Selective Killing of Adriamycin-Resistant (G2 Checkpoint-Deficient and MRP1-Expressing) Cancer Cells by Docetaxel

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Abstract
Chemotherapy of cancer is limited by toxicity to normal cells. Drug resistance further limits the therapy. Here, we investigated selective killing of drug-resistant cancer cells by antagonistic drug combinations, which can spare (because of drug antagonism) normal cells. We used paired cell lines that are resistant to Adriamycin due to either expression of MRP1 or lack of G2 checkpoints. The goal was to selectively kill Adriamycin-resistant cancer cells with Docetaxel (Taxotere), while protecting parental (Adriamycin-sensitive) cells, using cytostatic concentrations of Adriamycin. Taxotere kills cells in mitosis. Therefore, by arresting parental cells in G2, 20 to 40 ng/mL of Adriamycin prevented cell death caused by Taxotere. Also, Adriamycin prevented the effects of Taxotere in normal human lymphocytes. In contrast, Taxotere selectively killed MRP1-expressing leukemia cells, which did not undergo G2 arrest in the presence of Adriamycin. Also, in the presence of Adriamycin, HCT116-p21−/− cancer cells with a defective G2 checkpoint entered mitosis and were selectively killed by Taxotere. Finally, 20 ng/mL of Adriamycin protected normal FDC-P1 hematopoietic cells from Taxotere. Whereas parental cells were protected by Adriamycin, the mitogen-activated protein/extracellular signal-regulated kinase inhibitor PD90598 potentiated the cytotoxic effect of Taxotere selectively in Raf-1-transformed FDC-P1 leukemia cells. We propose a therapeutic strategy to prevent normal cells from entering mitosis while increasing apoptosis selectively in mitotic cancer cells.

Introduction
Microtubules represent one of the best drug targets identified to date (1–5). Docetaxel (Taxotere), a microtubule-stabilizing taxane, is widely used in the therapy of breast, ovarian, prostate, lung, head and neck, gastric, bladder and other cancers (6–9). Yet, microtubules are universal cellular structures that are necessary for all normal cells. As a consequence, all microtubule-active agents cause dose-limiting side effects (10, 11).

Microtubule dynamics is much faster during mitosis (M) than during interphase (G1, S, G2) of the cell cycle (2). At low concentrations, which do not cause polymerization of tubulin, taxanes inhibit mitotic progression (2, 12). Numerous studies indicate that inhibition of mitotic progression and mitotic arrest correlate with the cytotoxicity of microtubule-active drugs (13–21). When arrested in G2 and/or G2, cells are resistant to microtubule-active drugs (22–27). DNA-damaging agents cause G1 and/or G2 arrest (28). In particular, low concentrations of Adriamycin can cause G2 arrest without apoptosis. Therefore, low concentrations of Adriamycin can protect cells from microtubule-active drugs (24, 26). But how can we arrest normal cells in G2 without arresting cancer cells? First, cancer cells may have defective G2 checkpoints (29–31). Following DNA damage, such cancer cells continue to proliferate and enter mitosis (28). Second, cancer cells may acquire drug resistance, for instance, due to expression of MRP1 (32). Adriamycin is a substrate of MRP1, whereas Taxotere is not (33, 34). In the presence of low concentrations of Adriamycin, in theory, Taxotere could kill MRP1-expressing cells selectively. Here we investigated these scenarios. We showed that following pretreatment with low concentrations of Adriamycin, Taxotere selectively killed MRP1-expressing and G2 checkpoint-deficient cells. We also determined a protective window: namely, concentrations of Adriamycin that arrest cell cycle without causing cell death. Finally, the mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitor PD90859 potentiated the effects of Taxotere in Raf-1-transformed cells arrested in mitosis, whereas normal FDC-P1 hematopoietic cells were protected by Adriamycin.

Materials and Methods

Cell lines. HCT116 and cells lacking p21−/− (defective G1 and G2), Bax−/− and Securin−/−, were obtained from Bert Vogelstein (John Hopkins University). HL60 and HL/Adriamycin were described previously (35, 36). Hematopoietic FDC-P1 and Raf-1-transfected (FDC/Raf-1) cells were described previously (37, 28).

Lymphocytes. Human peripheral blood lymphocytes were isolated from healthy volunteers by venipuncture. Cells were maintained in culture and treated with 10 mg/mL phytohemagglutinin, as previously described (39).

Reagents. Docetaxel (Taxotere) was obtained from Aventis Pharmaceuticals, Inc. (Bridgewater, NJ). Adriamycin (Doxorubicin) and PD90859 were obtained from Sigma (St. Louis, MO).

Immunoblot analysis. Cells were lysed and soluble proteins were harvested in TNE buffer [50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, and 1% (v/v) NP40] containing protease inhibitors (20 mg/mL aprotinin, 20 mg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride). Proteins were resolved with either 12% SDS-PAGE (for Bcl-2) or with NuPAGE 4% to 12% Bis-Tris gel with MOPS running buffer (NOVEX, San Diego, CA) according to the manufacturer’s instructions. Immunoblotting was done with mouse monoclonal anti-p21 and anti-p53 (Oncogene Research, Calbiochem, La Jolla, CA), mouse monoclonal anti-human tubulin and actin (Sigma). Immunoblots were developed using a horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Richmond, CA) and a chemiluminescence detection kit (Dupont NEN, Boston, MA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Floating cells (20,000; HL60, HL/Adriamycin, FDC-P1, FDC/Raf-1, lymphocytes) or 5,000 adherent cells (HCT116 cells and their clones) were plated in 96-well flat-bottomed plates and then exposed to tested agents.
After 3 or 4 days, 20 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added to each well for 4 hours. After removal of the medium, 170 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance at 540 nm was determined using a BioTek Instruments plate reader as previously described (40). SD were determined in triplicate.

**Number of dead and live cells.** Cells were plated in 24-well plates in 1 mL of medium, or in 96-well plates in 0.2 mL, and were treated with drugs. Cells were incubated with trypan blue and the number of blue (dead) cells and transparent (live) cells were counted by a hemocytometer.

**Flow cytometry.** Cells were harvested, washed with PBS, and resuspended in 75% ethanol in PBS and kept at 4°C for at least 30 minutes. Cells were resuspended and incubated for 30 minutes in propidium iodide staining solution containing 0.05 μg/mL propidium iodide (Sigma), 1 mM EDTA, 0.1% Triton X-100, and 1 mg/mL RNase A in PBS. The suspension was then analyzed on a Becton Dickinson FACScan. DNA content frequency histograms were measured using a FACScan flow cytometer (Becton Dickinson Immunocytochemistry Systems, San Jose, CA). To calculate the percentage of cells in respective phases of the cell cycle, the DNA content frequency histograms were deconvoluted using the MultiCycle software (Phoenix Flow Systems, San Diego, CA).

**In situ DNA strand break labeling (terminal nucleotidyl transferase–mediated nick end labeling assay).** Cells were rinsed with PBS, fixed in 1% methanol-free formaldehyde for 15 minutes at room temperature and stored at 70% ethanol at −20°C for at least 1 hour. The cells were then rinsed twice with PBS for 5 minutes. DNA strand break labeling was done using the APO-BRDU kit provided by Phoenix Flow Systems. After washing with PBS, cells were stained with propidium iodide (5 μg/mL) and dissolved in PBS containing RNase A for 20 minutes. Cellular fluorescence was measured using a FACScan flow cytometer (Becton Dickinson).

**Mitotic index and apoptotic nuclei.** Cells were incubated with drugs for the indicated times. Cells washed with PBS, pelleted onto glass slides in a cytocentrifuge, fixed with 90% ethanol/10% glacial acetic acid. Nuclei were stained with 1 μg/mL of 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, Inc., Eugene, OR) in PBS and inspected under UV microscope (Nikon Microphot) as previously described (41, 42).

**Results**

**Premitotic arrest protects HL60 cells from Taxotere.** First, we compared the cytotoxicity of Taxotere in exponentially growing HL60 cells and nondividing HL60 cells that were arrested in G1 by high cell density (2,000,000 cells/mL). At concentrations >10 μM/mL, Taxotere was cytotoxic to proliferating HL60 cells. In contrast, quiescent HL60 cells were resistant to Taxotere-induced cytotoxicity (Fig. 1A), consistent with the notion that Taxotere kills dividing cells only. Next, we investigated whether pharmacologic arrest could also protect HL60 cells from Taxotere. Previously, we have found that 50 ng/mL Adriamycin causes G2 arrest in HL60 cells, thus preventing them from entering mitosis (41). As shown in Fig. 1A, pretreatment HL60 cells with 50 ng/mL Adriamycin abrogated cytotoxic effects of Taxotere. Because cell death and inhibition of proliferation cannot always be distinguished by the MTT cytotoxicity assay, we also measured the number of dead and live cells by trypan blue exclusion. By day 2, Taxotere killed all HL60 cells (Fig. 1B). Importantly, pretreatment with Adriamycin abrogated cell death caused by Taxotere (Fig. 1B).

**Selective killing MRP-expressing leukemia cells.** By pumping drugs out, Pgp and MRP provide very high levels of drug resistance (32). For example, HL60/MRP cells, a multidrug-resistant cell line that express MRP1, grow in the presence of 500 to 1,000 ng/mL Adriamycin (35, 43). In contrast, HL/MRP are sensitive to Taxotere (Fig. 2A), because Taxotere is not a substrate of MRP1. Because HL/MRP cells proliferate in the presence of Adriamycin, Taxotere will kill such cells. When cells were pretreated with Adriamycin, HL/MRP cells continue to grow. Taxotere efficiently kills HL/MRP (Fig. 2B). In contrast, Adriamycin protected HL60 cells. Thus, pretreatment with Adriamycin allowed Taxotere to kill MRP-expressing cells selectively (Fig. 2B).

In controls, both HL60 and HL/MRP cells were distributed in all phases of the cell cycle (Figs. 3 and 4). As expected, in both HL60 and HL/MRP cells Taxotere caused G2-M arrest (by flow cytometry) that was actually mitotic arrest by DAPI staining (Figs. 3 and 4). Taxotere-treated cells underwent apoptosis as evidenced by DNA strand breaks [terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) assay]. In the presence of Adriamycin, HL60 cells accumulated in the G2 phase but not in mitosis (no mitotic nuclei by DAPI staining; Fig. 3). Adriamycin and no apoptosis was detected by TUNEL. Most importantly, pretreatment with Adriamycin prevented mitotic arrest and apoptosis caused by Taxotere (Taxotere versus Adriamycin + Taxotere). Essentially, when treated with either Adriamycin alone or Adriamycin followed by Taxotere, HL60 cells were arrested in G2 and did not undergo apoptosis (Fig. 3). In brief, Adriamycin + Taxotere = Adriamycin.

Taxotere induced mitotic arrest and apoptosis in MRP-expressing HL60 cells (Fig. 4). In contrast, Adriamycin had no effect in these cells (Fig. 4). There was no difference between control and Adriamycin-treated HL/MRP cells (control versus Adriamycin). Therefore, the G2-M peak was purely mitotic in both Taxotere-treated and Adriamycin + Taxotere-treated cells (Fig. 4). In agreement, Adriamycin did not prevent apoptosis caused by Taxotere (Fig. 4). Essentially, when treated with either Taxotere alone or Taxotere following Adriamycin, MRP-expressing cells were arrested in mitosis and underwent apoptosis. In brief, Adriamycin + Taxotere = Taxotere.

**Protective and discriminating windows.** Thus, 50 ng/mL of Adriamycin protected HL60 cells from Taxotere. How wide is this protective window of Adriamycin concentrations? We treated...
HL60 and HL/MRP cells with increasing concentrations of Adriamycin (Fig. 5). After 16 hours, we changed the medium and added 60 nmol/L Taxotere. Taxotere alone (Adriamycin = 0) was toxic to both HL60 and HL/MRP cells. At cytostatic concentrations (20-80 ng/mL), Adriamycin protected parental HL60 cells. This protection was maximal at 40 ng/mL. At higher concentrations, Adriamycin was cytotoxic to HL60 cells. Therefore, a protective window was about 4-fold (20-80 ng/mL). At these concentrations, there was no protection of HL/MRP cells.

Approximately 100-fold higher concentrations of Adriamycin (3,000 ng/mL) affected HL/MRP cells. Simply, at high concentrations of Adriamycin, MRP cannot pump the drug out completely. Therefore, a discrimination window between HL60 and HL/MRP was about 100-fold (Fig. 5).

Figure 2. Pretreatment with Adriamycin protects parental but not MRP-expressing HL60 cells. A, cells were treated with Taxotere. MTT assay was done after 48 hours as described in Materials and Methods. B, cells were pretreated with 40 ng/mL Adriamycin. After 16 hours, cells were treated with Taxotere. MTT assay was done after 48 hours as described in Materials and Methods.

Prevention of Taxotere-induced mitotic arrest in human normal lymphocytes. Next, we wished to establish whether 50 ng/mL Adriamycin could prevent the effects of Taxotere in primary normal cells such as lymphocytes. Phytohemagglutinin-stimulated lymphocytes were either treated with 50 ng/mL Adriamycin or left untreated. After 8 hours, cells were either treated with 60 nmol/L Taxotere or left untreated for an additional 16 hours (Fig. 6). As expected, Taxotere arrested lymphocytes in mitosis (Fig. 6). This was followed by apoptosis. In contrast, no mitotic or apoptotic cells were detected in Adriamycin-pretreated lymphocytes, following treatment with Taxotere. This result confirmed that low concentrations of Adriamycin could arrest normal human lymphocytes without killing them and thus prevent the effects of Taxotere.

Selective killing of cancer cells lacking G2 checkpoint. Low concentrations of Adriamycin activate G2 checkpoint. Loss of cell cycle checkpoints is common in human cancer. Here we compared p21−/− cells lacking G2 checkpoint (28) with parental HCT116 cells having a proficient G2 checkpoint. In parental HCT116 cells, Adriamycin induced p21 (Fig. 7A). As expected, there was no induction of p21 in p21−/− cells (Fig. 7A). Taxotere alone was cytotoxic in both cell lines (Fig. 7B). Pretreatment with Adriamycin abrogated Taxotere-induced cytotoxicity in parental cells but not in cells lacking p21 (Fig. 7B).

In HCT116 cells, Adriamycin induced G1 and G2 arrest (Fig. 8). In p21−/− cells, Adriamycin caused accumulation of tetraploid (G2) cells (Fig. 8), which actually entered mitosis. Therefore, in p21−/− cells, pretreatment with Adriamycin did not prevent the cytotoxic effects of Taxotere. In the presence of Adriamycin, Taxotere arrested p21−/− cells in mitosis (Fig. 9). Furthermore, a sub-G1 apoptotic peak was evident in p21−/− cells treated with Adriamycin + Taxotere (Fig. 8A). In parental HCT116 cells that were arrested in G2 by Adriamycin (Fig. 8), Taxotere did not cause mitotic arrest (Fig. 9). We conclude that, whereas parental HCT116 cells were protected by Adriamycin, p21−/− cells lacking G2 checkpoint were selectively killed by Taxotere.

Figure 3. Cell cycle distribution and apoptosis: protection of parental HL60 cells. HL60 cells were incubated with 60 nmol/L Taxotere, 40 ng/mL Adriamycin, with 40 ng/mL Adriamycin (12 hours) followed by 60 nmol/L Taxotere, or left untreated. Flow cytometry and TUNEL (apoptotic) assay was done as described in Materials and Methods after 16 hours.
Selective effects on FDC-P1 and FDC/Raf-1 cells. Major side effects of Taxotere are due to its cytotoxicity to hematopoietic cells. Therefore, we investigated the protection of cytokine-dependent FDC-P1 hematopoietic cells from Taxotere. Low concentrations of Adriamycin (10-20 ng/mL) diminished the cytotoxicity of Taxotere (Fig. 10A). FDC/Raf-1 are cytokine-independent malignant cells (38). Raf-1 kinase inhibits apoptosis and renders cells resistant to cell cycle arrest. Therefore, FDC/Raf-1 cells were resistant to both Adriamycin and Taxotere (Fig. 10A). Taxotere was only marginally cytotoxic to these cells both in the absence (Fig. 10A, 0 ng/mL Adriamycin) and in the presence of Adriamycin (Fig. 10A, 10-200 ng/mL Adriamycin). We attempted to sensitize FDC/Raf-1 cells to Taxotere while protecting FDC-P1 cells with low concentrations of doxorubicin. First, 20 ng/mL Adriamycin protected parental cells from cell death caused by Taxotere (Fig. 10B). To potentiate the cytotoxicity of Taxotere in FDC/Raf-1 cells, we used PD98059,
an inhibitor of MEK, which is a downstream target of Raf-1. It has been shown that inhibition of MEK renders cells sensitive to apoptosis caused by microtubule-active drugs (37, 44–47). It has been shown that cells treated with paclitaxel followed by PD98059 exhibited a significant increase in apoptosis, whereas pretreatment of cells with PD98059 reduced cell lethality (48). This suggests that PD98059 is preferentially cytotoxic to mitosis-arrested cells but not G2-arrested cells. We confirmed this prediction. The addition of PD98059 after Adriamycin and Taxotere resulted in significant killing of FDC/Raf-1 cells, whereas parental cells were protected (Fig. 10B, Adriamycin + Taxotere + PD).

Discussion
The goal of cancer therapy is to kill cancer cells, without devastating side effects. Taxanes, at low concentrations, inhibit mitotic microtubules leading to mitotic arrest and, at higher concentrations, cause tubulin polymerization. Accordingly, there are two types of side effects due to (a) mitotic arrest in dividing cells (“mitotic” side effects) and (b) tubulin polymerization in nondividing neurons (neuropathy). As could be predicted, neuropathy occurs with higher doses of taxanes (10, 49, 50). For therapeutic effects, taxanes do not need to be used at doses and schedules that cause neurologic effects (50).

Figure 7. Adriamycin-induced p21 and Taxotere-induced cytotoxicity. A, Adriamycin induced p21 in parental HCT116 cells. Cells were treated with 50 ng/mL Adriamycin, then p21 and actin were measured after 16 hours. B, selective cytotoxicity of Adriamycin + Taxotere in p21−/− cells. Cells were treated with 50 ng/mL Adriamycin and 60 nmol/L Taxotere and MTT assay was done after 2 days, as described in Materials and Methods.

Figure 9. The combination Adriamycin + paclitaxel causes mitotic arrest selectively in p21−/− cells. Parental HCT116 cells and p21−/− cells were pretreated with 50 ng/mL Adriamycin for 12 hours, and then treated with 60 nmol/L Taxotere for 16 hours. DAPI nuclei staining reveals mitotic patterns.

Figure 8. Effects of Adriamycin and Taxotere on cell cycle distribution in parental and p21−/−-deficient HCT116 cells. HCT116 cells (parental) and HCT116-p21−/− (p21−/−) were pretreated, if indicated, with 50 ng/mL Adriamycin for 12 hours, and then treated with 60 nmol/L Taxotere. After 16 hours, flow cytometry was done.

Figure 10. Preferential killing of Raf-1-expressing FDC/Raf-1 cells while protecting parental FDC-P1. A, protective concentrations of Adriamycin. FDC-P1 and FDC/Raf-1 cells were pretreated with indicated concentrations (X-axis) of Adriamycin for 8 hours. At 0 ng/mL Adriamycin (X-axis), cells were left untreated. After 8 hours, the medium was changed and both Adriamycin-treated and untreated cells were treated with 60 nmol/L Taxotere. MTT assay was done after 64 hours. B, PD90598 sensitizes Taxotere-treated FDC/Raf-1 to cell death. When indicated, cells were pretreated with 20 ng/mL Adriamycin for 8 hours (ADR). Then, 60 nmol/L Taxotere was added (+TX). After 6 hours, cells were post-treated with 20 μmol/L PD90598. After 2 days, dead cells were counted with trypan blue. Cell numbers in thousands per well ±SEM.
Mitotic side effects are caused by damage of proliferating bone marrow cells (manifested as myelosuppression), epithelial cells in intestine, stomach, mouth (as diarrhea, nausea, and mucositis), hair follicles (as hair loss). Mitotic side effects may seem inevitable because therapeutic effects also depend on mitotic arrest (in cancer cells). Technically, mitotic side effects are markers of therapeutic doses. To prevent mitotic side effects, we suggest to selectively arrest normal cells in G2. Then, Taxotere is expected to cause mitotic arrest in cancer cells selectively. Here, we showed that low concentrations of Adriamycin arrested Adriamycin sensitive cells in G2, thus protecting them from Taxotere. In contrast, cancer cells with defective G2 checkpoints and MRP1-expressing cells were selectively killed by Taxotere.

Whereas normal cells are arrested in G2, it is important to ensure that cancer cells undergo cell death following Taxotere-induced mitotic arrest because Raf-1-transformed FDC-P1 cells are resistant to Taxotere-mediated cytotoxicity. Here we present the proof of principle of how to selectively increase the cytotoxic effects of Taxotere in such cells. For example, inhibitors of MEK increase apoptosis in cancer cells that are arrested in mitosis (37, 44–48). We showed that PD98059 sensitized FDC/Raf-1 cells to cell death caused by Taxotere. But to avoid sensitization of normal cells, they should first be prevented from entering mitosis. Because low concentrations of Adriamycin can arrest cells with normal G2 checkpoint, without arresting cancer cells, we can selectively attack cancer cells arrested in mitosis. The goal is (a) to prevent normal cells from entering mitosis by causing G2 arrest and (b) to prevent cancer cells from exiting mitosis by inducing apoptosis. It is important to emphasize that because low doses of Adriamycin will protect cells, they will not select for drug resistance. If anything, cancer cells that are sensitive to Adriamycin may be selected. Probably, due to tumor heterogeneity, some cancer cells with normal cell cycle checkpoints might be protected by Adriamycin.

Yet, most deranged and drug-resistant cancer cells will not be protected by Adriamycin and thus will be eliminated.

Animal studies may be useful for proofs of principle and to validate new protective agents. For example, Mdm-2 antagonists, which induce p53 without DNA damage, are of particular interest (51, 52). Yet, in the case of Adriamycin, animal studies may be neither particularly informative nor absolutely necessary. The crucial goal is protection of normal host cells, not merely killing of human tumor xenografts. Normal mouse and human cells differ in their sensitivities to DNA-damaging agents. Also, side effects in humans and mice are different (e.g., hair loss in humans). In addition, it may be difficult to translate in vitro concentrations to animal doses and then to patient doses. On the other hand, Adriamycin is a widely used anticancer drug with well-known dose-toxicity relationships. Thus, all information is available to design a clinical trial. For protection of normal cells, Adriamycin should be used just under its toxic doses. In patients with Adriamycin-resistant tumors, these doses are expected to decrease the side effects of Taxotere without protection of the tumor. The end point of such a clinical trial is a decrease of side effects caused by Taxotere. This is easily detectable. If reduction of side effects will be achieved, this will have far-reaching consequences in cancer therapy, allowing one to add synergistic agents to potentiate Taxotere selectively in cancer cells.

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