Biomedicine & Pharmacotherapy 108 (2018) 1553–1564

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

NQO1 induction mediated by photodynamic therapy synergizes with β-Lapachone-halogenated derivative against melanoma

Lamberti María Julia[a], Morales Vasconsuelo Ana Belén[a], Chiaramello Marianela[a], Ferreira Vitor Francisco[b], Macedo Oliveira Milena[c], Baptista Ferreira Sabrina[c], Rivarola Viviana Alicia[a,1], Rumie Vittar Natalia Belén[a,*,1]

[a] Departamento de Biología Molecular, Facultad de Ciencias Exactas Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta 36, Km 601, Río Cuarto, Córdoba, Argentina

[b] Departamento de Tecnologia Farmacêutica, Faculdade de Farmácia, Universidade Federal Fluminense, Rua Dr. Mário Viana, 523, Santa Rosa, Niterói, Rio de Janeiro, Brazil

[c] Departamento de Química Orgânica, Instituto de Química, Universidad Federal de Río de Janeiro, Avenida Athos da Silveira Ramos, 149 Bloco A -6° andar, CEP: 21941-909 Cidade Universitária, Rio de Janeiro, Brazil

ARTICLE INFO

Keywords:
NQO1
β-Lapachone
Naphtoquinones
Photodynamic therapy
Melanoma
Combinatorial therapies

ABSTRACT

The elevated expression of NQO1 in many human solid tumors along with its ability to activate quinone-based anticancer agents makes it an excellent target for enzyme-directed drug development. NQO1 plays an important role in melanogenesis and given its correlation with a poor patient outcome we propose this enzyme as an intriguing target for molecular-based therapeutic regimen against melanoma. Unfortunately, the natural product β-Lapachone (β-Lap), whose antitumor activity is based on NQO1, reported dose-limiting toxicity which hampered its pre-clinical and clinical use. Therefore, new effective and safe therapeutic NQO1-bioactivatable agents for melanoma treatment are desirable. Regarding NQO1, we demonstrated that halogenated β-Lap derivative named PFB is an excellent substrate and effective tumor-selective anticancer compound. In addition, PFB resulted more attractive than the parent β-Lap for treating metastatic-derived melanoma cells. In this context, it would be interesting to design strategies to induce NQO1 activity in cancer cells as a promising combinatorial approach with bioreductive drugs. In this sense, we had reported that photodynamic therapy (PDT) significantly upregulated NQO1 expression. Based on this event, here we demonstrated that the cytotoxic regimen consisting of PFB plus PDT improved synergistic therapeutic combination on melanoma cells. In conclusion, our contribution provides a strong rationale for using therapies that associate photo- and chemotherapy to effectively treat melanoma with modular NQO1 status.

1. Introduction

Melanoma is the most dangerous form of skin cancer and its incidence has been rising steadily over recent decades. Detection of the disease in early stage may be curable, but metastatic late stage disease has an extremely poor prognosis with a median survival of less than 10 months. This poor prognosis largely results from resistance to conventional chemotherapy. Molecular targeted therapies are being explored and they have reported successful in several tumors, but thus far have not shown this level of benefit in melanoma [1]. Therefore, the main goal is to identify novel treatment-associated melanoma biomarkers whose exploitation would confer greater therapeutic benefits.

In this context, recent studies demonstrated that NAD(P)H-quinone oxidoreductase 1 (NQO1) is overexpressed in many types of tumors,

Abbreviations: β-Lap, β-Lapachone; cDNA, complementary DNA; CI, combination index; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DRI, dose reduction index; GEPIA, gene expression profiling interactive analysis; GTEX, genotype-tissue expression; IC_{50}, 50% inhibitory concentration; Me-ALA, methyl-aminolevulinic acid; MMLV-RT, moloney murine leukemia virus reverse transcriptase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADH, nicotinamide adenine dinucleotide; NAD(P)H, nicotinamide adenine dinucleotide phosphate; NQO1, NAD(P)H dehydrogenase [quinone] 1; PBS, phosphate buffer saline; PDT, photodynamic therapy; PI, propidium iodide; RNA, ribonucleic acid; RTqPCR, reverse transcription polymerase chain reaction; SI, selectivity Index; SKCM, skin cutaneous melanoma; TCGA, the cancer genome atlas

* Corresponding author.
E-mail addresses: belenrumie@gmail.com, nrumievittar@exa.unrc.edu.ar (N.B. Rumie Vittar).

[1] Both authors contributed equally

https://doi.org/10.1016/j.biopha.2018.09.159
Received 23 August 2018; Received in revised form 26 September 2018; Accepted 26 September 2018

including melanoma [2–4]. Indeed, it has been reported that NQO1 catalyzes two-electrons reduction of β-Lapachone (β-Lap), a compound extracted from Lapacho trees, using either NADH or NAD(P)H as electron donor. The resultant hydroquinone is unstable and oxidized to the original form of β-Lap resulting in a futile cycling. The subsequent depletion of NADH or NAD(P)H together with oxidative stress trigger signal transduction for cell death. Therefore, the cytotoxicity of β-Lap is closely related to constitutive NQO1 expression to selective target tumor cells [5]. However, despite its significant anticancer activity, β-Lap underwent unsuccessful clinical trials in a variety of cancers given its dose limiting toxicity [6,7].

As a potential therapeutic options for the treatment of dermatological disorders, photodynamic therapy (PDT) is a noninvasive antitumor approach. It consists in the administration of a photosensitizer (PS) followed by *in situ* irradiation with visible light of specific wavelength(s). Excitation of the PS leads to a series of photochemical reactions and consequently the local generation of harmful reactive oxygen species (ROS) causing limited or non-systemic harmful effects. Its efficacy has been shown in non-melanoma skin cancers and other non-malignant skin lesions like vitiligo and psoriasis [8]. Even though several resistance mechanisms of melanoma to PDT had been reported [9], we consider that the understanding of modular molecular features could bypass these undesirable outcome.

In this context, we have previously demonstrated that PDT caused an upregulation of NQO1, thereby increasing local β-Lap induced cell damage [10]. Here, we propose complemental approaches to solve β-Lap and PDT failure. First, in order to identify NQO1-bioactivatable compounds as antitumor agents and to explore the more potent and selective chemotherapeutic, β-Lap analogues and derivatives were screened. A better comprehension of the interactions between simultaneously applied regimens and the rationale for combination therapy will provide new insights to improve therapeutic effect of conventional treatments alone. For this reason, secondly, we consider feasible to increase the NQO1 level in tumors by pre-exposing melanoma to photodynamic enhancing the cytotoxicity of β-Lap or its superior derivative investigated. This study provides the potential availability of photodynamic NQO1-modulation and β-Lap-halogenated derivative PFB as a novel combinatorial approach for the treatment of malignant melanoma.

2. Experimental section

2.1. Cell culture

SK-Mel 28, SK-Mel 2 and 1205Lu human melanoma, B16 murine melanoma and HaCaT normal keratinocytes were grown in complete medium DMEM (Dulbecco's modified Eagle medium high glucose 1X, Gibco) supplemented with 10% v/v fetal bovine serum (FBS) (PAA Laboratories), 1% v/v glutamine (GlutaMAXTM 100X Gibco), 1% v/v antibiotic (Penicillin 10,000 units/mL - streptomycin 10,000 µg/mL Gibco) and 1% v/v of sodium pyruvate 100 mM (Gibco). Cells were maintained in 5% CO_2 and 95% air at 37 °C in a humidified incubator. Stock cultures were stored in liquid nitrogen and used for experimentation within 5–7 passages. B16 cells constitutively expressing NQO1 construct (B16 NQO1) were generated by stable transfection of them with pEFIRES-NQO1. The plasmid was kindly provided by Dr Yosef Shaul (Weizmann Institute of Science, Rehovot, Israel)[11]. Transfection was performed using FuGENE® HD Transfection Reagent (Roche) according to manufacturer´s instructions. Stable transfected cells were selected in growth medium supplemented with 2 µg/ml of puromycin (Sigma). Individual colonies were isolated after 2–3 weeks of growth under selection using the "cloning ring" method and subsequently expanded into clonal cell lines [12].

2.2. NQO1 expression in skin cutaneous melanoma from the Cancer Genome Atlas (TCGA) data

Mining of NQO1 expression data in human skin cutaneous melanoma (SKCM) from the Cancer Genome Atlas (TCGA) was implemented with Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/), an online tool that delivered interactive and customizable functions to facilitate efficient expression analysis of TCGA and GTEx data [13]. We downloaded graphs of NQO1 expression in 461 SKCM tissues and 558 normal tissues as well as NQO1 expression in different clinical stages of SKCM from GEPIA.

2.3. Drugs

β-Lapachone and its derivatives and analogues: AMEB, PMEB, EA, BRONORBETA, PClB, PBB, PFB, PMEA, NorLap, AMEA, PFA, DIMERO, PClA, PBA, and EB, were synthetized by group of Prof. Sabrina Ferreira with collaboration by group of Prof. Vitor Ferreira as previously reported by them in the literature [14].

The compounds were dissolved in dimethyl sulfoxide (DMSO) to make a 6 mM stock solution stored at −20 °C. δ-aminoleavulinic acid methyl ester hydrochloride (Me-ALA) was purchased from Sigma and dissolved in sterile PBS to make a 100 mM stock solution stored at 4 °C. Dicumarol was purchased from Sigma and dissolved in sterile water to make a 5 mM stock solution stored at −20 °C. For the *in vitro* studies, drugs were diluted to desired concentrations in DMEM medium (Gibco) without FBS immediately before use.

2.4. Treatments

Exponential-phase melanoma or keratinocytes cells at a density of 1×10^5 cells. ml^{-1} 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) Chemotherapy: Cells monolayers were washed twice with phosphate buffer saline (PBS) to remove all traces of FBS and then β-Lapachone or its derivatives or analogues were added to each well or plate and incubated for 24 h (37 °C, 5% CO_2) in DMEM without FBS [10].

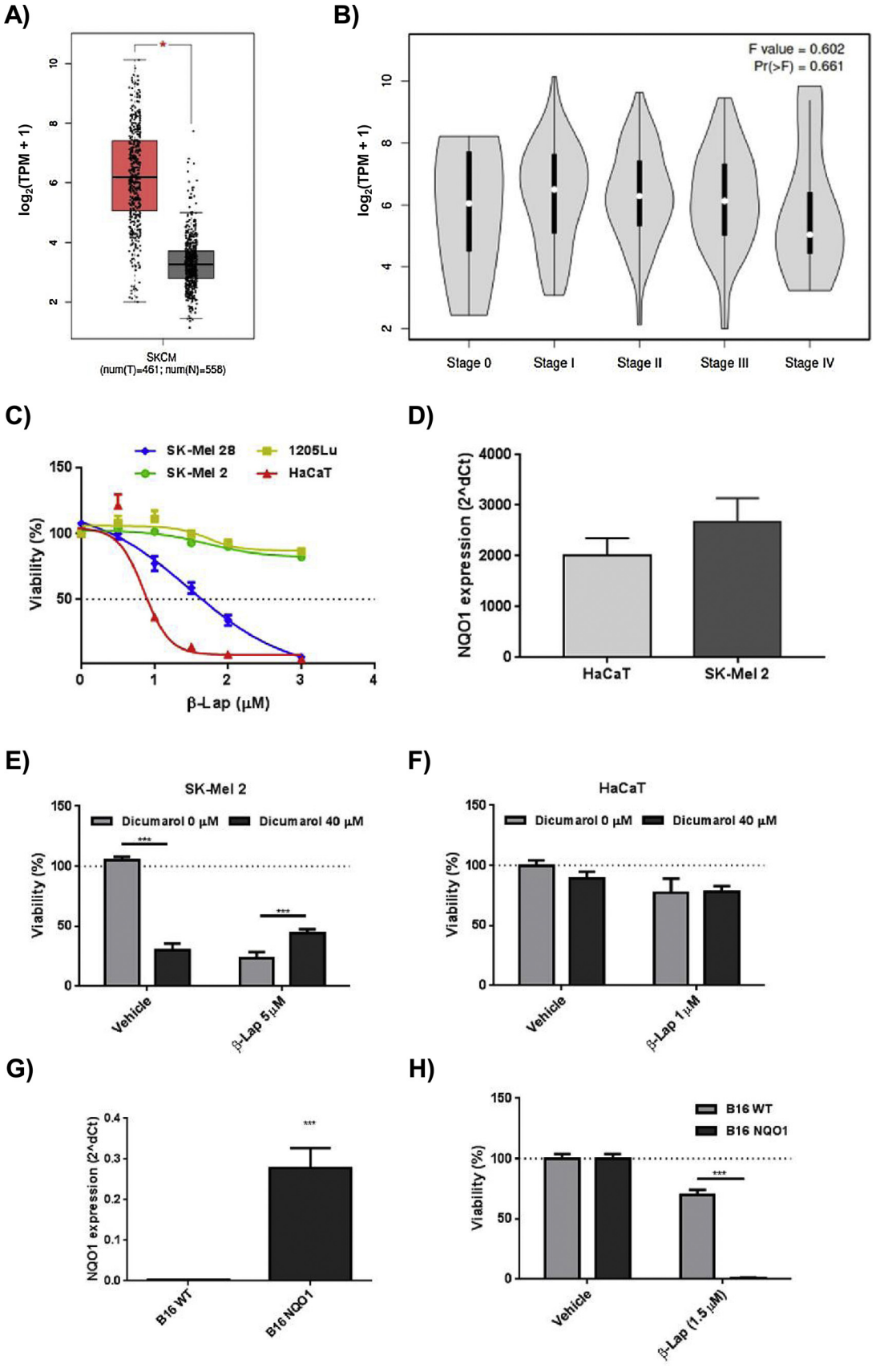
b) Photodynamic Therapy: Cells monolayers were washed twice with PBS to remove all traces of FBS and then Me-ALA was added to each well or plate and incubated for 4 h (37 °C, 5% CO_2) in DMEM without FBS. After that, cells were irradiated (0.5 J/cm^2) at room temperature with monochromatic light source (635 nm ± 17 nm) using a MultiLED system (coherent light). The fluence rate on the cell monolayer was 0.89 mW/cm^2 (as measured by Radiometer Laser Mate-Q, Coherent). Drug solution was then removed and replaced with fresh complete medium [10].

2.5. Cell viability determination

Cell viability was evaluated by 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay, which is reduced by mitochondrial dehydrogenases of viable cells to non-water soluble violet formazan crystals [10]. Twenty four hours post chemotherapy, MTT solution (5 mg/ml in PBS) was added for 3 h (dilution rate: 1/10). Then, DMSO was added to lyse the cells and solubilize the precipitated formazan product. Optical density of the resulting solution of formazan salt was read at 540 nm using ELISA reader plate (Thermo Scientific, Multiskan FC).

2.6. Real-Time RT-PCR (RTqPCR)

RNA was isolated using Trizol Reagent (Invitrogen). cDNA was made *via* manufacturer's instructions with M-MLV (Invitrogen). Real time PCR was performed on a Stratagene Mx3000PRO Real Time



(caption on next page)

Fig. 1. NQO1 implication in β-Lap cytotoxicity on melanoma and normal keratinocytes. (A) NQO1 expression patterns in 461 SKCM tissues (T) and 558 normal (N) tissues. Data obtained from GEPIA and expressed in log scale of transcripts per million (TPM). (B) NQO1 expression in different clinical stages of SKCM (stage 0-IV). Data obtained from GEPIA and expressed in log scale of transcripts per million (TPM). (C) Human melanoma cells: SK-Mel 28, SK-Mel 2 and 1205Lu, and normal keratinocytes: HaCaT cells were incubated with β-Lap (0–3 μM) for 24 h. Cell viability was evaluated by MTT assay and referred to cells incubated with DMSO (vehicle). The curves were fitted using a non-linear regression equation (dotted line: 50% viability). (D) Quantification of basal mRNA expression of NQO1 on HaCaT and SK-Mel 2 cells by RTqPCR. (E) Human melanoma cells SK-Mel 2 were incubated with β-Lap (5 μM) in the presence or absence of dicumarol (40 μM) for 24 h. Cell viability was evaluated by MTT assay and referred to cells incubated with DMSO (vehicle) (dotted line: 100% viability). (F) Human normal keratinocytes HaCaT were incubated with β-Lap (1 μM) in the presence or absence of dicumarol (40 μM) for 24 h. Cell viability was evaluated by MTT assay and referred to cells incubated with DMSO (vehicle) (dotted line: 100% viability). (G) Quantification of basal mRNA expression of NQO1 on B16 and B16 NQO1 by RTqPCR. (H) Murine melanoma cells B16 WT cells (wild type) and B16 NQO1 cells (with stable overexpression of NQO1) were incubated with β-Lap (1.5 μM) for 24 h. Cell viability was evaluated by MTT assay and referred to cells incubated with DMSO (vehicle) (dotted line: 100% viability).

machine using SYBR green incorporation. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative levels of gene expression using Stratagene MxPro QPCR software v3.00 (Stratagene). The housekeeping gene 18S (human) or GAPDH (mouse) expression was used as the internal control. A standard melting-curve cycle was used to confirm the quality of amplification. The reactions were performed in triplicate for each sample. The gene-specific primers were designed with the Primer BLAST software:

- NQO1 (human):
  - Forward: 5′ GGCGAGGCAGCTTGAGTTAA 3′
  - Reverse: 5′ CACCGCCTCGGCTTGTC 3′
- 18S (human):
  - Forward: 5′ AACCCGTTGAACCCCATTCGTGAT 3′
  - Reverse: 5′ AGTCAAGTTCGACCGTCTTCTCAG 3′
- NQO1 (mouse):
  - Forward: 5′ GTCCATTCCAGCTGACAACC 3′
  - Reverse: 5′ TCCTTTTCCCATCCTCGTGG 3′
- GAPDH (mouse):
  - Forward: 5′ TGCACCACCAACTGCTTAG 3′
  - Reverse: 5′ GGATGCAGGGATGATGTTC 3′

2.7. Analysis of apoptosis rate by Annexin V-FITC/PI assay

Twenty four hours after treatment, the percentage of apoptotic/necrotic cells was assessed using a standard flow cytometry Annexin-V-FITC binding assay (BD Pharmingen) according to manufacturer's instructions. Briefly, cells were disaggregated by trypsin digestion, and washed with PBS. The pellet was incubated at room temperature with 5 μg/ml Annexin V-FITC, 5 μg/ml propidium iodide (PI) and binding buffer for 15 min in the dark, according to manufacture's instruction. Annexin V and PI fluorescence were measured using a Millipore Guava Easycyte 6 2l cytometer. Early apoptotic cells are Annexin V-positive and PI-negative (Annexin V-FITC$^+$/PI$^-$), whereas late (end-stage) apoptotic cells are Annexin V/PI-double-positive (Annexin V-FITC$^+$/PI$^+$). Necrotic cells are Annexin V-negative and PI-positive (Annexin V-FITC$^-$/PI$^+$). Viable cells are Annexin V-negative and PI-negative (Annexin V-FITC$^-$/PI$^-$) [15]. Data was analyzed using FlowJo 10.0.7 software.

2.8. Analysis of combinatory treatment effect

For quantification of drug interaction, two measurements were employed: the combination index (CI) 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. 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 [16,17].

2.9. Statistical analysis

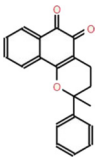
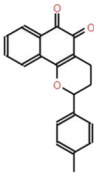
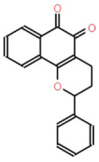
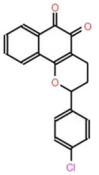
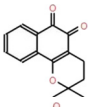
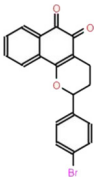
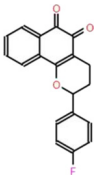
Differences between groups were tested by 1 or 2-way analysis of variance with Bonferroni post-hoc tests using Infostat software. Dose-response curves were fitted using a non-linear regression equation using GraphPad Prism 7 software. All the results are expressed as mean +/- standard error of at least three independent experiments, and $P < 0.05$ was considered statistically significant. References of figures: $P < 0.05$ = *; $P < 0.01$ = **; $P < 0.001$ = ***.

3. Results

3.1. β-Lapachone exhibited NQO1-dependent chemotherapeutic activity on melanoma cells

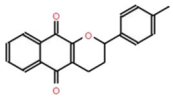
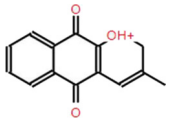
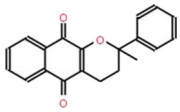
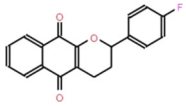
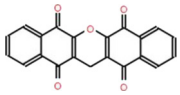
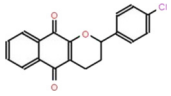
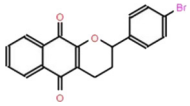
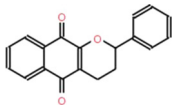
Although a lowered or absent NQO1 activity has been correlated with increased susceptibility for development of human cancers [18], several studies found that NQO1 is upregulated in a number of malignances [19,20]. In this context, RNA sequencing expression data of 461 melanoma tumors and 558 paired normal tissues from TCGA and GTEx projects were examined. Mining of data was implemented with Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/), an online tool that delivered interactive and customizable functions to facilitate efficient expression analysis [13]. The results indicated that NQO1 expression was remarkably higher in melanoma samples than in normal tissues (Fig. 1A). In addition, compared with stage I-III, NQO1 showed significantly lower level of expression in metastatic advanced clinical stages (stage IV) (Fig. 1B). Characterization of this expression pattern could be exploited as a therapeutic target involving anticancer drugs that are bioactivated by NQO1, such as β-Lapachone (β-Lap). To investigate the antitumor effect of β-Lap in melanoma, cell lines derived from primary or metastatic sites and normal keratinocytes were incubated with different concentration of the chemotherapeutic for 24 h and half maximal inhibitory concentration (IC_{50}) was determined. Metastasis-derived human melanoma cells (stage IV) exhibited higher resistance to β-Lap (IC_{50} SK-Mel 2 and 1205Lu: not reached) compared to primary-site derived melanoma cells (IC_{50} SK-Mel 28: 1.48 ± 0.22 μM) (Fig. 1C). Consistently, melanoma stage associated-NQO1 expression had a close correlation with the sensitivity to β-Lap treatment. Ideally, one of the criteria for a drug being safe is not exhibit any undesirable side-effects on normal cells. Surprisingly, drug sensitivity was not only linked to tumor cells because normal keratinocytes (IC_{50} HaCaT: 0.86 ± 0.02 μM) were more sensitive to β-Lap (Fig. 1C). These data were not associated with NQO1 expression, given that NQO1 mRNA level was higher in SK-Mel 2 than in HaCaT (Fig. 1D). Therefore, in order to evaluate the relevance of NQO1 in chemosensitivity of melanoma and normal cells, the cytotoxic effect of β-Lap was reversed with co-administration of dicumarol, a specific inhibitor of NQO1. Despite affecting cellular viability, inhibition of oxidoreductase activity only prevented cell death of metastasis-derived human melanoma cells (Fig. 1E). Dicumarol showed no protective effect in HaCaT cells (Fig. 1F), suggesting that β-Lap cytotoxicity was not NQO1-dependent on normal keratinocytes. To confirm the role of NQO1 in β-Lap toxicity on melanoma, we demonstrated that stable

Table 1
Characterization of derivatives and analogues of β/α-Lap.

Name		LogP	Molecular Weight	Molecular Formula	Structure	Polar Surface Area (Angstroms Squared)
β-Lap derivatives	AMEB	3.71	304.3392	C20H16O3		43.37
	PMEB	4.03	304.3392	C20H16O3		43.37
	EA	3.59	290.3127	C19H14O3		43.37
	BRONORBETA	2.95	307.1393	C14H11BrO3		43.37
	PClB	4.21	324.7577	C19H13ClO3		43.37
	β-Lap	2.66	242.2699	C15H14O3		43.37
	PBB	4.38	369.2087	C19H13BrO3		43.37
	PFB	3.75	308.3031	C19H13FO3		43.37

(*continued on next page*)

Table 1 (*continued*)

Name		LogP	Molecular Weight	Molecular Formula	Structure	Polar Surface Area (Angstroms Squared)
α-Lap derivatives	PMEA	4.03	304.3392	C20H16O3		43.37
	NorLap	3.00	228.2433	C14H12O3		54.37
	AMEA	3.71	304.3392	C20H16O3		43.37
	PFA	3.75	308.3031	C19H13FO3		43.37
	DIMERO	2.55	342.3011	C21H10O5		77.51
	PClA	4.21	324.7577	C19H13ClO3		43.37
	PBA	4.38	369.2087	C19H13BrO3		43.37
	EB	3.59	290.3127	C19H14O3		43.37

References:
LogP: Ratio of the concentrations of a solute between the two solvents (octanol/water), used to calculate lipophilic efficiency.
Polar Surface Area (PSA): Surface sum over all polar atoms, primarily oxygen and nitrogen, also including their attached hydrogen atoms. PSA is a commonly used medicinal chemistry metric for the optimization of a drug's ability to permeate cells (PSA < 90 angstroms is needed).

overexpression of NQO1 (B16 NQO1, Fig. 1G) completely restored chemosensitivity on B16 WT murine melanoma cell line which exhibited low basal NQO1 [21]) (Fig. 1H). Collectively, these observations suggested that β-Lap could be a potential chemotherapeutic agent for NQO1-expressing melanoma. However, non-specificity of this cytotoxic agent given its ability to damage normal as well as malignant cells represents the major disadvantage to be applied in melanoma treatment.

3.2. Halogenated β-Lap-derivative PFB as an excellent NQO1-substrate and tumor-selective anticancer agent against melanoma

To continue the efforts in developing novel compounds bioactivated by NQO1 as potential candidates to achieve melanoma-selective cytotoxicity, 14 naphtoquinones derivatives or analogues were screened (Table 1). Their anti-proliferative activities were evaluated in the melanoma cell line SK-Mel 28. α-Lap analogues failed to induce cell death, whereas only halogenated derivatives of β-Lap: PClB, PBB and PFB exhibited cytotoxicity and retained the same or higher magnitude of cell damage as the parental compound (Fig. 2A). Comparison of tumor viability after incubation with different concentrations (0–2 μM) of β-Lap and its halogenated derivatives over the period of 24 h revealed that PFB had higher cytotoxicity for SK-Mel 28 than the other chemotherapeutics had (Fig. 2B, Table 2). In addition, only PFB was more cytotoxic on melanoma cells than on normal keratinocytes cells (Fig. 2C, Table 2). The selectivity index (SI), defined by the ratio of IC_{50} on normal cells over IC_{50} on tumor cells, was determined to prioritize these 3 compounds. In this context, PFB displayed the highest selectivity index (SI: 1.32) (Table 2). To assess whether NQO1 is involved in β-Lap and its derivatives-mediated toxicity on melanoma, they were co-incubated with dicumarol. Inhibition of NQO1 suppressed the anti-proliferative activities of all tested compounds (Fig. 2D). Consistently, upregulation of NQO1 expression restored chemosensitivity (Fig. 2E). Based on these results, we proposed halogenated β-Lap derivative PFB as an excellent NQO1 substrate and melanoma-selective anticancer agent.

3.3. PFB increased chemotherapy efficiency on metastatic melanoma cells compared to β-Lap

We have previously showed that highly metastatic cell lines derived from melanoma (SK-Mel 2, 1205Lu) were more resistant to β-Lap than primary site-derived melanoma cells (SK-Mel 28) (Fig. 1C). This correlation between β-Lap therapy outcome and metastatic origin pointed the need to discover more efficient chemotherapeutic agents for the treatment of metastatic melanoma. Interestingly, the evaluation of the 50% inhibitory concentration (IC_{50}) of β-Lap and PFB for metastatic melanoma cell line SK-Mel 2 was 3.50 ± 0.05 μM and 2.89 ± 0.10 μM, respectively, after 24 h treatment (Fig. 3A). Blockade of NQO1 oxidoreductase activity by dicumarol inhibited PFB cytotoxicity on melanoma cells, but not on the normal keratinocytes cell line HaCaT (Fig. 3B), as observed with β-Lap (Fig. 1F). Considering the growth inhibitory effect of β-Lap and PFB on metastatic melanoma cells, we investigated whether these compounds induced apoptosis on

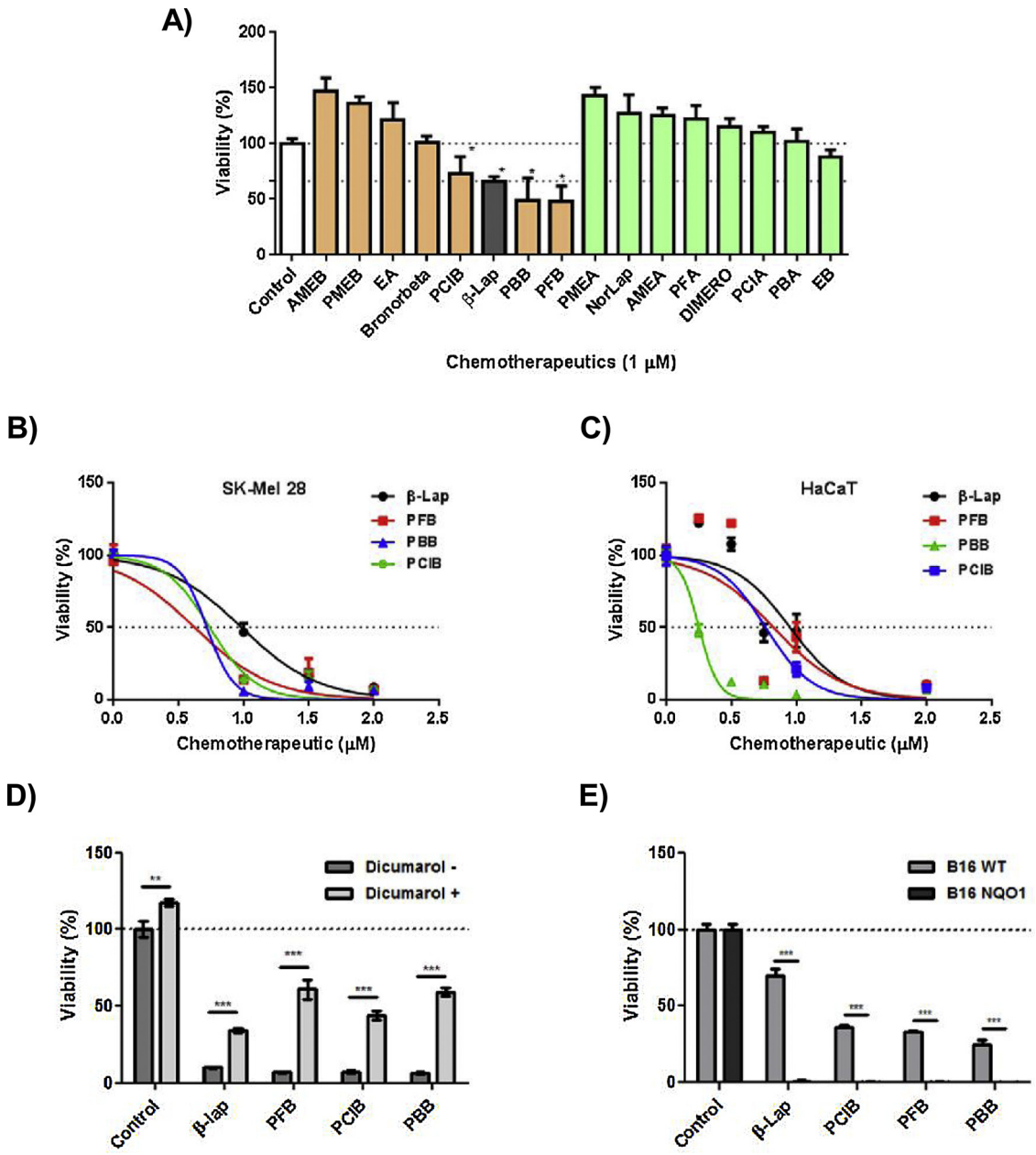


Fig. 2. Evaluation of β-Lap and its derivatives and analogues against melanoma. (A) Human melanoma cells SK-Mel 28 were incubated with β-Lap and its derivatives or analogues (1 μM) for 24 h. Cell viability was evaluated by MTT assay and referred to cells incubated with DMSO (vehicle). Orange columns represent β-Lap derivatives, green columns represent α-Lap derivatives and grey column represents b-Lap (upper dotted line: 100% viability; lower dotted line: 50% viability). (B) Human melanoma cells SK-Mel 28 were incubated with β-Lap and its halogenated derivatives (0–2 μM) for 24 h. Cell viability was evaluated by MTT assay and referred to cells incubated with DMSO (vehicle). The curves were fitted using a non-linear regression equation (dotted line: 50% viability). (C) Human normal keratinocytes HaCaT were incubated with β-Lap and its halogenated derivatives (0–2 μM) for 24 h. Cell viability was evaluated by MTT assay and referred to cells incubated with DMSO (vehicle). The curves were fitted using a non-linear regression equation (dotted line: 50% viability). (D) Human melanoma cells SK-Mel 28 were incubated with β-Lap and its halogenated derivatives (2 μM) in the presence or absence of dicumarol (40 μM) for 24 h. Cell viability was evaluated by MTT assay and referred to cells incubated with DMSO (vehicle) (dotted line: 100% viability). (E) Murine melanoma cells B16 WT cells (wild type) and B16 NQO1 cells (with stable overexpression of NQO1) were incubated with β-Lap and its halogenated derivatives (1.5 μM) for 24 h. Cell viability was evaluated by MTT assay and referred to cells incubated with DMSO (vehicle) (dotted line: 100% viability).

SK-Mel 2 cells. Both β-Lap and PFB triggered an apoptotic pathway that is dependent upon NQO1. In particular, PFB showed much stronger growth inhibitory effect through apoptosis in SK-Mel 2 than β-Lap (Fig. 3C–D). Overall, these data suggested that PFB possess NQO1-associated properties that make it more attractive than the parent β-Lap for treating metastatic-derived melanoma cells.

3.4. Synergistic antitumor effect by photodynamic NQO1 induction for melanoma treatment

Our preliminary results support the necessity of designing new strategies to induce NQO1 activity in cancer cells as a promising approach to increasing tumor-targeted therapies based on bioreductive anticancer drugs. In this context, we have previously reported that photodynamic therapy (PDT) incremented NQO1 protein expression in breast cancer cells [10]. Therefore, we evaluated the photodynamic

Table 2
Comparison of therapeutic efficiency of b-Lap, PBB and PClB.

Chemotherapeutic	IC50[a] on melanoma cells	Symbol	IC50 on keratinocytes	Selectivity index[b]
β-Lap	0.98 ± 0.04 μM	~	0.95 ± 0.05 μM	0.97
PFB	**0.62 ± 0.13 μM**	<	**0.82 ± 0.08 μM**	**1.32**
PBB	0.72 ± 0.08 μM	>	0.25 ± 0.02 μM	0.34
PClB	0.74 ± 0.13 μM	~	0.76 ± 0.17 μM	1.03

References:
[a] IC50: Half maximal inhibitory concentration.
[b] Selectivity index: Ratio of the IC50 values of the treatments on HaCaT cells to those in SkMel-28 cell line.

regulation of this oxidoreductase on B16 melanoma cells expressing low basal NQO1 [21]. B16 were pre-incubated with non-toxic Me-ALA during 4 h, to allow the formation of the photosensitizer protoporphyrin IX (PpIX) [8,22]. Later, they were exposed to monochromatic red radiation from light-emitting diode (LED). NQO1 mRNA was significantly upregulated in a dose-dependent manner (Fig. 4A), 5 h after treatment (Fig. 4B). Based on these results, we speculated that PDT-mediated induction of NQO1 might augment the effect of β-Lap or PFB on malignant melanoma cells. To test our hypothesis, B16 cells were sensitized with 0.1, 0.2 and 0.3 mM Me-ALA and subsequently irradiated (0.5 J/cm^2). Five hours afterwards, time required for NQO1 induction (Fig. 4A), melanoma cells were incubated with β-Lap or PFB at concentrations of 0.5, 2 and 3 μM for 24 h (Fig. 4C, experimental design). Dose response curves were constructed for each therapeutic alone and the combinatorial approaches (Fig. 4D-4E). Moreover, analysis of the nature of the interaction between photo- and chemotherapy (synergism, addition, or antagonism) was performed using Chou-Talalay method (Table 3). For the subsequent analysis, we set the concentration of the chemotherapeutics at 0.5 μM because it was not toxic for normal keratinocytes (Fig. 2C). The combination of PFB with sublethal dose of PDT exhibited the most synergic interaction (Fig. 4F, Table 3). The DRI values of PFB and β-Lap were up to 1, and increased proportionally with NQO1 expression after PDT (Fig. 4G–I). This mathematical method therefore demonstrated a strong relationship between NQO1 modulation and therapeutic interaction. In addition, PFB showed higher DRI than β-Lap in all PDT conditions tested. These data indicated that photodynamic upregulation of NQO1 was connected with the highly efficient combined effect of PDT plus PFB/β-Lap. In summary, PFB is the best candidate for NQO1-based combinatorial therapies using bioreductive anticancer drugs.

4. Discussion

The cytosolic flavoprotein NQO1 had been shown to play pleiotropic roles in melanoma biology. Several studies determined that NQO1 is involved in skin adaptation to environmental stress by positively regulating melanin synthesis through tyrosinase stabilization [23,24]. Even though NQO1 had been designated as a cancer preventer/detoxifying enzyme [25], constitutive up-regulation of this enzyme had been found in many types of cancers [19,20]. Regarding melanoma, it was observed that NQO1 was overexpressed in most melanoma cell lines with respect to melanocytes, and this upregulation was associated NF-kappaB-dependent cell proliferation [2,3]. Here, we reported that NQO1 expression in cutaneous melanoma is significantly higher than in adjacent normal tissues (Fig. 1A). In addition, the level of NQO1 abundance depends on melanoma stage (Fig. 1B). The correlation between the lower expression of NQO1 and melanoma metastatic (stage IV) is consistent with previous findings that postulate this feature as potential biomarker [4]. Indeed, the highly selective pattern of NQO1 expression can be strategically used as a therapeutic target for melanoma chemotherapy.

In this context, with its unique property of transferring two-electron by using either NADH or NADPH as reducing cofactor, NQO1 catalyzes the transformation of quinones into hydroquinones, which exerts cellular damage [26,27]. This is the basis of the cytotoxic activity of the chemotherapeutic β-Lap, a naturally occurring o-naphthoquinone present in the bark of the Lapacho tree (*Tabebuia avellanedae*) native of South America. Biochemical studies demonstrated that the hydroquinone forms of β-Lap spontaneously oxidizes back to the original β-Lap. This reaction creates a futile cycle between oxidized and reduced forms of β-Lap that leads to cell death mediated by reactive oxygen species (ROS) and a severe depletion of NAD(P)H and NADH [5]. Given its NQO1 dependence, the injury occurs specifically in cancer cells overexpressing the enzyme, while normal cells and tissue with low endogenous levels of the enzyme should be spared. Although the antitumor effect of β-Lap was reported in melanoma [28,29], here we have demonstrated the dependence of NQO1 for exert cytotoxicity: oxidoreductase activity suppression by dicumarol conferred resistance to the treatment (Fig. 1E) while NQO1 overexpression restored chemosensitivity (Fig. 1H). Moreover, the inability of β-Lap to inhibit the proliferation of metastatic-derived cell lines (Fig. 1C) could be associated with the lower expression of NQO1 in stage IV melanoma (Fig. 1B).

Although β-Lap is a promising agent from a mechanistic standpoint, its pre-clinical and clinical use (clinical form, ARQ501 or 761) is hampered by dose limiting toxicity in the form of hemolytic anemia, methemoglobinemia and non-specific drug distribution [6,7]. Our study supported the lack of tumor selectivity of β-Lap due to the high toxicity exerted on normal keratinocytes (Fig. 1C). However, the mechanism of cell death was not mediated by NQO1 (Fig. 1D and F). In this sense, β-Lap's action may involve multiple pathways and its NQO1-independent pharmacological activity could be attributed to its modulation of DNA repair inhibition [30,31], poly (ADP-ribose) polymerase (PARP1) inhibition [32,33], calcium [34], DNA topoisomerase inhibition [30], NF-κB inhibition [35] and mitochondria mechanism [36]. Further studies should be done in order to elucidate the molecular pathways involved in non-tumor β-Lap-associated damage.

Despite its effective antitumor activity, β-Lap had failed in advance clinical trial due to their high toxicity. Therefore, a major challenge is to design new drugs more selective for cancer cells, and thus having lesser side effects. In this context, we evaluated the performance of β-Lap derivatives and analogues that were rationally designed and synthetized to be metabolic activated by NQO1 (Table 1). In all cases, α-Lap derivatives exhibited the lowest antitumor effectiveness (Fig. 2A). In a preliminary screening, only β-Lap derivatives PFB, PClB and PPB inhibited melanoma cell growth (Fig. 2A). These compounds are structurally connected with halogen presence, and their antitumor activity linked with a previous report that demonstrated remarkable cytotoxicity of halogenated compounds [37]. Even the introduction of halogen atom(s) appeared to increase the affinity of drugs for the plasma membrane in erythrocytes [38], this feature did not correlate with LogP or PSA data of PFB, PClB and PPB (Table 1). Interestingly, these halogenated agents could undergo a similar NQO1-mediated metabolic bioactivation as with β-Lap (Fig. 2D and E). The low toxicity against normal keratinocytes exerted by PFB indicated that this compound showed some degree of selectivity for the melanoma cells studied (Fig. 2C, Table 2). On metastatic cell line, β-Lap cytotoxicity is more dependent on NQO1 since the effect of dicumarol is more evident than that of PFB (Fig. 3C and D). However, PFB exhibited a superior inhibition of metastatic melanoma cells growth in comparison to β-Lap induced-cell death (Fig. 3C and D).

Our results, when taken together, suggested that PFB is a novel NQO1-substrate with enhanced antitumor activity and higher selectivity to cancer cells than its parental β-Lap, which makes it an excellent candidate for metastatic and non-metastatic melanoma treatment.

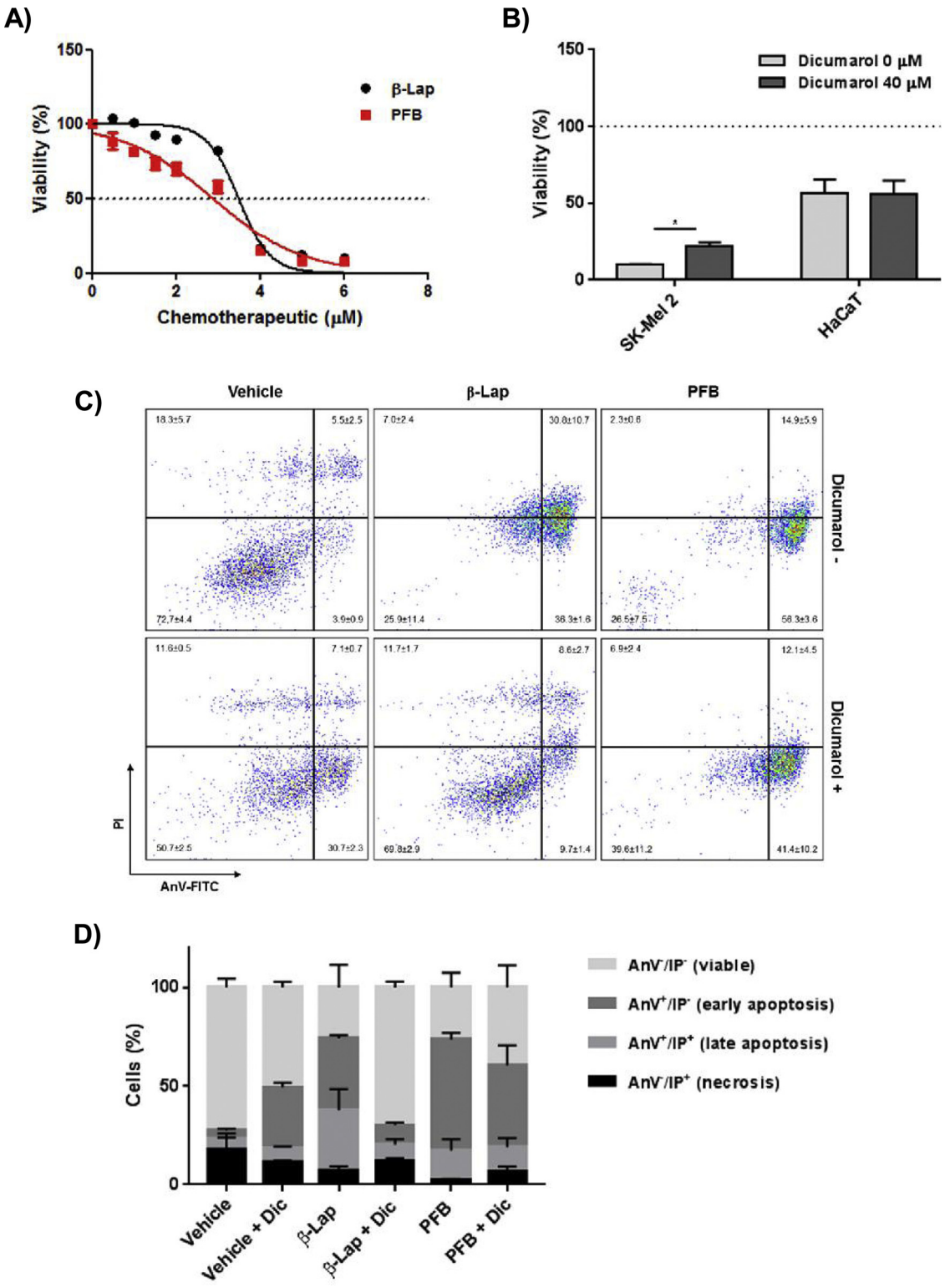


Fig. 3. Comparison of β-Lap and PFB antitumor efficiency against metastatic melanoma cells. (A) Human metastatic melanoma cells SK-Mel 2 were incubated with β-Lap and PFB (0–6 μM) for 24 h. Cell viability was evaluated by MTT assay and referred to cells incubated with DMSO (vehicle). The curves were fitted using a non-linear regression equation (dotted line: 50% viability). (B) Human melanoma cells SK-Mel 2 and human normal keratinocytes HaCaT were incubated with PFB (5 μM for SK-Mel 2 and 1.5 μM for HaCaT) in the presence or absence of dicumarol (40 μM) for 24 h. Cell viability was evaluated by MTT assay and referred to cells incubated with DMSO (vehicle) (dotted line: 100% viability). (C) Human melanoma cells SK-Mel 2 were incubated with β-Lap and PFB (5 μM) in the presence or absence of dicumarol (40 μM) for 24 h. Type of cell death was evaluated using Annexin V/PI staining by flow cytometry. The data generated by flow cytometry were plotted in two-dimensional dot plots in which PI is represented *versus* Annexin V-FICT. (D) Viable cells are Annexin V-negative and PI-negative (Annexin V^-/PI^-), early apoptotic cells are Annexin V-positive and PI-negative (Annexin V^+/PI^-), late (end-stage) apoptotic cells are Annexin V/PI-double-positive (Annexin V^+/PI^+) and necrotic cells are Annexin V-negative and PI-positive (Annexin V^-/PI^+) were quantified.

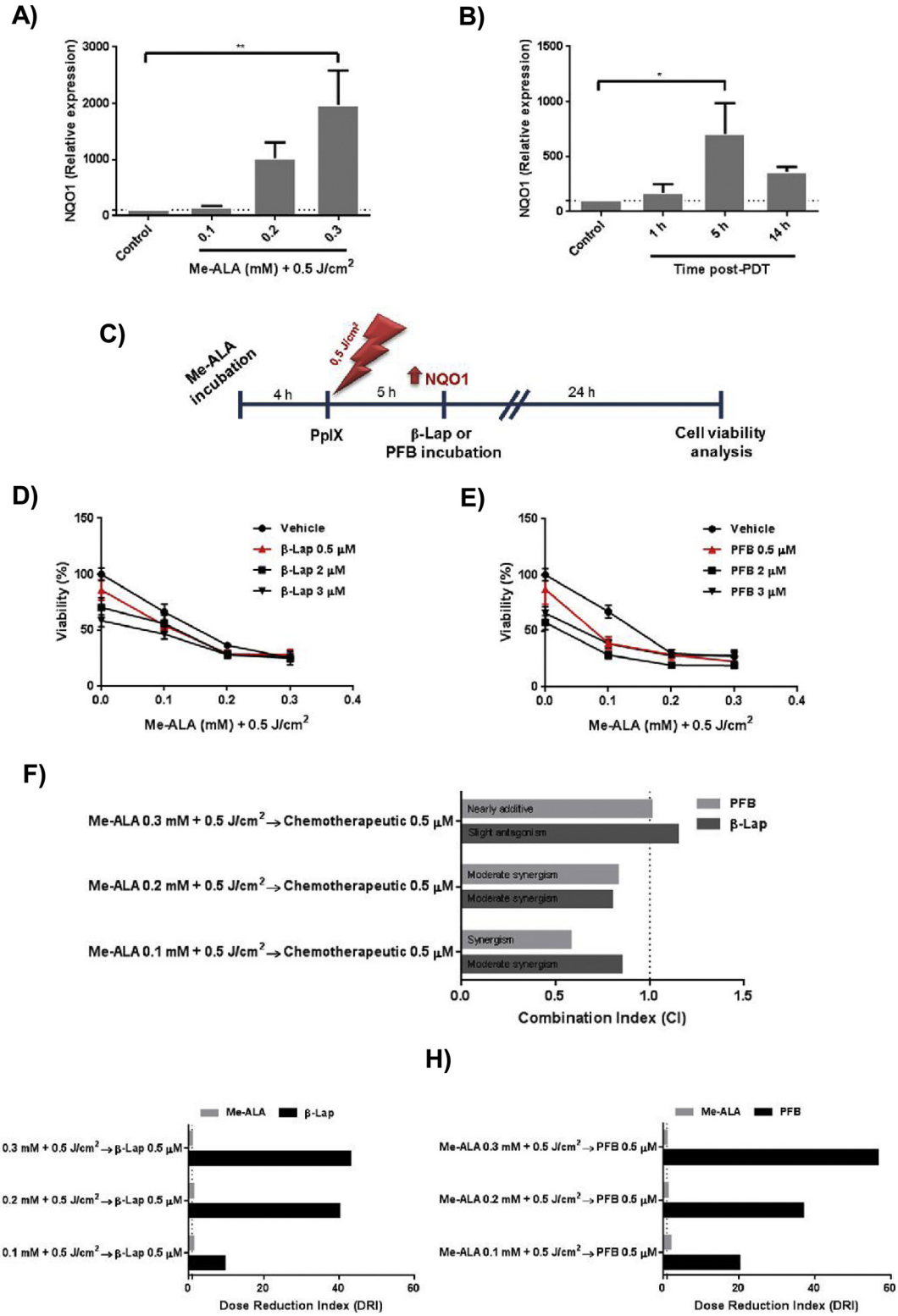


Fig. 4. Combined effects of PDT and β-Lap/PFB on the proliferation of melanoma cells. (A) Murine melanoma cells B16 were incubated with Me-ALA (0.1, 0.2 and 0.3 mM) for 4 h and then irradiated with 0.5 J/cm^2 light dose. Quantification of mRNA expression of NQO1 was performed 5 after treatment by RTqPCR. (B) Murine melanoma cells B16 were incubated with Me-ALA (0.3 mM) for 4 h and then irradiated with 0.5 J/cm^2 light dose. Quantification of mRNA expression of NQO1 was performed 1, 5 and 14 h after treatment by RTqPCR. (C) Experimental design: Murine melanoma cells B16 were incubated with Me-ALA (0.1, 0.2 and 0.3 mM) for 4 h and then irradiated with 0.5 J/cm^2 light dose. Following a 5 h incubation, β-Lap or PFB (0.5, 2 and 3 μM) were added for additional 24 h. Cell viability was evaluated by MTT assay and referred to cells incubated with DMSO (vehicle). (D) Dose-response curve for PDT/β-Lap combination. (E) Dose-response curve for PDT/PFB combination. (F) Combination index (CI) of non-constant dose rations showing interaction between PDT and β-Lap/PFB. A CI lower than 1 (dotted line) indicates synergism for combinations of PDT and β-Lap/PFB. (G–H) Dose Reduction Index (DRI) depending on combination schedule. A DRI greater than 1 (dotted line) indicates an enhanced cytotoxicity for combinations of PDT and β-Lap/PFB.

Table 3
Summary of synergistic, additive or antagonistic effects of combinations between β-Lap/PFB and PDT. CI values were interpreted as follows: < 0.1 very strong synergism, 0.1–0.3 strong synergism, 0.3–0.7 synergism, 0.7–0.85 moderate synergism, 0.85–0.90 slight synergism, 0.90–1.10 nearly additive, 1.10–1.20 slight antagonism, 1.20–1.45 moderate antagonism, 1.45–3.3 antagonism, 3.3–10 strong, > 10 very strong antagonism [16]. 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.

Me-ALA (mM)	β-Lap (μM)	PFB (μM)	Fa	CI	DRI β-Lap (μM)	DRI PFB (μM)	DRI Me-ALA (mM)
0.1	**0.5**	–	**0.46**	**0.85**	**9.60593**	–	**1.34797**
0.1	2.0	–	0.44	1.24	2.15199	–	1.28225
0.1	3.5	–	0.53	1.12	2.00805	–	1.60334
0.2	**0.5**	–	**0.71**	**0.80**	**40.2567**	–	**1.29490**
0.2	2.0	–	0.72	0.84	10.7574	–	1.33481
0.2	3.5	–	0.72	0.91	6.14708	–	1.33481
0.3	**0.5**	–	**0.72**	**1.15**	**43.0295**	–	**0.88987**
0.3	2.0	–	0.75	1.10	13.2608	–	0.97889
0.3	3.5	–	0.73	1.24	6.58015	–	0.91791
0.1	–	**0.5**	**0.61**	**0.57**	–	**20.1708**	**1.89642**
0.1	–	2.0	0.72	0.49	–	9.89724	2.57529
0.1	–	3.5	0.61	0.88	–	2.88154	1.89642
0.2	–	**0.5**	**0.71**	**0.83**	–	**37.0395**	**1.24933**
0.2	–	2.0	0.81	0.62	–	19.6478	1.75765
0.2	–	3.5	0.72	0.95	–	5.65557	1.28764
0.3	–	**0.5**	**0.77**	**1.01**	–	**56.6236**	**1.00980**
0.3	–	2.0	0.81	0.90	–	19.6478	1.17177
0.3	–	3.5	0.72	1.34	–	5.65557	0.85843

In the absence of an effective targeted monotherapy against melanoma, a better understanding of the interplay between biologic and cytotoxic anticancer agents will improve our ability to rationally design optimal combination regimens. There has been growing interest in the development of strategies to induce NQO1 activity in cancer cells for increasing the efficacy and selectivity of bioreductive anticancer drugs. Several reports have shown that ionizing radiation [39–41], cisplatin [42], hyperthermia [43–46], carnosic acid [47] and photodynamic therapy (PDT) [10] significantly upregulated NQO1 level in tumor cells, and sensitized them to β-Lap. As mentioned above, PDT is therapeutic involving light and a photosensitizing chemical substance with molecular oxygen to elicit *in situ* phototoxicity [8]. We have previously reported that PDT-mediated NQO1 upregulation significantly promoted therapeutic synergism in combination with β-Lap against breast cancer cell lines.

To better describe the effect of photodynamic induction of NQO1, here we have defined the transcriptional modulation of this enzyme over time and treatment conditions (Fig. 4A and 4B). Given that photodynamic effects are confined in the tumor site through spatially delivering both photosensitizer and light [8], PDT-mediated NQO1 upregulation *in situ* could certainly have occurred only on cancer cells. These data conferred the empirical guidelines for the design of combinatorial therapeutic regimens comprising PDT and NQO1-bioactivable drugs (Fig. 4C). Accordingly, the combination of chemotherapeutic plus PDT-based NQO1-inducing conditions increased therapeutic efficiency. We found that the sensitivity of malignant melanoma cells to β-Lap and PFB is closely tied to the extent of NQO1 expression. More importantly, the combination regimen worked better when chemotherapy was exerted by β-Lap-derivative PFB (Fig. 4).

In conclusion, our contribution provides a strong rationale for using therapies that combine photo- and chemotherapy to effectively treat melanoma with either high or low NQO1 expression. In addition, this combinatorial approach expands the limits of individual therapies, increasing the therapeutic possibilities against melanoma. On the basis of our exploratory data, PDT followed by low doses of PFB therapy should be both antitumor effective and extremely safe, not accompanied with normal tissue toxicity. Based on our findings, we emphasized NQO1 as a factor that deserves to be further investigated in order to provide a framework for improving melanoma treatment management.

Conflict of interest

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

Acknowledgements

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), CONICET-FAPERJ, Agencia Nacional de Promoción Científica y Tecnológica (AGENCIA-PICT), Secretaría de Ciencia y Técnica (SECyT), Universidad Nacional de Río Cuarto, Argentina. VAR and NBRV are members of the Scientific Researcher Career at CONICET. MJL hold graduate fellowship from CONICET. MC and ABMV hold undergraduate fellowship from CIN and INC, respectively.

References

[1] G. Mattia, R. Puglisi, B. Ascione, W. Malorni, A. Carè, P. Matarrese, Cell death-based treatments of melanoma:conventional treatments and new therapeutic strategies, Cell Death Dis. 9 (2018) 112.
[2] M. Garate, A. Wani, G. Li, The NAD(P)H:Quinone Oxidoreductase 1 induces cell cycle progression and proliferation of melanoma cells, Free Radic. Biol. Med. 48 (2010) 1601–1609.
[3] Y. Cheng, J. Li, M. Martinka, G. Li, The expression of NAD(P)H:quinone oxidoreductase 1 is increased along with NF-kappaB p105/p50 in human cutaneous melanomas, Oncol. Rep. 23 (2010) 973–979.
[4] J. Li, Z. Zhang, G. Li, Patient outcome prediction using multiple biomarkers in human melanoma : A clinicopathological study of 118 cases, Exp. Ther. Med. (2011) 131–135, https://doi.org/10.3892/etm.2010.169.
[5] J. Pink, S. Planchon, C. Tagliarino, M. Varnes, D. Siegel, D. Boothman, NAD(P)H:Quinone oxidoreductase activity is the principal determinant of beta-lapachone cytotoxicity, J. Biol. Chem. 25 (February (8)) (2000) 5416–5424.
[6] H. Khong, L. Dreisbach, H. Kindler, D. Trent, K. Jeziorski, I. Bonderenko, et al., A phase 2 study of ARQ 501 in combination with gemcitabine in adult patients with treatment naïve, unresectable pancreatic adenocarcinoma, J. Clin. Oncol. 25 (2017) 15017–15017.
[7] L. Hartner, L. Rosen, M. Hensley, D. Mendelson, A. Staddon, W. Chow, et al., Phase 2 dose multi-center, open-label study of ARQ 501, a checkpoint activator, in adult patients with persistent, recurrent or metastatic leiomyosarcoma (LMS), J. Clin. Oncol. 25 (2007) 20521–20521.
[8] P. Agostinis, K. Berg, K. Cengel, T. Foster, A. Girotti, S. Gollnick, et al., Photodynamic therapy of cancer: an update, CA Cancer J. Clin. 61 (2011) 250–281.
[9] Y. Huang, D. Vecchio, P. Avci, R. Yin, M. Garcia-Diaz, M. Hamblin, Melanoma resistance to photodynamic therapy: new insights, Biol. Chem. 394 (2013) 239–250.
[10] M. Lamberti, N. Rumie Vittar, F. de Carvalho da Silva, V. Ferreira, V. Rivarola, Synergistic enhancement of antitumor effect of B-Lapachone by photodynamic induction of quinone oxidoreductase (NQO1), Phytomedicine 20 (2013) 1007–1012.
[11] Y. Adamovicha, The Protein Level of PGC-10, a Key Metabolic Regulator, Is Controlled by NADH-NQO1, Mol. Cell. Biol. 33 (2013) 2603–2613.
[12] S. Mathupala, A. Sloan, An agarose-based cloning-ring anchoring method for isolation of viable cell clones, Biotechniques 46 (2009) 305–307.
[13] Z. Tang, C. Li, B. Kang, G. Gao, C. Li, Z. Zhang, GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses, Nucleic Acids Res. 45 (2017) W98–W102.
[14] F. Silva, S. Ferreira, C. Kaiser, A. Pinto, V. Ferreira, Synthesis of α-and β-lapachone derivatives from hetero diels-alder trapping of alkyl and aryl o-quinone methides, J. Braz. Chem. Soc. 20 (2009) 1478–1482.
[15] L. Ibarra, G. Porcal, L. Macor, R. Ponzio, R. Spada, C. Lorente, et al., Metallated porphyrin-doped conjugated polymer nanoparticles for efficient photodynamic therapy of brain and colorectal tumor cells, Nanomedicine (Lond) 13 (2018) 605–624.
[16] T.C. Chou, Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies, Pharmacol. Rev. 58 (2006) 621–681.
[17] M. Berenbaum, What is synergy? Pharmacol. Rev. 41 (1989) 93–141.
[18] R. Traver, T. Horikoshi, K. Danenberg, T. Stadlbauer, P. Danenberg, D. Ross, et al., NAD(P)H:quinone oxidoreductase gene expression in human colon carcinoma cells: characterization of a mutation which modulates DT-diaphorase activity and mitomycin sensitivity, Cancer Res. 52 (1992) 797–802.
[19] Y. Yang, Y. Zhang, Q. Wu, X. Cui, Z. Lin, S. Liu, et al., Clinical implications of high NQO1 expression in breast cancers, J. Exp. Clin. Cancer Res. 33 (2014) 14.
[20] Y. Ma, J. Kong, G. Yan, X. Ren, D. Jin, T. Jin, et al., NQO1 overexpression is associated with poor prognosis in squamous cell carcinoma of the uterine cervix, BMC Cancer 14 (2014) 414.

[21] Z.L. Jason, K. Yuebin, P.M. Hara, A.T. Michae, L.Y. Robert, Z. Hong, et al., Mechanistic studies of cancer cell mitochondria- and NQO1-mediated redox activation of beta-lapachone, a potentially novel anticancer agent, Toxicol. Appl. Pharmacol. 281 (2014) 285–293.
[22] R.C. Krieg, H. Messmann, J. Rauch, S. Seeger, R. Knuechel, Metabolic characterization of tumor cell-specific protoporphyrin IX accumulation after exposure to 5-aminolevulinic acid in human colonic cells, Photochem. Photobiol. 76 (2002) 518–525.
[23] T. Choi, K. Sohn, J. Kim, S. Kim, C. Kim, J. Hwang, et al., Impact of NAD(P)H:quinone oxidoreductase-1 on pigmentation, J. Invest. Dermatol. 130 (2010) 784–792.
[24] L. Marrot, C. Jones, P. Perez, J. Meunier, The significance of Nrf2 pathway in (photo)-oxidative stress response in melanocytes and keratinocytes of the human epidermis, Pigm. Cell Melanoma Res. 21 (2008) 79–88.
[25] A. Dinkova-Kostova, P. Talalay, NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector, Arch. Biochem. Biophys. 501 (2010) 116–123.
[26] D. Ross, J. Kepa, S. Winski, H. Beall, A. Anwar, D. Siegel, NAD(P)H:quinone oxidoreductase 1 (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms, Chem. Biol. Interact. 129 (2000) 77–97.
[27] D. Siegel, D. Gustafson, D. Dehn, J. Han, P. Boonchoong, L. Berliner, et al., NAD(P)H:quinone oxidoreductase 1: role as a superoxide scavenger, Mol. Pharmacol. 65 (2004) 1238–1247.
[28] J. Kee, Y. Han, D. Kim, J. Mun, S. Park, H. So, et al., β-Lapachone suppresses the lung metastasis of melanoma via the MAPK signaling pathway, PLoS One 12 (2017) e0176937.
[29] J. Li, Y. Ke, H. Misra, M. Trush, Y. Li, H. Zhu, et al., Mechanistic studies of cancer cell mitochondria- and NQO1-mediated redox activation of beta-lapachone, a potentially novel anticancer agent, Toxicol. Appl. Pharmacol. 281 (2014) 285–293.
[30] C. Li, L. Averboukh, A. Pardee, beta-Lapachone, a novel DNA topoisomerase I inhibitor with a mode of action different from camptothecin, J. Biol. Chem. 268 (1993) 22463–22468.
[31] D. Boothman, A. Pardee, Inhibition of radiation-induced neoplastic transformation by beta-lapachone, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 4963–4967.
[32] S. Villamil, D. Podestá, M. Molina Portela, A. Stoppani, Characterization of poly (ADP-ribose)polymerase from Crithidia fasciculata: enzyme inhibition by beta-lapachone, Mol. Biochem. Parasitol. 115 (2001) 249–256.
[33] A. Vanni, M. Fiore, R. De Salvia, E. Cundari, R. Ricordy, R. Ceccarelli, et al., DNA damage and cytotoxicity induced by beta-lapachone: relation to poly(ADP-ribose) polymerase inhibition, Mutat. Res. 401 (1998) 55–63.
[34] C. Tagliarino, J. Pink, G. Dubyak, A. Nieminen, D. Boothman, Calcium is a key signaling molecule in beta-lapachone-mediated cell death, J. Biol. Chem. 276 (2001) 19150–19159.
[35] S. Manna, Y. Gad, A. Mukhopadhyay, B. Aggarwal, Suppression of tumor necrosis factor-activated nuclear transcription factor-kappaB, activator protein-1, c-Jun N-terminal kinase, and apoptosis by beta-lapachone, Biochem. Pharmacol. 57 (1999) 763–774.
[36] Y. Li, C. Li, A. Pinto, A. Pardee, Release of mitochondrial cytochrome C in both apoptosis and necrosis induced by beta-lapachone in human carcinoma cells, Mol Med. 5 (1999) 232–239.
[37] M. Krawiecka, B. Kuran, J. Kossakowski, M. Cieślak, J. Kazmierczak-, K. Królewska, et al., Synthesis and Cytotoxic Properties of Halogen and Aryl- / Heteroarylpiperazinyl Derivatives of Benzofurans, Anticancer Agents Med. Chem. (2015) 115–121.
[38] Y. Kanaho, T. Sato, T. Fujii, The affinity of various phenothiazine drugs for membranes of intact human erythrocytes and their membrane-transforming activity, Mol. Pharmacol. 20 (1981) 704–708.
[39] E.K. Choi, K. Terai, I.-M. Ji, Y.H. Kook, K.H. Park, E.T. Oh, et al., Upregulation of NAD(P)H:Quinone oxidoreductase by radiation potentiates the effect of bioreductive beta-Lapachone on cancer cells, Neoplasia. 9 (2007) 634–642, https://doi.org/10.1593/neo.07397.
[40] M. Suzuki, M. Amano, J. Choi, H.J. Park, B.W. Williams, K. Ono, et al., Synergistic effects of radiation and beta-lapachone in DU-145 human prostate cancer cells in vitro, Radiat. Res. 165 (2006) 525–531.
[41] H. Park, K. Ahn, S. Ahn, E. Choi, S. Lee, B. Williams, et al., Susceptibility of cancer cells to beta-lapachone is enhanced by ionizing radiation, Int. J. Radiat. Oncol. Biol. Phys. 61 (2005) 212–219.
[42] K. Terai, G. Dong, E. Oh, M. Park, Y. Gu, C. Song, et al., Cisplatin enhances the anticancer effect of beta-lapachone by upregulating NQO1, Anticancer Drugs 20 (2009) 901–909.
[43] T. Hori, T. Kondo, H. Lee, C. Song, H. Park, Hyperthermia enhances the effect of β-lapachone to cause γH2AX formations and cell death in human osteosarcoma cells, Int. J. Hyperthermia 27 (2011) 53–62.
[44] G. Dong, H. Youn, M. Park, E. Oh, K. Park, C. Song, et al., Heat shock increases expression of NAD(P)H:quinone oxidoreductase (NQO1), mediator of beta-lapachone cytotoxicity, by increasing NQO1 gene activity and via Hsp70-mediated stabilisation of NQO1 protein, Int. J. Hyperthermia 25 (2009) 477–487.
[45] C. Song, J. Chae, E. Choi, T. Hwang, C. Kim, B. Lim, et al., Anti-cancer effect of bioreductive drug beta-lapachon is enhanced by activating NQO1 with heat shock, Int. J. Hyperthermia 24 (2008) 161–169.
[46] H.J. Park, E.K. Choi, J. Choi, K.-J. Ahn, E.J. Kim, I.-M. Ji, et al., Heat-induced up-regulation of NAD(P)H:quinone oxidoreductase potentiates anticancer effects of beta-lapachone, Clin. Cancer Res. 11 (2005) 8866–8871.
[47] N. Arakawa, A. Okubo, S. Yasuhira, K. Takahashi, H. Amano, T. Akasaka, et al., Carnosic acid, an inducer of NAD(P)H quinone oxidoreductase 1, enhances the cytotoxicity of β-lapachone in melanoma cell lines, Oncol. Lett. 15 (2018) 2393–2400.