Revista Brasileira de Farmacognosia 25 (2015) 634–640

Revista Brasileira de Farmacognosia
BRAZILIAN JOURNAL OF PHARMACOGNOSY
www.sbfgnosia.org.br/revista

Original Article

Perezone, from the gorgonian *Pseudopterogorgia rigida*, induces oxidative stress in human leukemia cells

Paula A. Abreu[a,b], Diego V. Wilke[a], Ana J. Araujo[a,c], José Delano B. Marinho-Filho[a,c], Elthon G. Ferreira[a], Carlos Margo R. Ribeiro[d], Leandro S. Pinheiro[d], Juliana W. Amorim[d], Alessandra L. Valverde[d], Rosângela A. Epifanio[d,†], Letícia V. Costa-Lotufo[a,e], Paula C. Jimenez[a,f,*]

[a] *Departamento de Fisiologia e Farmacologia, Universidade Federal do Ceará, Fortaleza, CE, Brazil*
[b] *INSERM U1113, Strasbourg University, Strasbourg, France*
[c] *Faculdade de Medicina, Universidade Federal do Piauí, Parnaíba, PI, Brazil*
[d] *Instituto de Química, Universidade Federal Fluminense, Niterói, RJ, Brazil*
[e] *Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brazil*
[f] *Departamento de Ciências do Mar, Universidade Federal de São Paulo, Santos, SP, Brazil*

ARTICLE INFO

Article history:
Received 1 May 2015
Accepted 20 July 2015
Available online 4 September 2015

Keywords:
Gorgonian coral
Marine natural products
Cytotoxicity
Quinone
ROS

ABSTRACT

Four bisabolanes **1**–**4**, including perezone (**1**) and triacetyl perezone (**2**), were isolated through a bioassay-guided fractionation of the extract obtained from the Caribbean gorgonian coral *Pseudopterogorgia rigida* collected during an expedition cruise to the Bahamas. All isolated compounds showed to be cytotoxic toward panel of four human tumor cell lines, as quantified by the MTT assay after 72 h incubation. Perezone (**1**), the most active one, was further analyzed, showing to be cytotoxic, but not selective, in a 12-cell line panel comprising tumor and non-tumor, as well as human and murine cells. Additionally, **1** was assayed for cytotoxicity against HL-60 leukemic cells. Pre-treatment with an acute free radical scavenger (L-NAC) before exposure of cells to perezone virtually eliminated the generation of intracellular ROS and lessened its severe cytotoxicity. The protective effect delivered by L-NAC evidences that the mechanism of perezone-induced cytotoxicity is partially associated to production of ROS and a consequent induction of oxidative stress.

Introduction

Natural or synthetic quinones perform various functions in organisms, as some are tightly involved in the cell's biochemical mechanisms (Asche, 2005). Nevertheless, quinones have been extensively studied for their cytotoxic potential and, currently, are an important group of anticancer drugs. Many quinoid compounds, such as the anthracyclines doxorubicin and daunorubicin and the antibiotic mitomycin, are successfully applied in various cancer chemotherapy schemes (Asche, 2005; Vásquez et al., 2010).

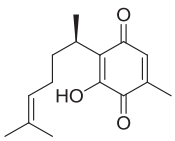
Perezone (**1**) was first isolated in 1852 from roots of *Perezia* mangrove plants (renamed *Accourtia*). This quinone showed insecticide activity against *Leptinotarsa decemlineata* and *Myzus persicae* (Burgueño-Tapia et al., 2008) and several pharmacological activities, such as laxative and anti-parasitic (Rubio et al., 1997). The study by García et al. (1995) evaluated the effects of **1** on intestinal contractility of smooth muscle of rats and showed that this quinone seems to increase the availability of intracellular Ca^{+2}. Conversely, De la Peña et al. (2001) disclosed **1** as an inhibitor of platelet aggregation and suggested that this feature was associated to the impairment in Ca^{2+} mobilization of internal stores. Furthermore, **1** was considered to be a hit in a 96 drug *in vitro* screen directed against neuroblastoma cells, a tumor that remains incurable in over 60% of cases (Gheeya et al., 2009). More recently, Sánchez-Torres et al. (2010) evaluated the cytotoxic properties of perezone on K562 cells and demonstrated that the apoptotic effects may be dependent or independent of caspases activation, depending on concentration.

The present study describes the isolation of **1**, along with other known compounds **2**–**4**, from the Caribbean gorgonian *Pseudopterogorgia rigida* and the respective cytotoxic activity on a panel of tumor cell lines. Additionally, we used HL-60 cells (acute promyelocytic leukemia) to characterize the respective mode of

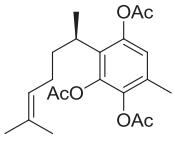
* Corresponding author.
E-mail: pcjimenez@unifesp.br (P.C. Jimenez).
† in memorian.

http://dx.doi.org/10.1016/j.bjp.2015.07.020
0102-695X/

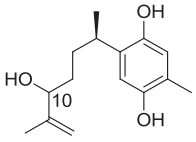
action, which largely involves the generation of reactive oxygen species.



1



2



3, 4 (epimers at C-10)

Materials and methods

General procedures

High-performance liquid chromatography was carried out using a semipreparative normal phase (silica gel) Dynamax column (15 cm length, i.d. 10 mm), a Waters M6000 pump and a Waters R401 differential refractometer. NMR spectra were recorded in $CDCl_3$ solution on a Varian Unity 500 MHz spectrometer. IR spectra were recorded on a Perkin-Elmer model 1600 (FTIR) spectrometer. Mass measurements were obtained on a HP5989A spectrometer. Vacuum liquid chromatography (VLC) was performed with silica gel for thin layer chromatography. Isolation procedures were monitored using thin-layer chromatography (TLC) on pre-coated silica gel plates (Merck, Kieselgel 60 F-254) through UV inspection and H_2SO_4/heat.

Collection and identification of biological material

Fourteen colonies of the gorgonian were collected by scuba diving at 15–20 m depth at Sweating Cays, Bahamas, during an expedition onboard the research vessel Seward Johnson in June 1996. The colonies were labeled and air-dried for 2 h. A small amount of each colony was extracted with CH_2Cl_2, and the extracts compared by TLC analysis were further classified as a single species, *P. rigida*, as identified by Dr. Frederick M. Bayer (National Museum of Natural History, Smithsonian Institution, and a voucher specimen (ATPH/MO/57) has been deposited at the Herbarium of the Department of Pharmacognosy and Chemistry of Natural Products, University of Athens.

Extracts, fractions and pure compounds from P. rigida

Gorgonian tissues (300 ml, about 25–30 g of dry weight) were cut in to small pieces and extracted with a mixture of MeOH–CH_2Cl_2, 1:1 (once), and pure CH_2Cl_2 (twice). The extracts were combined and evaporated under reduced pressure affording a brownish gum (12 g). The crude extract was fractionated by vacuum liquid silica gel chromatography (VLC), employing a gradient ranging from 0 to 100% of EtOAc in $(CH_3)_3CCH_2CH(CH_3)_2$ (TMP: 2,2,4-trimethylpentane) and MeOH to yield eleven fractions (F1–F11). Fraction 5 (2.67 g) was purified by successive re-crystallizations using mixtures of hexane and ethyl acetate, yielding pure perezone (**1**) (0.64 g) as intense yellow crystal with mp 102 °C. Fraction 7 (1.56 g) and 11 (39 mg) were further purified by normal phase semi-preparative HPLC using hexane or TMP and ethyl acetate mixtures to give, respectively, compound **2** (10.7 mg) and compounds **3** (8 mg) and **4** (9 mg).

Compound ***1*** (perezone; (*R*)-3-hydroxy-5-methyl-2-(6-methylhept-5-en-2-yl)cyclohexa-2,5-diene-1,4-dione). Mp 102 °C, ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.19 (3H, d, J = 7.0 Hz), 1.53 (3H, sl), 1.57 (1H, m), 1.65 (3H, d, J = 1.8 Hz), 1.82 (2H, m), 1.90 (1H, m), 2.06 (3H, d, J = 1.8 Hz), 3.05 (1H, m), 5.07 (1H, mt, J = 7.2 Hz), 6.48 (1H, q, J = 1.8 Hz), 7.00 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 14.6 (CH_3), 17.5 (CH_3), 18.1 (CH_3), 25.6 (CH_3), 26.6 (CH_2), 29.2 (CH), 34.0 (CH_2), 124.5 (CH), 124.5 (C), 131.3 (C), 135.5 (CH), 140.4 (C), 150.9 (C), 184.2 (C), 187.3 (C).

Compound ***2*** (triacetyl perezone; (*R*)-6-methyl-3-(6-methylhept-5-en-2-yl)benzene-1,2,4-triyl triacetate). Colorless oil, ^{1}H NMR ($CDCl_3$, 300 MHz): 1.20 (3H, d, J = 7.1 Hz), 1.54 (3H, sl), 1.59 (2H, m), 1.64 (3H, d, J = 1 Hz), 1.87 (2H, dd, J = 15.0 Hz; J = 7.5 Hz), 2.14 (3H, d, J = 0.7 Hz), 2.29 (3H, s), 2.29 (3H, s), 2.28 (3H, s), 2.82 (1H, ddq, J = 7.2 Hz; J = 7.2 Hz; J = 7.2 Hz), 5.04 (1H, tdq, J = 7.1 Hz; J = 1.4 Hz, J = 1.4 Hz), 6.82 (1H, d, 0.7 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.0 (CH_3), 17.5 (CH_3), 18.8 (CH_3), 20.2 (CH_3), 20.3 (CH_3), 21.0 (CH_3), 25.6 (CH_3), 26.2 (CH_2), 30.9 (CH), 35.2 (CH_2), 124.0 (CH), 129.5 (C), 129.9 (C), 131.7 (C), 139.2 (C), 141.4 (C), 146.3 (C), 167.6 (C), 167.8 (C), 168.9 (C). EI-MS m/z 376 (M+).

Compound ***3*** (2-((2*R*)-5-hydroxy-6-methylhept-6-en-2-yl)-5-methylbenzene-1,4-diol). Yellowish oil. IV (film, CH_2Cl_2) νmax 3339, 2958, 1650, 1423, 1191 e 1006 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.20 (3H, d, J = 7.0 Hz), 1.26 (1H, m), 1.48–1.59 (3H, m), 1.66 (3H, s), 2.16 (3H, s), 2.76 (1H, sl), 3.13 (1H, m), 4.14 (1H, m), 4.81 (1H, s), 4.94 (1H, s), 6.58 (1H, s) e 6.60 (1H, s); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 15.5 (CH_3), 17.6 (CH_3), 21.1 (CH_3), 30.8 (CH), 31.9 (CH_2), 34.8 (CH_2), 76.8 (CH), 110.9 (CH_2), 113.0 (CH), 118.4 (CH), 122.1 (C), 131.5 (C), 146.8 (C), 147.6 (C) e 147.8(C); HR-ESIMS (positive mode) m/z 273.1467 [M+Na$^+$] (calcd for $C_{15}H_{21}O_3$, 272.1388).

Compound ***4*** (2-((2*R*)-5-hydroxy-6-methylhept-6-en-2-yl)-5-methylbenzene-1,4-diol). Yellowish oil. IV (film, CH_2Cl_2) νmax 3334, 2956, 1651, 1442, 12195 e 1001 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.15 (3H, d, J = 7.0 Hz), 1.26 (1H, m), 1.42–1.55 (3H, m), 1.66 (3H, s), 2.14 (3H, s), 2.66 (1H, sl), 3.03 (1H, m), 4.05 (1H, m), 4.82 (1H, s), 4.93 (1H, s) e 6.57 (2H, s); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 15.6 (CH_3), 17.9 (CH_3), 21.2 (CH_3), 31.7 (CH), 32.1 (CH_2), 33.3 (CH_2), 76.2 (CH), 110.8 (CH_2), 113.2 (CH), 118.1 (CH), 122.1 (C), 131.5 (C), 146.7 (C), 147.2 (C) e 147.8(C); HR-ESIMS (positive mode) m/z 273.1467 [M+Na$^+$] (calcd for $C_{15}H_{21}O_3$, 272.1388).

Cytotoxicity assay

Human tumor cells (HL-60, SF 295, MDA MB435, MDA MB 231 and MX 1) and the murine cell line B16 were obtained from the National Cancer Institute, Bethesda, USA. HCT-8 cell line was donated by the Children's Mercy Hospital, Kansas City, MO, USA. Non-tumor cells, L929 e J744, were kindly donated by Dr. C.I. Oliveira (Centro de Pesquisa Gonçalo Muniz, Fiocruz, Bahia, Brazil). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 g/ml streptomycin at 37 °C with 5% CO_2. For all experiments, cells were plated in 96-well plates (10^5 cells/well for adherent cells or 0.5×10^5 cells/well for suspended cells in 100 μl of medium). After 24 h, crude extract, fraction and pure compounds were dissolved in DMSO, added to each well using a high-throughput screening system (Biomek 3000–Beckman Coulter, Inc. Fullerton, CA, USA), and incubated for 72 h. β-lapachone was used as a positive control. Negative control groups received the same amount of DMSO. Alternatively, perezone-treated cells were pre-incubated with L-NAC (*N*-acethyl-L-cystein), an antioxidant widely used as an experimental tool to increase intracellular free radical scavengers, during 1 h then washed with PBS prior to addition of the compound. In these circumstances, cells were subsequently exposed during 24 or 72 h. Cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to a purple formazan product. At the end of the incubation period, plates were centrifuged and then medium was replaced with fresh medium (150 μl) containing MTT (0.5 mg/ml). Three hours later, the plates were centrifuged, the MTT-formazan product was dissolved in 150 μl DMSO, and the absorbance was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada). The drug effect was quantified

as the percentage of the control absorbance of the reduced dye at 595 nm. Data are presented as mean ± S.E.M. The IC_{50} values and their 95% confidence intervals (CI 95%) were obtained by non-linear regression using the GraphPad Software 4.0 (Intuitive Software for Science).

Phenotypic characterization of perezone effects

Flow cytometry was used to further characterize the cytotoxicity of perezone on the human promyelocitic cell line HL-60. Perezone (**1**) was tested at 2, 4 and 8 μM. Doxorubicin 0.5 μM was used as the positive control and DMSO 1%, the dilution vehicle of all samples, served as the negative control.

Cell density and viability

HL-60 cells were seeded in a 24-well plate (3×10^5 cells/ml), cultured for 24 h to initiate cell growth and treated, as previously described. Cells were harvested after 3, 6, 12, 24, 28 and 72 h of exposure, centrifuged and resuspended in a 5 μg/ml propidium iodide (PI) solution (Sigma–Aldrich). After 5 min incubation in the dark, five thousand events were acquired using the Guava EasyCyte Mini flow cytometer (Guava Technologies) and the Guava Express Plus software on a gated region to exclude debris and doublets from the analysis. Concentration of cells in each treatment and membrane integrity were evaluated and plotted in an XY graph using GraphPad Software 4.0 (Intuitive Software for Science).

Cell cycle and DNA fragmentation

HL-60 cells were seeded in a 24-well plate (3×10^5 cells/ml), cultured for 24 h to initiate cell growth and treated, as previously described. After exposure for 3 or 24 h, cells were harvested, centrifuged and resuspended in a solution containing 5 μg/ml PI, 0.1% citrate and 0.1% Triton X–100. After 30 min incubation in the dark, five thousand events were acquired using the Guava EasyCyte Mini flow cytometer (Guava Technologies) and the Guava Express Plus software on a gated region to exclude debris and doublets from the analysis. Cell cycle histograms were acquired using the FL3 channel (excitation at λmax 488 nm and emission λmax 620 nM) to capture the red fluorescence emitted from PI–DNA complexes. The cell cycle profile (G_1/G_0–S–G_2/M) was analyzed in linear scale with the ModFit LT 4.0 software (Verity Software House). Sub-G_1 cells were pondered as having fragmented DNA. The differences between negative control and experimental groups were determined by analysis of variance (ANOVA) followed by Dunnet post-test on GraphPad Software 4.0 (Intuitive Software for Science). The minimal significance level was set at $p < 0.05$.

Analysis of the mechanisms involved in the cytotoxic activity of perezone

Further experiments were carried out to evaluate the influence of reactive oxygen species (ROS) in the cytotoxicity induced by **1** on human promyelocitic cell line HL-60. Perezone was tested at 2, 4 and 8 μM. β-lapachone 2 μM was used as the positive control and DMSO 1%, the dilution vehicle of all samples, served as the negative control. Before exposure, cells were pre-treated with L-NAC 5 μM during 1 h.

Morphological analysis with May–Grunwald–Giemsa staining

HL-60 cells were seeded in a 24-well plate (3×10^5 cells/ml), cultured for 24 h to initiate cell growth and pre-treated and treated, as previously described. After exposure for 24 h to all treatment types, cells were harvested, transferred to cytospin slides, fixed with methanol for 10 s and stained with May–Grunwald–Giemsa (Bioclin, Brazil). Slides were examined for morphological changes *via* light microscopy (Olympus, Tokyo, Japan).

Trypan blue exclusion

HL-60 cells were seeded in a 24-well plate (3×10^5 cells/ml), cultured for 24 h to initiate cell growth and pre-treated and treated, as previously described. After exposure for 24 h to all treatment types, cells were harvested, dyed with Trypan blue and differentially counted for viable (not dyed) or non-viable (dyed) counted in a Neubauer chamber. The differences between groups were determined by analysis of variance (ANOVA) followed by Student Newman–Keuls post-test on GraphPad Software 4.0 (Intuitive Software for Science). The minimal significance level was set at $p < 0.05$.

Measurement of reactive oxygen species generation

HL-60 cells were seeded in a 24-well plate (3×10^5 cells/ml), cultured for 24 h to initiate cell growth and pre-treated and treated, as previously described. After exposure for 1 h to all treatment types, cells were loaded with H_2-DCF-DA and incubated for 30 min in the dark at 37 °C. Cells were then harvested, washed, resuspended in PBS and immediately analyzed. Five thousand events were acquired using the Guava EasyCyte Mini flow cytometer (Guava Technologies) and the Guava Express Plus software on a gated region to exclude debris and doublets from the analysis, using excitation and emission wave lengths of 490 and 530 nm, respectively. The differences between groups were determined by analysis of variance (ANOVA) followed by Student Newman–Keuls post-test on GraphPad Software 4.0 (Intuitive Software for Science). The minimal significance level was set at $p < 0.05$.

Results

Bioassay-guided fractionation of P. rigida

The CH_2Cl_2/MeOH extract of *P. rigida* exhibited a strong cytotoxicity with IC_{50} values ranging from 8.8 μg/ml in SF-295 cells to 43.2 μg/ml in MDA-MB-435 cells (Table 1). Crude extract was purified by vacuum liquid chromatography in silica gel using a gradient of increasing polarity of TMP/EtOAc/MeOH yielding eleven fractions (F1–F11). Fractions F5 and F6 were the most active (Table 1) and analysis by TLC and ^{1}H NMR revealed the presence of an almost pure compound that when crystallized with hexane/ethyl acetate give pure intense yellow crystals with mp 102 °C. Spectrometric and spectroscopic data revealed that this compound is perezone (**1**). From F8, the major component was isolated by semi-preparative normal phase HPLC (EtOAc/hexane), yielding triacethyl perezone **2** as a colorless oil. IC_{50} for this compound ranged between 23.1 and 49.8 μM across a four human tumor cell line panel (Table 2). The most polar fraction, F11, was also purified by normal phase HPLC (EtOAc/TMP), yielding two pure bisabolanes **3** and **4**. ^{1}H and ^{13}C NMR data of these compounds are almost identical, and revealed them to be epimers at C-10 stereochemistry. The IC_{50} value calculated for **3** varied from 10.8 to 59.8 μM, while **4** retained nearly half the cytotoxicity strength of his epimer (Table 2).

Cytotoxicity of perezone

Perezone (**1**) showed cytotoxicity against all cell lines after 72 h incubation. For tumor cells, IC_{50} values ranged from 8.7 to 32.9 μM, while, for the normal murine cell lines L929 and J774, IC_{50} was calculated within that range, 16.3 and 11.5 μM, respectively (Table 2). Further studies on HL-60 cells showed that **1** induced a time and concentration decrease in cell density and viability (Fig. 1A and

Table 1
IC_{50} (CI95%) (μg/ml) of total extract and 11 fractions obtained from chemical fractionation of a *Pseudopterorgia rigida* organic extract on tumor cell lines in culture determined by the MTT assay over 72 h incubation.

Sample	HL-60	HCT-8	SF-295	MDA-MB435
P. rigida crude extract	14.73	9.28	8.80	43.12
	9.87–21.98	8.46–10.18	6.45–12.02	29.76–62.47
P. rigida F1	11.06	11.90	13.36	12.38
	n.d.	n.d.	9.87–18.08	7.93–19.33
P. rigida F2	>100,0	>100,0	>100,0	>100,0
P. rigida F3	>100,0	>100,0	>100,0	n.d.
P. rigida F4	65.41	37.32	29.62	79.83
	34.55–123.8	30.82–45.18	27.96–31.38	51.25–124.3
P. rigida F5	4.81	4.85	2.09	8.31
	4.27–5.43	4.31–5.47	1.86–2.35	7.72–8.94
P. rigida F6	1.75	4.12	2.03	8.91
	1.58–1.93	3.67–4.62	1.32–3.13	7.14–11.13
P. rigida F7	13.10	14.49	7.12	33.49
	9.42–18.22	11.86–17.70	5.878–8.62	18.56–60.44
P. rigida F8	2.28	8.43	20.35	5.96
	1.81–2.88	6.66–10.67	17.11–24.22	5.20–6.83
P. rigida F9	7.53	28.25	32.63	20.89
	5.61–10.10	22.99–34.72	27.88–38.18	19.38–22.52
P. rigida F10	41.37	47.20	59.38	33.86
	32.87–52.07	35.06–63.56	49.25–71.59	18.38–62.36
P. rigida F11	15.91	39.39	21.00	13.98
	13.59–18.62	33.15–46.79	16.71–26.38	12.38–15.79

n.d., not determined or not rated.

Table 2
IC_{50} (μM) and 95% confidence intervals of compounds **1**–**4** obtained from a *Pseudopterorgia rigida* organic extract on tumor cell lines in culture determined by the MTT assay over 72 h incubation. The results are presented as IC_{50} (μM) values, with 95% confidence intervals. β-lapachone was used as positive control.

Cel line	Histotype	IC_{50} (95% CI) μM				
		1	**2**	**3**	**4**	β-lapachone
HL-60	Promyelocytic leukemia	8.7	33.2	23.7	38.4	1.6
		7.5–10.2	n.d.	4.6–7.7	30.9–47.8	1.5–1.8
HCT-8	Colon	17.9	23.1	59.8	89.4	0.8
		14.5–22.3	18.8–28.4	36.5–97.9	43.1–185.4	0.7–0.9
SF 295	Brain	15.4	49.6	10.8	16.9	0.9
		13.8–17.1	34.2–71.7	8.2–14.2	12.4–23.2	0.7–1.1
MDA MB 435	Breast cancer	22.0	30.6	32.4	66.9	0.2
		18.7–25.9	26.1–35.9	27.8–37.8	55.2–81.1	0.2–0.3
MDA MB 231	Breast cancer	13.2	n.d.	n.d.	n.d.	n.d.
		11.3–15.5				
MX 1	Breast cancer	25.2	n.d.	n.d.	n.d.	n.d.
		n.d.				
B 16	Murine melanoma	24.2	n.d.	n.d.	n.d.	n.d.
		19.1–30.8				
J774	Murine macrophage	11.5	n.d.	n.d.	n.d.	n.d.
		9.8–13.5				
L929	Murine fibroblast	16.32	n.d.	n.d.	n.d.	n.d.
		11.7–22.82				

n.d., not determined or not rated.

B). Precisely, during the first 24 h, cultures exposed to **1** suffered a rapid decline in cell number and membrane integrity, especially under higher concentrations. Alterations in the cell cycle of perezone-treated cells with 4 and 8 μM were detectable after 24 h, and showed a significant and rather striking increase of the G_2/M phase, accompanied by a decrease in the S phase (Fig. 1C). Internucleosomal fragmentation, however, was detected only in cells treated with **1** at 8 μM (Fig. 1D).

Involvement of ROS generation in cytotoxicity induced by perezone

Interestingly, cytotoxic activity of **1** on HL-60 cells proved stronger after a shorter exposure time, as IC_{50} value increased a 3.5-fold between 24 and 72 h. Nevertheless, when HL-60 cells were pre-incubated with L-NAC, those became less sensitive to perezone-induced cytotoxicity, as IC_{50} increased about 8 times after 24 h treatment and, after 72 h, toxicity was undetectable (Table 3).

Substantial morphological changes were observed in HL-60 cells treated with **1** or β-lapachone, the later considered, herein, as the positive control (Fig. 2A). In β-lapachone-treated cells, vacuoles in the cytoplasm could be observed, along with further characteristics of apoptosis, such as reduced volume, membrane blebbing and nuclear fragmentation. Additionally, a considerable number cells resembling necrosis, were also observed after 24 h treatment. Perezone-treated cells showed volume reduction and some cellular aggregation. Little DNA fragmentation and some membrane rupture were detected. Moreover, picnotic cells were widespread in slides. Such characteristics seamed to retain concentration dependency. Cells pre-treated with L-NAC prior to exposure to **1** showed fewer signs of toxicity, especially those related to apoptosis, at all tested concentrations.

Cell viability was assessment by exclusion of trypan blue dye indicated that **1** reduced the number of viable HL-60 cells and

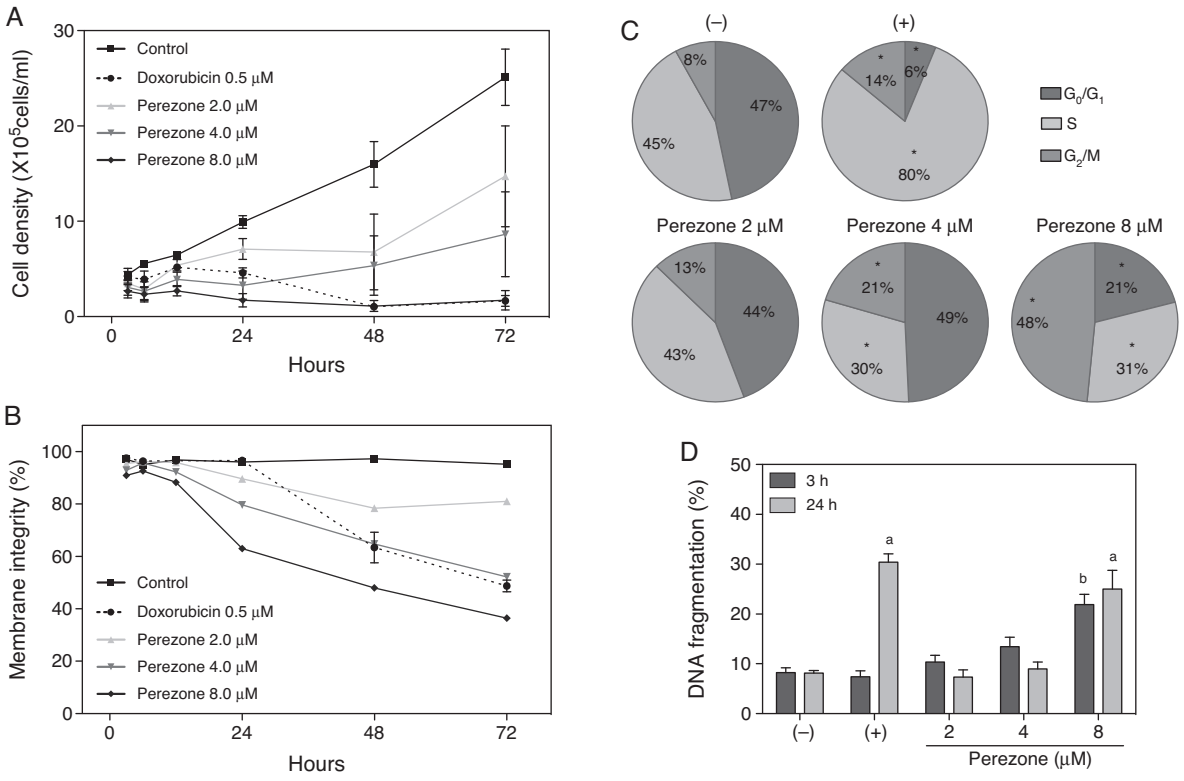


Fig. 1. Characterization of perezone (**1**) cytotoxicity on HL-60 cells. (A) Depicts the effects on cell growth after 3, 6, 12, 24, 48 and 72 h exposure; (B) the effects on membrane integrity after 3, 6, 12, 24, 48 and 72 h; (C) the effects on cell cycle after 24 h exposure; and (D) the effects on internucleossomal fragmentation after 3 and 24 h exposure. Cells were either treated with DMSO 1% (−), 0.5 μM doxorubicin (+), or with **1** at 2, 4 or 8 μM, and analyzed by flow cytometry after staining with PI. Data are presented as mean values ± standard error of the mean (S.E.M.) from three independent experiments, each conducted in triplicate. Five thousand events were acquired for each replicate and compared with the respective controls by ANOVA and followed by Dunnett's comparison test. In (C), * indicates statistically significantly different from (−) ($p < 0.05$). In (D), a, indicates statistically significantly different from (−) at 24 h ($p < 0.05$); and b, indicates statistically significantly different from (−) at 3 h ($p < 0.05$).

Table 3
Cytotoxicity of perezone evaluated by the MTT assay in HL-60 cells after 24 or 72 h exposure, pre-treated or not pre-treated for 1 h with L-NAC. The results are presented as IC_{50} (μM) values, with confidence interval 95%.

Treatment	24 h	72 h
Perezone	2.45 2.01–2.98	8.747.46–10.18
Perezone + NAC	20.3313.29–30.6	>25

increased the number of non-viable ones in a concentration-dependent manner (Fig. 2B). Pre-incubation with L-NAC for 1 h was effective in lessening perezone-induced cytotoxicity, but not sufficient to reverse the effects prompted especially by higher concentrations on neither viable and non-viable cell count. It is noteworthy that the present data showed a reduction in the number of viable cells in control groups when incubated only with L-NAC, which indicates that this antioxidant itself has a mild cytotoxicity.

Intracellular reactive oxygen species (ROS) accumulation was monitored using 2,7-dichlorodihydrofluorescein diacetate (H_2-DCF-DA), which is converted to the highly fluorescent dichlorofluorescein (DCF) in the presence of intracellular ROS. Perezone (**1**) showed to be a strong producer of intracellular ROS, and this effect could be perceived after merely 1 h exposure (Fig. 2C). Pre-incubation of cells with L-NAC, as expected, weakened most of this effect.

Discussion

Marine secondary metabolites containing interesting biomedical properties have been isolated from many organisms, including bacteria, algae, sponges, bryozoans, mollusks, ascidians, and cnidarians (Costa-Lotufo et al., 2009). From a chemical standpoint, within the cnidarians, the greatest attentions in this group are sessile and soft body organisms, such as corals, gorgonians and zoanthids. Marine Gorgonacea Octocorals are known to be a rich source of biologically active terpenoids, which are an important part in ecological relationships (Martins and Epifanio, 1998). Several studies carried out with pseudopterosin, a diterpene glycoside isolated from *Pseudopterogorgia elisabethae* species, demonstrated a potent anti-inflammatory activity (Ata et al., 2003; Mayer et al., 1998).

The Caribbean gorgonian *P. rigida* (Bielschowsky, 1929) belongs to the phylum Cnidaria, class Anthozoa and it is known for producing a number of sesquiterpenes, of which most are quinones and hydroquinones (McEnroe and Fenical, 1978; Freyer et al., 1997; D'Armas et al., 2000). Some of these compounds seem to play important ecological roles in the host organism, such as defense against predation. In fact, curcuphenol and curcuhydroquinone proved to be responsible for the feeding deterrent activity of the crude extract obtained from *P. rigida* against generalist fishes (Harvell et al., 1988). In plants, bisabolanes are also allelopatic compounds with herbicidal and antifeedant activities, hence recruiting agrochemical interests (Burgueño-Tapia et al., 2008).

Perezone (**1**) has been previously reported in extracts of *P. rigida* (Georgantea et al., 2013), besides being isolated from the dry roots of *Perezia* spp. (Enrıquez et al., 1980). Its spectral data are also identical to those previously described (Joseph-Nathan et al., 1972). Compound **2** has been described before as a semisynthetic derivative of **1**, and is reported herein for the first time as a natural product. The NMR data are according to the literature (Joseph-Nathan et al., 1977). Compounds **3** and **4** have recently been isolated

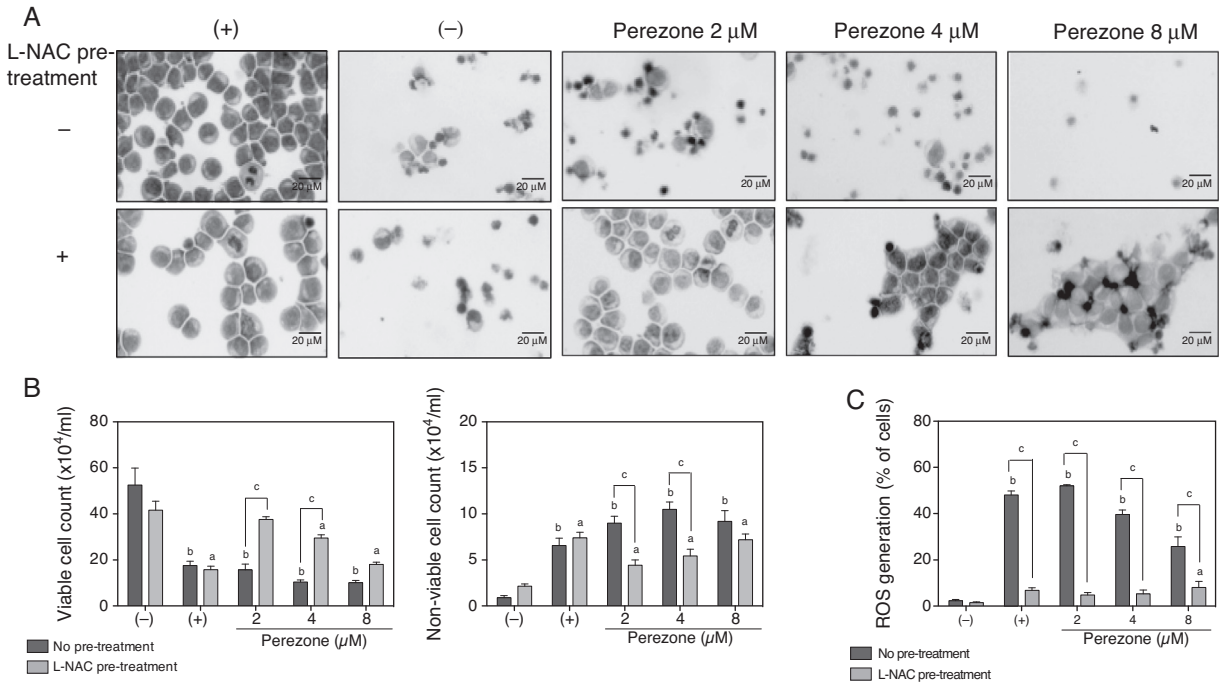


Fig. 2. Influence of reactive oxygen species (ROS) on the cytotoxicity induced by perezone (**1**) on HL-60 cells with or without 1 h pre-treatment with 5 μM L-NAC 5. (A) Depicts representative structural effects on cell morphology by May–Grümwald–Giemsa staining after 24 h exposure; (B) the effects on cell viability by exclusion of trypan blue dye after 24 h exposure; and (C) the effects on ROS generation by H_2-DCF-DA staining and flow cytometry analysis after 1 h exposure. Cells were either treated with DMSO 1% (−), 2 μM β-lapachone (+), or with **1** at 2, 4 or 8 μM. Data are presented as mean values ± standard error of the mean (S.E.M.) from three independent experiments, each conducted in triplicate, and compared by ANOVA followed by the Student Newman–Keuls test. a, indicates statistically significantly different from (−) with L-NAC pre-treatment ($p < 0.05$); b, indicates statistically significantly different from (−) without L-NAC pre-treatment ($p < 0.05$); and c, indicates statistically significantly different from their respective treatment counter part ($p < 0.05$).

from this specie and the NMR data are also according to the literature (Georgantea et al., 2014).

A natural product screening carried out by the National Cancer Institute (NCI) has identified that quinones are an important pharmacological element with cytotoxic activity (Pérez-Sacau et al., 2007). Accordingly, **1** showed to be cytotoxic in a four-human tumor cell line panel. Furthermore, bisabolanes **2**–**4** also showed the same kind of activity, however to a lesser potency (Table 2). Perezone (**1**) was not selective between tumor and non-tumor cells, and its cytotoxicity seemed to be related to apoptosis inducing properties in leukemia cells. These findings are in agreement with a previous study from Sánchez-Torres et al. (2010) showing that **1** is cytotoxic to K562 leukemic cells and can cause mitochondrial depolarization in a concentration dependent manner.

Quinoidal structures are widespread in nature and are highly redox active molecules. Such reactivity leads to the production of ROS (reactive oxygen species), a normal mitochondrial metabolic by-product, which, in excess, triggers cellular oxidative stress through the oxidation of macromolecules (Bolton et al., 2000). These proprieties, nonetheless, can be explored for the development of new drugs in cancer therapy. Production of ROS is involved in induction of apoptosis and this death pathway is considered the most appropriate when it comes to provoking tumor cells death (Bironaite et al., 2004). One of the main features responsible for quinone cytotoxicity is the alkylation of cellular nucleophiles, such as DNA, lipids and proteins, and therefore, their ability to generate ROS. The one electron reduction from a quinone compound produces a semi-quinone radical, which, in turn, generates ROS while altering mitochondrial transmembrane potential. This alteration can interfere in some cell signs associated with death pathways (Tudor et al., 2003). Perezone (**1**) has been shown to be both, an electron-donor and an electron-acceptor, and these oxido-reduction proprieties inhibit mitochondrial electron transport at 50 μM (Carabez and Sandoval, 1988).

Glutathione is an acute free radical neutralizer due to its role as a substrate to intracellular ROS scavenging enzymes (Circu and Aw, 2008). L-NAC is a precursor of glutathione and, therefore, an important antioxidant. Thus, L-NAC has been widely used as tool for investigating the involvement of ROS in cytotoxicity (Sun, 2010). Perezone (**1**) is a potent inducer of intracellular ROS in HL-60 cells, and this effect can be observed after as little as 1 h exposure (Fig. 2C). Pre-treatment with L-NAC, on the other hand, showed competence to virtually eliminate measurable ROS induction by **1** and β-lapachone. Actually, L-NAC desensitized HL-60 to perezone-induced cytotoxicity, and IC_{50} increased at least 8-fold after 24 h exposure (Table 3). The protective effect delivered by L-NAC is evidence that the mechanism of perezone-induced cytotoxicity is partially associated to generation of ROS and a consequent induction of oxidative stress.

Generation of ROS can be linked with activation of apoptosis pathways (Ozben, 2007) and many quinones share this mechanism. Perezone-treated cells displayed structural features compatible with the induction of apoptosis and, also, of necrosis. However, these severe morphological alterations declined in the presence of L-NAC. Conversely, cells exposed to β-lapachone were clearly not shielded by pre-treatment with L-NAC, although generation of ROS was completely reversed in the later condition. Moreover, in positive and negative controls alike, there were a number of cells with reduced volume and highly stained nucleus. The decrease in L-NAC protective effect at the higher concentration of **1** suggests that necrosis, rather than apoptosis, may be the prevailing death pathway in such circumstances, in which there is a smaller influence of ROS (Zong and Thompson, 2006).

Other quinones, such as that isolated from the roots of *Cordia leucocephala*, also induce apoptosis and necrosis by a mechanism involving oxidative stress. This naphthoquinone, rapidly induced ROS-related death in HL-60 cells after only 3 h exposure (Marinho-Filho et al., 2010). Studies also suggest that several cytotoxic agents applied clinically in cancer chemotherapy, like doxorubicin, bleomycin and cisplatin, induce stress through ROS generation in tumor cells (Tiligada et al., 2002).

Conclusion

Perezone (**1**), isolated herein from the bisabolane-rich tropical gorgonian *P. rigida*, is a cytotoxic quinone with low selectivity that induces cells death trough a mechanism that is associated, though incompletely, with the excessive generation of intracellular reactive oxygen species and consequent induction of oxidative stress.

Conflicts of interest

The authors declare no conflicts of interest.

Author contribution

CMRR, LSP, JWA, ALV and RAE conducted the extract fractionation, natural product isolation and structure elucidation efforts, including the MS and NMR studies. PAA, PCJ, DVW, JDBMF, AJA and EGF conducted biological assays with fractions and pure compounds, including cytotoxicity, flow cytometry and microscopy analysis. PCJ, ALV, CMRR, LVCL and RAE developed the project and interpreted the results. All authors contributed to the writing.

Acknowledgements

We thank Dr. Frederick Bayer, National Museum of Natural History Smithsonian Institution, for the gorgonian identification, and the staff at the Mass Spectrometry Center, University of California-Riverside, for providing HRMS. We also thank Dr. William Fenical, from Scripps Institution of Oceanography, University of California San Diego, for structural support during the collection expedition and plentiful scientific discussions. This work was supported by grants from the United States National Science Foundation (NSF) and the Brazilian funding agency Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

Asche, C., 2005. Antitumor quinones. Mini Rev. Med. Chem. 5, 449–467.
Ata, A., Kerr, R.G., Moyab, C.E., Jacobs, R.S., 2003. Identification of anti-inflammatory diterpenes from the marine gorgonian *Pseudopterogorgia elisabethae*. Tetrahedron 59, 4215–4222.
Bironaite, D., Kalvelyte, A.V., Imbrasaite, A., Stulpinas, A., 2004. The intracellular antioxidant balance of HL-60 cells and its implication in the apoptosis induced by quinoidal compounds. Biologia 1, 48–51.
Bolton, J.L., Trush, M.A., Penning, T.M., Dryhurst, G., Monks, T.J., 2000. Role of quinones in toxicology. Chem. Res. Toxicol. 13, 135–160.
Burgueño-Tapia, E., Castillo, L., Gonzalez-Coloma, A., Joseph-Nathan, P., 2008. Antifeedant and phytotoxic activity of the sesquiterpene p-benzoquinone perezone and some of its derivatives. J. Chem. Ecol. 34, 766–771.
Carabez, A., Sandoval, F., 1988. The action of the sesquiterpenic benzoquinone perezone, on electron transport in biological membranes. Arch. Biochem. Biophys. 260, 293–300.
Circu, M.L., Aw, T.Y., 2008. Glutathione and apoptosis. Free Radic. Res. 42, 689–706.
Costa-Lotufo, L.V., Wilke, D.V., Jimenez, P.C., Epifanio, R.A., 2009. Organismos marinhos como fonte de novos fármacos: histofico & perspectivas. Quim. Nova 32, 703–716.
D'Armas, H.T., Mootoo, B.S., Reynolds, W.F., 2000. An unusual sesquiterpene derivative from the Caribbean gorgonian *Pseudopterogorgia rigida*. J. Nat. Prod. 63, 1593–1595.
Enrıquez, R., Ortega, J., Lozoya, X., 1980. Active components in *Perezia* roots. J. Ethnopharmacol. 2, 389–393.
De la Peña, A., Izaguirre, R., Baños, G., Viveros, M., Enriquez, R.G., Fernández-G, J.M., 2001. Effect of perezone, aminoperezone and their corresponding isomers isoperezone and isoaminoperezone upon *in vitro* platelet aggregation. Phytomedicine 8, 465–468.
Freyer, A.J., Patil, A.D., Kilmer, L., Zuber, G., Meyer, C., Johnson, R.K., 1997. Rigidone, a sesquiterpene o-quinone from the gorgonian *Pseudopterogorgia rigida*. J. Nat. Prod. 60, 309–311.
García, X., Alcántara-Sarabia, G., Cartas-Heredia, L., Gijón, E., 1995. Actions of perezone on rat smooth muscle. Gen. Pharmacol. 26, 1741–1745.
Georgantea, P., Ioannou, E., Vagias, C., Roussis, V., 2014. Bisabolane and chamigrane sesquiterpenes from the soft coral *Pseudopterogorgia rigida*. Phytochemistry. Lett. 8, 86–91.
Georgantea, P., Ioannou, E., Vagias, C., Roussis, V., 2013. Perezoperezone and curcuperezone: bisabolane dimers from the soft coral *Pseudopterogorgia rigida*. Tetrahedron Lett. 54, 6920–6922.
Gheeya, J.S., Chen, Q.R., Benjamin, C.D., Cheuk, A.T., Tsang, P., Chung, J.Y., Metaferia, B.B., Badgett, T.C., Johansson, P., Wei, J.S., Hewitt, S.M., Khan, J., 2009. Screening a panel of drugs with diverse mechanisms of action yields potential therapeutic agents against neuroblastoma. Cancer Biol. Ther. 8, 2386–2395.
Harvell, C.D., Fenical, W., Greene, C.H., 1988. Chemical and structural defenses of Caribbean gorgonians (*Pseudopterogorgia* spp). 1. Development of an *in situ* feeding assay. Mar. Ecol. Prog. Ser. 49, 287–294.
Joseph-Nathan, P., González, M.P., Rodríguez, V.M., 1972. Terpenoids of *Perezia hebeclada*. Phytochemistry 11, 1803–1808.
Joseph-Nathan, P., Mendoza, V., García, E., 1977. The reaction mechanism of the perezonepipitzol transformation. Tetrahedron 33, 1573–1576.
Marinho-Filho, J.D., Bezerra, D.P., Araújo, A.J., Montenegro, R.C., Pessoa, C., Diniz, J.C., Viana, F.A., Pessoa, O.D., Silveira, E.R., Moraes, M.O., Costa-Lotufo, L.V., 2010. Oxidative stress induction by (+)-cordiaquinone J triggers both mitochondria-dependent apoptosis and necrosis in leukemia cells. Chem. Biol. Interact. 183, 369–379.
Martins, D.L., Epifanio, R.A., 1998. A new Germacrane Sesquiterpene from the Brazilian Endemic Gorgonian *Phyllogorgia dilatata* Esper. J. Braz. Chem. Soc. 9, 586–590.
Mayer, A.M.S., Jacobson, P.B., Fenical, W., Jacob, R.S., Glasei, K.B., 1998. Pharmacological characterization of the pseudopterosins: novel anti-inflammatory natural products isolated from the Caribbean soft coral, *Pseudopterogorgia elisabethae*. Life Sci. 62, 401–407.
McEnroe, F.J., Fenical, W., 1978. Structures and synthesis of some new antibacterial sesquiterpenoids from the gorgonian coral *Pseudopterogorgia rigida*. Tetrahedron 125, 1661–1664.
Ozben, T., 2007. Oxidative stress and apoptosis: impact on cancer therapy. J. Pharm. Sci. 96, 2181–2196.
Pérez-Sacau, E., Díaz-Peñha, R.G., Estévez-Braun, A.E., Ravelo, A.G., García-Castellano, J.M., Pardo, L., Campillo, M., 2007. synthesis and pharmacophore modeling of naphthoquinone derivatives with cytotoxic activity in human promyelocytic leukemia HL-60 cell line. J. Med. Chem. 50, 696–706.
Rubio, M., Ramirez, G.G., Jimenez, F.G., Salcedo, R., Belmont, M.A., 1997. About perezone derivatives, a theoretical approach. J. Mol. Struct. (Theochem) 397, 239–248.
Sánchez-Torres, L.E., Torres-Martinez, L.A., Godínez-Victoria, M., Omar, J.M., Velasco-Bejarano, B., 2010. Perezone and its isomer isoperezone induce caspase-dependent and caspase-independent cell death. Phytomedicine 17, 614–620.
Sun, S-Y., 2010. *N*-acetylcysteine, reactive oxygen species and beyond. Cancer Biol. Ther. 9, 109–110.
Tiligada, E., Miligkos, V., Delitheos, A., 2002. Cross-talk between cellular stress, cell cycle and anticancer agents: mechanistic aspects. Curr. Med. Chem. Anti-cancer Agents 2, 553–566.
Tudor, G., Gutierrez, P., Aguilera-Gutierrez, A., Sausville, E.A., 2003. Cytotoxicity and apoptosis of benzoquinones: redox cycling, cytochrome c release, and BAD protein expression. Biochem. Pharmacol. 65, 1061–1075.
Vásquez, D., Rodríguez, J.A., Theoduloz, C., Calderon, P.B., Valderrama, J.A., 2010. Studies on quinones. Part 46. Synthesis and *in vitro* antitumor evaluation of aminopyrimidoisoquinolinequinones. Eur. J. Med. Chem. 45, 5234–5242.
Zong, W-X., Thompson, C.B., 2006. Necrotic death as a cell faith. Genes Dev. 20, 1–15.