B. Stagman, Chem. Biol. Interact. 121 (1999) 177–197.
[17] J. Verrax, J. Stockis, A. Tison, H.S. Taper, P. Buc Calderon, Biochem. Pharmacol. 72 (2006) 671–680.
[18] J. Verrax, M. Delvaux, N. Beghein, H.S. Taper, B. Gallez, P. Buc Calderon, Free Radic. Res. 39 (2005) 649–657.
[19] J. Verrax, S. Vanbever, J. Stockis, H.S. Taper, P. Buc Calderon, Int. J. Cancer 120 (2007) 1192–1197.
[20] J.A. Valderrama, J. Benites, M. Cortés, H. Pessoa-Mahana, E. Prina, A. Fournet, Tetrahedron 58 (2002) 881–886.
[21] J.A. Valderrama, J. Benites, M. Cortés, H. Pessoa-Mahana, E. Prina, A. Fournet, Bioorg. Med. Chem. 11 (2003) 4713–4718.
[22] A.F. Barrero, E. Alvarez-Manzaneda, M. Herrador, R. Chahboun, P. Galera, Bioorg. Med. Chem. Lett. 9 (1999) 2325–2328.
[23] S. Loya, R. Tal, Y. Kashman, A. Hizi, Antimicrob. Agents Chemother. 10 (1990) 2009–2012.
[24] M.L. Bourguet-Kondracki, A. Longeon, E. Morel, M. Guyot, Int. J. Immunopharmacol. 13 (1991) 393–399.
[25] J.D. Duerksen, I.J. Paul, Nucleic Acids Res. 3 (1976) 2277–2291.
[26] H.S. Taper, J. de Gerlache, J.M. Lans, M. Roberfroid, Int. J. Cancer 40 (1987) 575–579.
[27] B.F. Jordan, V. Gregoire, R.J. Demeure, P. Sonveaux, O. Feron, J. O'Hara, V. Vanhulle, N. Delzenne, B. Gallez, Cancer Res. 62 (2002) 3555–3561.
[28] V.F. Traven, Frontier Orbitals and Properties of Organic Molecules, Ellis Horwood Limited, New York, 1992.
[29] G. Tudor, P. Gutiérrez, A. Aguilera-Gutiérrez, E.A. Sausville, Biochem. Pharmacol. 65 (2003) 1061–1075.
[30] M.B. Hampton, S. Orrenius, FEBS Lett. 414 (1997) 552–556.
[31] J.A. Hardy, J. Lam, J.T. Nguyen, T. O'Brien, J.A. Wells, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 12461–12466.
[32] X. Wang, B. Thomas, R. Sachdeva, L. Arterburn, L. Frye, P.G. Hatcher, D.G. Cornwell, J. Ma, Proc. Natl. Acad. Sci. U.S.A. 13 (2006) 3604–3609.
[33] Y. Ishihara, D. Shiba, N. Shimamoto, Free Radic. Res. 39 (2005) 163–173.
[34] F. Wroblesky, J. Ladue, Proc. Soc. Exp. Biol. Med. 90 (1955) 210–213.
[35] O. Lowry, N. Rosebrough, L. Farr, R. Randall, J. Biol. Chem. 183 (1951) 265–275.
[36] V.H. Cohn, J.A. Lyle, Anal. Biochem. 14 (1966) 434–440.