Phytomedicine xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Synergistic enhancement of antitumor effect of β-Lapachone by photodynamic induction of quinone oxidoreductase (NQO1)

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ARTICLE INFO

Keywords:
β-Lapachone
Photodynamic therapy
NQO1
Synergy
Isobologram
MCF-7c3 cells

ABSTRACT

β-Lapachone is a phytochemotherapeutic originally isolated from Lapacho tree whose extract has been used medicinally for centuries. It is well known that NAD(P)H:quinone oxidoreductase (NQO1) activity is the principal determinant of β-Lapachone cytotoxicity. As NQO1 is overexpressed in most common carcinomas, recent investigations suggest its potential application against cancer. Photodynamic therapy (PDT) is a clinically approved and rapidly developing cancer treatment. PDT involves the administration of photosensitizer (PS) followed by local illumination with visible light of specific wavelength. In the presence of oxygen molecules, the light illumination of PS can lead to a series of photochemical reactions and consequently the generation of cytotoxic reactive oxygen species (ROS). It has been reported that β-Lapachone synergistically interacts with ionizing radiation, hyperthermia and cisplatin and that the sensitivity of cells to β-Lapachone is closely related to the activity of NQO1. So, the present study aimed to investigate the feasibility of PDT to increase the anticancer effect of β-Lapachone by up-regulating NQO1 expression on breast cancer MCF-7c3 cells. NQO1 expression was evaluated by Western blot analysis at different times after PDT using ME-ALA as PS. The cytotoxicity of the photodynamic treatment and β-Lapachone alone or in combination was determined by MTT assay and the combination index (CI)-isobologram method and the dose reduction index (DRI) analysis were used to assess the effect of drug combinations. Our studies for the first time demonstrated that the expression of NQO1 is induced 24 h after photodynamic treatment. The sensitivity of cancer cells to β-Lapachone treatment increased 24 h after PDT and a synergistic inhibitory effect on MCF-7c3 cells was showed. Taken together, these results lead us to conclude that the synergistic interaction between β-Lapachone and PDT in killing cells was consistent with the up-regulation of NQO1. The combination of β-Lapachone and PDT is a potentially promising modality for the treatment of cancer.

Introduction

Among women worldwide, breast cancer appears as the most common malignant neoplasm, as well as being a significant cause of dying by cancer. The abundance of clinical research surrounding anticancer agents, together with ongoing cancer biology research, is expected to further increase the available pool of therapeutic options for breast cancer. Nowadays, alternative cytotoxic combination strategies are being explored both clinically and preclinically to identify mechanisms for synergy between agents (Luu et al. 2011).

β-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]pyran-5,6-dione) is an ortho naphthoquinone originally obtained from the leaves and inner bark of the Lapacho tree (*Tabebuia avelanedae*) in South America. Compounds containing the quinone moiety are found in numerous natural products and often are associated with different pharmacological activities, such as fungicide (Freire et al. 2010), antimalarial (Sharma et al. 2013), trypanocidal (Carneiro et al. 2012), antitubercular (Ferreira et al. 2010) and antibacterial (Francisco et al. 2010).

More specifically, β-Lapachone has demonstrated cytotoxic activity against a variety of cancer cells both *in vitro* and *in vivo*, even at micromolar doses, so it was postulated as a novel antitumor agent (Pardee et al. 2002). It is well known that the enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1) is the principal

Abbreviations: NQO1, NAD(P)H:quinone oxidoreductase 1; PDT, photodynamic therapy; PS, photosensitizer; ME-ALA, methyl aminolevulinate acid; PpIX, protoporphyrin IX; ROS, reactive oxygen species; CI, combination index; DRI, dose reduction index; ARE, antioxidant response element; HO-1, heme oxygenase 1; Nrf2, nuclear factor E2-related factor 2.

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http://dx.doi.org/10.1016/j.phymed.2013.04.018

determinant of β-Lapachone cytotoxicity. NQO1 catalyzes the redox cycling of β-Lapachone through the generation of an unstable hydroquinone (HQ), which under aerobic conditions, is rapidly oxidized back to the parent quinine (Q). This futile cycling between Q and HQ forms of β-Lapachone using NADH or NAD(P)H as electron sources results in severe depletion of intracellular reducing power, leading to a rapid increase in intracellular calcium, mitochondrial membrane depolarization, loss of ATP, DNA fragmentation and apoptosis (Siegel et al. 2012). It was previously reported that cisplatin (Terai et al. 2009), ionizing radiation (Park et al. 2005a; Suzuki et al. 2006; Choi et al. 2007) and hyperthermia (Park et al. 2005b; Song et al. 2008; Dong et al. 2009; Hori et al. 2011) sensitizes cancer cells to β-Lapachone treatment by up-regulating NQO1 activity.

Photodynamic therapy (PDT) is a minimally invasive therapeutic modality approved for clinical treatment of several types of cancer and non-oncological disorders. PDT is based on tumor specific accumulation of a compound with photosensitizing properties (photosensitiser, PS), followed by irradiation with visible light, resulting in cell death and tumor ablation. 5-Aminolevulinic acid (ALA) and its derivative methyl aminolevulinate (ME-ALA) are the photosensitizing precursor agents most widely used in clinical practice. These are considered to be "prodrugs" which enter in the heme biosynthetic pathway to be converted to protoporphyrin IX (PpIX) that acts as an endogenous PS (Wachowska et al. 2011). Unlike traditional chemotherapy, selectivity is derived from both the ability of useful PSs to localize in neoplastic lesions and the precise delivery of light to the treated sites. Activated PSs transfer energy to molecular oxygen, generating singlet oxygen ($^1O_2$) and other highly reactive oxygen species (ROS), such as superoxide radical anion ($O_2\cdot-$), hydrogen peroxide (H_2O_2) or hydroxyl radical ($HO\cdot$). These cytotoxic photoproducts start a cascade of biochemical events that induces damage and death of neoplastic cells (Agostinis et al. 2011).

In the present report, we investigated the cytotoxic effect of β-Lapachone in combination with PDT using ME-ALA as PSs (ME-ALA/PDT) *versus* phytochemotherapy or photochemotherapy alone for treatment of human breast cancer MCF-7c3 cells. The combination index (CI)-isobologram method and the dose reduction index (DRI) analysis were used to assess the effect of drug combinations. The purpose of this study was to test the hypothesis that ME-ALA/PDT-induced ROS might provoke upregulation of NQO1 in cancer cells. It was observed that ME-ALA/PDT significantly upregulates NQO1 in cancer cells, thereby markedly increasing the sensitivity of MCF-7c3 cells to β-Lapachone treatment.

Materials and methods

Materials

β-Lapachone was synthetized by Prof. Vitor F. Ferreira (Universidade Federal Fluminense, Niterói, Brazil) and dissolved in dimethyl sulfoxide (DMSO) to make a 6 mM stock solution stored at −20 °C. γ-Aminoleavulinic acid methyl ester hydrochloride (ME-ALA) was purchased from SIGMA and dissolved in sterile water to make a 10 mM stock solution stored at 4 °C. For the *in vitro* studies, both drugs were diluted to desired concentrations in DMEM medium (Gibco) without fetal bovine serum (FBS) immediately before use.

Cell cultures

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 (Gibco) containing 10% FBS (Gibco), 2 mM glutamine (Sigma), and antibiotic–antimycotic mixture (Gibco). Cells were maintained in a humidified atmosphere with 5% CO_2/95% air at 37 °C.

Treatments

Exponential-phase MCF-7c3 cells at a density of 1×10^5 cells/ml were plated onto 96-well plates, using 100 μl per well. Following an overnight incubation, cells were subjected to different treatments as detailed below:

(a) β-Lapachone (phytochemotherapy): β-Lapachone (1–6 μM) was added and incubated for 4 h (37 °C, 5% CO_2) in DMEM without FBS. Drug solutions were then removed and replaced with fresh medium and cells were incubated at 37 °C, 5% CO_2/95% air for additional 24 h (Fig. 1A).
(b) ME-ALA/PDT (photochemotherapy): ME-ALA (0.5 mM) was added and incubated for 4 h (37 °C, 5% CO_2) in DMEM without FBS. After that, cells were irradiated with different light doses (0.25–2 J/cm^2) at room temperature with monochromatic light source (635 nm ± 17 nm) using a MultiLED system (coherent light). The fluence rate on the cell monolayer was 1.6 mW/cm^2 (as measured by Radiometer Laser Mate-Q, Coherent). Drug solution was then removed and replaced with fresh medium and cells were incubated at 37 °C, 5% CO_2/95% air for additional 24 h (Fig. 1A).
(c) Combination treatments (Combs 1–15, Table 1): Cells were incubated firstly with ME-ALA 0.5 mM for 4 h and then irradiated with 1, 1.5 and 2 J/cm^2 (ME-ALA/PDT). Following 24 h incubation, β-Lapachone was added at various concentrations (2, 2.3, 2.8, 3 and 3.5 μM) for 4 h. Drug solutions were then removed and replaced with fresh medium and cells were incubated at 37 °C, 5% CO_2/95% air for additional 24 h (Fig. 3A).

Cell viability determination

Cell viability was then determined following 24 h of treatment by quantization of the reduction of the tetrazolium salt MTT (3-(4,5-dimethylthiazolil-2)-2,5-diphenyltetrazolium bromide, Sigma) by mitochondrial dehydrogenases. 10 μl MTT solution (5 mg/ml in phosphate buffer saline, PBS) was added and incubated for 3 h. Then, 100 μl DMSO was added to each well to lyse the cells and solubilize the precipitated formazan crystals product. Optical density of the resulting solution of formazan salt was read at 540 nm. Control cells, without irradiation or drug, were treated under the same conditions. The proliferation inhibitory rates were calculated using the following formula:

$$\text{Inhibitory rate } (\%) = \frac{1 - (\text{absorbance of treated well} - \text{absorbance of blank})}{(\text{absorbance of control} - \text{absorbance of blank}) \times 100}$$

Analysis of combinatory treatment effect

MTT assays as described above were used to evaluate the inhibitory efficacy of the mixtures of the agents. For quantification of synergy two measurements were employed: the combination index (CI)-isobologram method and the dose reduction index (DRI), using CompuSyn software (ComboSyn Inc., Paramus, NJ), based on the median–effect equation derived from the mass-action law. Briefly, the median–effect equation describes dose–effect relationships, allows the construction of the median–effect plot and provides parameters for the calculation of CI and DRI. When two or more drugs are combined and the CI is calculated, CI < 1, =1, and >1 indicates synergism, additive effect, and antagonism, respectively. Due to the fact that this study design was made with non-constant

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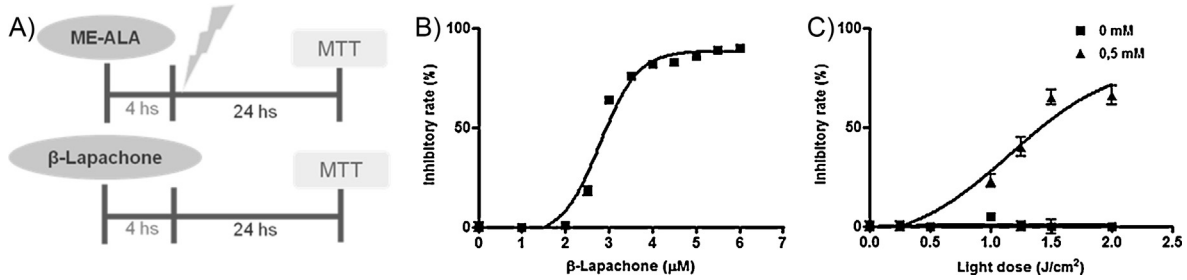

Fig. 1. The inhibitory effect of β-Lapachone or ME-ALA/PDT on MCF-7c3 cells. (A) Experimental design: MCF-7c3 cells were treated with β-Lapachone or ME-ALA/PDT alone. Exponentially growing cells in culture (1×10^5 cells/ml) were treated with 0–6 μM of β-Lapachone (B) or 0.5 mM ME-ALA (C) for 4 h. After incubation with ME-ALA, cells were irradiated with different light doses (0.25–2 J/cm^2). Drug solutions were then removed and replaced with fresh medium and cells were incubated for additional 24 h. The proliferation inhibitory rates were evaluated by MTT assay.

dose rations, a normalized isobologram was constructed after calculating CI for each of the drugs and was related to the IC50 for each of the separately administered drugs. This was graphed on the X [$D_{\text{ME-ALA}}/(\text{IC50})_{\text{ME-ALA}}$] and Y [$D_{\beta\text{-Lapachone}}/(\text{IC50})_{\beta\text{-Lapachone}}$] axes; $D_{\text{ME-ALA}}$ and $D_{\beta\text{-Lapachone}}$ are the doses that, when combined, produce a %X of inhibited proliferation. The oblique line indicates the theoretical doses that produce an additive effect. If the points obtained from the experimental agent combinations were below the line, the interaction was considered to be synergistic and if they were above the line it was considered to be antagonistic. On the other hand, the DRI measures how many folds the dose of each drug in a synergistic combination may be reduced to a given effect level; the greater DRI value indicates a greater dose reduction (Berenbaum 1989; Chou 2006).

Immunoblot analysis

Expression of NQO1 was analyzed by Western blotting. Total cell lysates from control and photodynamic-treated cells were extracted with lysis buffer containing 20 mM HEPES pH 7.5; 1.5 mM KCl; 1 mM EDTA; 1 mM EGTA; 0.15% Triton-X100; 1 mM PMSF; 1 mM DTT; and a cocktail of protease inhibitors (Sigma). The protein content of the lysate was measured using BCA protein assay reagent (Pierce). Aliquots containing 30 μg of protein were separated by 12% SDS-PAGE and then transferred onto PVDF membranes (Sigma). Blots were blocked with 5% nonfat dry milk in PBS Tween 0.1% (PBST), incubated with anti-NQO1 antibody (1:2000 dilution in 5% nonfat dry milk in PBST, Santa Cruz Biotech), and treated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:3000 dilution in 5% nonfat dry milk in PBST, Cell Signaling). Immunoreactive bands detection was carried out using the enhanced chemoluminescence (ECL) kit (Amersham) according to the manufacturer's instructions. Equal sample loading was confirmed by reprobing the same blots with anti-α-tubulin (1/4000 dilution in PBST, Sigma). Densitometric analysis was performed using the public domain Java image processing program ImageJ (developed at the National Institutes of Health and available at http://rsb.info.nih.gov/ij/index.html). Control and post-treatment signals were normalized to the α-Tubulin signals. The normalized target signals were then compared to the normalized control signal for fold-change (normalized sample signal/normalized control signal).

Statistical analyses

All assays were carried out in triplicate. All data are expressed in mean ± standard deviation. The treatments conditions required to inhibit 50% of the cell growth was determined from dose–response curves and defined as the IC50 value. Graphs were drawn using GraphPad Prism® software (GraphPad Prism® 5.01, GraphPad Software Inc., CA, USA). One-way analysis of variance and Bonferroni's *post hoc* test were used to analyze differences between the sets of data. A p-value less than 0.05 was considered significant.

Table 1
Summary of synergistic, additive or antagonistic effects of combinations between β-Lapachone and ME-ALA/PDT. CI values were interpreted as follows: 0.7–0.85 moderate synergism (++), 0.90–1.10 nearly additive (±), 1.10–1.20 slight antagonism (−), 1.20–1.45 moderate antagonism (− −), 1.45–3.3 antagonism (− − −) (Chou, 2006). DRI (Dose Reduction Index) represents the order of magnitude (fold) of dose reduction that was allowed in combination for a given degree of effect as compared with the dose of each component alone. Bold values indicate synergistic interaction.

Combination	ME-ALA 0.5 mM (J/cm^2)	β-Lapachone (μM)	Fa	CI – symbol	DRI β-Lapachone	DRI ME-ALA
Comb 1	1	2	0.06	3.13 (− − −)	0.69	0.60
Comb 2	1.5	2	0.22	2.49 (− − −)	1.04	0.65
Comb 3	2	2	0.86	1.14 (−)	2.44	1.38
Comb 4	1	2.3	0.11	2.73 (− − −)	0.72	0.74
Comb 5	1.5	2.3	0.37	2.1 (− − −)	1.11	0.84
Comb 6	2	2.3	0.97	**0.71** (++)	3.36	2.40
Comb 7	1	2.8	0.23	2.33 (− − −)	0.75	1.00
Comb 8	1.5	2.8	0.64	1.63 (− − −)	1.24	1.21
Comb 9	2	2.8	0.96	**0.85** (++)	2.54	2.17
Comb 10	1	3	0.45	1.80 (− − −)	0.93	1.39
Comb 11	1.5	3	0.70	1.56 (− − −)	1.24	1.33
Comb 12	2	3	0.94	1.03 (±)	2.06	1.83
Comb 13	1	3.5	0.70	1.45 (− −)	1.06	1.97
Comb 14	1.5	3.5	0.74	1.59 (− − −)	1.13	1.42
Comb 15	2	3.5	0.93	1.17 (−)	1.69	1.74

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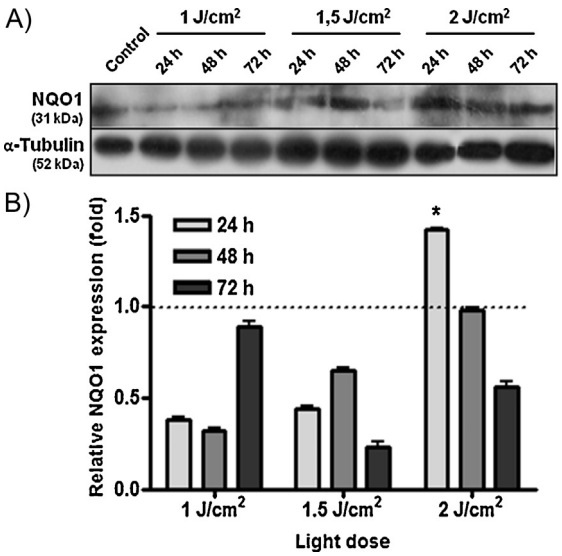

Fig. 2. Induction of NQO1 in MCF-7c3 cells by ME-ALA/PDT. Exponentially growing cells in culture (1×10^5 cells/ml) were treated with 0.5 mM ME-ALA for 4 h and then irradiated with different light doses (1, 1.5 y 2 J/cm^2). (A) Western blot analyses of NQO1 expression. (B) Densitometric analysis represented the average signal intensity of NQO1 protein and was normalized to α-tubulin. $^*p < 0.05$. Dotted line indicates control value.

Results

Effect of β-Lapachone or ME-ALA/PDT on inhibition of MCF-7c3 cell proliferation

Firstly, the inhibitory rate of ME-ALA/PDT and β-Lapachone individually on MCF-7c3 was established, measuring the ability of viable cells to reduce MTT and convert it to nonwater-soluble violet formazan crystals (Fig. 1). The observed inhibitory effect was dose-dependent for both treatments. Regarding photodynamic treatment, cell viability was not affected by light or 0.5 mM ME-ALA alone (Fig. 1A and B). For to reach the IC50 value was used 2.93 ± 0.03 μM of β-Lapachone and 0.5 mM/1.40 ± 0.01 J/cm^2 of PDT dose (ME-ALA/PDT).

Effect of PDT on NAD(P)H:quinone oxidoreductase levels in MCF-7c3 cells

Western blot analysis showed that a considerable amount of constitutive NQO1 exists in MCF-7c3 (Fig. 2A), as previously observed (Pink et al. 2000). Photodynamic therapy with 0.5 mM ME-ALA and 2 J/cm^2 caused a significant increase in NQO1 expression. At 24 h after treatment, the expression of NQO1 was about 1.5 times that in control cells and then began to decline. The remaining light doses evaluated (1 J/cm^2 and 1.5 J/cm^2) failed to induce the expression of NQO1 (Fig. 2A and B).

Effect of β-Lapachone in combination with photodynamic therapy on MCF-7c3 cell proliferation

MCF-7c3 cells were incubated with 0.5 mM ME-ALA followed by irradiation at a light dose of 1, 1.5 and 2 J/cm^2. 24 h afterwards, time required for NQO1 induction, the cells were treated with β-Lapachone at concentrations of 2, 2.3, 2.8, 3 and 3.5 μM and incubated for another 24 h (Fig. 3A). The inhibitory effect was greater when cells were firstly photosensitized with 1.5 and 2 J/cm^2 and then treated with β-Lapachone (Fig. 3B). The IC50 values of β-Lapachone combined with ME-ALA/PDT (0.5 mM + 1 or 1.5 J/cm^2) were 3.14 ± 0.01 μM and 2.48 ± 0.01 μM, respectively. On the contrary, when cells were photosensitized with 2 J/cm^2, the percentage of inhibition was higher than 50%, so the IC50 value could not

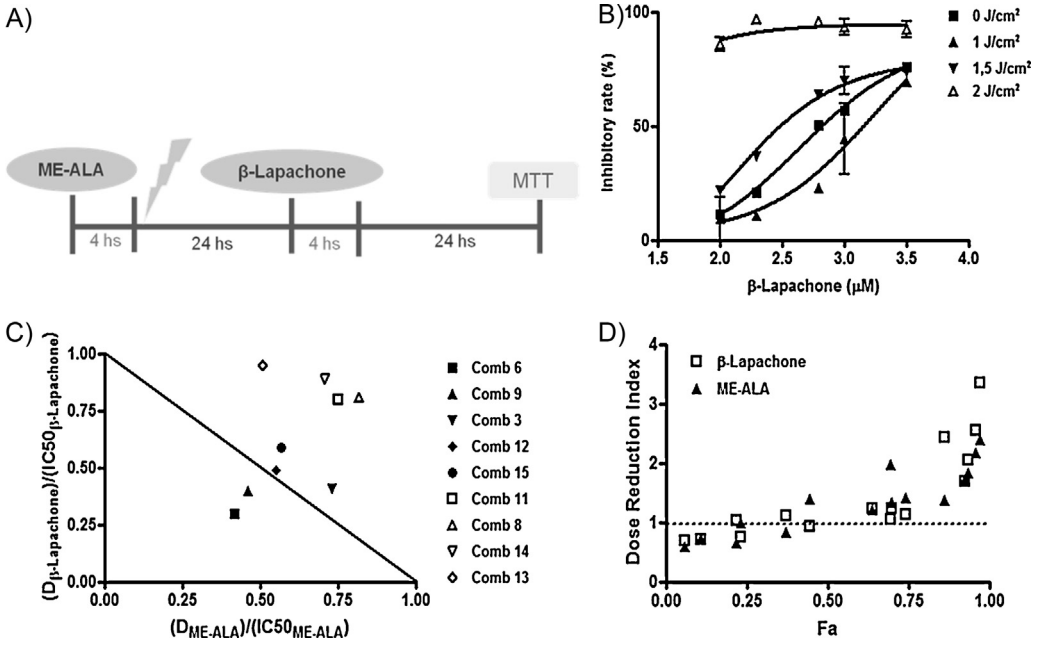

Fig. 3. Combined effects of ME-ALA/PDT and β-Lapachone on the proliferation of MCF-7c3 cells. (A) Experimental design: MCF-7c3 cells were treated with both β-Lapachone and ME-ALA/PDT. (B) Exponentially growing cells in culture (1×10^5 cells/ml) were treated with 0.5 mM ME-ALA for 4 h and then irradiated with different light doses (1, 1.5 y 2 J/cm^2). Following a 24 h incubation, β-Lapachone (2, 2.3, 2.8, 3 and 3.5 μM) was added. Drug solutions were then removed and replaced with fresh medium and cells were incubated for additional 24 h. The proliferation inhibitory rates were evaluated by MTT assay. (C) Normalized ED50-isobologram of non-constant dose rations showing interaction between ME-ALA and β-Lapachone. The oblique line between the *X* and *Y* axes indicates the theoretical doses that produce an additive effect. The points below the line represented synergistic interactions. The point above the line was considered to be antagonistic. For the legend, see Table 1. (D) Dose reduction index (DRI) depending on the fraction affected (Fa). A DRI greater than 1 (dotted line) indicates an enhanced cytotoxicity for combinations of β-Lapachone and ME-ALA/PDT.

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be determined for this treatment condition. This magnitude of inhibition was observed in both single and combined therapeutic modalities.

To quantify synergism, the isobol-curve method was used and the CI values were calculated. The derived isobologram indicated that drug interactions ranged from antagonism through addition to synergy (Fig. 3C). Moreover, CI values for the interaction are between 0.71 and 3.13 over the entire range of Fa values tested (0.06–0.97) indicating interactions involving antagonism (Combs 1, 2, 4, 5, 7, 8, 10, 11 and 14), moderate antagonism (Comb 13), slight antagonism (Comb 3 and 15), nearly additive (Comb 12), and moderate synergism (Combs 6 and 9) (Table 1).

Experimental combination therapy data points plot well below the expected additive line when MCF7-c3 were photosensitized with ME-ALA/PDT 2 J/cm^2 and then treated with 2.3 μM (CI = 0.71, Comb 1) and 2.8 μM (CI = 0.85, Comb 9) of β-Lapachone, indicating moderate synergism (Fig. 3C and Table 1).

Using these data, the DRI was then calculated for each Fa value (Fig. 3D and Table 1). The DRI values of β-Lapachone and ME-ALA/PDT to achieve more than 50% inhibition effect were up to 1 (Combs 3, 6, 8, 9, 11, 12, 13, 14 and 15). This mathematical method therefore demonstrates a strong interaction between each drug over a wide range of therapeutic doses and cytotoxic effect levels.

The results suggest that natural compound β-Lapachone plus ME-ALA/PDT combination possesses a synergistic effect on MCF-7c3 cell proliferation.

Discussion

Synergistic combinations of drugs offer favorable outcomes, such as increasing the efficacy of the therapeutic effect, decreasing the dosage but increasing or maintaining the same efficacy to avoid toxicity and minimizing or slowing down the development of drug resistance (Chou 2006). For these therapeutic benefits, drug combinations represent a good strategy to treat the most dreadful diseases including cancer. A better understanding of the molecular interactions between cytotoxic agents, combined with increasing knowledge of the molecular mechanisms underlying drug resistance and sensitivity, has enabled the rational design of effective combination regimens for the treatment of patients (Wagner and Ulrich-Merzenich 2009). In this sense, the enzyme NQO1 has emerged as a promising target for the development of cancer chemotherapeutics. NQO1 content in most human tumors including breast, colon, and lung cancers, is intrinsically greater than that in adjacent normal tissues (Jaiswal 2000). This observation suggests that drugs that are activated by NQO1 such as β-Lapachone should show significant tumor-specific activity. It was demonstrated that β-Lapachone-mediated cell death required about 90 enzymatic units of NQO1 and that endogenous level of NQO1 proportionally affects the sensitivity of cells to β-Lapachone (Pink et al. 2000; Li et al. 2011). MCF-7 cells exhibit a high level of NQO1 compared to other breast cancer cell lines such as MDA-MB or T47D (Pink et al. 2000), which explains at least in part its high sensitivity to β-Lapachone treatment.

A number of divergent agents have been reported to be able to activate NQO1 in cancer cells and investigations are in progress to identify ideal inducing agents for NQO1 to enhance the antitumor effect of β-Lapachone. Cisplatin (Terai et al. 2009), hyperthermia (Park et al. 2005b; Song et al. 2008; Dong et al. 2009; Hori et al. 2011) and ionizing radiation (Park et al. 2005a; Suzuki et al. 2006; Choi et al. 2007) have proven to increase the expression of NQO1 and consequently sensitize cells to treatment with β-Lapachone. Coincidentally, these therapeutic agents produce a variety of ROS that damage cells, initiate signal transduction pathways, and alter gene expression (Cook et al. 2004; Brozovic et al. 2010). Cellular exposure to oxidative stress initiated by ROS leads to the coordinated induction of genes encoding antioxidant enzymes including NQO1, NRH:quinone oxidoreductase 2 (NQO2), glutathione S-transferase Ya subunit (GST Ya subunit), heme oxygenase 1 (HO-1), glutamate cysteine ligase (GCL) and so on. Promoter analysis identified a DNA element designated as "antioxidant response element" (ARE) that regulates basal expression and coordinated induction of these genes. Upon exposure of cells to oxidative stress, nuclear factor E2-related factor 2 (Nrf2) is phosphorylated in response to the protein kinase C, phosphatidylinositol 3-kinase, and MAP kinase pathways. After phosphorylation, Nrf2 translocates to the nucleus, binds AREs, and activates detoxifying and antioxidant enzymes gene expression including NQO1 (Jaiswal 2004; Nguyen et al. 2009). These data support the view that modulation of intracellular redox state could be an alternative approach to enhance cancer cell sensitivity to β-Lapachone. Thus, we hypothesize that ME-ALA/PDT induces an overexpression of NQO1 because it has been observed a significant accumulation of intracellular ROS after its application (Blázquez-Castro et al. 2012). Indeed, we observed an unprecedented NQO1 overexpression 24 h after application of high-dose of ME-ALA/PDT, which could be assigned to Nrf2 activation in response to oxidative stress generated by ROS. Although this is the first study involving NQO1 with PDT, previous reports have shown that PDT is capable of inducing expression of other genes by activating Nrf2, such as HO-1 (Kocanova et al. 2007; Hagiya et al. 2008).

The percentaje of inhibition of cell viability caused by β-Lapachone treatment applied 24 h after 1.5 or 2 J/cm^2 of ME-ALA/PDT was greater than that caused by β-Lapachone treatment alone. However, synergism between these two therapies was only observed when MCF-7c3 cells were photosensitized with the condition that induce an upregulation of NQO1 previous exposure to β-Lapachone. We may conclude that the increase in NQO1 activity 24 h after 2 J/cm^2 ME-ALA/PDT sensitized the cells to β-Lapachone. Interestingly, when MCF-7c3 cells were subjected with sublethal doses (0.5 mM + 1 J/cm^2) of ME-ALA/PDT, they acquired resistance to subsequent chemotherapy treatment. This is consistent with findings of other authors that show that a photodynamic treatment with low ME-ALA concentrations can be used to promote a moderate production of endogenous ROS, which efficiently stimulates cell growth (Blázquez-Castro et al. 2012). This suggests that under non-cytotoxic conditions PDT could submit potential photoactivating effects on cell proliferation that consequently may confer resistance to other treatments.

In this study, the results indicated that the combination of β-Lapachone and ME-ALA/PDT could be synergistic depending on the redox state to which tumor cells are subjected to and the impact it has on the expression of NQO1. Although this mechanism appear plausible for explaining the synergism, more detailed studies are required in understanding the molecular profile involved. Moreover, further studies are required with animal *in vivo* systems to better known the pharmacokinetics and pharmacodynamics of the corresponding drug combinations.

In the absence of an effective targeted monotherapy, a better understanding of the interplay between biologic and cytotoxic anticancer agents will improve our ability to rationally design optimal combination regimens. The results of the present study strongly suggest that NQO1 activity in tumors may be further and selectively elevated using local photodynamic therapy to improve the cytotoxicity of β-Lapachone against cancer cells.

Conflict of interest

None of the authors have financial relationship with a commercial entity that has an interest in the content of this study.

Please cite this article in press as: Lamberti, M.J., et al., Synergistic enhancement of antitumor effect of β-Lapachone by photodynamic induction of quinone oxidoreductase (NQO1). Phytomedicine (2013), http://dx.doi.org/10.1016/j.phymed.2013.04.018

Acknowledgements

The authors are grateful to Dra. Jarunee Hongrapipat for their generous assistance in the analysis of drug interactions. This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (PICT), Secretaría de Ciencia y Técnica (SECyT), Universidad Nacional de Rio Cuarto, Argentina. VR and NBRV are members of the Scientific Researcher Career at CONICET. MJL hold fellowship from CONICET.

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