Manumycin Enhances the Cytotoxic Effect of Paclitaxel on Anaplastic Thyroid Carcinoma Cells

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ABSTRACT

Despite the current multimodal approach to treatment of anaplastic thyroid cancer (ATC), the prognosis for patients with the disease is poor. New effective therapy for ATC is desperately needed. Thus, we investigated the effects of manumycin (a farnesyl:protein transferase inhibitor), alone and in combination with other drugs frequently used to treat ATC, in six human ATC cell lines: ARO, C643, DRO, Hth-74, KAT-4, and KAT-18. By means of a formazan dye-based spectrophotometric assay of cell viability and light microscopy, manumycin was shown to decrease the number of viable cells in all six of the cell lines though to a lesser degree in DRO and C643 cells than in ARO, Hth-74, KAT-4, and KAT-18 cells. In combination, manumycin enhanced the effect of paclitaxel in all six of the cell lines. The mechanism of cell death was investigated by measuring caspase-3 activity, immunoblottting with anti-poly-(ADP-ribose)polymerase (PARP) antibody and electrophoresis of DNA. After an 18-h incubation, manumycin plus paclitaxel caused enhanced activation of caspase-3 activity, cleavage of PARP into M, 89,000 and 28,000, fragments, and internucleosomal fragmentation of DNA (all of which are characteristic of apoptotic cell death). In contrast, neither manumycin alone, paclitaxel alone, doxorubicin alone, nor doxorubicin plus manumycin produced significant specific cleavage of PARP and internucleosomal DNA fragmentation after 18 h of incubation. The in vitro effect and toxicity of combined manumycin and paclitaxel treatments were evaluated in a nude mouse xenograft model using ARO and KAT-4 cells. Drugs were injected i.p. on days 1 and 3 of a 7-day cycle for three cycles. Both manumycin (7.5 mg/kg/dose) and paclitaxel (20 mg/kg/dose) had significant inhibitory effects on tumor growth. Combined manumycin and paclitaxel treatments seemed as effective as manumycin against ARO cells and more effective than either manumycin or paclitaxel alone against KAT-4 cells. No significant morbidity or mortality was caused by the treatments. In conclusion, manumycin can inhibit the growth of ATC both in vitro and in vivo. Manumycin plus paclitaxel has enhanced cytotoxic effects and increased apoptotic cell death in ATC cells in vitro compared with either drug by itself. The combination of manumycin and paclitaxel is also effective in vivo with no significant toxicity observed. The lack of synergy observed in this in vitro experiment may be due to a ceiling effect, and further experimentation is warranted to ascertain the optimal way to combine these two agents for maximal therapeutic effects.

INTRODUCTION

ATC is one of the most aggressive solid tumors, and patients with ATC have a poor prognosis with a mean survival time of 2–6 months. Surgery, radiotherapy, and chemotherapy do not meaningfully improve survival (1–3). Current chemotherapy for ATC is based primarily on doxorubicin (4, 5) and cisplatin (5). The use of paclitaxel is under clinical investigation (6). Consequently, there is a desperate need for new effective therapeutic modalities.

One possibility is the use of a group of compounds with potential cancer therapeutic effect called FPT inhibitors (7, 8). These compounds were originally developed with the intention of blocking ras oncogene function. Ras, the protein product of the ras proto-oncogenes, is synthesized as a cytosolic precursor, and it requires post-translational modification by conjugation of a farnesyl (15-carbon isoprenyl group) moiety to the COOH-terminal region. After farnesylation, Ras is localized to the inner surface of the cell membrane, in which it becomes functional in transducing the mitogenic signals of tyrosine kinase receptors.

There are many isoprenylated proteins other than Ras. Each isoprenylated protein has the characteristic COOH-terminal sequence of CAAX, XXCC, or XCXC, in which C is cysteine, A is any aliphatic amino acid, and X is any other amino acid (9–11). Four posttranslational processing steps have been described for the CAAX sequence: (a) addition of either a 15-carbon farnesyl group or a 20-carbon geranylgeranyl group to the cysteine residue; (b) proteolytic cleavage of the AAX peptide; (c) carboxymethylation of the farnesylcysteine; and (d) except for K-Ras, palmitoylation of cysteine residues located upstream of the CAAX motif. Proteins with the XXCC or XCXC motif are modified by geranylgeranylation and do not require an endopeptidase step. K-Ras and N-Ras can be alternatively geranylgeranylated when farnesylation is inhibited (12, 13), but nonfarnesylated oncogenic H-Ras can exert a dominant negative effect and, therefore, inhibit the function of membrane-bound Ras in some circumstances. Thus, although inhibition of FPT would be expected to affect only H-Ras, the dominant negative effect would inhibit the Ras-transforming pathway. Because wild-type Ras does not display the dominant negative phenotype, the observed inhibition would be selective for tumor cells. Interestingly, soluble complexes of nonfarnesylated oncogenic H-Ras and Raf can be isolated from cells, thus supporting the concept that a dominant negative Ras may sequester Ras effector proteins and prevent them from interacting with membrane-associated Ras.

FPT inhibitors abolish the function of Ras and block the mitogenic action of ras oncogenes (9–11). One example is manumycin A, a natural product of Streptomyces, identified by random screening. This inhibitor has competition with farnesyl PP i (FPP) groups as its mechanism of inhibition and has shown antitumor activity in cell culture (14–16) and in nude mouse xenograft models (17).

However, FPT inhibitors may also exert anticancer activity via mechanisms other than blocking the function of ras. For example, in a cell culture study, 31 of 42 cancer cell lines derived from various tumor types and of various oncogenic make-ups (including wild-type ras) were sensitive to a peptidomimetic FPT inhibitor (L-744832; 18). In a study using NIH3T3 cells transfected with various oncogenes, manumycin A exhibited action against cells transformed by non-ras oncogenes (19). The authors of that study also concluded that the antiproliferative effect of manumycin was not necessarily directly related to interference with Ras processing. In addition, more than 10 unidentified isoprenylated proteins are affected by FPT inhibitors (19). Among the known farnesylated CAAX proteins other than Ras,
the G-proteins (for example, rap-1, rab, and rho), lamin A and B, and inositol triphosphate 5-phosphatase type I may have relevance to intracellular signaling and apoptosis. At present, however, the roles of these proteins in the antineoplastic activity of FPT inhibitors are not clear.

To explore the potential application of manumycin A in the therapy of ATC, we investigated the drug’s antiproliferative effect on six human ATC cell lines when used alone and in combination with paclitaxel, cisplatin, and doxorubicin, which are chemotherapeutic drugs frequently used for ATC. The hypothesis that these drug treatments cause apoptosis in ATC cells was tested by assessing the cells for increase in caspase-3 activity, intermembranous DNA fragmentation, and specific cleavage of PARP in drug-treated cells. The potential for therapeutic application was also assessed using a nude mouse xenograft model with particular attention to the possibility of increased toxicity when manumycin and paclitaxel were combined.

MATERIALS AND METHODS

Materials. Manumycin A, cisplatin, doxorubicin, and paclitaxel were purchased from Sigma. Manumycin A, doxorubicin, and paclitaxel were dissolved in DMSO (tissue-culture grade, Sigma) at appropriate concentrations before dilution in tissue culture medium such that the final concentration of DMSO in culture medium would not exceed 0.1% (v/v).

Cell Culture. Six human ATC cell lines were used: ARO, DRO, Kat-4, Kat-18, C643, and Htt-h4. All of the cells were cultured in RPMI 1640 with heat-treated bovine serum (10%), penicillin (50 units/ml), streptomycin (50 μg/ml), MEM nonessential amino acids (1X), pyruvate (1 mM), glutamine (2 mM), and amphotericin (2.5 μg/ml) at 37°C in a water-saturated atmosphere with 5% CO2.

Late log-phase cultures were trypsinized, and 750-1500 cells were plated in each well of a 96-well tissue culture plate. The next morning, medium in each well was replaced with fresh medium or medium containing various concentrations of drugs, and the cells were then incubated for specified periods of time. In experiments that involved protein or DNA isolation, cells were cultured in six-well plates, and experimental treatments were applied when the cells were about 70–80% confluent.

Colorimetric Measurement of Viable Cells. The number of viable cells was measured by a colorimetric technique based on the cleavage of tetrazolium salts added to the culture medium [Cell Proliferation Kit II (WST-1), Boehringer-Mannheim]. In this technique, the tetrazolium salt WST-1 is broken down to the colored product, formazan, by the “succinate-tetrazolium reductase” system of the mitochondrial respiratory chain in viable cells. Thus, the amount of formazan dye, measured colorimetrically as absorbance at wavelength 450 nm (A450 nm) with reference at wavelength 690 nm (A690 nm), directly correlates with the number of metabolically active cells.

During the last h of experimental treatment, 10 μl of WST-1 dye was added to 100 μl of culture medium in each well. After 1 h of incubation at 37°C, A450 nm–A690 nm was measured in each well using a microtiter plate spectrophotometer (Dynex Tech.). All of the experiments were performed with the absorbance values within the linear range of this colorimetric assay. Viability was defined as follows:

\[ \text{Viability} = \left( \frac{A_{\text{experimental}} - A_{\text{background}}}{A_{\text{control}} - A_{\text{background}}} \right) \times 100\% \]

where Aexperimental is the absorbance of the experimental sample, Acontrol is the absorbance of the control sample and Abackground is the absorbance of samples with heat-killed cells.

Colorimetric Assay of Caspase-3 Activity. The CPP32/caspase-3 colorimetric protease assay kit from Chemicon International, Inc. (Temecula, CA) was used. Two × 10^5 cells were plated in each vessel. After attachment to the vessels overnight, the experimental groups were treated with manumycin, paclitaxel, or manumycin plus paclitaxel, and the control group was treated with DMSO 0.1% in culture medium. After cell lysis, caspase-3 activity was measured in each cytosolic extract according to the manufacturer’s protocol using DEVD-pNA as substrate. Absorbance was measured at 405 nm using a microplate spectrophotometer (Dynex Tech.).

SDS-PAGE and Immunoblotting. After experimental treatments, cells floating in the culture medium were pelleted by centrifugation. Cells that had become attached to the well were rinsed with PBS. The cell pellet and the cells attached to the well were lysed in a total of 150 μl of sample buffer [25 mm Tris (pH 6.8), 6 M urea, 2% 2-mercaptoethanol, 1% SDS, 0.002% bromophenol blue, 10 mm EDTA, 1 mm phenylmethylsulfonylfluoride, and Complete Protease Inhibitor Mix (Boehringer-Mannheim, 1 tablet per 50 ml)]. The DNA in the lysate was sheared by rapidly passing the lysate five times through a 23-gauge needle. SDS-PAGE was performed with standard methods. Kaleidoscope prestained standards (Bio-Rad) were used for molecular weight calibration. Immunoblotting (western blotting) was performed using supported nitrocellulose membranes. Blocking was performed in 0.3% Tween 20 (V/V) and 5% nonfat dry milk in PBS. The primary antibody was rabbit polyclonal anti-PARP serum (Boehringer-Mannheim), and the secondary antibody was antirabbit IgG-peroxidase conjugate (Boehringer-Mannheim). Washing was performed with 0.3% Tween 20 (V/V) in PBS. Kodak X-AR film was used to record the image generated by enhanced chemiluminescence using the ECL kit (Amersham).

DNA Isolation and Electrophoresis. The cell samples were processed for observation of DNA fragmentation according to standard methods. Briefly, the cells were trypsinized and detached from the culture well. Both detached cells and cells floating in the medium were pelleted by centrifugation, and the cell pellet was washed with PBS. Then the cell pellet was lysed in 40 μl of buffer containing 0.25% Triton X-100, 10 mm Tris-HCl (pH 7.4), 1 mm EDTA, and 2 mg/ml RNase A. After gentle mixing and incubation at 37°C for 20 min, 5 μl of protease K (20 mg/ml) was added to the sample and further incubated at 37°C until the solution cleared. Then loading buffer was added and the DNA was electrophoresed in a 2% agarose gel at 36 V in a buffer containing 40 mm Tris acetate and 2 mm EDTA. Ethidium bromide-stained DNA was visualized by transillumination with UV light, and the image was recorded by a digital video gel documentation system (Fotodyne Foto/Analyst Visionary gel documentation system).

Nude Mouse Xenograft Model. One × 10^6 human ATC cells suspended in RPMI 1640 were injected s.c. on a flank of each 7-week-old nude mouse (nu/nu BALB-c mice bred at the animal facility of University of Texas M. D. Anderson Cancer Center). The mice were housed in barrier facilities on a 12-h light-dark cycle with food and water available ad libitum. s.c. tumors were measured every 2–3 days with calipers. Tumor volumes were calculated by the formula: \[ a^2 \times b 	imes 0.4 \], where a is the smallest diameter and b is the diameter perpendicular to a. After the tumors reached at least 10 mm^3, the mice were randomly assigned into experimental or control groups.

Drug solutions were injected i.p. on days 1 and 3 of a 7-day cycle for three cycles. The drugs were dissolved in tissue culture grade DMSO before dilution in tissue culture medium. The final concentration of DMSO was 0.1%. Mice in the control group received 2 injections with DMSO 0.1% in tissue culture medium. Mice in the manumycin group received 1 injection with DMSO 0.1% in tissue culture medium and 1 injection with manumycin. Mice in the paclitaxel group received 1 injection with DMSO 0.1% in tissue culture medium and 1 injection with paclitaxel. Mice in the manumycin plus paclitaxel group received 1 injection with manumycin and 1 injection with paclitaxel.

The tumor volume in each animal was calculated every 2 to 3 days. The logarithm of the tumor volume divided by the original tumor volume was calculated and averaged in each group and plotted against time since treatment began. The body weight, feeding behavior and motor activity of each animal were monitored as indicators of general health. At the end of the experiments when the animals were killed, blood samples were collected by intracardiac puncture, and lactate dehydrogenase, aspartate and alanine aminotransferases, alkaline phosphatase, and complete blood count with differential were measured in the veterinary clinical laboratory at University of Texas M. D. Anderson Cancer Center.

Statistical Analysis. The statistical significance of differences between two groups was assessed using Student’s t test with P < 0.05. The statistical significance of interaction between two treatments (i.e., nonadditivity) was assessed using two-way ANOVA. The computer software, SigmaStat for Windows 95 (Version 2.0, Jandel Scientific), was used to facilitate calculations. Results were reported at the 95% confidence level.

The cytotoxic interaction between manumycin and paclitaxel was also
assessed using the median-effect method of Chou and Talalay (20). The method has been described in detail elsewhere (21). Briefly, dose-response curves were obtained for manumycin and paclitaxel, and for multiple dilutions of a fixed-ratio combination of the two drugs. The CI was the ratio of the combination dose to the sum of the single-agent doses at an isoeffective level. Therefore, CI < 1, synergy; CI > 1, antagonism; and CI = 1, additivity.

RESULTS

ATC cells incubated in vitro with manumycin plus paclitaxel had a higher incidence of apoptosis than did cells incubated with either drug alone. It also appeared that manumycin and paclitaxel were effective against ATC in vivo.

Correlation of Viable Cell Number with Absorbance. In experiments done to correlate cell numbers with absorbance obtained by spectrophotometric assay of viable cells and to define the linear range of the assay, the number of viable cells and \( A_{450 \text{ nm}} - A_{690 \text{ nm}} \) formed a tight correlation up to about 50,000 cells per well (data not shown). The number of heat-killed cells per well (killed by incubating at 70°C for 15 min) caused no significant change in the absorbance. Therefore, the spectrophotometric method using WST-1 dye conversion by mitochondrial enzymes was a valid technique for measuring the number of viable cells. All of the experiments performed were within the linear range of the assay.

Effect of Manumycin on Cell Number and Shape. The effect of manumycin on ATC cells was studied in cell culture, with the number of viable cells being measured spectrophotometrically as described above. At the end of the treatments, the cells were also observed under a microscope and photographed. In brief, manumycin inhibited the growth and decreased the number of viable cells in all six of the ATC cell lines, as confirmed by both spectrophotometry and microscopy. Increasing concentrations of manumycin induced morphological changes in the ATC cells (data not shown). Most control cells, which had not been exposed to manumycin, grew attached to the well, but a significant portion (i.e., rounded cells) grew on top of the other cells without directly attaching to the well. As the concentration of manumycin increased, the number of cells seen per high-power field decreased. At concentrations that affected the viability of the cells (> 5 \( \mu \text{M} \)), the cells became rounded. Additional increases in the concentration of manumycin led to a condensed appearance of the cells.

Effects of Antineoplastic Drugs on Viability of ATC Cells. Manumycin affected the viability of ATC cell lines, as shown by the dose-response curves in Fig. 1A. The viability of each cell line (as defined in “Materials and Methods”) was plotted against the concentration of manumycin used in a 48-h treatment, with each data point representing the geometric mean of at least three independent experiments. In brief, manumycin decreased viability in all six of the ATC cell lines tested. However, at the concentration of 54 \( \mu \text{M} \), DRO and C643 cells were the least sensitive of all of the cell lines. All of the curves began to diverge from baseline at about 5 \( \mu \text{M} \), a finding that agrees with the concentration (IC\(_{50}\)) of manumycin to inhibit 50% activity of farnesyl:protein transferase as reported in the literature (22).

The effects of the chemotherapeutic agents currently used to treat ATC (paclitaxel, cisplatin, and doxorubicin) were also studied to provide a baseline for comparing the effects of manumycin with these agents and detecting their interaction if any. As shown by dose-response curves of paclitaxel on the ATC cell lines in Fig. 1B, paclitaxel decreased viability in all six of the ATC cell lines. Paclitaxel achieved >50% cell kill at 27 \( \mu \text{M} \) in all of the cell lines except KAT-4. As shown in Fig. 1C, cisplatin had little effect on KAT-4 and KAT-18 cells (only about 10% of cells were killed). The dose-response curves for ARO, Hth-74, and C643 cells were almost identical to one another. DRO cells, however, were particularly sensitive to cisplatin. As shown in Fig. 1D, doxorubicin decreased viability in all six of the ATC cell lines. DRO and KAT-18 cells were the most susceptible (about 70% of cells were killed at a concentration of 10

Fig. 1. Dose-response curves for manumycin (A), paclitaxel (B), cisplatin (C), and doxorubicin (D). The viability of each cell line (as defined in “Materials and Methods”) was plotted against the concentration of each drug used in a 48-h treatment. Each data point represents the geometric mean of at least three independent experiments.

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Effects of Manumycin in Combination with Paclitaxel, Cisplatin, or Doxorubicin. To explore whether manumycin could enhance the effect of the chemotherapeutic agents currently used to treat ATC, the effects of 48-h treatments with manumycin plus paclitaxel, manumycin plus cisplatin, and manumycin plus doxorubicin were examined. For each drug combination, the effect was compared with the effects on ATC cells treated with either drug alone and on control cells incubated without the drugs. Each drug in a pair was used at a less than maximal concentration so that its effect would not mask enhancement. In brief, manumycin enhanced the effect of paclitaxel (Fig. 2A). The interaction (nonadditivity) of the two drugs was significant (P < 0.05) in all six of the cell lines (Fig. 2). In contrast, manumycin enhanced the effect of cisplatin (Fig. 2B) only in DRO and KAT-18 cells and the effect of doxorubicin (Fig. 2C) only in C643, Hth-74, and KAT-4 cells. The enhanced cytotoxic effect of manumycin plus paclitaxel was also observed in PANC-1 and SK-BR3, a pancreatic carcinoma cell line and a breast carcinoma cell line, respectively (data not shown).

Another way to demonstrate synergism is the shifting of dose-response curves of one drug in the presence or absence of another drug. In all of the 6 ATC cell lines, the dose-response curve of manumycin is shifted to the left by paclitaxel, and vice versa. (The dose-response curves of manumycin in the presence or absence of paclitaxel and the dose-response curves of paclitaxel in the presence or absence of manumycin for two of the ATC cell lines are shown in Fig. 3.) We conclude, therefore, that paclitaxel and manumycin enhanced the cytotoxic effect of each other.

Using four serial (1:3) dilutions of manumycin, paclitaxel, and a mixture of manumycin and paclitaxel at a chosen fixed ratio (manumycin 54 μM:paclitaxel 22 μM), the existence of synergism between manumycin and paclitaxel was evaluated using the median-effect method of Chou and Talalay (20). Four ATC cell lines (ARO, DRO, C643, and Hth-74), a pancreatic cancer cell line (PANC-1), and a breast cancer cell line (SK-BR3) were evaluated using this method. The CI was <1 for these six cell lines (Fig. 4), which indicated synergism in the combination of manumycin and paclitaxel.

Induction of Apoptosis by Manumycin plus Paclitaxel. To test the hypothesis that manumycin plus paclitaxel would increase cell death by inducing apoptosis, three characteristics of apoptosis (activation of caspase-3, specific cleavage of PARP, and internucleosomal cleavage of DNA) were evaluated in ARO cells treated for 18 h with either no drugs or with manumycin (54 μM), paclitaxel (22 μM), or manumycin (54 μM) plus paclitaxel (22 μM). The ARO cell line was chosen because it is one of the cell lines that was more sensitive to manumycin than the others. The doses of manumycin and paclitaxel chosen were close to their respective 75% inhibitory concentrations (IC75). ARO cells treated with doxorubicin (10 μM) or manumycin (54 μM) plus doxorubicin (10 μM) were also included for comparison in some of these experiments.

Manumycin plus paclitaxel caused enhanced activation of caspase-3 (Fig. 5). Manumycin plus paclitaxel activated caspase-3 to a higher degree than manumycin by itself, whereas paclitaxel did not significantly increase caspase-3 activity compared with the control. Two-way ANOVA showed that the interaction between manumycin and paclitaxel was significant (F test, P < 0.01).

Manumycin plus paclitaxel caused cleavage of PARP into a M[alpha] 89,000 fragment (Fig. 6, upper panel) and a M[alpha] 24,000 fragment (data not shown) as detected by immunoblotting with anti-PARP antiserum.

In contrast, neither paclitaxel alone nor doxorubicin alone had any significant effect on PARP (i.e., no change from the “no-drugs” control). Manumycin alone and manumycin plus doxorubicin produced a pattern of PARP cleavage that was not characteristic of apoptosis. Internucleosomal cleavage of DNA to form a DNA ladder was observed in the cells treated with manumycin plus paclitaxel (Fig. 6, lower panel). The control and other treatments (manumycin, doxorubicin, paclitaxel, manumycin plus paclitaxel) showed no such ladder.

1 P < 0.05 by F test for interaction between two factors in two-way ANOVA.
rubicin, manumycin plus doxorubicin, and paclitaxel) did not cause significant internucleosomal cleavage of DNA after an 18-h incubation, as detected by ethidium bromide staining. However, manumycin or paclitaxel alone did cause DNA laddering detectable by ethidium bromide staining after incubating for 48 h (data not shown). This specific cleavage of PARP and internucleosomal cleavage of DNA supported the hypothesis that manumycin plus paclitaxel increased cell death by enhancing apoptosis.

In Vivo Effect of Manumycin and Paclitaxel against Human ATC Xenografts in Nude Mice.

These studies were performed using the ARO and KAT-4 cell lines. Nude mice bearing s.c. ATC xenografts were randomized into 4 groups: control, manumycin, paclitaxel, and manumycin plus paclitaxel. Both manumycin and paclitaxel had a significant antitumor effect against ARO (Fig. 7, left panel) and KAT-4 cells (Fig. 7, right panel). The combination of manumycin plus paclitaxel seemed to have an effect on tumor inhibition similar to that of manumycin against ARO cells but showed an additional inhibitory effect against KAT-4 cells compared with either drug alone.

The body weight of the mice remained stable, and there were no significant differences among the treatment groups. Motor activity and feeding behavior of the mice were all normal. Complete blood counts (with differential) did not reveal any significant myelosuppression in any of the treatment groups. Liver enzyme panel demonstrated a slight rise in lactate dehydrogenase in all of the treatment groups. No mortality was observed. Overall, surveillance of morbidity and mortality did not reveal any significant toxicity of the drug combination.

DISCUSSION

Because manumycin A affected the viability of six human ATC cell lines tested in a dose-dependent manner, manumycin A seems to be a...
promising agent for the treatment of ATC. This conclusion is based on both direct observation of cell numbers per high-power field under a microscope and spectrophotometric measurement of mitochondrial dehydrogenase activity, which correlates directly with the number of viable cells. It is possible, however, that manumycin simply has a nonspecific toxic effect on all living cells (for example, acts as a metabolic poison that inhibits mitochondrial enzymes). Thus, an ideal control experiment would be to incubate the cancer cells with a compound structurally similar to manumycin but devoid of inhibitory activity against FPT. Unfortunately, no such compound is available.

Nevertheless, several observations argue against a nonspecific toxic effect on living cells in general: (a) different degrees of sensitivity to manumycin were manifested by different ATC cell lines (Fig. 1A); (b) the manumycin concentration range that inhibits the viability of ATC cells correlates with the concentration range reported in the literature for inhibition of FPT. The IC50 of manumycin against FPT is about 5 \( \mu \text{M} \) (22), and the inhibitory effects of manumycin on ATC cells in the present study began to emerge at about 5 \( \mu \text{M} \), as evident on the dose-response curves (Fig. 1A); and (c) manumycin induced minimal toxicity in nude mice in an in vivo study using xenograft models (17) and in this report. Therefore, the effect of manumycin on the ATC cells is likely to be, at least in part, mediated by its blocking of Ras function and, thus, the proliferation signal through the tyrosine kinase receptor pathway.

Manumycin also consistently enhanced the effect of paclitaxel in vitro in all six of the human ATC cell lines tested. In contrast, combinations of manumycin with doxorubicin and cisplatin resulted in synergism in some but not all of the ATC cell lines. Enhancement of the cytotoxic effect of paclitaxel by a different peptidomimetic farnesyltransferase inhibitor (called L-744832) has been reported in other cell lines (23), and our results corroborate and extend those findings. However, manumycin inhibits FPT by competition against farnesylpyrophosphate, whereas L-744832 inhibits FPT by mimicking the CAAX amino acid sequence. Nevertheless, their distinctly different chemical structures imply that potentiation of paclitaxel is caused by farnesyltransferase inhibition. This potentiation has been observed previously in four breast cancer cell lines and one prostate cancer cell line, and now in six ATC cell lines, another breast cancer cell line, and one pancreatic cancer cell line. This phenomenon of enhancement between FPT inhibitors and paclitaxel may be general and, therefore, important because this synergistic combination of drugs may have potential application in a wide variety of tumors.

Our study also showed that manumycin plus paclitaxel induced apoptosis in ATC cells after an 18-h incubation, whereas neither drug alone induced a detectable degree of apoptosis as assessed by observing internucleosomal DNA fragmentation and specific cleavage of PARP in drug-treated cells. Synergistic activation of caspase-3 activity by manumycin plus paclitaxel was also observed. These observations suggest that the synergistic decrease in cell viability seen when manumycin and paclitaxel are combined may be accounted for, at least in part, by the synergistic induction of apoptosis after treatment with this drug combination. Paclitaxel induces apoptosis in many types of tumors, generally after 24–36 h (24, 25), and FPT inhibitors also induce apoptosis in cancer cells (26, 27). However, whereas the pathway by which paclitaxel induces apoptosis has been a focus of apoptosis research (25, 28–33), little is known about the pathway by which FPT inhibitors induce apoptosis. It is open to speculation as to whether manumycin and paclitaxel perturb different regulators of apoptosis and lead to synergistic induction of apoptosis.

Whether manumycin plus paclitaxel would cause severe toxicity to normal cells is a critical question that needed to be addressed. We addressed this question by the in vivo studies using the nude mouse xenograft model. Toxicity to normal cells and the animal as a whole was carefully monitored by the observation of motor and feeding behavior, measurement of body weight, complete blood count with differential, and liver enzyme panel. The combination of manumycin and paclitaxel did not result in increased toxicity to normal cells or the host animal as a whole.

At least in the KAT-4 cell line, the combination of manumycin and paclitaxel produced more antitumor effect in vivo than either drug alone. With the particular dose regimen and administration schedule,
synergism was not observed in vivo. One possible explanation for the lack of synergism in vivo may be the ceiling effect. Additional in vivo experimentation is justified to define the optimal way to combine manumycin and paclitaxel to maximize the therapeutic efficacy. In the search for more efficacious therapies for ATC, the discovery of synergism between manumycin and paclitaxel is one small step forward, and efficacy in vivo may justify future clinical trials.

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