Tamoxifen Modulation of Cisplatin Sensitivity in Human Malignant Melanoma Cells

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ABSTRACT

Tamoxifen (TAM) markedly increases the response rate of malignant melanoma to treatment with cisplatin (DDP), carmustine, and dacarbazine, and we have previously reported that there is a highly synergistic interaction between TAM and DDP with respect to the cytotoxic effect against the human melanoma cell line T-289 (E. F. Mc Clay et al., Cancer Res., 52: 6790-6796, 1992). The mechanism underlying synergy was investigated by examining the effect of selection for either DDP or TAM resistance on the magnitude of the synergy quantitated by median effect analysis. The combination index at 50% cell kill was 0.26 ± 0.02 (SD) for parental T-289 cells (indicating marked synergy), 0.54 ± 0.14 for cells selected for low-level DDP resistance (indicating moderate synergy), and 1.39 ± 0.20 for cells selected for low-level TAM resistance (indicating antagonism). Thus, factors that determine DDP sensitivity have a moderate effect on reducing the DDP/TAM synergy, but determinants of TAM sensitivity have a major effect. The known biochemical effects of TAM include antagonism of estrogen at the estrogen receptor (ER) and inhibition of calmodulin and protein kinase C activity. T-289 cells contained undetectable amounts of ER by the dextran-coated charcoal assay and expressed only trace amounts of ER mRNA, and another more avid ER antagonist, droloxifene, failed to interact synergistically with DDP. N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), a potent calmodulin and protein kinase C antagonist, did not alter the cell cycle phase perturbation produced by exposure to DDP alone. We conclude that the synergy between TAM and DDP is not mediated by the effects of TAM on the ER, calmodulin, protein kinase C, or cell cycle regulation. However, the factors that determine cellular sensitivity to TAM also determine whether TAM interacts synergistically with DDP.

INTRODUCTION

Recent clinical studies indicate that the addition of TAM to the combination of DDP, carmustine, and dacarbazine markedly increases the response rate in patients with malignant melanoma (1, 2). We have been investigating the nature of the interaction between TAM and the other components of this regimen in vitro. These studies have identified a truly synergistic interaction between TAM and DDP in the human melanoma cell line T-289 (3) using median effect analysis (4). In contrast, TAM was antagonistic with carmustine and an activated form of dacarbazine. The mean CI10 was 0.26 ± 0.02 (SD) for TAM and DDP. More importantly, this synergism was observed at concentrations of both agents that are clinically achievable.

Investigation of the effect of TAM on the biochemical pharmacology of DDP demonstrated that TAM did not alter any of the parameters commonly believed to determine cellular sensitivity to DDP (3). Specifically, TAM had no effect on the uptake of the DDP analogue [3H]dichloro(ethylenediamine)platinum(II), and there was no effect on the formation or repair of DDP intrastrand DNA adducts. Similarly, TAM had no effect on the intracellular concentrations of glutathione or metallothionein IIA. The DDP/TAM interaction was found to be unique in that the interaction still occurred when TAM is added up to 48 h after the exposure to DDP (3).

TAM is best known for its ability to bind to and inhibit the function of the ER. However, TAM has been reported to have two other biochemical effects in cells, inhibition of calmodulin function (5, 6) and protein kinase C activity (7, 8). Here we report additional studies on the mechanism of the synergistic interaction between TAM and DDP focusing on the known biochemical effects of TAM. We have determined that the synergistic effect of TAM is critically dependent on the sensitivity of the cell to TAM but that DDP resistance has a smaller effect on the magnitude of the synergistic interaction. The effect of TAM is not dependent on the presence of detectable ER, nor can it be mimicked by other inhibitors of calmodulin or modulators of PKC. Likewise, TAM does not alter the magnitude or duration of the cell cycle arrest induced by DDP. We conclude that the biochemical determinants of cellular sensitivity to the cytotoxic effect of TAM are also determinants of its potential to interact synergistically with DDP.

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 (9). Cells were cultured on 75-cm² flasks (Corning, Corning, NY) in RPMI 1640 (Irvine 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 hydrocortisone, 5 μg/ml insulin, 5 μg/ml human transferrin, 10 mM estradiol, and 5 mg/ml selenium (Sigma Chemical Co., St. Louis, MO). The “Brown” melanoma cell line, received from Genetics Institute (Cambridge, MA), was cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM l-glutamine. The 2.9-fold resistant 289/DDP(2) cells were developed by serially passing T-289 melanoma cells in the continuous presence of increasing concentrations of DDP. Eight-fold TAM-resistant [289/ TAM(6)] cells were developed in a similar manner by selection with TAM.

Drugs and Chemicals. DDP (clinical formulation) was obtained from Bristol-Myers Squibb (Evaston, IL), and unlabeled dichloro(ethylenediamine)platinum(II), an analogue of DDP, was from Alfa Ventrón (Danvers, MA). [3H]dichloro(ethylenediamine)platinum(II) was synthesized as previously reported (10). Carboplatin (clinical formulation) was obtained from Bristol-Myers (Evansville, IN). TAM was obtained from ICI Pharmaceuticals (Macclesfield, England). Droloxifene, a TAM analogue, was generously supplied by Klingen Pharma (Munich, Germany). W-7 and TPA were obtained from Applied Science Laboratories (Pottsgrove, PA), (9). Cells were cultured on 75-cm² flasks (Corning, Corning, NY) in RPMI 1640 supplemented with 10% fetal bovine serum, 50 μg/ml gentamicin (Gemini Bio-products, Calabasas, CA), 2 mM l-glutamine, 10 mM hydrocortisone, 5 μg/ml insulin, 5 μg/ml human transferrin, 10 mM estradiol, and 5 mg/ml selenium (Sigma Chemical Co., St. Louis, MO). The “Brown” melanoma cell line, received from Genetics Institute (Cambridge, MA), was cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM l-glutamine. The 2.9-fold resistant 289/DDP(2) cells were developed by serially passing T-289 melanoma cells in the continuous presence of increasing concentrations of DDP. Eight-fold TAM-resistant [289/ TAM(6)] cells were developed in a similar manner by selection with TAM.

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 once to remove drug, and resuspended in 5 ml complete media 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 prepared 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 4000 cells/ml, aliquoting this suspension into drug-containing tubes, and then seeding these onto 35-mm dishes as described above.

Median Effect Analysis. Median effect analysis was used to determine the nature of the interaction between TAM and DDP (4). CI was determined from colony-forming assays at increasing levels of cell kill. CI values 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.

Estrogen Receptor Assay. The ER content of the T-289 cell line was determined by Nichols Institute (San Diego, CA) from xenograft-generated tissue slices using a quantitative dextran-coated charcoal method and expressed as fmol receptor/mg protein (11). Expression of ER mRNA was determined by Northern blot analysis. Five μg of total cellular RNA were harvested and electrophoresed in formaldehyde gels and then transferred to Nitroplus membranes (Micron Separations, Inc., Westborough, MA). These were probed sequentially with 32P-labeled complementary DNA probes for β-actin (12) and the human ER.

Cell Cycle Phase Distribution. T-289 cells were seeded onto 60-mm dishes and allowed to attach for 24 h. DDP was then added for 1 h, either alone or concurrently with TAM. After 1 h, the dishes were aspirated and washed. Regular or TAM-containing medium was added back to the appropriate dishes. Drugs were present at concentrations equal to the IC50 for each drug, in a ratio that yielded synergy in the median effect analysis. At each time point, the cells were trypsinized, centrifuged, washed, and resuspended in ice-cold 70% ethanol at a concentration of 1 X 10^6 cells/ml. Cells were resuspended in 50 μm propidium iodide and 1000 units/ml RNase A in phosphate-buffered saline. After a 30-min incubation at 37°C, the cells were analyzed on a CytoFluorograf (Ortho Diagnostics Systems, Raritan, NJ). Multicycle Cell Cycle Software (Phoenix Flow Systems, San Diego, CA) was used to determine the fraction of cells in each phase of the cell cycle.

Calmodulin Content. Monoclonal antibodies directed against bovine brain CAM (UBI, Lake Placid, NY) were used to quantify CAM content in T-289 cells and TAM-selected T-289 variants. Cells were harvested by trypsinization, washed and permeabilized in 93% methanol, and then incubated with 10 μg/ml mouse anti-CAM antibody overnight at 4°C. The cells were then washed in phosphate-buffered saline, incubated in fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) for 2 h, and then washed again. Immunofluorescence was determined using a CytoFluorograf (Ortho Diagnostics Systems) with excitation and emission settings of 495 and 528 nm, respectively.

RESULTS

Effect of Sensitivity to DDP on the Magnitude of Synergy. One approach to identifying the biochemical mechanism(s) underlying the synergistic interaction between DDP and TAM is to determine whether they are the same or different from those that determine cellular sensitivity to DDP. T-289 cells were selected for resistance to DDP by growing them in progressively higher concentrations of DDP. The T-289/DDP(3) subline grew well in 3 μM DDP. The IC50 for these cells following exposure to DDP for 1 h was 27.5 ± 3.5 (SD) μM, which is approximately 9.2-fold higher than the IC50 of 2.98 ± 0.41 (SD) for the parental T-289 cells. Fig. 1 shows that median effect analysis of the interaction of DDP and TAM using the T-289/DDP(3) cells yielded a CI50 of 0.54 ± 0.14 (SD), demonstrating the persis-

![Figure 1](attachment:combination_index.png)

Fig. 1. Combination index plots for the interaction between DDP and TAM for T-289 (A), T-289/DDP(3) (B), and T-289/TAM(6) (C) cells. Each point represents the mean of a minimum of 2 experiments, and each was carried out with triplicate cultures. Bars, SD.
tence of a high degree of synergism. However, this value is 2.1-fold greater than the CI_{50} for TAM/DPD in the parent cells, indicating that the extent of synergy in the DPD-resistant cells was substantially reduced.

Effect of Sensitivity to TAM on the Magnitude of Synergy. TAM-resistant 289/TAM(6) cells were developed by serially passaging T-289 cells in the continuous presence of tamoxifen. The IC_{50} of the T-289 cells was 1.09 ± 0.21 (SD) μM, whereas the IC_{50} of the 289/TAM(6) cells was 9.09 ± 0.61 (SD) μM, indicating that the latter were 8.3-fold resistant to TAM when tested in clonogenic assays. Median effect analysis of the DDP/TAM combination in the 289/TAM(6) cells yielded antagonism (Fig. 1). The CI_{50} value of 1.39 ± 0.20 (SD) was 5.3 times greater than the CI_{50} for DDP/TAM in the parent T-289 cell line. Thus selection for TAM resistance completely obliterated synergistic interaction, and the DDP/TAM combination was actually antagonistic in the T-289/TAM(6) cells.

As previously reported, the Brown cell line is a human melanoma cell line that is 4.6-fold resistant to TAM (IC_{50} = 5.02 μM) as compared to the T-289 cells. This intrinsic resistance was slightly less than that of the T-289/TAM(6) cells. Despite this somewhat lower level of resistance to TAM, median effect analysis of the TAM/DPD combination yielded a CI_{50} value of 1.3, which was similar to that of the 289/TAM(6) cells. This value is consistent with antagonism. These data provide further support to the hypothesis that the biochemical or molecular determinants of sensitivity to the cytotoxic effect of TAM also play a decisive role in mediating the synergistic interaction between DDP and TAM in melanoma cells.

Role of the Estrogen Receptor in Mediating Synergy. TAM is an ER antagonist, and several investigators have demonstrated the presence of ER in melanoma cells (11). If the effect of TAM on sensitivity to DDP was being mediated via the ER, one would expect that the T-289 cells would contain significant amounts of this receptor and that other ER antagonists would also demonstrate synergy with DDP. However, when the ER content was measured by the dextran-coated charcoal method on T-289 xenografts, it was found to be negligible. The ER content was <3 fmol/mg protein, while the progesterone receptor content was <5 fmol/mg protein. Such levels are characteristic of estrogen-nonresponsive tumors (13). Nevertheless, Northern blot analysis using a full-length ER complementary DNA (14) probe did demonstrate low-level expression of ER mRNA in the T-289 cells grown in tissue culture. Thus an additional approach was needed to exclude a role for the ER.

Droloxifene is a TAM analogue that binds to and blocks the ER even more avidly than TAM and is less estrogenic (15). Thus, if the synergy between TAM and DDP were mediated via the ER, one would expect that droloxifene would also be synergistic with DDP. The IC_{50} for droloxifene was 2.74 ± 0.19 (SD), which is 2.5-fold higher than the IC_{50} for TAM. Fig. 2 shows plots of the CI as a function of the extent of cell kill for the interaction between TAM and DDP and droloxifene and DDP. In the case of the TAM/DPD interaction, all values of CI are less than 1, and the CI_{50} was 0.26 ± 0.02 (SD), indicating a high degree of synergy. Fig. 3 shows the CI plot for the interaction between droloxifene and DDP, indicating that the interaction was additive rather than synergistic. To further explore whether TAM was involved in DDP/TAM synergy, we determined the effect of TAM on CAM content in both the parental T-289 and the chronically TAM-selected 289/TAM(6) cell lines. T-289 cells were exposed to 1.0 μM TAM for 48 h, and CAM content was quantitated by immunohistochemistry and flow cytometry using an antibody specific for CAM. Fig. 4 shows that TAM exposure had no effect on CAM content in T-289 cells. Chronic TAM selection, however, decreased CAM content in 289/TAM(6) cells by more than 20% (P < 0.007).

Role of Protein Kinase C in Mediating Synergy. TAM has been reported by several investigators to inhibit PKC activity (7, 8). If PKC inhibition is the mechanism by which TAM sensitizes cells to DDP, activation of PKC would be expected to have the opposite effect, that is to increase resistance to DDP. To determine the effect of PKC activation on DDP cytotoxicity, we treated T-289 cells with TPA, a known PKC activator, for 1 h concurrently with DDP and assessed the interaction by median effect analysis. Fig. 5 shows that TPA and DDP were synergistic with a CI_{50} of 0.48 ± 0.10 (SD), contradicting the hypothesis that PKC inhibition might be the mechanism by which TAM enhances DDP cytotoxicity.

Cell Cycle Phase Distribution. Previous work demonstrated that TAM was still synergistic with DDP even when applied up to 48 h after a 1-h DDP exposure, indicating that TAM was affecting events occurring late in the cellular response to DNA damage (1). Exposure of mammalian cells to DDP produces cell cycle arrest, predominantly in G2. Interference with the functioning of the G2 checkpoint sensitizes cells to DDP (17). To determine whether TAM was altering the ability of cells to arrest in G2, T-289 cells were exposed to DDP and TAM on the schedule on which synergy was observed by median effect analysis, and the cell cycle phase distribution of the cells as a function of time was analyzed by flow cytometry. Fig. 6a shows the changes that occurred in the cell cycle phase distribution over a 72-h
TAMOXIFEN MODULATION OF CISPLATIN SENSITIVITY IN MELANOMA

Fig. 3. Combination index plot for the interaction between W-7 and DDP in T-289 melanoma cells. Each point represents the mean of 2 experiments. Bars, SD.

Fig. 4. CAM content of T-289 exposed to 1.0 μM TAM for 48 h (left) and T-289/TAM(6) cells grown chronically in TAM (right). Data are expressed relative to the CAM content of untreated T-289 cells. Each histogram represents the mean of 3 experiments. Bars, SD.

Fig. 5. Combination index plots for the interaction between TPA and DDP in T-289 melanoma cells. Each point represents the mean of 3 experiments. Bars, SD.

DISCUSSION

Our clinical studies have demonstrated that TAM is an important component of the combination chemo/hormonal therapy regimen consisting of TAM, DDP, carmustine, and dacarbazine for the treatment of patients with metastatic melanoma (1, 2). In in vitro studies, we were able to demonstrate that there was a marked synergism between TAM and DDP in the human melanoma cell line T-289 (3). In an attempt to understand the nature of this interaction, we first explored the effect of TAM on those factors reported to determine sensitivity to DDP. However, TAM failed to alter DDP uptake, DNA adduct formation or repair, glutathione, or metallothionein levels (3). Furthermore, we demonstrated antagonism between TAM and the other agents in the regimen, suggesting that it was the synergy between TAM and DDP that was responsible for the clinical success of this regimen.

The results reported here indicate that, despite the fact that there was no identifiable effect of TAM on the biochemical pharmacology of DDP in T-289 cells, the determinants of the DDP sensitivity of these cells are also determinants of the extent of the synergistic interaction. In the parental T-289 cells the CI50 was 0.26, whereas it was 0.54 in the 3-fold DDP-resistant T-289/DDP(3) cells, reflecting a 2.4-fold less extensive synergistic interaction as assessed by median effect analysis. Nevertheless, a CI50 of 0.54 still indicates a very significant synergistic interaction, permitting the conclusion that as low-level DDP resistance evolves it reduces but does not obliterate the cytotoxic synergy. It is important to note, however, that even this low-level DDP resistance may be of clinical significance. The in vitro data predict that higher plasma levels of both DDP and TAM would be required to achieve synergy. Specifically, plasma levels of DDP greater than 3.5 μM and TAM levels of greater than 1 μM are required for 50% cell kill.

In contrast to the situation with DDP resistance, the evolution of low-level TAM resistance did completely eliminate the synergistic interaction. Median effect analysis demonstrated no synergy between TAM and DDP in the T-289/TAM(6) cell line, and the importance of TAM sensitivity was confirmed using the Brown melanoma cell line, which also demonstrated intrinsic low-level resistance to TAM. In the latter cell line, no synergy was observed between TAM and DDP by median effect analysis, and in fact for both TAM-resistant cell lines there was a small degree of antagonism. Thus the determinants of cellular sensitivity to the cytotoxic effect of TAM have a major effect on the extent of synergy between TAM and DDP.

The mechanisms by which cells become resistant to the antiproliferative effect of TAM are not well defined. Changes that have been reported to be associated with the in vitro or in vivo development of resistance include loss of ER, failure of the autocrine inhibitory functions of transforming growth factor β1, and altered pharmacokinetics.
TAMOXIFEN MODULATION OF CISPLATIN SENSITIVITY IN MELANOMA

Fig. 6. Effect of TAM on the perturbation of the cell cycle phase distribution of T-289 melanoma cells produced by DDP as a function of time. A, DDP alone; B, TAM alone; C, TAM and DDP in combination.

of TAM (18–21). In addition to its well-recognized ability to antagonize the effect of estrogen at the ER, TAM has also been reported to inhibit CAM function (6, 16) and PKC activity (7, 22), although whether these inhibitory effects change during the evolution of TAM resistance is unknown.

Although in estrogen-dependent tissues blockade of the ER is presently accepted as the major mechanism of the antiproliferative effect of TAM (18), we were unable to identify a role for the ER in the synergistic interaction between DDP and TAM. Although ER has been detected in some melanomas, using a standard dextran-charcoal assay, as well as an enzyme-linked immunosorbent assay, we were unable to demonstrate the presence of ER in T-289 xenografts. Nevertheless, Northern blot analysis showed that the T-289 cells expressed low levels of estrogen receptor mRNA. The importance of this low-level ER expression remains to be determined. In the clinical situation, ER activity as demonstrated by the dextran-coated charcoal technique is closely correlated with the clinical ability of TAM to inhibit tumor growth (18). However, since T-289 cells were not completely devoid of ER as detected by Northern blot, we could not, on the basis of ER content alone, completely eliminate the possibility that the ER was involved in the synergy. Nevertheless, the results of the experiments with droloxifene provide further evidence that it was not. Despite the fact that droloxifene is an even more avid inhibitor of the ER than TAM, it was antagonistic of rather than synergistic with DDP.

If inhibition of CAM function were essential to the synergy between DDP and TAM, then one would expect that other CAM antagonists would also be synergistic with DDP. We selected W7, an extensively used and potent inhibitor of CAM activity, to test this hypothesis. Median effect analysis of the interaction between DDP and W7 yielded a CI50 of 1.00 ± 0.01 (SD), indicating additivity only, favoring rejection of the hypothesis. To further explore whether TAM could be modulating CAM levels independently of activity, we measured the effect of TAM on CAM content in the T-289 TAM-sensitive and -resistant cells. Following a 48-h exposure to 0.5 and 1 μM TAM, the CAM content of the T-289 cells was not different from that of untreated controls. A small but statistically significant suppressive effect of TAM was observed in the T-289/TAM(6) cells, the significance of which is unknown.

The role of PKC inhibition in the synergistic interaction was approached indirectly in these studies because of the difficulty in identifying alternative PKC inhibitors that do not also have effects on other biochemical processes in the cell. If the ability of TAM to inhibit PKC is central to the synergy, then one would expect an increase in PKC activity to antagonize the synergistic interaction. However, activation of PKC with TPA, rather than being antagonistic, was also highly synergistic, with a CI50 of 0.48. This result obtained in the T-289 cells is consistent with high degrees of synergy between PKC activators and DDP in human ovarian carcinoma and HeLa cell lines (23, 24). This result suggests that PKC inhibition is not an important component of the synergy between TAM and DDP, but our study has not addressed the possible differential role of the various isoforms of PKC, and further studies are needed to confidently exclude a role for PKC.

The integrity of the G2 checkpoint in cell cycle regulation influences cellular sensitivity to DNA-damaging drugs including DDP, and pharmacological suppression of G2 arrest enhances DDP cytotoxicity (17). We have shown that DDP alters cell cycle phase distribution and induces both an early S-phase and a later G2-phase arrest in the T-289 cell line. It was hypothesized that TAM might increase the cytotoxicity of DDP by perturbing these cell cycle effects. However, TAM had no effect on either the extent or duration of growth arrest in T-289 cells, arguing that it is not by altering cell cycle control systems that TAM is exerting its synergistic effect on DDP sensitivity.

Recently Coconni et al. (25) have published clinical data that suggest that there may be an important interaction between TAM and DTIC. In a prospective randomized clinical trial they compared the response rate and survival of patients with metastatic melanoma treated with DTIC ± TAM and reported an improvement in both the overall response rate and survival for the combination. We have specifically evaluated the ability of TAM to synergize with an active metabolite of DTIC (5-(methyltrazeno)imidazole-4-carboxamide) in the T-289 melanoma cell line (3). Our data suggest that, if anything, there was low-level antagonism with the combination of TAM and
DTIC. One possible explanation for this discrepancy is the fact that the response rate for the group of patients treated with DTIC alone in the Coconni study was much lower than that reported in the world literature (12% versus 21%) (26). If the response rate in the Coconni study for the patients treated with single-agent DTIC was 20% or greater then the statistical advantage for the combination would not have been observed. Therefore, it is possible that the positive clinical results were the result of small patient numbers and that there was no true interaction between TAM and DTIC in these patients. It is also important to note that our data examined this interaction in only one melanoma cell line and that synergy may be observed in other melanoma cell lines.

In summary, our data have clearly demonstrated that TAM and DDP are synergistic in the human melanoma cell line T-289. The magnitude of the synergistic interaction is influenced to some extent by determinants of DDP sensitivity but very strongly by determinants of TAM sensitivity. The currently available evidence argues that synergy was not mediated by effects of TAM on the ER, CAM activity or level, PKC activity, or cell cycle phase distribution perturbations produced by DDP. The results reported here serve to focus particular attention on those factors that determine cellular sensitivity to TAM as being important to the synergy between DDP and TAM.

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