ORIGINAL ARTICLE

Combinatorial effects of thymoquinone on the anti-cancer activity of doxorubicin

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Abstract

Purpose Doxorubicin is a mainstay of cancer chemotherapy despite its clinical limitations that arise from its cardiotoxicity and the high incidence of multi-drug resistance. Recent studies revealed a protective effect of thymoquinone, a non-toxic constituent of the essential oil of *Nigella sativa*, against doxorubicin-induced cardiotoxicity. We now investigated the influence of thymoquinone on various other effects exerted by doxorubicin in human cancer cells.

Methods Doxorubicin, thymoquinone and equimolar mixtures of both were tested for cytotoxicity on human cells of HL-60 leukaemia, 518A2 melanoma, HT-29 colon, KB-V1 cervix, and MCF-7 breast carcinomas as well as multi-drug-resistant variants thereof and on non-malignant human fibroblasts (HF). Apoptosis induction was analysed via DNA fragmentation, activity studies of the caspases-3, -8 and -9, determination of changes in the mitochondrial membrane potential and in the ratio of the mRNA expressions of pro- and anti-apoptotic proteins bax and bcl-2. The generation of reactive oxygen species (ROS) was assessed by the NBT assay.

Results Thymoquinone improved the anti-cancer properties of doxorubicin in a cell line-specific manner. We found a significant rise of the growth inhibition by doxorubicin in HL-60 and multi-drug-resistant MCF-7/TOPO cells when thymoquinone had been added. The mode of action of both drugs and of their mixture was mainly apoptotic. In HL-60 cells, the drug mixture caused an additional concentration maximum of effector caspase-3 not observed for either of the pure drugs. The impact of the drug mixture on the mitochondria of HL-60 cells was also greater than those of the individual quinones alone. In addition, the drug mixture led to a higher concentration of reactive oxygen species in HL-60 cells.

Conclusions In summary, thymoquinone is a booster for the anti-cancer effect of doxorubicin in certain cancer cell lines. Distinct improvements on efficacy, selectivity, and even breaches of multi-drug resistance were observed for equimolar mixtures of doxorubicin and thymoquinone.

Introduction

A great deal of naturally occurring quinones have pharmacologically interesting properties including anti-tumoural activity [1–3]. The anthraquinone doxorubicin (DOX, **1**) is a *Streptomyces* metabolite that is clinically used in the treatment of human tumours such as soft tissue sarcomas, lymphomas and various types of carcinomas. However, the clinical application is hampered by an early onset of resistance and by dose restrictions necessary to prevent cumulative cardiac toxicity [4]. The *para*-benzoquinone thymoquinone (TQ, **2**) is the active principle of thyme essential oil and of the volatile oil of black seed (*Nigella sativa*), responsible for the anti-oxidant and anti-neoplastic effects of these spices. It has a low general toxicity, but a promising anti-tumour activity [5–8]. It induces apoptosis in cancer cells in p53-dependent and p53-independent ways, and it was found to delay tumour growth by induction of cell cycle arrest in certain xenograft models [9] (Scheme 1).

As part of a project aimed to improve the efficacy of anti-cancer drugs in resistant tumour cells, we previously prepared

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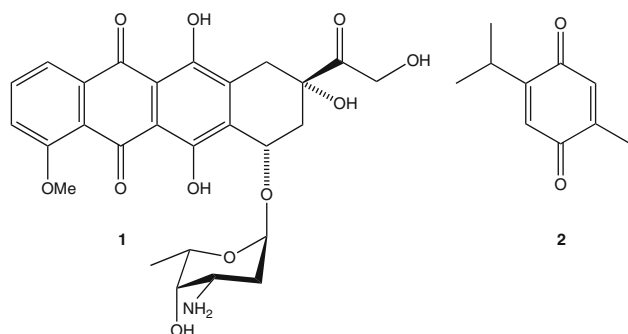
Scheme 1 Two biologically active natural products with *para*-quinone motif: doxorubicin (DOX, **1**) isolated from *Streptomyces peucetius* var. *caesius* and thymoquinone (TQ, **2**) a constituent of the essential oil of black seed (*Nigella sativa*)

covalent conjugates of both DOX and TQ with fatty acid and terpene carriers [10–12]. In some cases, we saw dramatically greater anti-proliferative effects of the conjugates when compared to those of the parent quinones. For instance, 6-(hencosahexaenyl)thymoquinone, derived from the reaction of TQ with docosahexaenoic acid (DHA), was efficacious in multi-drug-resistant MCF-7/TOPO breast carcinoma cells at an IC_{50} (72 h) <50 nM while TQ itself required an IC_{50} (72 h) $\approx$ 30 μM [10]. Conjugation to betulinic acid resulted in a compound with IC_{50} (72 h) $\approx$ 130 nM in these cancer cells [11]. In the case of DOX, similar conjugates with improved efficacy and a potential to overcome multi-drug resistance were developed. Some of them had a mechanism of action different from that of DOX [12].

Inspired by studies proving that TQ can indeed mitigate or even prevent doxorubicin-induced cardiomyopathy [13–15], we now investigated the influence of TQ on the magnitude and mechanism of the various effects exerted by DOX in human cancer cells. We tested both substances and an equimolar mixture of them for growth inhibition on the human cancer cell lines HL-60 leukaemia and 518A2 melanoma, HT-29 colon, KB-V1 cervix, MCF-7 breast carcinomas as well as in their multi-drug-resistant variants and non-malignant fibroblasts (HF). The impact of the test compounds and their mixture on the mitochondrial membrane potential of HL-60 and 518A2 cells and on the cellular levels of various apoptosis-relevant species such as caspases, bax and bcl-2 mRNA, and reactive oxygen species (ROS) was also scrutinised.

Materials and methods

Chemicals

Thymoquinone was purchased from Sigma–Aldrich, Munich (Germany), doxorubicin was a gift from Ribo-sepharm GmbH, Gräfelfing (Germany). Both drugs were

kept at 4°C as 10 mM stock solutions in DMSO. Appropriate test concentrations were obtained by dilution with cell culture medium immediately before use.

Cell lines and culture conditions

The HL-60 leukaemia cells were obtained from the German Collection of Biological Material (DSMZ), Braunschweig (Germany), the 518A2 melanoma cells from the Department of Oncology and Haematology of the Martin-Luther University, Halle (Germany), the KB-V1 cervix and the MCF-7 breast carcinoma cells from the Institute of Pharmacy of the University of Regensburg (Germany), and the HT-29 colon carcinoma cells as well as the human foreskin fibroblasts (HF) from the University Hospital of Erlangen (Germany). The multi-drug-resistant cells were bred by repeated treatment with vinblastine sulphate (KB-V1/VBL: 340 nM), topotecan hydrochloride (MCF-7/TOPO: 550 nM), colchicine (HT-29/COLC: 62.5 nM) and doxorubicin (KB-V1/DOX: 200 nM, MCF-7/DOX: 55 nM, HT-29/DOX: 75 nM). The HL-60 and HT-29 cells were grown in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS), 100 IU mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin sulphate, 0.25 μg mL^{-1} amphotericin B and 250 μg mL^{-1} gentamycine (all from Gibco, Egenstein, Germany). The 518A2 and the KB-V1 cells as well as the HF were cultured in D-MEM medium (Gibco) containing 10% FCS, 100 IU mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin sulphate, 0.25 μg mL^{-1} amphotericin B and 250 μg mL^{-1} gentamycine. The MCF-7 cells were grown in E-MEM medium (Sigma) supplemented with 2.2 g L^{-1} NaHCO$_3$, 110 mg L^{-1} sodium pyruvate and 5% FCS. The cells were maintained in a moisture-saturated atmosphere (5% CO$_2$) at 37°C in 75-mL culture flasks (Nunc, Wiesbaden, Germany). They were serially passaged following trypsinisation by 0.05% trypsin/0.02% EDTA (PAA laboratories, Cölbe, Germany). Mycoplasma contamination was routinely monitored, and only mycoplasma-free cultures were used.

Inhibition of cell growth and metabolic activity (MTT assay)

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (ABCR, Karlsruhe, Germany) was used to identify viable cells which reduce it to a violet formazan. HL-60 leukaemia cells (5 × 10^5 mL^{-1}) and cells (5 × 10^4 mL^{-1}) of 518A2 melanoma, KB-V1 cervix, MCF-7 breast and HT-29 colon carcinoma, and HF foreskin fibroblasts were seeded out in 96-well tissue culture plates and cultured for 24 h [16]. Inhibitors of P-pg, BCRP or MRP 1 (P-gp overexpressing KB-V1/VBL cells: 24 μM verapamil hydrochloride, BCRP-rich MCF-7/TOPO cells: 1.2 μM

fumitremorgin C, MRP 1-rich HT-29/COLC cells: 10 μM MK571) were optionally added. Incubation (5% CO_2, 95% humidity, 37°C) of the cells following treatment with the test compounds was continued for up to 72 h. Blank and solvent controls were treated identically. MTT in phosphate-buffered saline (5 mg mL^{-1}) was added to a final concentration of 0.05% (HL-60, 518A2, HF) or 0.1% (KB-V1, MCF-7, HT-29). After 2 h, the formazan precipitate was dissolved in 10% sodium dodecylsulphate in DMSO containing 0.6% acetic acid in the case of the HL-60 cells. For the adherent 518A2, KB-V1, MCF-7, HT-29 and HF cells, the microplates were swiftly turned to discard the medium before adding the solvent mixture. The microplates were gently shaken in the dark for 30 min, and the absorbance at 570 and 630 nm (background) was measured with a Tecan F200 plate reader. All experiments were carried out in quadruplicate; the percentage of viable cells was calculated as the mean ± SD with controls set to 100%.

Caspase activity assay

Caspase activities were determined by luminometric assays (Caspase-Glo 3/7, 8 and 9, Promega, USA), according to the manufacturer's instructions. Cellular proteins were extracted from HL-60 and 518A2 cells following treatment with 5 μM of the test compounds or a combination of both compounds at equal 5 μM concentrations in a lysis buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton-X-100 and 1× EDTA-free protease inhibitor mix (Calbiochem). Cell lysates were incubated at 4°C for 15 min, centrifuged at 800g for 10 min, and the precipitates were discarded. Protein concentrations were measured using the Bradford Reagent (Sigma) and bovine serum albumin as a standard. Cell lysates (15 μg cellular protein) were analysed for caspase activities using the homogeneous luminescent assay. After cleavage of the luminogenic substrate containing a caspase-specific peptide sequence, the luminescence was quantified with a Tecan Genios Plus plate reader and assumed to be proportional to the caspase activities in the cell lysates [17].

Real-time PCR for detection of bcl-2 and bax mRNA expression

HL-60 and 518A2 cells were treated with 5 μM of the test compounds or a combination of both compounds at equal concentrations (5 μM) for up to 24 h. The total RNA was extracted using peqGOLD RNAPure (peqLab, Erlangen, Germany) according to the manufacturer's instructions. The synthesis of cDNA was conducted in a 21-μL reaction mixture starting with 80 ng total RNA using the reverse transcription system (Promega, Madison, WI, USA). Quantitative PCR was performed using the SYBR GREEN

PCR-Kit LightCycler fast start DNA Master for a Light-Cycler 2.0 System (Roche Diagnostic, Mannheim, Germany) and threshold numbers were determined using the LightCycler Software, version 3.5. All genes examined were normalised to a housekeeping gene encoding GAPDH [18]. The primers were obtained from Qiagen: GAPDH (Hs_Gapd_2_SG QuantiTect Primer Assay, Cat. No.: QT01192646), Bax (Hs_Bax_2_SG QuantiTect Primer Assay, Cat. No.: QT00031192), Bcl-2 (Hs_Bcl2_1_SG QuantiTect Primer Assay, Cat. No.: QT00025011).

Mitochondrial membrane potential

Changes in mitochondrial membrane potential were determined by the Mitochondrial Membrane Detection Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's procedure. Following treatment, cell samples were centrifuged at 400g for 5 min. The pellets were resuspended in 500 μL JC-1 solution (0.1×), incubated at 37°C for 15 min (HL-60) or 35 min (518A2) and then centrifuged again for 5 min at 400g. After washing, the pellets were resuspended in 100 μL PBS and transferred to a well of a black 96-well plate. The red ($\lambda_{ex} = 585$ nm, $\lambda_{em} = 590$ nm) and green ($\lambda_{ex} = 510$ nm, $\lambda_{em} = 527$ nm) fluorescence intensities were measured with a Tecan F200 plate reader, and their ratio was calculated [19].

Generation of ROS (NBT assay)

HL-60 cells (5×10^5 mL^{-1}) were plated in 96-well tissue culture plates, and test compounds were added after 24 h of incubation at 37°C to achieve a final concentration of 5 μM. Incubation (5% CO_2, 95% humidity, 37°C) of cells following treatment with the test compounds was continued for 24 and 72 h. After removal of the cell medium by centrifugation, the cells in each well were resuspended in 100 μL 0.1% NBT, and the plates were placed into the incubator for 1 h. The reduced NBT was solubilised with 100 μL 2 M KOH and 130 μL DMSO for 30 min. The absorbance was measured for each well at 630 and 405 nm (background) using an ELISA plate reader. The adherent 518A2 cells (5×10^4 mL^{-1}) were seeded out in 96-well tissue culture plates after trypsinisation and incubation for 24 h at 37°C to allow attachment, then treated similarly, only the medium was removed prior to incubation with NBT for 4 h. All experiments were carried out in quadruplicate [20].

Mathematical analysis of combined drug effects

The combined effects of DOX and TQ on the 518A2, HL-60, HT-29/COLC, KB-V1/VBL and MCF-7/TOPO cells were quantified using a known "multiple-drug effect analysis" based on the median effect principle and isobologram

technique by Chou and Talalay [21, 22]. This model allows the determination of CI values (combination indices) based on the median dose–effect and combination index equation. We interpreted the combination of DOX and TQ as synergistic when CI < 1, additive when CI = 1 and antagonistic when CI > 1.

Statistical analysis of measurement results

The results are expressed as means ± standard deviation (SD). The Student–Newman–Keuls test was used to determine statistical significance with a P value <0.05 considered significant (*).

Results and discussion

Inhibition of cell growth

Compounds **1** (DOX), **2** (TQ), and an equimolar mixture of both were tested for their growth inhibition in cells of human HL-60 leukaemia and 518A2 melanoma, P-gp-rich KB-V1/VBL cervix carcinoma, BCRP-rich MCF-7/TOPO breast carcinoma and MRP 1-rich HT-29/COLC colon carcinoma as well as their parental, sensitive variants (KB-V1, MCF-7 and HT-29) and in non-malignant human foreskin fibroblasts (HF). The IC$_{50}$ values after 72 h exposure to the test compounds are summarised in Table 1 [16].

In sensitive HL-60 cells, DOX was efficacious at IC$_{50}$ (72 h) = 80 nM [12]. The equimolar mixture of DOX and TQ was even fourfold more active in these cells. An opposite activity lowering effect of adding TQ to DOX was found for the 518A2 melanoma cells.

Somewhat surprising, the non-malignant foreskin fibroblasts (HF) were affected most by the drug mixture, which was growth inhibiting at an IC$_{50}$ value ca. 20-times lower than that of DOX alone. A possible explanation for this could be developed along the following line. There were reports that DOX induces apoptosis in normal and tumour cells via different mechanisms [23]. While a transcriptional activation of p53 is crucial for a DOX-induced apoptosis in tumour cells, the apoptosis of normal cells is initiated mainly by a rise in caspase-3 and in ROS. Should a mixture of **1** and **2** lead to a significantly higher ROS level in the fibroblasts, they would be hit harder than the tumour cells (see below).

For multi-drug-resistant MCF-7/TOPO cells, a doubling of growth inhibition by the mixture of the two quinones over that by DOX alone was observed. The multi-drug-resistant KB-V1/VBL cells responded equally poorly to DOX and to the mixture of DOX and TQ. Furthermore, a co-incubation of multi-drug-resistant HT-29/COLC colon carcinoma cells with DOX and TQ resulted in a decrease in growth inhibition compared to DOX alone.

To quantitatively analyse the combined drug effects of DOX and TQ against the above-mentioned cells, the

Table 1 Inhibitory concentrations IC$_{50}$ in μM of DOX (**1**), TQ (**2**) and an equimolar mixture of **1** and **2** when applied for 72 h to 518A2, HL-60, KB-V1, MCF-7, HT-29 cancer cells and foreskin fibroblasts (HF)

Compound	**1**	**1**[a]	**2**	**2**[a]	**1 + 2**	**1 + 2**[a]
518A2	0.12 ± 0.04[c]	–	28.3 ± 9.2[b]	–	0.33 ± 0.03	–
HF	6.9 ± 3.7	–	32.6 ± 19.6[b]	–	0.29 ± 0.09	–
HL-60	0.08 ± 0.01[c]	–	27.8 ± 6.0[b]	–	0.02 ± 0.01	–
HT-29	0.85 ± 0.11	–	46.8 ± 18.7	–	1.1 ± 0.4	–
HT-29/COLC	0.69 ± 0.10	0.10 ± 0.03	57.2 ± 27.9	77.5 ± 14.1	0.83 ± 0.22	0.17 ± 0.03
HT-29/DOX	0.42 ± 0.12	–	39.0 ± 12.6	–	0.35 ± 0.21	–
KB-V1	0.05 ± 0.01	–	25.8 ± 13.4	–	0.18 ± 0.02	–
KB-V1/VBL	18.3 ± 7.6[c]	1.0 ± 0.04[c]	32.3 ± 6.0[b]	24.1 ± 3.5[b]	20.9 ± 3.5	0.44 ± 0.16
KB-V1/DOX	1.8 ± 0.6	–	18.7 ± 1.6	–	5.6 ± 1.3	–
MCF-7	0.09 ± 0.01	–	20.1 ± 2.0	–	0.26 ± 0.02	–
MCF-7/TOPO	1.0 ± 0.4[c]	0.08 ± 0.01[c]	26.7 ± 5.6[b]	23.1 ± 2.5[b]	0.64 ± 0.22	0.18 ± 0.01
MCF-7/DOX	1.7 ± 0.4	–	40.2 ± 2.3	–	5.6 ± 1.2	–

Values are derived from concentration–response curves obtained by measuring the percentual absorbance of viable cells relative to untreated controls (100%) after 72 h exposure of 518A2 melanoma, HL-60 leukaemia, KB-V1 cervix carcinoma, MCF-7 breast carcinoma and HT-29 colon carcinoma cells as well as human foreskin fibroblasts (HF) to the test compounds in the MTT assay. Values represent means of four independent experiments ± standard deviations

[a] With 24 μM verapamil hydrochloride added for KB-V1/VBL, with 1.2 μM fumitremorgin C added for MCF-7/TOPO and with 10 μM MK-571 added for HT-29/COLC cells

[b] Values taken from [11]

[c] Values taken from [12]

multi-drug effect analysis based on the median effect principle and the isobologram technique by Chou and Talalay was used [21, 22]. DOX and TQ exhibited synergy (CI < 1) for HL-60 and MCF-7/TOPO cells, they were additive (CI = 1) for HT-29/COLC cells and antagonistic (CI > 1) for KB-V1/VBL and 518A2 cells at 72 h.

In order to check whether TQ can help overcome the DOX-related multi-drug resistance of cancer cells, we measured the IC$_{50}$ (72 h) values of the individual quinones and a combination of both in HT-29/COLC, KB-V1/VBL and MCF-7/TOPO cells, in the absence and in the presence of specific inhibitors of their respective ABC transporters. To this end 10 μM MK571 was added to the MRP 1-rich HT-29/COLC cells, 24 μM verapamil hydrochloride was added to the P-gp overexpressing KB-V1/VBL cells, and 1.2 μM fumitremorgin C was added to the BCRP-rich MCF-7/TOPO cells. The interference of the ABC transporters with the applied drugs was specified by the ratio of the IC$_{50}$ (72 h) values with and without the addition of the specific inhibitors (R$_{+/-}$) in MTT assays. This ratio is typically close to unity for drugs that are not substrates of these ABC efflux pumps. This was approximately the case for TQ when applied to KB-V1/VBL (R$_{+/-}$ = 0.75) and to MCF-7/TOPO cells (R$_{+/-}$ = 0.87). In contrast and as expected, DOX was a good substrate for the transporters MRP 1, P-gp and BCRP, respectively, of these three cell lines, apparent from ratios R$_{+/-}$ < 0.15. For the breast and colon carcinoma cells MCF-7/TOPO and HT-29/COLC, this ratio could be distinctly increased to R$_{+/-}$ = 0.28 by incubating them with an equimolar mixture of the two quinones.

In addition, the ratio (R$_{r/s}$) of the IC$_{50}$ (72 h) values in the multi-drug-resistant cells and the parental sensitive cells was determined. R$_{r/s}$ could be improved by adding TQ to DOX by a factor of nearly five in the case of MCF-7 and at least by a factor of three for the KB-V1 cells. In HT-29 cells, no difference was observed between resistant and sensitive cells.

We also tested DOX and TQ individually and in combination with KB-V1, MCF-7 and HT-29 cells that had been desensitised beforehand with clinically relevant doses of DOX (MCF-7/DOX: 55 nM, KB-V1/DOX: 200 nM, HT-29/DOX: 75 nM). We found a 1.2-fold higher efficacy of the mixture of DOX and TQ in the HT-29 cells desensitised for DOX.

Thus, the addition of TQ can improve the growth inhibitory effect of DOX in various cancer cells, including DOX refractory ones.

Induction of apoptosis

The induction of apoptosis by the quinones **1** and **2** had already been visualised via DNA fragmentation [9, 12, 24].

Additionally, we now probed the involvement of the apoptosis-relevant caspases-3, -8 and -9. HL-60 and 518A2 cells were treated with 5 μM of DOX or TQ or an equimolar mixture of both, which is a clinically relevant concentration resembling the peak plasma concentrations observed in patients after standard bolus infusions of DOX [25, 26]. When applied at markedly higher concentrations (>20 μM), the cytotoxicity of DOX is no longer correlated with its cardiotoxicity and its generation of reactive oxygen species [12]. The changes in caspase activities were then analysed at regular intervals between 1 and 24 h by a substrate-cleaving luminometric assay based on specific dye-tagged oligopeptides (Caspase-Glo Assay, Promega) [17].

Incubation of 518A2 cells with DOX and co-incubation with DOX and TQ gave similar caspase kinetics, while for HL-60 cells different kinetics were observed (Fig. 1). The levels of initiator caspase-8 and effector caspase-3 in HL-60 cells treated with DOX alone both reached a maximum after 12 h, while application of TQ caused only a maximum of caspase-3 after 2 h. Treating HL-60 cells with a mixture of TQ and DOX led to two maxima for the activities of either caspase. There was a simultaneous first maximum of caspases-3 and -8 after 2 h and a second maximum of caspase-8 activity after 12 h and of caspase-3 activity after 18 h. In effect, it means that mixtures of DOX and TQ give rise to two waves of pro-apoptotic effector
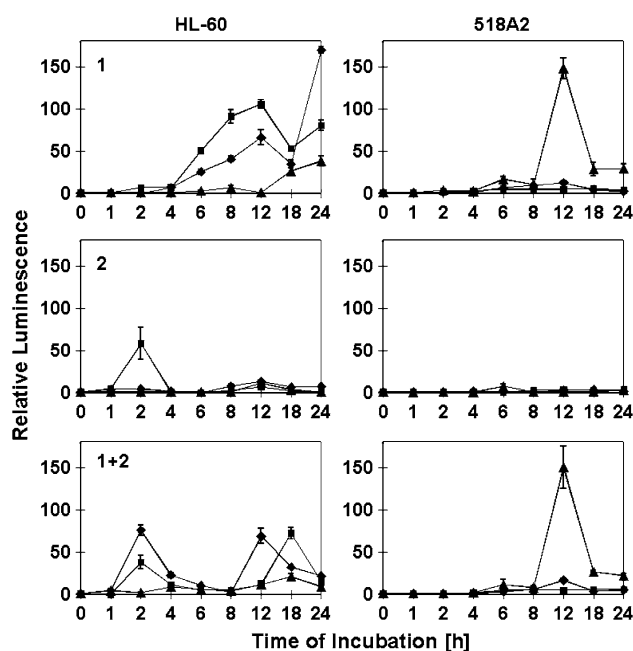

Fig. 1 Caspase activation in HL-60 and 518A2 cells treated with 5 μM of DOX (**1**), TQ (**2**) or **1** + **2** for up to 24 h. The activities of caspases-3 (*square symbol*), -8 (*lozenge symbol*) and -9 (*triangle symbol*) were quantified by a luminometric assay and are reported as relative luminescence intensities (I_{rel}). Data shown are means ± SD of two independent experiments
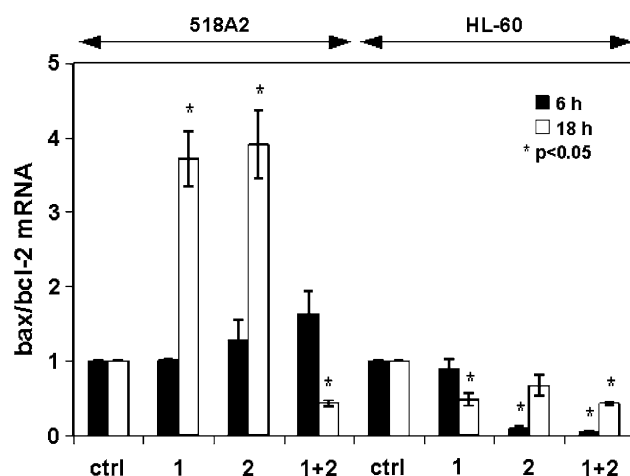
Fig. 2 Bax/bcl-2 mRNA expression in 518A2 and HL-60 cells after exposure to 5 μM of DOX (**1**), TQ (**2**) or **1** + **2** for 6 or 18 h. Conditions as stated in the experimental section. *Column heights* represent means of two independent experiments, *bars* indicate SD. Results from the Student's *t* test are given on the *top of the bars*. *P values of <0.05 vs. control were considered to be significant

caspase-3 activity in HL-60 leukaemia cells. This effect should contribute to the efficacy boost observed in the MTT tests with HL-60 cells upon addition of TQ to DOX.

Next, we measured the mRNA expression of anti-apoptotic bcl-2 and pro-apoptotic bax proteins in 518A2 and HL-60 cells upon incubation with 5 μM of DOX, TQ or an equimolar mixture of these quinones for up to 18 h (Fig. 2) [18]. Exposure of 518A2 cells to either DOX or TQ for 18 h led to a significant increase in the bax to bcl-2 mRNA ratio while their incubation with an equimolar mixture of DOX and TQ caused a sharp drop in this ratio due to a greater mRNA expression of anti-apoptotic bcl-2 ($P < 0.05$). HL-60 cells had earlier been reported to react by an immediate rise of the bax/bcl-2 ratio to a short-term treatment (2–6 h) with either TQ [9] or DOX [24]. This is in line with the caspase kinetics for TQ and its mixture with DOX as described above. Upon exposure of HL-60 cells to the test compounds for more than 6 h, the bax/bcl-2 ratio was now found to decrease again (Fig. 2). In summary, both 518A2 melanoma and HL-60 leukaemia cells respond to TQ and DOX in a pro-apoptotic manner, the former respond more slowly than the latter. The combination of TQ and DOX seems to accelerate the rise and subsequent fall of the pertinent bax/bcl-2 mRNA ratio.

Mitochondrial membrane potential and generation of ROS

The intrinsic pathway of apoptosis is associated with a temporal change not only of the caspase levels but also of the mitochondrial membrane potential $\Delta\Psi_m$, which we analysed using a kit from Stratagene that is based on the

fluorescent cationic dye JC-1 [19]. 518A2 and HL-60 cells were again treated with 5 μM DOX or TQ or their equimolar mixture for 6 and 18 h and then stained with JC-1. The ratio of red (JC-1 aggregates in intact mitochondria) to green fluorescence (JC-1 monomers in the cytosol) is decreased in apoptotic cells. Incubation of HL-60 cells with a mixture of DOX and TQ for 6 h resulted in a decrease of intact mitochondria relative to untreated cells, which was more pronounced than that observed for incubation with DOX alone. However, this difference dwindled upon prolonged incubation: DOX (6 h: 83%; 18 h: 28%), TQ (6 h: 77%; 18 h: 63%), DOX + TQ (6 h: 64%; 18 h: 32%), $P < 0.05$. In 518A2 cells, TQ exerted no additional effect.

Finally, we assessed the ability of the test compounds to initiate the generation of ROS in p53-negative HL-60 and p53-mutated 518A2 cells by means of the colorimetric nitroblue–tetrazolium (NBT) assay which is based on the selective reduction of a yellow, water-soluble tetrazolium chloride to an insoluble violet diformazan by superoxide ($O_2^{\cdot-}$) [20]. The *p*-quinones DOX and TQ can act either as anti-oxidants or as pro-oxidants depending on the given redox environment. The anti-proliferative and apoptotic effects of TQ in human colon cancer cells but not in normal intestinal cells were shown by El-Najjar et al. [27] to be induced via the generation of ROS. Similarly, different apoptosis-triggering mechanisms in normal and tumour cells were established for DOX [23]. However, unlike TQ which is non-toxic in normal tissues, DOX-initiated ROS generation seems to contribute both to the desired anti-cancer effect and to the unwanted cardiotoxicity [23, 26]. Table 2 also lists the results of the NBT assays for incubation periods of 24 and 72 h as in the MTT assays. This allows a correlation of ROS generation and cell growth inhibition. In both cancer cell lines, the mixture of DOX and TQ caused an increase in ROS generation when compared to the effect of DOX alone ($P < 0.05$). In 518A2 cells, we noticed a doubling of the ROS level, for the HL-60 cells the increase was even more than sevenfold.

This over-additive effect is remarkable. It might be due to a coupled redox pair of both *para*-quinones that can possibly tap other or more NAD(P)H-oxidoreductases than DOX alone and so generate intermediate semiquinones and eventually ROS more effectively and in higher concentrations [26]. Anyway, there is some coherence between the growth inhibitory effects of the test compounds and their ROS initiation in the two cancer cell lines examined.

Interestingly, for the non-malignant HF foreskin fibroblasts, no enhanced generation of ROS was observed upon treatment with either DOX or TQ or the mixture of both for 24 and 72 h. Thus, the stronger growth inhibition of HF observed in the MTT assay for the mixture of TQ and DOX remains inexplicable on the basis of the different impact of ROS on normal and cancer cells.

Table 2 Intact mitochondria (%) and increase of ROS (%) upon treatment of 518A2 melanoma and HL-60 leukaemia cells with 5 μM of DOX (**1**) or TQ (**2**) or an equimolar mixture of **1** and **2**

Compound	Mitochondria (%)[a]				ROS (%)[b]			
	518A2		HL-60		518A2		HL-60	
	6 h	18 h	6 h	18 h	24 h	72 h	24 h	72 h
1	98 ± 10[d]	74 ± 19[d]	83 ± 8[d,*]	28 ± 4[d,*]	2.5 ± 0.3[d,*]	15 ± 2[d,*]	2.8 ± 0.8[d,*]	5.6 ± 0.8[d,*]
2	106 ± 10	94 ± 9	77 ± 1*	63 ± 8*	0.8 ± 0.2[c]	1.2 ± 0.1[c]	1.0 ± 0.0[c]	1.0 ± 0.0[c]
1 + 2	99 ± 14	71 ± 12	64 ± 4*	32 ± 8*	6.2 ± 0.4*	26 ± 5*	12 ± 2*	40 ± 6*

[a] Ratio of red to green fluorescence relative to untreated controls (100%) after 6- and 18-h exposure of 518A2 melanoma and HL-60 leukaemia cells to 5 μM of the test compounds. Determined with the Mitochondrial Membrane Detection Kit (Stratagene, La Jolla, CA, USA). Values represent means of four independent experiments ± standard deviations

[b] NBT reduction as determined from per cent absorbance of formazan relative to untreated controls (1%) after 24 and 72 h exposure of 518A2 melanoma and HL-60 leukaemia cells to the test compounds. Values represent means of four independent experiments ± standard deviations

[c] Values taken from [10]

[d] Values taken from [12]

* Significant decrease of intact mitochondria/ROS generation vs. untreated controls ($P < 0.05$)

Protective effects of administration of high doses of TQ, i.e. 10 mg kg^{-1} d^{-1} [14], against DOX-induced cardiotoxicity have been observed when starting several days before application of DOX. The potential of TQ to act as a superoxide radical scavenger [28] and to inhibit lipid peroxidation was believed to be responsible. However, so far, the superoxide scavenging properties of TQ were established only in cell-free model systems. Further experiments, especially with cardiomyocytes, are necessary to elucidate the precise role of ROS mediation by TQ in its cardioprotective effect when administered in combination with DOX. This work is already under way.

Conclusion

We showed that the in vitro efficacy and selectivity of doxorubicin can be improved by adjuvant administration of the black seed constituent thymoquinone. In multi-drug-resistant MCF-7/TOPO breast carcinoma cells, the growth inhibition by DOX could be doubled by adding equimolar amounts of TQ (CI < 1). In HL-60 cells, the efficacy of DOX was even quadrupled in this way (CI < 1). In contrast, a diminished growth inhibitory effect of the 1:1-mixture of the two quinones was observed for 518A2 melanoma and HT-29/COLC colon cancer cells. In multi-drug-resistant KB-V1/VBL cervix carcinoma cells, no difference between DOX and its mixture with TQ was noticed. The interference of DOX with the BCRP transporter of the MCF-7/TOPO cells, as characterised by the ratio (R$_{+/-}$) of IC$_{50}$ (72 h) values in the presence and in the absence of the specific inhibitor fumitremorgin C, was attenuated by a factor of ca. 3.5 upon co-incubation with equimolar amounts of TQ. In the case of the HT-29/COLC

cells, similar experiments with the specific MRP 1 inhibitor MK-571 revealed a less pronounced effect of TQ. The mixture of DOX and TQ was also more efficacious than DOX alone against HT-29 cells that had been desensitised by repeated exposure to DOX (CI < 1). This is remarkable since TQ itself exhibited the same low activity in DOX-sensitive and DOX-resistant HT-29 cells.

The mode of action of both drugs and of their equimolar mixture was mainly apoptotic. Treatment of HL-60 cells with DOX, TQ or its equimolar mixture caused an induction of apoptosis by activating caspase-3 and -8. The mixture of the two quinones led to a greater decrease of intact mitochondria in HL-60 cells than the pure compounds. These beneficial effects of TQ could explain the observed quadruplication of growth inhibition and the rise of ROS in HL-60 cells treated with a mixture of TQ and DOX. As judged by the caspase kinetics, there seem to be two consecutive pro-apoptotic impacts that prevent the recovery of shattered cells. In 518A2 cells, DOX and its mixture with TQ gave rise to similar caspase kinetics, and no beneficial effect of TQ on the mitochondrial membrane potential $\Delta\Psi_m$ was seen when applied in combination with DOX. Either of the two quinones gave a pro-apoptotic effect in 518A2 cells when applied alone, whereas their mixture evoked an anti-apoptotic effect due to a higher level of anti-apoptotic bcl-2. 518A2 melanoma cells are known to have a bcl-2-related blockade in their mitochondrial pathway of apoptosis resulting in caspase-8 inhibition [29, 30]. When apoptosis eventually occurs, it mainly relies on caspase-9 that can be activated by a cytochrome c translocation which is independent of a mitochondrial transmembrane depolarisation [31]. As in HL-60 cells, the combination of DOX and TQ caused an increase of reactive oxygen species (ROS) in 518A2 melanoma cells, eventually leading to growth inhibition.

In summary, thymoquinone is a potential booster for the in vitro anti-cancer effect of doxorubicin. Depending on the cancer cell line, distinct improvements on efficacy, selectivity, and breach of multi-drug resistance were observed for equimolar mixtures of doxorubicin and thymoquinone.

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