Interactions of doxycycline with chemotherapeutic agents in human breast adenocarcinoma MDA-MB-231 cells

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Commonly used chemotherapeutic agents for breast cancer treatment include cisplatin, doxorubicin, and paclitaxel. Unfortunately, these effective antiproliferative agents are limited by their toxicities. Previously, we have shown that doxycycline can substantially reduce tumor burden in an animal model of breast cancer bone metastasis. The purpose of this study was to examine the effect of doxycycline in combination with chemotherapy. Human breast adenocarcinoma MDA-MB-231 cells were treated *in vitro* with each drug individually and in combination with doxycycline. Cell survival was determined using the clonogenic survival assay. Doxycycline in combination with doxorubicin or paclitaxel yielded therapeutic antagonism at all effect levels. Combinatory treatment with cisplatin, however, yielded a biphasic interaction, at low combinatorial doses, the effect was quantified as nearly additive, whereas higher doxycycline–cisplatin doses yielding greater than 50% cell inhibition resulted in synergistic effects. Cell cycle profiles were determined and showed that treatment with doxycycline, cisplatin, and doxorubicin resulted in G_1-phase, S-phase, and G_2/M-phase arrests, respectively. Upon addition of doxycycline to doxorubicin, the G_2/M-arrest characteristic of doxorubicin-only treatment was abrogated, which may account for the observed antagonism. Cells treated with doxycycline and cisplatin showed a further increase in S-phase arrest, also observed with cisplatin alone, which may be responsible for the additive and synergistic effects on cell survival. We clearly show that doxycycline in combination with paclitaxel or doxorubicin treatment resulted in antagonism; however, combining doxycycline with cisplatin led to synergistic interactions at higher effect levels. The increased potency of cisplatin may warrant dose reduction and thus decrease toxicity *in vivo*. *Anti-Cancer Drugs* 20:115–122 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Breast cancer is the most common cancer and the second leading cause of cancer death in women in the Western world, including North America. Despite recent advances in early detection and the understanding of the molecular basis of breast cancer biology, bone is the most frequent site of metastasis in patients with advanced breast cancer, occurring in approximately 70% of all cases. The clinical consequences of osteolytic bone metastases often associated with breast cancer include severe bone pain, pathologic fractures, nerve-compression syndromes, and hypercalcemia [1], because of dysregulation of normal bone remodeling processes related to the presence of metastases. Currently, there are no effective curative treatment options for women with bone metastases.

In an animal bone metastasis model of human breast cancer, we have shown that doxycycline (DCY) can profoundly decrease the tumor burden in bone and induce a substantial increase in bone formation [2]. DCY belongs to the tetracycline family of antibiotics, which have been shown to disrupt mitochondrial protein synthesis in various mammalian cells [3,4] leading to proliferation arrest in the G_1 phase of the cell cycle [5]. The multiple biological effects exerted by tetracyclines, in addition to their natural osteotropic property, make these antibiotics ideal candidates to treat breast cancer patients who are at risk of developing bone metastases [2,6].

Antiproliferative agents, such as cisplatin (*cis*-diammine-dichloridoplatinum, DDP), doxorubicin (adriamycin, ADR), and paclitaxel (taxol, TAX), have long been used to treat breast cancer. DDP and other platinum-based drugs, such as carboplatin and oxaliplatin, are widely used to treat breast, testicular, ovarian, lung, head and neck, esophageal, and cervical cancer [7]. DDP preferentially interacts with the N7 position of purine bases, resulting in the formation of 1,2-intrastrand and 1,3-intrastrand DNA adducts and, to a lesser extent, interstrand cross-links [8], directly inducing cell death [8,9]. The anticancer activity of ADR and other anthracyclines can be most persuasively explained by their inhibition of topoisomerase II (topo II) [10,11]. Topo II catalyzes topological rearrangements by forming a transient double-strand break in DNA essential to numerous nuclear processes [12,13]. The inhibition of topo II in

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ADR-treated cells leads to a G_2/M growth arrest, followed by apoptotic cell death [11,14,15]. The primary cellular target of TAX is the β-tubulin subunit of polymerized microtubules, preventing microtubule disassembly [16,17]. As the normal dynamic reorganization of the microtubules is essential for cell proliferation, cells treated with TAX arrest at the metaphase–anaphase transition of mitosis [16,18]. Prolonged exposure to TAX induces apoptotic cell death, an event considered to be a consequence of the protracted arrest at the mitotic phase [19].

DDP, ADR, and TAX are effective antiproliferative agents, but unfortunately cause severe toxicity to nontarget tissues [20]. DCY shows cytostatic and cytotoxic effects toward tumor cells [4,5,21] and may therefore be effective against cancer cells in bone where concentrations of the drug may reach sufficiently high levels. Little is known about the therapeutic effects of DCY in combination with chemotherapy. The purpose of this study is to determine the impact of DCY on cellular proliferation when administered in combination with TAX, DDP, or ADR. To this end, human breast adenocarcinoma MDA-MB-231 cells were treated with DCY and the anticancer agents, individually and in combination, and the clonogenic survival and the cell cycle profiles were determined. The results may suggest combinations of drugs with greater clinical efficacy or combinations that should be used with caution.

Materials and methods

Cell culture

Mycoplasma-free human breast adenocarcinoma MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA) and cultured in Dulbecco's modified Eagle media (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin G sodium, 100 µg/ml of streptomycin sulfate, and 0.25 µg/ml of amphotericin B (all obtained from Invitrogen, Burlington, Ontario, Canada) at 37°C, 5% CO_2, and humidified atmosphere.

Treatment

Stock solutions of DCY hyclate (7.68 mmol/l; Sigma-Aldrich, St Louis, Missouri, USA) and TAX (Bristol-Myers Squibb, Montreal, Quebec, Canada) were prepared fresh with MilliQ water and filter-sterilized using a 0.2-µm syringe filter. Stock solutions of ADR (Bedford Laboratories, Bedford, Ohio, USA) and DDP (Sigma-Aldrich) were prepared freshly with media. Three sets of drug combinations [DCY–TAX (60 000 : 1), DCY–DDP (384 : 1), and DCY–ADR (12 000 : 1)] were freshly prepared by serial dilution with media to reach end concentrations ranging from 60 to 960 µmol/l for DCY, 1 to 16 nmol/l for TAX, 0.16 to 2.5 µmol/l for DDP, and 5 to 80 nmol/l for ADR.

Colony-forming assay

MDA-MB-231 cells (150/well) were seeded onto 6-well plates in 2 ml of medium and allowed to adhere by incubating at 37°C and 5% CO_2 for 5 h. The medium was aspirated and replaced with 1 ml of the appropriate drug solutions in the medium (either alone or in combination) in triplicate wells. Control wells were replenished with 1 ml of medium. After a 24-h incubation period, the medium was aspirated, followed by a gentle wash with phosphate-buffered saline (PBS) and the addition of 2 ml of fresh DMEM to each well. The plates were incubated for an additional 5 days, after which the medium was aspirated and the colonies were stained with 0.5% methylene blue in 70% methanol. Colonies of at least 10 cells were counted and represent the cells unaffected by drug treatment. The survival is expressed as a percentage of the corresponding untreated controls (f_u). Experiments were repeated three times for each set of drug combinations.

Analysis of the combinatorial response

The fraction of cells affected ($f_a = 1–f_u$) for the corresponding doses of each drug alone and in combination, carried out in triplicate, were averaged and analyzed using CalcuSyn software Version 1.2 (Biosoft, Cambridge, UK). CalcuSyn uses the median-effect principle to calculate the potency of each drug, individually and in combination, in addition to extrapolating the dose for any given effect (and the effect for any given dose) [22] according to the following equation:

$$f_a/f_u = (D/D_m)^m$$

where D is the dose, D_m is the dose required to achieve 50% effect (IC_{50}), f_a is the fraction affected by D, and m is the coefficient of sigmoidicity of the dose–effect curve. For $m > 1$, the dose–effect curve is sigmoidal; for $m < 1$, it is negative sigmoidal; and for $m = 1$, it is hyperbolic. Median-effect analysis that yields a linear correlation coefficient greater than 0.90 ($r > 0.90$) shows that the measured dose–effect data conform to the median-effect principle and were included in the analysis. CalcuSyn uses the extrapolated dose–effect data obtained from the median-effect equation, in concert with the multiple-drug effect equation, to generate combination index (CI) values [22]. The CI was calculated based on the assumption of mutually nonexclusive drug interactions. Mean CI values from at least three experiments were subjected to a Student's t-test to determine the combination as synergistic (CI significantly lower than 1) or antagonistic (CI significantly higher than 1).

Cell cycle analysis

MDA-MB-231 cells (5×10^5) were seeded into 100-mm culture plates and were incubated at 37°C and 5% CO_2 for 5 h. The medium was aspirated and replaced with 5 ml of media containing appropriate drug concentrations. Three sets of drug combinations were freshly prepared by serial dilution with media. The control plate was replenished with 5 ml of DMEM. The plates were incubated for an

additional 24 h. The cells were washed with PBS and subsequently trypsinized using 2% trypsin–EDTA (Invitrogen). The cells were fixed in 70% ethanol in PBS and incubated at 40°C for 1 h. The fixed cells were subsequently washed with PBS and centrifuged at 2000 rpm for 5 min. The cells were resuspended in propidium iodide staining solution (50 μg/ml PBS) containing 0.5 mg/ml RNase A and incubated in the dark at room temperature for 30 min [23]. The fluorescence distribution of 2.5×10^4 cells was analyzed on an Epics flow cytometer (Beckman Coulter, Fullerton, California, USA). The data were analyzed by the Cylchred Software (Cardiff University, UK) to determine G_1-phase, S-phase, and G_2/M-phase populations. Data were tested for differences between means using a two-tailed Student's *t*-test. Differences were considered significant at P value of less than 0.05.

Results

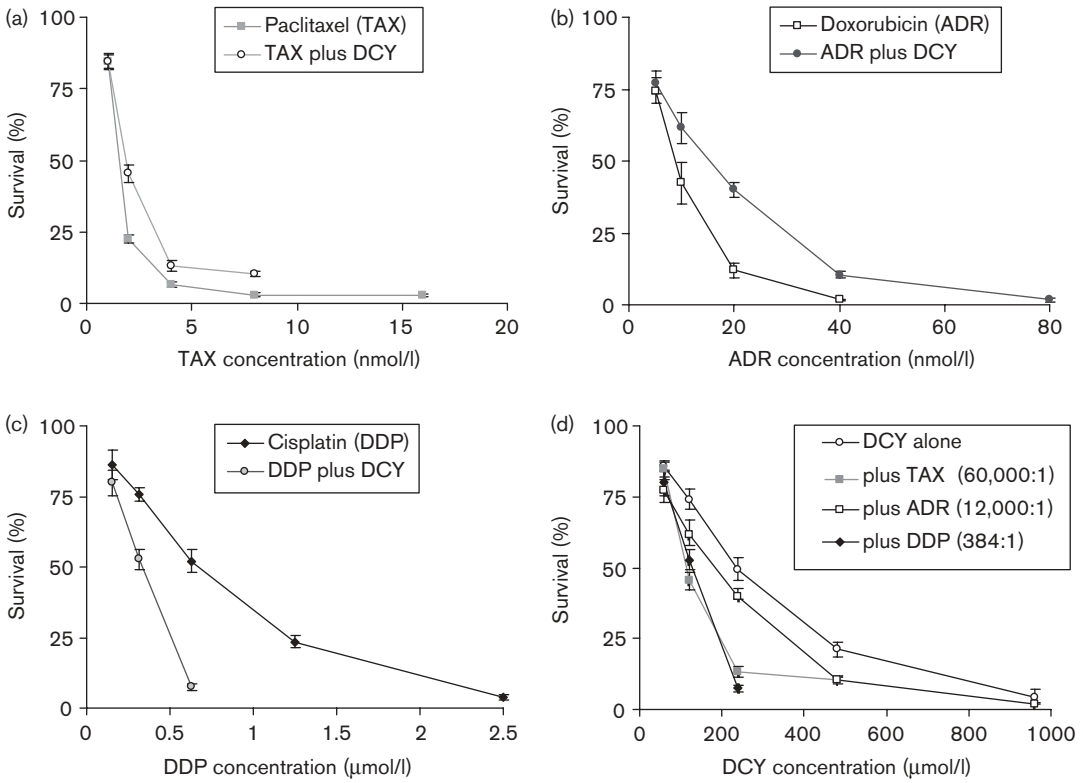
Single response

The dose–effect relationships for DCY and each of the anticancer drugs (DDP, ADR, or TAX) were determined by the clonogenic survival of MDA-MB-231 cells after 24 h exposure and are presented in Fig. 1. The data were subjected to the median-effect analysis to determine potency (IC_{50}) and the linear correlation coefficient (r). The IC_{50} (D_m) for each drug administered was calculated using CalcuSyn software, along with the coefficient of sigmoidicity (m) of the dose–response curves (Table 1).

Combinatorial response

The clonogenic survival of MDA-MB-231 cells was measured after 24 h treatment of the cells with a combination of DCY and one of the three chemotherapeutic drugs at concentrations equalling their approximate IC_{25}, IC_{50}, and IC_{75} values. The dose–effect relationships were determined (Fig. 1d) and median-effect analyses for all drug combinations were calculated. The measured dose–effect data conformed to the median-effect principle for all drug combinations, and were therefore extended to generate CI values (Fig. 2). Combined treatments are defined as antagonistic (CI > 1), additive (CI = 1), and/or synergistic (CI < 1). In each case, the potency of DCY in combination with TAX, ADR, or DDP was increased, showing that less DCY

Fig. 1



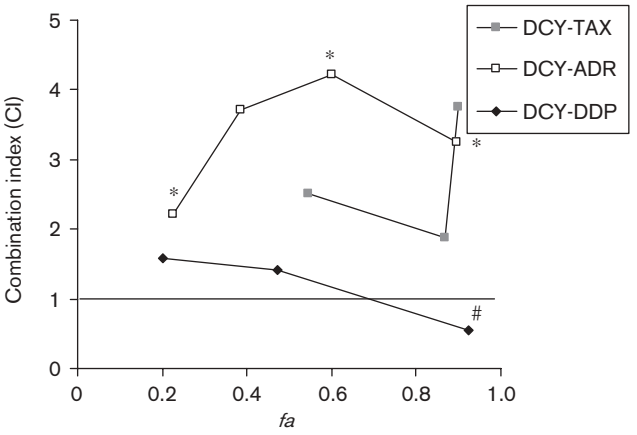
Dose–response curves of clonogenic survival of human mammary adenocarcinoma MDA-MB-231 cells after 24 h of treatment with (a) paclitaxel (TAX) alone, and in combination with doxycycline (DCY) and (b) doxorubicin (ADR) alone, and in combination with DCY and (c) cisplatin (DDP) alone, and in combination with DCY, and (d) DCY alone and in combination with each of the three anticancer drugs at the indicated ratios. The number of colonies is represented as the percentage of treated over corresponding untreated control. Data represent means of at least three independent experiments carried out in triplicate.

Table 1 Dose–effect analysis after treatment of human mammary adenocarcinoma MDA-MB-231 cells with DCY and anticancer agents (TAX, DDP, or ADR) individually, and in combination for 24 h

Treatment Drug	IC_{50} (95% confidence interval)			
	Doxycycline (DCY) (μmol/l)	Doxorubicin (ADR) (nmol/l)	Cisplatin (DDP) (μmol/l)	Paclitaxel (TAX) (nmol/l)
Single treatment	203.04 (183.16–225.07)	8.05 (6.60–9.82)	0.54 (0.42–0.71)	1.49 (1.02–2.19)
r ; *m*	0.96; 1.69	0.98; 2.67	0.97; 1.91	0.91; 1.92
DCY–ADR combination	145.97 (121.55–175.30)	12.16 (10.13–14.61)	–	–
r ; *m*		0.97; 1.76		
DCY–DDP combination	106.78 (95.55–119.32)	–	0.28 (0.25–0.31)	–
r ; *m*			0.98; 2.84	
DCY–TAX combination	110.57 (85.73–142.60)	–	–	1.84 (1.43–2.38)
r ; *m*				0.96; 1.88

Median-effect analysis was carried out using the averaged survival data obtained from three separate colony forming assays and the linear correlation coefficient (*r*), and the coefficient of sigmoidicity (*m*) were calculated for individual and combination treatments. For the combination, *r* and *m* are presented once with ADR, DDP, or TAX.

Fig. 2



Combination index (CI) plotted against the surviving fraction of MDA-MB-231 cells (f_a) after treatment with doxycycline (DCY) in combination with each of the three anticancer drugs. CI values represent means of at least three independent experiments carried out in triplicate. Combination treatments were determined to be synergistic (#CI significantly lower than 1) or antagonistic (*CI significantly higher than 1) by a one-sided *t*-test ($P<0.05$) using the mean CI values. ADR, doxorubicin; DDP, cisplatin; TAX, paclitaxel.

was needed to achieve 50% proliferative inhibition. The IC_{50} of DCY in combination with DDP decreased by almost two-fold, from 203.04 to 106.78 μmol/l. Similarly, the potency of DDP, when given in combination with DCY also increased, from 0.54 to 0.28 μmol/l. Conversely, both ADR and TAX showed decreased potency when combined with DCY, as indicated by an increase in their combinatorial IC_{50} values. With respect to cytotoxicity, DCY treatment in combination with TAX interacted antagonistically, as the calculated CI values for all drug combinations were considerably greater than 1. Similarly, DCY and ADR also proved significantly less effective at inducing MDA-MB-231 toxicity, as the CI values for three out of four combinatorial doses indicated antagonism ($CI > 1$). The combination treatment of DCY together with DDP, however, displayed a biphasic response. The lower two DCY–DDP combinations, corresponding to survival inhibition below 50%, yielded a response suggestive of near additivity ($CI = 1$). The CI value of the highest DCY and DDP dose was 0.55 and significantly less than 1, thereby showing that these drugs interacted synergistically ($CI < 1$) at higher dose–effect levels.

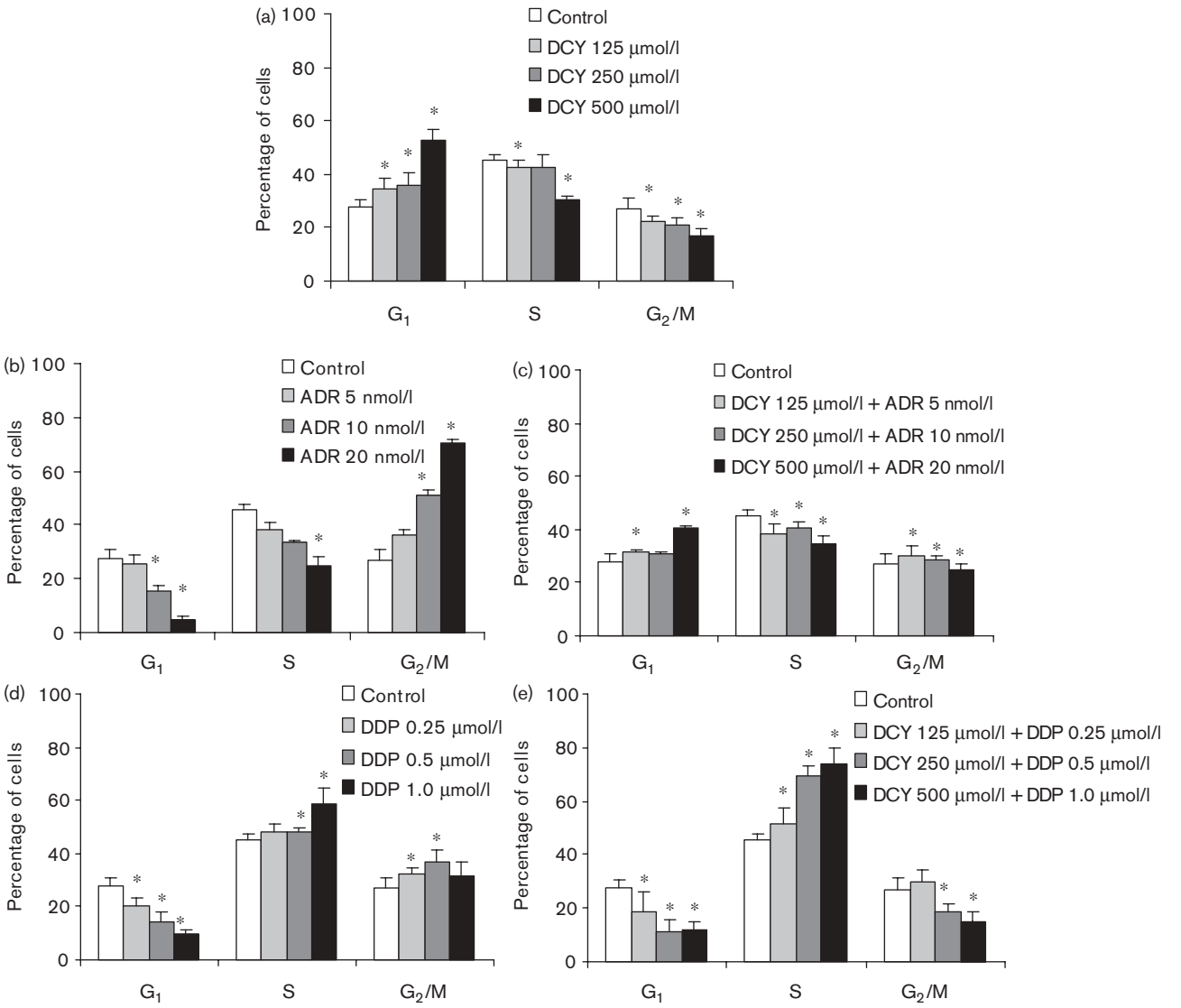
Cell cycle analysis

The cell cycle profile of the MDA-MB-231 cells was determined by flow cytometric analysis after 24-h exposure to DCY and anticancer agents (DDP and ADR) alone and in combination. The doses for individual and combination treatments represented the approximate IC_{25}, IC_{50}, and IC_{75} values, measured for each drug administered alone. Flow cytometric analysis of MDA-MB-231 cells treated with DCY showed a change in the cell cycle distribution from the untreated control. Cells treated with increasing doses of DCY showed a sequential increase in G_1 accumulation (Fig. 3a). MDA-MB-231 cells treated with increasing doses of ADR showed considerable accumulation of cells in the G_2/M phase of the cell cycle, and subsequently showed a progressive decline in the G_1-phase and S phase populations when compared with the control (Fig. 3b). Interestingly, DCY and ADR combination treatment relieved the pronounced G_2/M arrest originally observed with ADR only, consequently shifting the entire population toward a cell cycle profile similar to DCY-only treatment (Fig. 3c). DDP-treated MDA-MB-231 cells, however, displayed a trend to accumulate in the S phase with increasing concentrations (Fig. 3d). Compared with DDP-only treatment, it was observed that DCY plus DDP treatment further elevated the number of cells in the S phase (Fig. 3e). Unfortunately, TAX-treated MDA-MB-231 cells produced inconsistent results with regard to their cell cycle profiles.

Discussion and conclusion

We have previously shown that DCY can profoundly decrease the tumor burden in bone and induce a substantial increase in bone formation in an animal model

Fig. 3



Effect of different treatments on the MDA-MB-231 cell cycle profile as determined by flow cytometric analysis. Treatment occurred with (a) doxycycline (DCY) alone, (b) doxorubicin (ADR) alone, and (d) cisplatin (DDP) alone, and (c and e) their respective combinations. Values represent means (± SD) of at least three independent experiments carried out in triplicate. Treatments resulting in subpopulations significantly differing from their respective untreated controls ($P<0.05$) are indicated with an asterisk (*). Treatment with a combination of DCY and DDP resulted in a statistically significant difference ($P<0.05$) compared with DCY alone for all combined doses and cell cycle phases, except for G_2/M at the two highest concentrations. Treatment with a combination of DCY and DDP resulted in a statistically significant difference in the cells in S and G_2/M cell cycle phases when compared with DDP alone for the combinations at the two highest concentrations. Treatment with a combination of DCY and ADR resulted in a statistically significant difference compared with DCY alone for the combination at the highest concentration and at all cell cycle phases, and for all combinations for the G_2/M cell cycle phase. Treatment with a combination of DCY and ADR resulted in a statistically significant difference compared with ADR alone for the combinations at the two highest concentrations in all cell cycle phases, and for the G_1 cell cycle phase at 125 μmol/l DCY and 5 nmol/l ADR.

of human breast cancer bone metastasis [2] and is an effective cytotoxic agent in bone-metastatic cancer cells *in vitro* [21]. The therapeutic effects of chemotherapy agents, alone or in combination with DCY, were determined using clonogenic survival of human breast cancer MBA-MB-231 cells *in vitro*. Our results are in accordance with comparable studies using human breast cancer cells *in vitro*. Treatment with ADR and TAX resulted in IC_{50}s of 8.05 and 0.15 nmol/l, respectively, which are close to reported IC_{50} values of 16 nmol/l [24] and 2 nmol/l [25] found using human estrogen receptor-positive breast cancer MCF-7 cells. Using human breast cancer MDA-MB-468 cells, the IC_{50} of DDP amounted to 2 μmol/l after 1 h treatment [26], compared with the IC_{50} of 0.55 μmol/l DDP obtained in this study, after 24 h treatment. Flow cytometric analysis of MDA-MB-231

cells treated with DCY showed a significant change in the cell cycle distribution from the untreated control cells. The cells showed a dose-dependent increase in G_1 accumulation (Fig. 3a). This confirms previous studies also showing a pronounced arrest in the G_1 phase of the cell cycle upon treatment with tetracyclines [5]. DCY is known to disrupt the mitochondrial respiratory function, consequently reducing cellular ATP production and preventing cells from advancing beyond the G_1 energetic checkpoint [3,5].

MDA-MB-231 cells treated with increasing doses of ADR showed significant accumulation in the G_2/M phase of the cell cycle, and a concomitant progressive decline in the G_1-phase and S-phase populations when compared with control cells (Fig. 3d), consistent with previous reports [11,14]. The addition of DCY to the ADR treatment relieved the pronounced G_2/M arrest, consequently shifting the entire population toward a cell cycle profile similar to DCY-only treatment (Fig. 3e), which may account for the strong antagonism observed between DCY and ADR (Fig. 2). The cytotoxicity of topo II-targeting agents, including ADR, is specific at certain stages of the cell cycle. These agents cause the greatest strand breakage in the G_2/M phase and the least in the G_1 phase [11], most likely because of the cell cycle-dependent expression of topo II, which is also highest in the G_2/M phase [27]. Therefore, it is feasible that the increased accumulation at G_1 observed with DCY–ADR cotreatment, relative to ADR alone, may be accompanied with decreased levels of topo II expression, consequently decreasing the extent of ADR-mediated DNA damage and enabling greater cell survival. Further investigations into the activity of topo II enzymes and the extent of DNA damage are, however, necessary to confirm this. Alternatively, the antagonism observed upon addition of DCY to ADR treatment (Fig. 2) may be a consequence of the dynamics of topo II activity. Topo II relieves torsional strain on DNA by cleaving double-stranded DNA, passing a second double-stranded DNA segment through the double-strand break to finally religate the cleaved break, a catalytic process shown to be ATP dependent [12,13]. Several studies have consistently reported that the cytotoxicity of topo II poisons is antagonized by inhibitors of respiration [28–31]. Thwarting the formation of topo II–DNA cleavage complexes reduces the cell killing potential of topo II poisons, which is associated with the inhibition of the religation step of topo II catalysis [13]. In accordance, it has been reported that cells nearly devoid of ATP are almost completely protected against single-strand DNA breaks using several topo II poisons, including ADR [31]. Therefore, it is also possible that the inhibition of ATP production by DCY exposure could have reduced the rate of topo II activity, thereby abating the DNA-damaging effects of ADR and yielding an antagonistic drug interaction.

DDP treatment resulted in a tendency of the cells to accumulate in the S phase of the cell cycle with increasing concentrations (Fig. 3b). Sorenson and Eastman [32] showed that DDP-treated Chinese hamster ovary cells proficient in nucleotide excision repair pathway, exhibit inhibition of DNA synthesis and accumulate in the S phase, whereas deficient cells show a marked G_2/M arrest. Therefore, the tendency of the MDA-MB-231 cell population to accumulate in the S phase after exposure to DDP suggests that MDA-MB-231 cells are proficient in nucleotide excision repair. Compared with DDP-only treatment, we observed that the addition of DCY to the treatment further elevated the numbers of cells in the S phase (Fig. 3c). It is possible that DCY may have sufficiently reduced ATP generation, thereby impeding the energy-driven nucleotide excision repair pathway from effectively removing DDP–DNA adducts, and thus further disabling the cells' ability to circumvent this arrest. We speculate that increased levels of accumulated DNA damage may account for the enhanced anticancer effects of DCY and DDP combination treatment [9]. Alternatively, DDP-induced mitochondrial DNA [33] and electron transport chain damage [34], in concert with inhibition of mitochondrial protein synthesis by DCY, could be responsible for the additive and synergistic drug interactions.

Combining DCY with the microtubule-stabilizing agent TAX was less effective in decreasing MDA-MB-231 cell survival than treatment with TAX alone. The drug combination was defined as antagonistic (Fig. 2), even though microtubule disassembly is a well-characterized ATP-dependent process. Exposing cells to inhibitors of metabolism reduces the available ATP pools, consequently promoting tubulin polymerization and stabilizing microtubules, resulting in mitotic arrest and apoptosis. It has been shown that the addition of TAX to an energy-deprived cell further suppresses microtubule dynamics and enhances the anticancer effects of TAX [35,36]. The debilitating effects of DCY on metabolism [3,5], in combination with TAX, could act in accordance with previous studies to synergistically inhibit cell survival. However, we did not observe this effect, which could be due to several factors, including the use of different cell models and survival assays. In addition, DCY does not strictly affect mitochondrial ribosomes to inhibit protein synthesis, but also interacts with multiple other cellular targets [37,38].

In this study, we clearly show that the potency of DCY in combination with TAX, ADR, or DDP increased considerably, as less DCY was required to achieve a given degree of inhibition (Table 1). Similarly, the potency of DDP measured in combination treatment had also increased. Conversely, the potency of TAX and ADR, in combination with DCY, experienced a marginal reduction. Superficially, decreasing the dose of DCY in combination

appears promising, even when antagonistically coupled with TAX and ADR. Clinically, however, reducing the dose for a particular degree of effect is primarily used to alleviate drug-induced toxicities. As discussed earlier, DCY is a well-tolerated drug and thus, reducing its dose in combination chemotherapy is not a priority. Rather, the more attractive alternative is to reduce the dose of anticancer agents with narrow therapeutic indices, such as TAX, ADR, and DDP [20]. Our results using breast cancer cells *in vitro* show that DCY in combination with TAX or ADR adjuvant therapy is not beneficial, as their combinatorial outcome – antagonism and decreased potency of TAX and ADR – is unfavorable. In contrast, we also showed that DCY in combination with DDP worked synergistically at higher effect levels. The increased potency of DDP may warrant dose reduction and thus decreased toxicity *in vivo*. Recently, when cyclophosphamide, which is, like DDP, a DNA-alkylating agent commonly used in the clinical management of metastatic breast cancer [7], was used in conjunction with DCY using MCF-7 cells, it was shown that DCY potentiates both in-vivo tumor regression and the in-vitro cytotoxicity of cyclophosphamide [39], suggesting that a strategy using combination therapy may improve the treatment of breast cancer. Similarly, this study observed that the clonogenic inhibition of MDA-MB-231 cells treated with DCY and DDP was nearly additive below 50% proliferative inhibition, with a shift to synergism above this effect level (Table 1).

Because of the acute sensitivity of in-vitro clonogenic survival assays, this study, like many others, was only successful in assessing the effects of combination treatments at relatively low doses. Clinically relevant drug concentrations are 25–250 nmol/l of ADR [10], 2–12 μmol/l of DDP [40], and 5–200 nmol/l of TAX [41]. Therefore, to extrapolate from the current in-vitro data, it needs to be taken into account that clinically relevant concentrations of anticancer drugs are several fold higher than the concentrations used in this study. For instance, the highest measurable combinatorial dose of 8 nmol/l for TAX represents a mere 8% of the upper effective plasma concentrations observed in cancer patients [41]. Therefore, the antagonism observed with DCY plus TAX or ADR may not accurately reflect the interaction of these drugs *in vivo*. The same logic holds true for the additivity and synergy observed upon DCY plus DDP treatment. In contrast, DCY is a well-tolerated drug and pharmacological plasma concentrations can reach approximately 4.5 μmol/l [42]. These levels can be expected to be at least several-fold higher in bone, as tetracyclines have long been known to be osteotropic. In the context of bone metastasis, it would be more relevant to show the sensitivity of tumor cells to concentrations of DCY achievable in the bone combined with classical antiproliferative agents at steady-state levels.

This study provides insight into drug interactions occurring *in vitro* that we are further exploring *in vivo*. We have clearly shown that DCY in combination with TAX or ADR treatment leads to antagonism; however, we also show the evidence that DCY in combination with DDP worked synergistically at higher effect levels. The increased potency of DDP may warrant dose reduction and thus decreased toxicity *in vivo*.

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References

1 Mundy GR. Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat Rev Cancer* 2002; **2**:584–593.
2 Duivenvoorden WC, Popovic SV, Lhotak S, Seidlitz E, Hirte HW, Tozer RG, *et al*. Doxycycline decreases tumor burden in a bone metastasis model of human breast cancer. *Cancer Res* 2002; **62**:1588–1591.
3 Riesbeck K, Bredberg A, Forsgren A. Ciprofloxacin does not inhibit mitochondrial functions but other antibiotics do. *Antimicrob Agents Chemother* 1990; **34**:167–169.
4 Van den Bogert C, Dontje BHJ, Holtrop M, Melis TE, Romijn JC, van Dongen JW, *et al*. Arrest of proliferation of renal and prostate carcinomas of human origin by inhibition of mitochondrial protein synthesis. *Cancer Res* 1986; **46**:3283–3289.
5 Van den Bogert C, van Kernebeek G, de Leij L, Kroon AM. Inhibition of mitochondrial protein synthesis leads to proliferation arrest in the G1-phase of the cell cycle. *Cancer Lett* 1986; **32**:41–51.
6 Stepensky D, Kleinberg L, Hoffman A. Bone as an effect compartment: models for uptake and release of drugs. *Clin Pharmacokinet* 2003; **42**: 863–881.
7 Decatris MP, Sundar S, O'Byrne KJ. Platinum-based chemotherapy in metastatic breast cancer: current status. *Cancer Treat Rev* 2004; **30**:53–81.
8 Jamieson ER, Lippard SJ. Structure, recognition, and processing of cisplatin-DNA adducts. *Chem Rev* 1999; **99**:2467–2498.
9 Henkels KM, Turchi JJ. Induction of apoptosis in cisplatin-sensitive and -resistant human ovarian cancer cell lines. *Cancer Res* 1997; **57**:4488–4492.
10 Gewirtz DA. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. *Biochem Pharmacol* 1999; **57**:727–741.
11 Potter AJ, Rabinovitch PS. The cell cycle phases of DNA damage and repair initiated by topoisomerase II-targeting chemotherapeutic drugs. *Mutat Res* 2005; **572**:27–44.
12 Osheroff N. Eukaryotic topoisomerase II. Characterization of enzyme turnover. *J Biol Chem* 1986; **261**:9944–9950.
13 Wang JC. Cellular roles of DNA topoisomerases: a molecular perspective. *Nat Rev Mol Cell Biol* 2002; **3**:430–440.
14 Fornari FA Jr, Jarvis DW, Grant S, Orr MS, Randolph JK, White FK, *et al*. Growth arrest and non-apoptotic cell death associated with the suppression of c-myc expression in MCF-7 breast tumor cells following acute exposure to doxorubicin. *Biochem Pharmacol* 1996; **51**:931–940.
15 Perego P, Corna E, De Cesare M, Gatti L, Polizzi D, Pratesi G, *et al*. Role of apoptosis and apoptosis-related genes in cellular response and antitumor efficacy of anthracyclines. *Curr Med Chem* 2001; **8**:31–37.
16 Manfredi JJ, Horwitz SB. Taxol: an antimitotic agent with a new mechanism of action. *Pharmacol Ther* 1984; **25**:83–125.
17 Rao S, He L, Chakravarty S, Ojima I, Orr GA, Horwitz SB. Characterization of the Taxol binding site on the microtubule. Identification of Arg(282) in beta-tubulin as the site of photoincorporation of a 7-benzophenone analogue of Taxol. *J Biol Chem* 1999; **274**:37990–37994.
18 Jordan MA, Wendell K, Gardiner S, Derry WB, Copp H, Wilson L. Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. *Cancer Res* 1996; **56**:816–825.
19 Wang TH, Wang HS, Soong YK. Paclitaxel-induced cell death: where the cell cycle and apoptosis come together. *Cancer* 2000; **88**:2619–2628.
20 Doroshow JH, Pollock RE, Doroshow JH, Khayat D, Nakao A, O'Sullivan B. *Principles of medical oncology*. 8th ed. New Jersey: John Wiley & Sons; 2004.

21 Duivenvoorden WC, Hirte HW, Singh G. Use of tetracycline as an inhibitor of matrix metalloproteinase activity secreted by human bone-metastasizing cancer cells. *Invasion Metastasis* 1997; **17**:312–322.

22 Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* 2006; **58**:621–681.

23 Walker PR, Kwast-Welfeld J, Gourdeau H, Leblanc J, Neugebauer W, Sikorska M. Relationship between apoptosis and the cell cycle in lymphocytes: roles of protein kinase C, tyrosine phosphorylation, and AP1. *Exp Cell Res* 1993; **207**:142–151.

24 Engel D, Nudelman A, Levovich I, Gruss-Fischer T, Entin-Meer M, Phillips DR, *et al*. Mode of interaction between butyroyloxymethyl-diethyl phosphate (AN-7) and doxorubicin in MCF-7 and resistant MCF-7/Dx cell lines. *J Cancer Res Clin Oncol* 2006; **132**:673–683.

25 Liebmann JE, Fisher J, Teague D, Cook JA. Sequence dependence of paclitaxel (Taxol) combined with cisplatin or alkylators in human cancer cells. *Oncol Res* 1994; **6**:25–31.

26 Dixit M, Yang JL, Poirier MC, Price JO, Andrews PA, Arteaga CL. Abrogation of cisplatin-induced programmed cell death in human breast cancer cells by epidermal growth factor antisense RNA. *J Natl Cancer Inst* 1997; **89**: 365–373.

27 Larsen AK, Skladanowski A, Bojanowski K. The roles of DNA topoisomerase II during the cell cycle. *Prog Cell Cycle Res* 1996; **2**:229–239.

28 Fry DW. Cytotoxic synergism between trimetrexate and etoposide. Evidence that trimetrexate potentiates etoposide-induced protein-associated DNA strand breaks in L1210 leukemia cells through alterations in intracellular ATP concentrations. *Biochem Pharmacol* 1990; **40**:1981–1988.

29 Kaufmann SH. Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. *Cancer Res* 1989; **49**: 5870–5878.

30 Shibuya ML, Buddenbaum WE, Don AL, Utsumi H, Suciu D, Kosaka T, *et al*. Amsacrine-induced lesions in DNA and their modulation by novobiocin and 2,4-dinitrophenol. *Cancer Res* 1991; **51**:573–580.

31 Sorensen M, Sehested M, Jensen PB. Effect of cellular ATP depletion on topoisomerase II poisons. Abrogation of cleavable-complex formation by etoposide but not by amsacrine. *Mol Pharmacol* 1999; **55**:424–431.

32 Sorenson CM, Eastman A. Influence of cis-diamminedichloroplatinum(II) on DNA synthesis and cell cycle progression in excision repair proficient and deficient Chinese hamster ovary cells. *Cancer Res* 1988; **48**:6703–6707.

33 Olivero OA, Chang PK, Lopez-Larraza DM, Semino-Mora MC, Poirier MC. Preferential formation and decreased removal of cisplatin–DNA adducts in Chinese hamster ovary cell mitochondrial DNA as compared to nuclear DNA. *Mutat Res* 1997; **391**:79–86.

34 Murata T, Hibasami H, Maekawa S, Tagawa T, Nakashima K. Preferential binding of cisplatin to mitochondrial DNA and suppression of ATP generation in human malignant melanoma cells. *Biochem Int* 1990; **20**: 949–955.

35 Martin DS, Stolfi RL, Colofiore JR, Nord LD. Marked enhancement in vivo of paclitaxel's (taxol's) tumor-regressing activity by ATP-depleting modulation. *Anticancer Drugs* 1996; **7**:655–659.

36 Martin KJ, Vassallo CD, Teicher BA, Kaddurah-Daouk R. Microtubule stabilization and potentiation of taxol activity by the creatine analog cyclocreatine. *Anticancer Drugs* 1995; **6**:419–426.

37 Amin AR, Attur MG, Thakker GD, Patel PD, Vyas PR, Patel RN, *et al*. A novel mechanism of action of tetracyclines: effects on nitric oxide synthases. *Proc Natl Acad Sci U S A* 1996; **93**:14014–14019.

38 Attur MG, Patel RN, Patel PD, Abramson SB, Amin AR. Tetracycline upregulates COX-2 expression and prostaglandin E2 production independent of its effect on nitric oxide. *J Immunol* 1999; **162**:3160–3167.

39 Chhipa RR, Singh S, Surve SV, Vijayakumar MV, Bhat MK. Doxycycline potentiates antitumor effect of cyclophosphamide in mice. *Toxicol Appl Pharmacol* 2005; **202**:268–277.

40 Nagai N, Kinoshita M, Ogata H, Tsujino D, Wada Y, Someya K, *et al*. Relationship between pharmacokinetics of unchanged cisplatin and nephrotoxicity after intravenous infusions of cisplatin to cancer patients. *Cancer Chemother Pharmacol* 1996; **39**:131–137.

41 Blagosklonny MV, Fojo T. Molecular effects of paclitaxel: myths and reality (a critical review). *Int J Cancer* 1999; **83**:151–156.

42 Stoller NH, Johnson LR, Trapnell S, Harrold CQ, Garrett S. The pharmacokinetic profile of a biodegradable controlled-release delivery system containing doxycycline compared to systemically delivered doxycycline in gingival crevicular fluid, saliva, and serum. *J Periodontol* 1998; **69**:1085–1091.