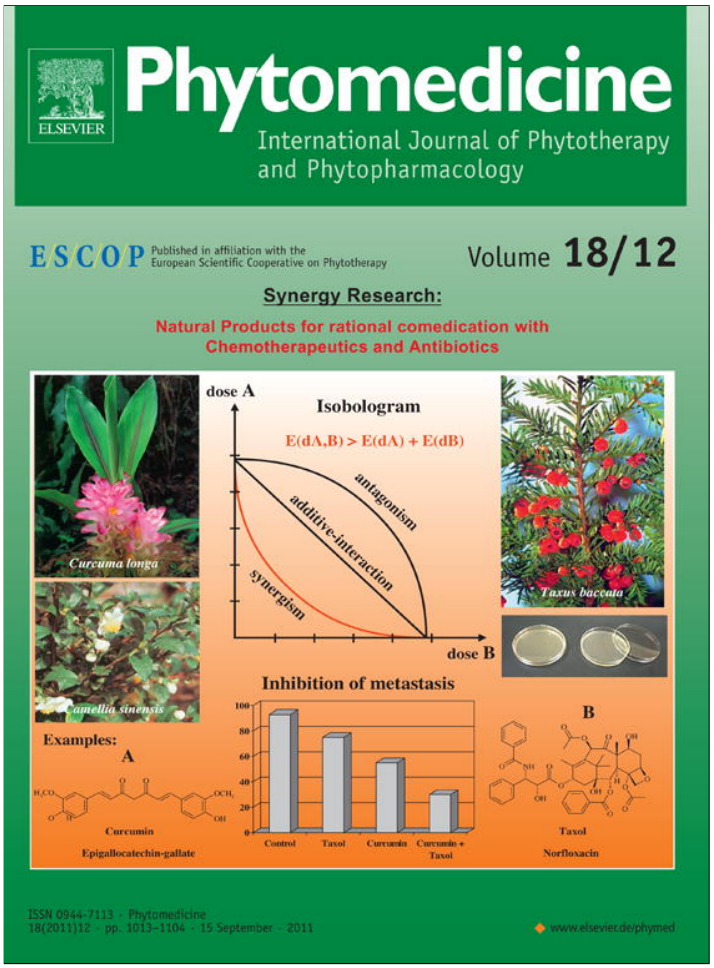

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Short communication

Photodynamic activity of anthraquinones isolated from *Heterophyllaea pustulata* Hook f. (Rubiaceae) on MCF-7c3 breast cancer cells

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ABSTRACT

Searching for agents that could be effective in the treatment of cancer, special highlight has focused on the study of numerous plant-derived compounds. We previously demonstrated that anthraquinones (AQs) isolated from a vegetal species: *Heterophyllaea pustulata* Hook f. (Rubiaceae), such as rubiadin, rubiadin-1-methyl ether, soranjidiol, soranjidiol-1-methyl ether exhibit photosensitizing properties without antecedents as photodynamic agents in malignant cells. In the present study, we investigated the potential role of these AQs as a phototoxic agent against human breast carcinoma using MCF-7c3 cells. All AQs exhibited significant photocytotoxicity on cancer cells at the concentration of 100 μM with 1 J/cm^2 light dose, resulting soranjidiol-1-methyl ether in complete cell destruction. The observed cellular killing by photoactivated AQs exhibited close relation with singlet oxygen production, except for soranjidiol-1-methyl ether, where cell viability decrease is in relation to uptake by tumor cells.

Introduction

Photodymanic therapy (PDT) is based in the cytotoxic effect induced for action combined of ultraviolet or visible light (UV–vis), molecular oxygen and photosensitizer compounds (PS) incorporated selectively into tumor. Thereby, these compounds are activated for the light producing ROS such as superoxide anion ($O_2^{\bullet -}$) and/or singlete oxygen ($^1O_2$) (Gomer et al. 1991; Henderson and Dougherty 1992). These species cause oxidation of several biological molecules with consequent destruction of tumor (Alvarez et al. 2004, 2005).

Among the plant secondary metabolites, AQs have been extensively studied with respect to their UV/vis absorption characteristics and their photosensitizing properties in photodynamic reactions (Gollnick et al. 1992; Gutierrez et al. 1997). Particularly, these sensitizing properties in aminoanthraquinone derivatives have been shown to promote efficient cancer cell photosensitization that is suitable for use in PDT (Pawlowska et al. 2003).

Heterophyllaea pustulata Hook f. (Rubiaceae), native of the mountainous region of the Northwest of Argentina and Bolivia (Bacigalupo 1993), is a phototoxic vegetal species popularly known as "cegadera" (Hansen and Martiarena 1967), which has been the subject of several studies in our group. We succeeded in the isolation and identification of nine AQs: rubiadin 1-methyl ether and soranjidiol 1-methyl ether, damnacanthal, damnacanthol, heterophylline, pustuline and 5,5′-bisoranjidiol (Núñez Montoya et al. 2003, 2006). We have previously demonstrated that these AQs exhibit photosensitizing properties by generation of $O_2^{\bullet -}$ and/or $^1O_2$ (Núñez Montoya et al. 2005; Comini et al. 2007).

Based on its photosensitizing abilities, the aim of this study was to examine the photodynamic activity induced by the majority AQs isolated from *H. pustulata* (rubiadin and soranjidiol) and its methylated derivates (rubiadin 1-methyl ether and soranjidiol 1-methyl ether) such as antitumoral agents against human breast cancer cell line.

Materials and methods

Anthraquinones

Four known AQs: rubiadin (1,3-dihidroxy-2-methyl AQ), rubiadin 1-methyl ether (3-hidroxy-1-methoxy-2-methyl AQ), soranjidiol (1,6-dihidroxy-2-methyl AQ) and soranjidiol 1-methyl ether (6-hidroxy-1-methoxy-2-methyl AQ) were isolated and purified from the stem and leaves of *H. pustulata* by combination of several chromatographic techniques. The identification was made by application of different spectroscopic/spectrometric tech-

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Table 1
Photodynamic properties of natural anthraquinones and quantum yields for $^1O_2$ generation.

Compounds	Concentration (μM)	Cell viability (%)		LD_{50} (μM)	ϕ_Δ
		0 J/cm^2	1 J/cm^2		
Rubiadin	0	101 ± 2	100 ± 5	74	0.34 ± 0.04[a]
	1	100 ± 4	100 ± 3.6		
	50	100 ± 2	68.48 ± 3.4		
	100	100 ± 2	34.24 ± 3		
Rubiadin 1-methyl ether	0	100 ± 2	100 ± 2	98.7	0.00[a]
	1	98.8 ± 8	93 ± 10		
	50	98 ± 8	96 ± 6.3		
	100	98 ± 8	49 ± 10		
Soranjidiol	0	100 ± 2	100 ± 1	37	0.47 ± 0.04[a]
	1	98 ± 4	98 ± 4		
	50	98 ± 5	30.5 ± 8		
	100	98 ± 4	15.5 ± 3		
Soranjidiol 1-methyl ether	0	100 ± 2	100 ± 1	29.6	0.07 ± 0.01[a]
	1	100 ± 10	98 ± 8		
	50	100 ± 5	10 ± 0.5		
	100	100 ± 3	6 ± 1.3		

[a] See Núñez Montoya et al. (2005).

niques (Núñez Montoya et al. 2003). A stock solution of each AQ (2×10^{-3} M) was prepared in dimethylformamide (DMF).

Cells and culture conditions

The human breast cancer MCF-7 (WS8) cell line transfected with the pBabepuro retroviral vector encoding procaspase-3 cDNA (here referred to as MCF-7c3 cells) was provided by Dr. C.J. Froelich (Northwestern University, Evanston, IL). The cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and antibiotics. Cells were maintained in a humidified atmosphere with 5% CO_2/95% air at 37 °C.

Irradiation system

We selected for irradiation the absorption bands of AQs between 410 and 420 nm. The irradiation system comprised a 20 W Phillips actinic lamp (380–480 nm, 0.65 mW/cm^2) with a maximum of 420 nm.

Photodynamic treatment and cell viability determination

Different concentrations of AQs (1–100 μM in PBS) were added to a confluent monolayer of MCF-7c3 cells (2×10^5 cells/dish), incubated overnight (37 °C, 5% CO_2) in DMEM with 10% FBS, and immediately irradiated with 1 J/cm^2 at room temperature. After that, AQ solution was removed and replaced with fresh medium and cells were incubated at 37 °C for additional 24 h. Cell viability was then determinate by quantization of the cleavage of the tetrazolium salt MTT (3-(4,5-dimethylthiazolil-2)-2,5-diphenyltetrazolium bromide – Sigma) by mitochondrial deshydrogenases (Denizot and Lang 1986). Optical density of the resulting solution of formazan salt was read at 540 nm, after subtraction of the blank. Control cells, without irradiation or AQ, were treated under the same conditions. Results are presented as percentage of survival, taking control as 100%. All experiments were carried out in triplicate.

Identification and quantification of anthraquinones uptaken by MCF-7c3 tumor cells

Cells were exposed to 100 μM of each AQ. After incubation at 37 °C for 25 min, the cells were collected by scrapping and immediately lysed by adding 2 ml of 2% sodium dodecyl sulfate. The control samples were processed without AQ under the same working conditions. To extract the AQs from the cells, the samples were firstly treated with hydrochloric acid (HCl) pH 2.0, and then partitioned with chloroform ($CHCl_3$), the resulting solution was called intracellular fraction. The fraction was evaporated to dryness and dissolved in methanol for subsequent analysis by HPLC. A Varian Pro Star chromatograph (model 210, series 04171), equipped with a UV–vis detector and a Microsorb-MV column 100-5 C-8 (250 × 4.6 mm i.d., Varian) was used. The mobile phase was MeOH–H_2O (8:2) at constant flow (1 ml/min). The detection was performed at 269 nm.

Identification of each AQ from intracellular fraction was carried out by comparison of the HPLC retention times (t_R) with the corresponding standards. The AQs were quantified using the external calibration method (Núñez Montoya et al. 2008). Using the calibration curves, the concentration of each AQ in the intracellular fraction was calculated by interpolating the under each peak for each compound. The seven pointed constructed curves ($n = 3$) were linear (correlation coefficients >0.99).

Results

Photodynamic activity and cell viability determination

The photodynamic effect of rubiadin, rubiadin 1-methyl ether, soranjidiol and soranjidiol 1-methyl ether against MCF-7c3 cell line is shown in Table 1. All AQs tested sensitizing irradiated cells to die in a concentration-dependent manner showing to be innocuous without light. Maximal decrease of cell viability was observed a 100 μM with soranjidiol 1-methyl ether (94%), soranjidiol (84.5%), rubiadin (65.76%) and rubiadin 1-methyl ether (51%) (Table 1). The loss of 50% cell population (LD_{50}) was occasioned when incubated cultures with 74 μM rubiadin and were exposed to light, whereas their methylated derivate (rubiadin 1-methyl ether) needed 98.7 μM (Table 1). However, soranjidiol and soranjidiol 1-methyl ether were more photodynamically active for to reach LD_{50}, 37 μM and 29.6 μM, respectively (Table 1). Clearly, complete loss of cell viability could be observed with soranjidiol 1-methyl ether at the higher dose (100 μM) and 1 J/cm^2 irradiation.

Identification and quantification of anthraquinones uptaken by MCF-7c3 tumor cells

Uptake of AQs by tumoral cells was determined by HPLC. The chromatograms corresponding to the intracellular fractions from treated cells with each AQ compared with the chromatograms of

standard AQ and cell control, showed that the four AQs were able of incorporate into cancerous cells.

In addition, HPLC determination showed the amount of each AQ accumulated into cells. The *t*-test was used to determinate the degree of statistical difference between the AQs uptake percentages into cancerous cells. Differences were considered significant at $p < 0.01$. Data analysis demonstrated that soranjidiol 1-methyl ether has the larger incorporation percentage ($34.27 \pm 1.18\%$), followed by soranjidiol ($20.79 \pm 2.37\%$) and rubiadin ($23.20 \pm 2.21\%$) with similar values and finally rubiadin 1-methyl ether with the lowest incorporation percentage ($9.31 \pm 0.52\%$). In spite of the differences observed in the incorporation percentage of each AQ into cancerous cells, all of them produce photodynamic activity *in vitro*.

Discussion

One of the major findings in this study is that the tested AQs exhibit an anti-proliferative effect on MCF-7c3 cancer cells, and only possess the mentioned effect under visible irradiation (380–480 nm), that means under PDT regimen. To further analyze these results, we also observed that soranjidiol 1-methyl-ether was a potent inhibitor of MCF-7c3 cells growth, mediated via phototoxic reaction, more than soranjidiol and similarly rubiadin and rubiadin 1-methyl-ether on the basis of LD_{50}.

The analysis of Table 1 allows us to estimate that photodynamic activity of these AQs in cancer MCF-7c3 cells would be mediated by the production of $^1O_2$ in agreement with Dalla Via and Magno (2001), whom reported that PDT effect of most photosensitizers is mediated by the production of $^1O_2$ since its electrophilic nature renders it very efficient in producing oxidized forms of biomolecules thus initiating the damage. The observed cell killing induced by photoactivated AQs has close relation with $^1O_2$ production (Table 1), except for soranjidiol 1-methyl ether ($LD_{50} = 29.6\ \mu M$; $\phi_\Delta = 0.07$). It is also accepted that PS can generate $^1O_2$ both in cell culture medium and within cells, depending on the location of the PS. However, in the first case, very few of the generated $^1O_2$ actually reach the cells and the chances of cellular alterations are limited ($^1O_2$ exists 4 μs in aqueous solution and during this time it diffuse only about 100 nm) (Kochevar and Redmond 2000). Therefore, the photocytotoxicity exhibited by soranjidiol 1-methyl ether could be explained by AQ uptake found in cancer cells ($34.27 \pm 1.18\%$), statistically greater than the remaining AQs ($p < 0.01$), which denotes that intracellular AQ would be the main cause of the anti-cancer effect *in vitro*.

Our results confirmed that AQ internalization is essential for PDT-mediated by $^1O_2$ photodynamic action in MCF-7C3 cells, due to the fact that soranjidiol and rubiadin have similar uptake ($p > 0.01$), but soranjidiol, with high $^1O_2$ production, shows a potent antitumor effect on human cancer cell line.

In addition, rubiadin 1-methyl ether reported the lesser photodynamic effect ($LD_{50} = 98.7\ \mu M$), which would be related not only by absent of chemical structural conditions in order to pushing $^1O_2$ yield up (Table 1), but also to exhibiting the minimal uptake by tumor cells ($9.31 \pm 0.52\%$). The moderate photodynamic effect would be associated with $O_2^{\bullet -}$ production (Núñez Montoya et al. 2005).

The data from the present study allow us to conclude that the effect of AQs photoactivated would be close related to $^1O_2$ production leaving evidence the relevance of PS intracellular localization.

In summary, these AQs tested, isolated from *H. pustulata*, could be promising chemotherapeutic candidates as anticancer PDT agents, highlighting soranjidiol 1-methyl for having the best antiproliferative effect on breast cancer in humans *in vitro*. Further studies are still required to reveal the detailed mechanism of cell death induction.

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