Synergy between Tamoxifen and Cisplatin in Human Melanoma Cells Is Dependent on the Presence of Antiestrogen-binding Sites

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ABSTRACT

We have demonstrated previously that cisplatin (DDP) and tamoxifen (TAM) act synergistically to kill human melanoma T-289 cells, and that the observed synergy is lost in the 3-fold TAM-resistant subline, 289/TAM₆. We have identified the intracellular antiestrogen-binding sites (AEBSs), defined by their ability to bind antiestrogens while having no affinity for estrogen, as a possible mediator of this synergy. We report here that [³H]TAM binds to AEBSs, as defined by the ability of N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine·HCl, an AEBS-specific ligand, to compete with [³H]TAM binding. Furthermore, we have characterized the number of binding sites and their affinity for [³H]TAM by Scatchard analysis in whole-cell lysates, microsomal fractions, and nuclear fractions of both cell lines by competing [³H]TAM binding with increasing concentrations of unlabeled TAM. These data demonstrate that the loss of a high-affinity AEBS from the nuclear fraction of the 289/TAM₆ cell line correlates with the loss of synergy between DDP and TAM in these cells. This implicates AEBSs as a critical component of the mechanism that mediates the synergistic interaction of DDP and TAM in human melanoma cells.

INTRODUCTION

Recent clinical success with a combination chemotherapy regimen for the treatment of metastatic melanoma has resulted in the identification of a novel interaction between TAM² and DDP (1, 2). When TAM was removed from a four-drug combination that included TAM, DDP, carmustine, and dacarbazine, the overall response rate fell from more than 50% to 10% (3). Reincorporation of TAM into the regimen resulted in a return of the overall response rate to 50% (4). Lattanzi et al. have confirmed these observations and demonstrated a survival advantage for those patients treated with the TAM-containing regimen (5). Additional studies with melanoma have demonstrated that TAM can overcome established clinical resistance to DDP in patients with melanoma, and that concurrent TAM treatment can delay the development of acquired DDP resistance (6, 7).

Our laboratory investigations have confirmed that a novel synergistic cytotoxic interaction exists between TAM and DDP in human melanoma cells, which is likely responsible for the improved response rate observed in the clinical studies (8). Using the T-289 human melanoma cell line as a model, we have demonstrated that the synergy is not due to an effect of TAM on any of the factors most commonly associated with DDP resistance. In in vitro studies, there was no effect of TAM on cellular uptake of the DDP analogue [³H]dichloro(ethyl-enediamine)platinum(II) on the formation or repair of DDP intranuclear adducts. Similarly, there was no effect of TAM on the intracellular nucleophiles glutathione and metallothionein. Furthermore, the synergy was found not to be dependent on the presence of estrogen receptors, nor on a TAM effect on calmodulin or protein kinase C (9).

Recent attention has been focused on AEBSs as possible mediators of the synergistic interaction between DDP and TAM. These quantitatable cellular drug binding sites are distinct from the estrogen receptor in that, in addition to antiestrogens, AEBSs also bind various chemical compounds, such as phenothiazines, cytochrome P-450 inhibitors and oxygenated sterols without, however, binding estrogens (10). AEBSs were first identified by Sutherland and Foa and have been found to be distributed ubiquitously in many human and animal tissues (11). Although the natural ligand for these receptors has not been identified, the binding of agents such as TAM or ethanamine results generally in the inhibition of cell proliferation by a mechanism that remains to be identified (12-14).

Brandes and Hermonat synthesized the TAM analogue, DPPE, which binds AEBSs with high affinity and specificity, in a fashion similar to TAM but has no effect on calmodulin, calcium channels, protein kinase C activity, or estrogen receptor binding (15, 16). We hypothesized that, if the synergistic interaction between TAM and DDP were mediated via AEBSs, other agents that bind AEBSs should also be synergistic with DDP and should compete with TAM for binding to AEBSs. In an attempt to determine what role AEBSs may play in the synergy between TAM and DDP, we have conducted experiments to (a) determine whether the cytotoxicity of DPPE mimics that of TAM in being synergistic with DDP in the T-289 cells but not in the 3-fold TAM-resistant 289/TAM₆ cells; (b) identify the number and location of AEBSs in human melanoma cells; and (c) determine whether TAM and DPPE compete for the same binding site in these cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The T-289 melanoma cell line was derived from a tumor explant and has been passaged in culture for more than 7 years (17). Cells were cultured in RPMI 1640 containing 2 ng/mL glutamine and supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA), 10 ng/mL hydrocortisone, 5 μg/mL insulin, 5 μg/mL human transferrin, 10 ng/mL estradiol, 5 ng/mL selenium, and 50 μg/mL gentamicin (Sigma Chemical Co. St. Louis, MO). The 3-fold TAM-resistant 289/TAM₆ cells were developed by serially passaging T-289 melanoma cells in the continuous presence of increasing concentrations of TAM until they were growing at normal rates in the continuous presence of 6 μM TAM. Both cell lines are estrogen receptor negative, as determined previously (9).

Drugs and Chemicals. DDP (clinical formulation) was obtained from Bristol-Myers Squibb Co. (Evaston, IL). Unlabeled TAM and [³H]TAM were purchased from Sigma Chemical Co. (St. Louis, MO) and Amersham Life Science, Inc. (Arlington Heights, IL), respectively. SeaPlaque low-melting temperature agarose was purchased from FMC BioProducts (Rockland, ME). DPPE was synthesized and provided generously by Lorne Brandes, M.D. (University of Manitoba, Winnipeg, Manitoba, Canada).

Competitive Binding Studies and Scatchard Analysis. Cell extracts derived from whole cells, nuclei, or microsomes of the T-289 and 289/TAM₆
cells were used in competitive binding studies and Scatchard analysis. Whole-cell extracts were prepared by lysing approximately $1 \times 10^7$ cells with 100 strokes of a dounce homogenizer in binding buffer, which consisted of 10 mM Tris-Cl (pH 7.4) and 1 mM EDTA. Microsomes could then be isolated by centrifuging the whole-cell lysates at 1250 x g for 10 min at 4°C to remove nuclei and unbroken cells. The nuclei were isolated similarly to those described previously by Miller and Katzenellenbogen (18) and Kendra and Katzenellenbogen (19). The protein content of the cell lysate was determined by the Bradford method (20).

Competition assays were carried out by preincubating the cell extract for 30' at 4°C in the presence of 1.5 nM [3H]TAM and 1 µM 17-β-estradiol in a total volume of 250 µl of binding buffer. Following the initial incubation, an additional 245 µl of binding buffer were added along with increasing concentrations of unlabeled competitor (TAM, 0.01 nM—5 µM; DPPE, 0.01 nM—100 µM; and histamine, 0.01 nM—100 µM). The cell extract was incubated overnight at 4°C. The following day, 100 µl of dextran-coated charcoal were added, and the incubation was continued for 30 min to allow binding of any unbound TAM. The charcoal was then removed by centrifugation at 8000 x g for 6 min. The bound [3H]TAM remaining in the supernatant was counted in a Beckman LS6500 scintillation counter (Beckman Instruments, Inc., Schamburg, IL), and the number of binding sites as well as the specificity of binding were then calculated by Scatchard analysis. The background binding was determined by averaging the data points that fell within the plateau of the competitive binding curve. At this point, the continued addition of cold competitor produced no further reduction in specific [3H]TAM binding. The number of AEBs and the $K_d$ of the binding site were calculated using the Scatchard method by comparing the ratio of bound TAM to free TAM, with concentration of bound TAM expressed in nmoles/liter.

**Colonies-forming Assays.** Colony-forming assays were performed by incubating 4000 cells/ml in DDP for 1 h at 37°C or by incubating 4000 cells/ml in a continuous exposure to DPPE in 1 ml of complete medium containing 0.2% low-melting temperature agarose plated on a basement layer of solidified 1% agarose, in triplicate. The cell-containing layer was allowed to solidify at 4°C for 10 min, and the dishes were then incubated at 37°C in humidified 5% CO₂ atmosphere for 7 days. Colonies greater than 125 µm in diameter were counted.

**Median Effect Analysis.** Median effect analysis was used to determine the nature of the interaction between DDP and DPPE resulting from a 1-h exposure to DDP followed by continuous exposure to DPPE (21). The drugs were combined at increasing concentrations in ratios of their IC₅₀ values as determined by clonogenic assay. The combination results were then compared to the cytotoxicity of each drug alone using median effect analysis (22, 23). CI values of less than 1 indicate that the drugs are working synergistically, CI values greater than 1 indicate drug antagonism, and CI values of approximately 1 indicate drug additivity.

**RESULTS**

**DPPE/DDP Synergy.** Synergy between DPPE and DDP was sought by performing median effect analysis of survival data for the parental T-289 melanoma cell line and its TAM-resistant subline 289/TAM₆, generated using colony-forming assays in a manner similar to the studies conducted previously with TAM and DDP (8, 9). Fig. 1 shows the CI plot generated by the median effect analysis for both the T-289 and the 289/TAM₆ cell lines. The data clearly demonstrate strong synergism between DPPE and DDP in the parent T-289 cells and a loss of synergy in the TAM-resistant 289/TAM₆ cell line. The CI₅₀ for DPPE in the T-289 cells was 0.30 ± 0.00 (n = 2), and in the TAM-resistant 289/TAM₆ cells it was 1.53 ± 0.62 (n = 2). Thus, an agent that putatively binds specifically to AEBSs interacts synergistically with DDP in a manner closely analogous to the interaction between TAM and DDP.

**AEBs.** The presence of TAM-bound AEBs was quantitated in the parental T-289 and TAM-resistant 289/TAM₆ cell lines. Scatchard analysis of competitive binding was done in whole-cell homogenates as well as microsomal and nuclear fractions of each cell line in the presence of excess estrogen. Table 1 presents the data on the number and affinity of the AEBs determined by [3H]TAM binding in the presence of unlabeled TAM. AEBs TAM binding was identifiable in all fractions of both cell lines. The number of AEBs in the whole-cell homogenates of the 289/TAM₆ cells did not differ from the parental T-289 cells. However, in the 289/TAM₆ cells, the number of AEBs in the nuclear fraction increased 34% and in the microsomal fraction increased 42% as compared to the parental cell line. Because of the experimental variability inherent in conducting Scatchard analysis, these data failed to reach statistical significance; however, these data may be consistent with an increase in AEB number in these fractions, as compared to the parental cell line. The $K_d$ for the binding sites in the whole-cell homogenates of the T-289 cells was 7.4 ± 1.5 nM. The $K_d$ in the 289/TAM₆ cells, as compared to the parental T-289 cells, was 41% higher in the whole-cell homogenates, 74% higher in the nuclear fraction, and 34% higher in the microsomal fraction. Despite the fact that the changes were not statistically significant for all of the fractions, the changes were consistent with a general decrease in the affinity of TAM for AEBs in the 289/TAM₆ cells. In the case of the nuclear fraction, the change in $K_d$ observed did reach statistical significance. This indicates the loss of a high-affinity AEBs from the nuclear fraction in the 3-fold-resistant 289/TAM₆ cells ($P = 0.025$).

**Competitive DPPE Binding.** Competitive binding assays between TAM and DPPE were performed on the nuclear fractions of the T-289 and 289/TAM₆ cells to determine whether these agents were binding to the same molecules. Fig. 2A depicts [3H]TAM-specific binding remaining as a fraction of function of DPPE concentration in the nuclear fractions of both cell lines. The fact that the specific [3H]TAM binding was inhibited by unlabeled DPPE suggests that both molecules compete for the same target.

**Competitive Histamine Binding.** It has been suggested by Brandes et al. (24, 25) that AEBs are a subset of the histamine family of receptors. To determine whether histamine affected the specific binding of TAM to AEBs, we carried out competitive binding studies using [3H]TAM and increasing concentrations of unlabeled histamine with the microsomal fraction of the parental T-289 cells. Fig. 2B demonstrates that histamine did not compete for the [3H]TAM-specific binding sites, even at micromolar concentrations.

**DISCUSSION**

The major findings of this study provide additional evidence that AEBs play a role in the mechanism underlying the synergistic cytotoxic interaction between DDP and TAM. First, the loss of
DPD/TAM synergy in the TAM-resistant 289/TAM<sub>α</sub> melanoma cell line was found to be associated with a general decrease in the affinity of AEBSs for TAM. This was associated specifically with the statistically significant 1.74-fold increase in \(K_d\) of a high-affinity binding site in the nucleus of these cells. Secondly, DPPE, a compound believed to be specific for AEBSs, mimicked the exact same pattern of synergy with DDP, as described previously with DDP and TAM (8); it was highly synergistic with DDP in killing the T-289 cells (CI at 50% cell kill, 0.30), whereas, in the 289/TAM<sub>α</sub> cells, this synergy was lost completely (CI at 50% cell kill, 1.53). Finally, DPPE and TAM appear to compete for binding to AEBSs.

Sutherland and Foo have described AEBSs as quantitatively distinct binding sites that bind compounds falling into the family of antiestrogens, while not binding estrogen itself (11). DPPE is a diphenylmethane derivative synthesized by Brandes and Hermonat, which binds selectively to AEBSs while not binding the estrogen receptor (15, 16). Its availability permitted an analysis of whether TAM was binding specifically to AEBSs or some other as yet unidentified binding site. The binding of \(^{3}H\)TAM was competed by the addition of unlabeled DPPE, suggesting that the two compounds can bind to the same molecule. This type of experiment does not permit the conclusion that they are binding to exactly the same site, because it is conceivable that the binding of DPPE could alter the conformation of the target so as to reduce the affinity of TAM for another site in another domain.

Brandes <em>et al.</em> have hypothesized that AEBSs are a novel low-affinity histamine receptor distinct from the H<sub>1</sub>, H<sub>2</sub>, and H<sub>3</sub> histamine receptors based on their work demonstrating that DPPE can inhibit \(^{3}H\)histamine binding in rat cerebral cortex and rat liver microsomes (14, 25). Furthermore, they have shown that \(^{3}H\)DPPE binding could be competed with (in order of strength): DPPE, TAM, hydroxyazine, and pyrilamine. Because the latter two compounds are well-known histamine H<sub>1</sub> receptor ligands, we looked at the ability of histamine to compete for \(^{3}H\)TAM binding to AEBSs. We were unable to detect any competition. This may indicate that AEBSs are a novel binding site distinct from histamine receptors. We cannot, however, rule out the possibility that TAM and histamine both bind AEBSs at different sites that do not lead to allosteric changes in the molecule and therefore do not affect the binding of either compound. Nor can we rule out the possibility of rapid histamine degradation through histamine decarboxylase activity, which may render histamine unsuitable to compete for binding. Clearly, further investigation is needed to determine the exact role of AEBSs in these cells.

Although the molecular nature of the entity that constitutes the AEBS has not been elucidated, Poirot <em>et al.</em> (13, 26) and Chailleux <em>et al.</em> (27) have purified a candidate Mr 40,000 protein that demonstrates TAM-specific binding. Further understanding of the mechanism of synergy between DDP and TAM will be aided by sequencing of the protein or its gene and the generation of specific antibodies with which to characterize its function. Recent studies have demonstrated that proteins involved in DNA mismatch repair (28–30) and nucleotide excision repair (31) play an important role in the recognition of DDP adducts in DNA, and we speculate that nuclear AEBSs may constitute one of the proteins involved in these functions.

### REFERENCES


Fig. 2. Competition between DPPE (A) and histamine (B) and \(^{3}H\)TAM for binding to AEBSs. T-289 cells; ○, 289/TAM<sub>α</sub> cells.
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