Geldanamycin and its 17-Allylamino-17-Demethoxy Analogue Antagonize the Action of Cisplatin in Human Colon Adenocarcinoma Cells: Differential Caspase Activation as a Basis for Interaction

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ABSTRACT

Several chaperone-binding drugs based on geldanamycin (GA) have been synthesized, and one of them, 17-allylamo-17-demethoxygeldanamycin (17-AAG), is being developed in the clinic. Interest in the use of 17-AAG in combination with cytotoxic drugs led us to study both GA and 17-AAG with cisplatin (DDP) in the human colon adenocarcinoma cell lines HCT116. We performed isobologram analysis of combinations of DDP with GA or 17-AAG in these cell lines using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to evaluate cell survival. In HCT116, the effects of GA and 17-AAG with DDP were additive and schedule dependent. In HT29 both GA and 17-AAG antagonized DDP effects resulting in cytotoxicity less than expected. We hypothesized that the antagonism in HT29 cells might be a consequence of altered p53 function in this cell line. Accordingly, we tested GA/17-AAG and DDP in combination in the HCTp5.2 cell line, which expresses a dominant-negative form of p53. In these cells too, the GA analogues antagonized DDP, suggesting a role for p53 in the observed effects. Investigation of the DDP-induced signaling pathways revealed that ansamycins block the activation of mitogen-activated protein kinase and c-Jun NH2-terminal kinase pathways and c-Jun expression in HT29 cells while exerting incomplete inhibitory effects in HCT116 and HCTp5.2 cell lines. Therefore, effects on signaling are thought not to underlay the antagonism in the latter model. The ansamycins inhibited DDP-induced activation of caspases 8 and 3 in HT29 and HCTp5.2 but not in HCT116 cells, which we postulate to be the basis for higher survival of p53-deficient cells when treated with combinations of the two drugs.

INTRODUCTION

Specific inhibitors targeted to a variety of signal transduction pathways are in clinical development as potential anticancer agents (1, 2). Several have activity as single agents, but studies in animal models suggest that most will be considerably more active in combination with chemotherapeutics (3). Among the inhibitors in clinical development are agents targeted to the molecular chaperone Hsp 90 (4, 5). The benzoquinone ansamycins, herbimycin A and GA, were first described as inhibitors of tyrosine kinases and were shown to reverse cell transformation by oncogenic kinases such as Src, Abl, and ErbB (6). Later, it was shown that ansamycins do not affect kinases directly but instead serve as inhibitors of Hsp 90 (7), a chaperoning protein responsible for proper protein folding and an important participant in a variety of cellular processes (8). GA acts by blocking the binding of ATP to Hsp 90 (9), which leads to destabilization of Hsp 90 complexes with its client proteins rendering them available for proteasomal degradation (10, 11). The ability of GA to deplete Raf1, ErbB2, and mutant p53 in breast cancer cells was found to correlate with its antiproliferative activity (12), making it a plausible candidate for use in cancer treatment. However, GA caused liver toxicity in preclinical studies (13). A search for more tolerable derivatives has yielded 17-AAG, a less toxic analogue that retains the tumoroidal features of GA. Like its parent compound, 17-AAG inhibits several signaling pathways through binding to Hsp 90, which results in destabilization of signaling complexes and degradation of its client proteins by the proteasome in a variety of cell lines (14–16). Treatment with 17-AAG has been shown to inhibit tumor growth and induce apoptosis in colon cancer, glioblastoma, and breast cancer cell lines (16–18). These studies led to the active clinical development of 17-AAG as a potential anticancer drug (19, 20).

Recent work suggests that 17-AAG enhances the cytotoxic effects of paclitaxel in non-small cell lung cancer cell lines and xenografts (21) and of Taxol and doxorubicin in breast cancer cell lines (22), making it a promising candidate for combination treatment of solid tumors. Doxorubicin exerts its cytotoxicity by causing DNA strand breaks as a consequence of topoisomerase II inhibition (23). Enhancement of its action by ansamycins prompted us to evaluate the effects of GA and 17-AAG in colon tumor cell lines when used in combination with another DNA-damaging drug, DDP [cis-diaminedichloroplatinum (II)], which exhibits a broad spectrum of activity against several tumor types and is routinely used for the treatment of solid tumors (24, 25). DDP treatment leads to formation of DNA platinum adducts, DNA damage, cell cycle arrest, and cell death, which occurs primarily by apoptosis, although under certain circumstances DDP treatment can cause death by necrosis (26, 27). DDP treatment activates caspase cascades regulated both by cellular receptors such as Fas/CD95 (28) and mitochondrial release of cytochrome c (29). Cell lines expressing either wild-type or mutated p53 protein were shown to demonstrate growth arrest and were able to undergo apoptosis after DDP exposure (30, 31). DDP also activates signaling pathways resulting in the induction of MAPKs. The roles of MAPK, JNK, and p38 kinase in mediating DDP effects are under active investigation, and there are numerous conflicting reports concerning their input in both enhancement and attenuation of DDP cytotoxicity (32–36). These mechanisms may also be critical to an interaction with ansamycins because we have previously shown that GA was able to inhibit signaling pathways using Hsp 90 and its analogues as chaperones (37).

Our results show that when added simultaneously, DDP and GA/17-AAG have an additive effect in HCT116 colon cancer cell line but are antagonistic in HT29 cells. Our initial hypothesis that these phenotypes reflect altered p53 status in these cells was supported by antagonism of DDP and ansamycins in the HCT-116-derived cell line, HCTp5.2, expressing dominant-negative form of p53. Additional dissection on the basis of observed antagonism indicated that it results from inhibition of DDP-induced Fas-mediated apoptosis in this cell line.
MATERIALS AND METHODS

Cell Lines and Reagents. Colon cancer cell lines HT29 (functionally p53-deficient, mismatch repair proficient) and HCT116 (opposite characteristics) were purchased from American Type Culture Collection (Manassas, VA). Cells were cultivated in Eagle’s MEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 units/ml), and fungizone (Life Technologies, Inc., Grand Island, NY). The HCTp5.2 cell line was generated by transfection with pcDNA3 plasmid expressing a dominant-negative form of p53 (gift from Dr. Wafik El-Deiry, University of Pennsylvania, Philadelphia, PA) followed by isolation of stable transfectant. Cultures were maintained in a humidified incubator at 37°C in 5% CO2–95% air. GA was purchased from Life Technologies, Inc., and 17-AAG was kindly provided by Dr. Edward Sausville (DTP; National Cancer Institute, Bethesda, MD). Stock solutions of ansamycins (1 mM) were prepared in DMSO, aliquoted, and stored at −20°C. DDP (Sigma Chemical Co., St. Louis, MO) was dissolved in sterile PBS at a final concentration of 1 mg/ml (3.33 mM).

MTT Assay and Isobologram Analysis. HT29, HCT116, and HCTp5.2 cells were plated in 96-well plates at a density of 2000 cells/well in 140 μl of medium 20–24 h before addition of drugs. DDP was then added in concentrations of 0, 1, 3, 6, 8, and 10 μM from left to the right side of the plate (over two plates, each concentration of DDP in quadruplicate). GA or 17-AAG was added cross-wise in concentrations of 0–50 and 0–75 nm, respectively. Cytotoxicity was measured using the standard MTT assay after a 72-h exposure to drugs (38). In sequential experiments, cells were treated totally for 96 h, with the second compound in combination introduced with a 24-h delay. Results were quantified using ELx800 Universal Microplate Reader (Bio-Tek Instruments, Inc.) at 595 nm wavelength, and control absorbance was designated as 100%, with percentage of it as a cell survival. MTT results were analyzed as described by Tsai et al. (39). Briefly, after each experiment, survival curves were generated, two for DDP and GA or 17-AAG alone and 13 for the 42 pairs of drug combinations. The IC50 for each drug in combination were determined, and IC50 units were derived as ratio of IC50 for DDP and GA/17-AAG in this particular drug combination relative to IC50 of drug alone (this value was designated as 1) for each cell line. Isobolograms were generated at IC50 levels. Each plot represents values generated in at least three independent experiments for simultaneous treatment of cells and two experiments for sequential drug addition. The method of Chou et al. (40) was also used to determine CIs for drugs.

Cell Treatment, Protein Extracts Preparation, and Western Blotting. Cells were treated for 24 h with 0.01% DMSO (control), 500 nm GA or 17-AAG alone, 30 μM DDP alone, and with combinations of DDP with GA or 17-AAG. Protein extracts were prepared as described previously (37). Extracts were diluted with lysis buffer to obtain equal protein concentration, aliquoted, and stored at −70°C. Samples for electrophoresis were prepared, separated in discontinuous SDS gels, and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Western blotting was carried out according to the manufacturer recommendations, using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). The following antibodies were used: mouse monoclonal antibodies against p21, p53, phospho-ERK, phospho-c-Jun, and phospho-activating transcription factor-2 and rabbit polyclonal antibodies raised against ERK, JNK, and p38 from Santa Cruz Biotechnology; rabbit polyclonal antibodies against phosphorylated forms of p53, JNK, and p38 from Cell Signaling Technology (Beverly, MA); and goat polyclonal antibodies raised against β-actin (Santa Cruz Biotechnology). Mouse monoclonal antibodies against caspase 8 were purchased from Oncogene Research Products (Boston, MA).

Caspase Activity Assay. ApoTarget caspase colorimetric protease assay kits were purchased from BioSource International, Inc. (Camarillo, CA). Cells were plated in 10-cm Petri dishes and subjected to drug treatment as described above. After 20 h, cellular extracts were isolated, and assays were carried out according to manufacturer recommendations. Synthetic peptides conjugated with chromophore (p-nitroaniline) served as substrates for measuring the activity of caspases, which subsequently was quantified by measuring the absorbance of light by free chromophore using microtiter plate reader at 405 nm. Comparison of the absorbance of p-nitroaniline from apoptotic sample with an untreated control allowed determination of the fold increase in caspase activity. All experiments were performed at least twice.

RESULTS

DDP and GA/17-AAG Exhibit Additive Effects in H116 and Antagonize Each other in HT29 Cells. Treatment of colon cancer cell lines with DDP, GA, and its analogue, 17-AAG, results in dose-dependent cytotoxicity. Fig. 1 demonstrates survival curves of HT29 and HCT116 cell lines treated with DDP, GA, and 17-AAG with IC50 ranging from 8.8 to 4.5 μM for DDP, 6.8 to 12 nm for GA, and from 18 to 50 nm for 17-AAG, respectively. To evaluate the impact of the combined treatment with DDP and GA/17-AAG on human colon adenocarcinoma cell lines, HT29 and HCT116 cells were treated with various concentrations of drugs alone and in combination followed by survival assessment (MTT assay) and isobologram analysis. We used the method described by Tsai et al. (39) to create isobolograms at the IC50 level: IC50 unit values of GA or 17-AAG < 1 were plotted against corresponding IC50 unit values of DDP. The distribution of dots along the line connecting values of 1 constitutes an additive effect of two drugs while scattering below or above represents synergism and antagonism, respectively. As Fig. 2 demonstrates, the effect of GA/17-AAG and DDP on cell survival is
additive in HCT116 cells, whereas in the HT29 cell line, these drugs exhibit antagonism. In addition, CIs at the IC₅₀ of DDP and of GA/17-AAG were calculated by the method of Chou et al. (40) for all experiments performed. The CI values < 0.9 or > 1.1 reflect synergistic or antagonistic interaction of drugs, respectively. Results of all experiments performed are summarized in Table 1.

Because the importance of the schedule of drug administration was demonstrated for the combination of Taxol and 17-AAG (22) in breast cancer cell lines, we examined if sequential addition of DDP and ansamycins to HCT116 and HT29 cells would change the outcome observed with simultaneous treatment. Experiments with the second drug in combination added with a 24-h delay were carried out, and the results analyzed as described above. The effects of GA and 17-AAG antagonized those of DDP in both cell lines irrespective of the order in which drugs were added (Table 1).

Stable Expression of Dominant-negative p53 in HCT116 Recapitulates the Antagonistic Phenotype of Combinations of DDP with Ansamycins. The importance of p53 in cellular responses to DNA damage is well established (41). Because HT29 and HCT116 differ in p53 status, we hypothesized that loss of p53 function in HT29 could be the basis of the observed antagonism between DDP and ansamycins. Using HCT116 as the parental line, we generated HCTp5.2, a cell line stably expressing a dominant-negative form of p53 for DDP cytotoxicity in this model. Isobologram analysis revealed partial recapitulation of antagonism between ansamycins and DDP in HCTp5.2 cell line (Fig. 3B), with CIs higher than these in HCT116 cells (Table 1).

Ansamycins Exert Distinct Effects on DDP Signaling through MAPK Pathways in HT29 and HCT116 Cells. DDP has been shown to induce MAPK-signaling pathways in various cell lines, whereas ansamycins, on the other hand, are known inhibitors of signaling. We investigated the effect of GA and 17-AAG on DDP-induced signaling through MAPKs in HT29, HCT116, and HCTp5.2 cell lines. Fig. 4 demonstrates that in HT29 cells, ansamycins block DDP-induced activation of ERK and JNK, causing profound inhibition of phosphorylation of activating transcription factor-2 and c-Jun and blockade of c-Jun synthesis. In HCT116 and HCTp5.2, the effects of GA and 17-AAG antagonized those of DDP in both cell lines irrespective of the order in which drugs were added (Table 1).

Table 1. Effect of combination of GA/17-AAG and cisplatin in colon cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>DDP + GA</th>
<th>DDP + 17-AAG</th>
<th>GA → DDP</th>
<th>17-AAG → DDP</th>
<th>DDP → GA</th>
<th>DDP → 17-AAG</th>
</tr>
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<tbody>
<tr>
<td>HT29</td>
<td>1.38 ± 0.22</td>
<td>1.19 ± 0.09</td>
<td>1.24 ± 0.22</td>
<td>1.25 ± 0.1</td>
<td>1.14 ± 0.1</td>
<td>1.24 ± 0.09</td>
</tr>
<tr>
<td>HCT116</td>
<td>0.98 ± 0.02</td>
<td>1.02 ± 0.07</td>
<td>1.18 ± 0.14</td>
<td>1.26 ± 0.19</td>
<td>1.12 ± 0.06</td>
<td>1.14 ± 0.09</td>
</tr>
<tr>
<td>HCTp5.2</td>
<td>1.21 ± 0.06</td>
<td>1.09 ± 0.06</td>
<td>N/A*</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* Combinatorial indices at IC₅₀ for colon cancer cell lines treated with DDP and GA/17-AAG are shown; values represent results from at least three independent experiments.

* N/A, not available.
of ansamycins on signaling pathways are less pronounced, resulting in partial inhibition, the degree of which seems to be independent of p53 function. Thus, the antagonism of DDP and ansamycins in HCTp5.2 cannot be attributed to altered DDP signaling through MAPK pathways in this cell line.

**Higher Survival of HCTp5.2 Cells Treated with DDP Is because of Abrogated Apoptosis.** One of the reasons for the greater survival of HCTp5.2 treated with DDP and ansamycins could be the abrogation of DDP-induced p53-dependent apoptosis in this cell line. The apoptotic DNA ladder analysis (Fig. 5A) confirmed that assumption. In addition, it was supported by the observations of cell morphology after treatment with high concentrations of DDP and 17-AAG alone and in combination for 24 h, whereas HCTp5.2 cells did not exhibit significant visible changes, HCT116 cells demonstrated such characteristic features of cell death as cell shrinkage, membrane blebbing, and formation of apoptotic bodies. We were unable, however, to detect any significant difference in contents neither of several Bcl-2 family members, nor AKT in HCT116 and HCTp5.2 cells (data not shown). However, in accordance with the loss of p53 transcriptional activation function in HCTp5.2, induction of Fas by DDP was greatly reduced (Fig. 5B) in this cell line. The increase of Fas expression in DDP-treated HCT116 was not affected by the presence of ansamycins and was accompanied by higher degree of procaspase 8 processing than that in HCTp5.2 cells (Fig. 5B). Taken together, these data initially localized the effect of the drug combination in HCTp5.2 cells to the Fas-mediated apoptotic pathway. It was confirmed by measuring the activation of caspases in HT29, HCT116, and HCTp5.2 cells lines after combined treatment with DDP and 17-AAG. The results revealed marked differences in the activation of caspases: the presence of 17-AAG inhibited DDP-induced activation of caspase 8 in HT29 cells and, to a lesser degree, in the HCTp5.2 cell line, whereas the HCT116 cell line demonstrated enhanced activation (Fig. 6A). The same dynamic was observed for caspase 3 (Fig. 6B). These results confirmed our conclusion that the loss of functional p53 protein in HCTp5.2 cell line was associated with inhibition of DDP-induced apoptosis in the presence of ansamycins.

**DISCUSSION**

The aim of this study was to investigate the effects of combined treatment of colon cancer cell lines with GA/17-AAG and DDP. Because among the known effects of ansamycins are inhibition of signaling through MAPK pathways, we also wished to determine its role in the interaction of these Hsp 90 inhibitors and DDP, the cytotoxicity of which depends upon DNA damage-induced signaling (42, 43). We observed additivity between ansamycins and DDP in HCT116 colon cancer cells but were surprised that this combination was antagonistic in HT29 cells. The demonstration of antagonism led to an investigation of its cause.

We show that in HT29 cell line activation of JNK associated with DDP-induced apoptosis was blocked by ansamycins, leading to complete inhibition of c-Jun expression. The depletion of this major component of the activator protein 1 transcription factor makes the effect of ansamycins on DDP signaling in HT29 cells even more profound, expanding it to a multitude of not only JNK targets but also to activator protein 1-regulated genes, known to participate in cell growth, proliferation, and death pathways (44). Because p53 function is absent in this cell line, there is a possibility that signaling inhibition may contribute to the antagonism but only through p53-independent mechanisms, which we are currently investigating. In the HCT116 line, MAPK signaling was less affected by ansamycins, which together with the presence of functional p53 could contribute to additive effects of drugs in this cell line; this assumption was confirmed by occurrence of the antagonistic phenotype in the HCT116-derived HCTp5.2 cells, where it was accompanied by the attenuation of...
apoptosis. It was critical to explore this interaction further if one is to use such combinations in clinical trials.

The significance of scheduling in treatment of cells with Taxol and 17-AAG was shown previously: pretreatment with 17-AAG abrogated Taxol-induced apoptosis (22, 45). In our experiments, alternative scheduling not only did not affect antagonism of ansamycins and DDP in HT29 cells but led to the antagonism of these drugs in the HCT116 cell line as well. Because the effects of ansamycins (when added first) on cell cycle distribution and multiple signaling pathways occurred before DDP addition, it could contribute to the observed antagonism. Recently the binding of DDP to Hsp 90 leading to conformational change was described (46). The consequence of this phenomenon are not completely understood, but it is possible to speculate that they could have a part in antagonism of DDP and ansamycins when DDP was introduced first in scheduling experiments.

The cytotoxicity of DDP is the result of its binding to DNA, followed by formation of intra- and interstrand DNA adducts, which are poorly repaired in human cells. The formation of DNA adducts consequently leads to inhibition of transcription, cell cycle arrest, and apoptosis (49). The tumoricidal effects of ansamycins used alone have consequently leads to inhibition of transcription, cell cycle arrest, and apoptosis (49). The tumoricidal effects of ansamycins used alone have

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