Determinants of Tamoxifen Sensitivity Control the Nature of the Synergistic Interaction between Tamoxifen and Cisplatin

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ABSTRACT

The cytotoxic effect of tamoxifen (TAM) was investigated in the T-289 melanoma cell line, as well as the 289 DDP, cisplatin (DDP)-resistant and the 289 TAM, TAM-resistant variant melanoma cell lines to determine the effect of drug resistance on synergy. T-289 melanoma cells were made DDP or TAM resistant through chronic exposure to increasing concentrations of the respective drugs. Whereas DDP resistance could be overcome by increasing the concentration of TAM, the development of TAM resistance completely abolished synergy. TAM resistance was not related to the development of estrogen receptors, decreased TAM uptake, or the increased expression of the mdr-1 gene. TAM did not inhibit the action of Topoisomerase 1; however, TAM did induce apoptosis in the 289 melanoma cells. In contrast, TAM did not induce apoptosis in the TAM-resistant variant 289 TAM6 cells. To our knowledge, these are the first data associating TAM resistance with the inhibition of apoptosis.

INTRODUCTION

Our previous clinical studies have demonstrated that the four-drug combination of DDP,2 dacarbazine, carmustine, and TAM has an overall response rate of approximately 50% in patients with metastatic melanoma (1, 2). Additional clinical studies have suggested that the addition of TAM was responsible for the clinical success, likely by preventing and/or overcoming resistance to DDP (3–5).

To further explore the nature of the interaction between TAM and DDP, we have conducted a series of in vitro studies, the results of which confirm the fact that TAM and DDP exhibit synergistic cytotoxicity as defined by a CI50 of <1 in the MEA equation (6, 7). The synergistic interaction is not based on any of the previously recognized mechanisms of DDP resistance (8, 9). Specifically, synergy is not dependent on an effect of TAM on the uptake of DDP, the expression of metallothionein IIA or glutathione, or the formation or repair of DDP-DNA adducts (8). Additionally, synergy is not dependent on the presence of ERs or PRs, the amount or activity of calmodulin, or the inhibition of PKC by TAM (9).

To further investigate the effect of TAM resistance on the interaction with DDP, T-289 parental cells were serially passaged in the continuous presence of TAM, resulting in a cell line (289 TAM3) that was 8-fold TAM resistant. The development of TAM resistance resulted in the complete loss of synergy (9). The CI50 for the parental line was 0.26 ± 0.02 (SD) compared with a CI50 of 1.39 ± 0.20 for the 289 TAM6 cell line (9). In a similar manner, a DDP-resistant variant (T-289/DDP3) was developed and tested using MEA. Unlike the effect of TAM resistance, DDP resistance only minimally affects synergy. That is, synergy was also observed in the DDP-resistant cells; however, it required an increase in the concentration of TAM (1 order of magnitude). Increasing either the TAM or DDP concentration failed to restore a synergistic interaction in the 289 TAM6 cells.

Because it was clear that synergy was dependent on the cell retaining sensitivity to TAM, we attempted to determine the mechanism of TAM resistance in the 289 TAM6 cells. We report here that there is a differential apoptotic response between the 289 and 289 TAM6 cells; i.e., TAM induces apoptosis in the T-289 and not the 289 TAM6 cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The T-289 melanoma cell line was derived from a tumor explant of a patient with metastatic disease and has been passaged in culture for more than 7 years (10). Cells were cultured in 75-cm² flasks (Corning Glass, Corning, NY) in RPMI 1640 (Irving Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin (Gemini Bio-Products, Calabasas, CA) 2 mm L-glutamine, 10 mm hydrocorti- sone, 5 μg/ml insulin, 5 μg/ml human transferrin, 10 nm estradiol, and 5 ng/ml selenium (Sigma Chemical Co., St. Louis, MO).

Drugs and Chemicals. DDP (clinical formulation) was obtained from Bristol-Myers Squibb (Evaston, IL). TAM was obtained from ICI Pharmaceuticals (Macclesfield, England). 3H-labeled TAM was obtained from Amersham. SeaPlaque low melting temperature agarose was obtained from FMC Bioproducts (Rockland, ME). N,N-diethyl-2-(4-phenylmethyl-phenoxyl)- ethanesulfonamide HCl was the generous gift of L. Brandes (Hamilton, Ontario, Canada). Topo and dX174 RF DNA were obtained from Life Technologies, Inc. (Gaithersburg, MD). CPT (NSC 94600-J) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

Colony-forming Assay. Colony-forming assays using a 1-h drug exposure were performed by seeding cells onto 60-mm tissue culture dishes at 20,000 cells/dish and allowing 2 h for them to attach. Drug was added to the dishes and incubated for 1 h; then, the dishes were washed and the cells were harvested by trypsinization, washed twice to remove the drug, and resuspended in 5 ml complete medium containing 0.2% low melting temperature agarose at 37°C. The cell suspension was mixed well and then aliquoted at 1 ml/dish, in triplicate, onto preprepared 35-mm dishes containing a basement layer of solidified 1% agarose. The cell-containing layer was allowed to solidify at room temperature, and the dishes were incubated at 37°C in humidified 5% CO2. Colonies greater than 125 μm were counted after 5 days. Colony-forming assays using continuous exposure were performed by resuspending cells in 0.2% agarose at 4,000 cells/ml, aliquoting this suspension into drug-containing tubes, and then seeding these onto 35-mm dishes as described above.

MEA. MEA was used to determine the nature of the interaction between TAM and DDP (6). The CI was determined from colony-forming assays at increasing levels of cell kill. CI values of less than or greater than 1 indicate synergy and antagonism, respectively, whereas a CI value of 1 indicates additivity of the drugs. Drugs were combined at the ratio of the IC50 values for TAM and DDP as determined by clonogenic assay. The combination was compared to the cytotoxicity of each drug alone in every experiment, and each experiment was performed using triplicate cultures for each data point.
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**Topo 1 Supercoil Relaxation Assay.** This assay employs the use of φX174 RF DNA, which is naturally in a supercoiled state. When exposed to Topo 1, single-strand nicks in the DNA are introduced, allowing the DNA to relax into a circular form. The two forms (supercoiled and relaxed) can be separated on an agarose gel. To perform this assay, 0.5 μg of supercoiled φX174 RF DNA was incubated in 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 0.5 mM DTT, 0.1 M EDTA, 30 μg/ml BSA, Topo 1 (10 units), and the appropriate concentration of either vehicle control, CPT, or TAM. The above reagents were combined in a 50-μl volume and reacted at 37°C for 30 min. The reaction was stopped by adding buffer, and the mixture was then loaded onto a 1% agarose gel and run overnight at 10 V. The DNA was then stained using ethidium bromide.

**Apoptosis.** Apoptosis was quantified by using the cellular DNA fragmentation enzyme-linked immunosassay (Boehringer Mannheim, Indianapolis, IN). Briefly, T289 cells and the TAM-resistant variant 289 TAM₆ were incubated for 16 h in 10 μM BrdUrd. They were then trypsinized and plated at 11,000 cells/well in a 96-well tissue culture-treated microtiter plate. After 4 h of attachment, they were exposed to 0, 3, 6, 12, 18, and 24 μM TAM for 24 h. At the end of the drug exposure, 100 μl of culture medium were transferred to a 96-well microtiter plate containing an anti-DNA antibody fixed to the well. The remainder of the medium was discarded, the cells were lysed, and 100 μl of the cell lysate were also transferred to a 96-well plate containing an anti-DNA antibody fixed to the well. After incubation, the DNA binding plates were washed, and anti-BrdUrd antibody was added. The amount of the anti-BrdUrd binding was quantitated using an anti-idiotypic secondary antibody conjugated with peroxidase. Substrate solution was added, and after a short incubation, the plates were read in a Dynatech MRX (Dynatech Laboratories, Inc., Sullyfield, VA) microplate reader at 450 nm with a reference wavelength of 630 nm. The amount of DNA fragmentation quantitated from the supernatant and cell lysate was expressed as absorbance (450–630 nm) versus TAM concentration.

**RESULTS**

**MEA.** The effect of DDP resistance on the synergistic interaction between TAM and DDP was investigated by performing MEA using the 9-fold DDP-resistant 289 DDP₆ melanoma cells. As can be seen in Fig. 1, despite DDP resistance, the interaction between TAM and DDP remains synergistic. The CI₅₀ for this interaction was 0.53 ± 0.14 (mean ± SD), which represents a reduction when compared to the CI₅₀ for the parent cell line (0.26 ± 0.02; Ref. 11). Additionally, the concentration of TAM that was required to induce synergy increased from 0.1 μM to 1.0 μM.

**Selection for DDP or TAM Resistance.** Cells resistant to DDP or TAM were selected by growing the T-289 cells in the continuous presence of DDP or TAM. To start the cells at the highest concentration that they can survive, drug concentrations of IC₅₀, IC₅₀, and IC₅₀ were chosen as the initial concentrations. Thus, the initial concentrations were 0.5, 1.0, and 2.0 μM, and 1.0, 2.0, and 4.0 μM for DDP and TAM, respectively. The cells were allowed to grow to confluence, at which time successful cultures were split and re-exposed to the same concentration of drug. Cells were treated in this manner for a total of three exposures to the same concentration of drug, after which the concentration of each drug was increased by 10–20% for an additional three exposures. At each time point where the concentration of drug was increased, a sample of cells was split out and grown for 2 weeks drug free. These cells were then used in the colony-forming assays as described above to determine the new IC₅₀.

**ER/PR Receptor Assay.** The ER/PR status of the 1-289 melanoma cell lines was determined by the Nichols Institute (San Juan Capistrano, CA) from cells grown under identical culture conditions using a quantitative dextran coated charcoal method, and expressed as fmol of receptor per mg protein.

**3H-labeled TAM Uptake.** T-289 and 289 TAM₆ cells were split into 60-mm culture dishes and allowed to grow for 2 days. The medium was aspirated and replaced with medium containing the appropriate amount of 3H-labeled TAM (0.1 μCi/ml) or DMSO (control), and the cells were incubated for the appropriate time period. The cells were then harvested, washed with ice-cold PBS, and then digested overnight in 1 N NaOH. The next day, a portion of the digest was used to measure the protein content, while the remainder was counted in a scintillation counter (Beckman) for TAM content.

**Fig. 1.** MEA plot for TAM and DDP in the DDP-resistant 289 DDP₁ cells. CI₅₀ = 0.53 ± 0.14 (mean ± SD). Fa, fraction affected.

**Fig. 2.** MEA plot for TAM and DDP in the TAM-resistant 289 TAM₆ cells. CI₅₀ = 1.36 ± 0.15 (mean ± SD). Fa, fraction affected.

**Fig. 3.** TAM uptake in the 289 and 289 TAM₆ melanoma cells as a function of time. Cells were exposed to 1 μM TAM (0.1 μCi/ml) for 0–240 min.
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Similarly, the effect of TAM resistance on the synergistic interaction between TAM and DDP was investigated by performing MEA using the 8-fold TAM-resistant 289 TAM<sub>r</sub> melanoma cells. As can be seen on Fig. 2, the emergence of TAM resistance resulted in the complete loss of synergy between TAM and DDP. The Cl<sub>50</sub> increased from 0.26 ± 0.02 to 1.36 ± 0.15, indicating antagonism.

**Hormone Receptor Analysis.** Because the 289 melanoma line is without either ER or PR, one mechanism of TAM resistance might be the expression of ER in previously ER-negative cells capable of competing for TAM and acting as a sink binding TAM and reducing its ability to reach the target essential for the synergistic interaction. Using standard dextran-coated charcoal analysis, both the parental cell line and the 289 TAM<sub>r</sub> cell lines failed to express measurable amounts of either of the receptors. The ER content was found to be <3 fmol/mg, and the PR content was <5 fmol/mg in both cell lines.

**TAM Uptake.** Altered uptake of cytotoxic drugs is a well-described mechanism of drug resistance exhibited by a variety of malignant cells (12). To test whether or not altered TAM uptake was related to TAM resistance, 289 and 289 TAM<sub>r</sub> cells were exposed to <sup>3</sup>H-labeled TAM (5 μM) from 0 to 240 min. Fig. 3 demonstrates that there was no significant difference in the uptake of TAM between the parental and TAM-resistant variants.

**Topo 1 Assay.** TAM has been shown to form DNA adducts by binding to the minor groove in DNA (13, 14). Chen et al. (15) have demonstrated that many drugs that bind to the minor groove act by inhibiting the action of Topo 1, similarly to CPTs. The ability of TAM to act in a manner similar to CPT was investigated using the Topo 1 supercoil relaxation assay. This assay employs the use of supercoiled φX174 RF DNA, determining the ability of an agent to inhibit Topo 1-induced relaxation as measured by differential mobility on a gel. As can be seen in Fig. 4, the addition of TAM to φX174 RF DNA resulted in the complete relaxation of the DNA. The addition of CPT resulted in a dose-dependent inhibition of this relaxation. In contrast, TAM at concentrations up to 40 μM failed to inhibit Topo 1 activity.

**MDR.** The expression of the MDR phenotype in melanoma cells has provided at least a partial explanation for the well-documented drug resistance of this malignancy (16). It has also been shown that TAM is both a substrate and an inhibitor of this energy-dependent P-glycoprotein cell membrane efflux pump (17–19). TAM resistance, therefore, may be the result of the increased expression of the mdr-1 gene, resulting in active efflux of TAM from the resistant cells.

Using a modification of the Noonan protocol, quantitative PCR analysis was employed to determine the expression of mdr-1 in the parent 289 and TAM-resistant 289 TAM<sub>r</sub> cell lines (20). The GRC-1, P-glycoprotein-overexpressing ovarian cancer cell was employed as a positive control. Quantitative PCR analysis demonstrated a 500-fold expression of mdr-1 in the GRC-1 cell line compared to the 289 melanoma cell line. This finding suggests that the increased expression of the mdr-1 gene may play a role in TAM resistance in melanoma cells.
positive control. As seen in Fig. 5, there was no increased expression of mdr-1 mRNA in either the 289 parental or 289 TAM<sub>6</sub> cell lines.

**Apoptosis.** The effect of TAM resistance on the ability of TAM to induce apoptosis in the 289 and 289 TAM<sub>6</sub> cell lines was evaluated using a DNA fragmentation assay. The cells were incubated overnight in BrdUrd, followed by a 24-h exposure to TAM at increasing concentrations. Fig. 6 demonstrates that TAM induced apoptosis in a dose-dependent fashion in the 289 cells. In comparison, doses of TAM up to 24 μM failed to induce a significant apoptotic response in the 289 TAM<sub>6</sub> cells.

**DISCUSSION**

Previous studies have failed to demonstrate a relationship between synergy and any of the recognized effects of TAM (9). To further study this interaction, a TAM-resistant variant of the 289 melanoma cell was developed to determine whether TAM resistance played a role in synergy. The data from this study demonstrate that the synergistic cytotoxicity observed between TAM and DDP is critically dependent on a determinant that controls cellular sensitivity to TAM. Although the development of DDP resistance did not abrogate the synergistic nature of the interaction between TAM, the development of TAM resistance resulted in the complete loss of the synergy.

Studies were initiated to continue the investigation of the mechanism of TAM cytotoxicity and resistance. Altered drug uptake and enhanced drug efflux, the latter frequently related to the development of the MDR phenotype, are common mechanisms of drug resistance (12, 21). In these cell lines, neither mechanism played a significant role. There was no difference in the uptake of TAM between the parental and TAM-resistant variant cell lines. Likewise, neither the 289 nor the 289 TAM<sub>6</sub> cell lines expressed the mdr-1 gene to any significant level.

TAM has been shown to bind to the minor groove of DNA. It is, therefore, possible that TAM acts in a manner similar to CPT by inhibiting Topo 1 activity. To investigate this activity, the ability of TAM to inhibit Topo 1 action on the supercoiled <i>φX174</i> RF DNA was evaluated. This assay is dependent on the separation of supercoiled and circular (relaxed) forms of this DNA. Topo 1 will produce DNA single-strand nicks, relaxing the supercoil, resulting in all-circular DNA that migrates through an agarose gel at a slower rate. The addition of an inhibitor of Topo 1 results in the production of two bands, corresponding to both circular (Topo 1 activity) and supercoiled (inhibited Topo 1 activity) DNA. Although CPT clearly inhibited Topo 1 activity in a dose-dependent fashion, concentrations of TAM up to 40 μM failed to exhibit similar activity (Fig. 4).

The nature of TAM cytotoxicity, aside from the effect it has on ERs, has not been well established. However, several recent studies have shown that TAM induces apoptosis in breast cancer cell lines (22, 23). Perry et al. (22) demonstrated that TAM induced classic morphological and biochemical changes associated with apoptosis in MCF-7 (ER<sup>+</sup>) as well as the MDA-231 (ER<sup>-</sup>) breast cancer cells. Welsh (23) studied the effect of vitamin D resistance in MCF-7 cell lines and showed that TAM induced apoptosis regardless of the vitamin D effect (23). To determine the effect of TAM resistance on TAM-induced apoptosis, 289 and 289 TAM<sub>6</sub> cells were exposed to increasing concentrations of TAM, and apoptosis was evaluated in a DNA fragmentation assay. TAM induced a dose-dependent increase in the percentage of fragmented DNA, consistent with the induction of apoptosis, in the 289 cells. Under the same conditions, there was essentially no increase in fragmented DNA in the 289 TAM<sub>6</sub> cells. To our knowledge, these are the first data to demonstrate a resistance to apoptosis as a mechanism of TAM resistance. The mechanism of TAM-induced apoptosis as well as the mechanism of TAM resistance is currently under investigation in this laboratory.

The above data clearly demonstrate the importance of TAM sensitivity to the synergistic cytotoxic effect observed between TAM and DDP. As demonstrated previously, this synergistic interaction is not confined to melanoma cells but can be observed in other tumors, such as ovarian and small cell lung cancer (24). Thus, it will continue to be important to determine the mechanism of TAM effect. Once the mechanism is identified, it should be possible to develop an in vitro test that will predict which patients will respond to this combination, allowing the selection of patients <i>a priori</i> for treatment.

**REFERENCES**


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