Synergy between 3′-Azido-3′-deoxythymidine and Paclitaxel in Human Pharynx FaDu Cells

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Purpose. We recently demonstrated simultaneous targeting of telomere and telomerase as a novel cancer therapeutic approach, and that telomerase inhibitors such as 3′-azido-3′-deoxythymidine (AZT) significantly enhanced the antitumor activity of paclitaxel, which causes telomere erosion, in telomerase-positive human pharynx FaDu tumors *in vitro* and *in vivo* (1). The present study evaluated the synergy between AZT and paclitaxel to identify optimal combinations for future clinical evaluation.

Methods. FaDu cells were incubated with or without AZT for 24 h and then treated with AZT with or without paclitaxel for an additional 48 h. Under these conditions, single agent paclitaxel produced a 60% maximum reduction of cell number (IC_{50} was 7.3 nM), and single agent AZT produced a 97% reduction (IC_{50} was 5.6 μM). Synergy was evaluated using fixed-concentration and fixed-ratio methods, and data were analyzed by the combination index method.

Results. The results indicate a concentration-dependent synergy between the two drugs; the synergy was higher for combinations containing greater paclitaxel-to-AZT concentration ratios and increased with the level of drug effect. For example, in combinations containing 1 μM AZT, synergy was 1.3-fold at the 20% effect level and 3.1-fold at the 60% effect level. Because the major antitumor activity, determined by comparing the posttreatment cell number to the pretreatment cell number, was antiproliferation at the 20% effect level and cell kill at the 60% effect level, our results suggest that AZT mainly enhances the cell kill effect of paclitaxel.

Conclusion. In summary, the present study demonstrates a synergistic interaction between paclitaxel and AZT and supports a combination using a low and nontoxic AZT dose in combination with a therapeutically active dose of paclitaxel.

KEY WORDS: paclitaxel; AZT; synergy; telomere; telomerase.

INTRODUCTION

Telomeres are specific DNA structures at the ends of chromosomes that protect chromosomes from end-to-end fusion, maintain chromosome integrity, reversibly repress transcription of neighboring genes, and play a role in chromosome positioning in the nucleus (1). Because of the inability of DNA polymerases to replicate the 3′ end of chromosomes, telomeres are shortened by 50 to 200 bp per cell division in normal somatic cells. Loss of telomeres to below a threshold value is believed to induce senescence. Telomerase is a ribonucleoprotein DNA polymerase that synthesizes telomeric repeats *de novo* and is involved in multiple cellular processes, including cell differentiation, proliferation, inhibition of apoptosis, tumorigenesis, and possibly DNA repair and drug resistance (3–6). Telomerase is present in nearly all immortal cell lines, germ-line cells, stem cells, and about 90% of human tumors, but seldom in normal somatic cells (7).

The selective expression of telomerase in tumor cells makes telomerase an attractive therapeutic target. However, telomerase inhibition results in cytotoxicity only after a significant lag time. For example, telomerase inhibitors resulted in cytotoxicity in HeLa cells after 23 to 26 cell doublings (8). The long lag time may also allow for activation of the telomerase-independent alternative mechanism of telomere maintenance (9). These concerns limit the therapeutic potential of telomerase inhibitors.

We recently demonstrated simultaneous targeting of telomere and telomerase to be a novel approach to targeting cancer cells that use telomerase for telomere maintenance. We further showed that telomerase inhibitors [i.e., antisense to the RNA component of telomerase and 3′-azido-3′-deoxythymidine (AZT)] significantly enhanced the antitumor activity of paclitaxel, which causes telomere erosion, in telomerase-positive human pharynx FaDu tumors *in vitro* and *in vivo* (1). The present study evaluated the concentration-dependent synergy between AZT and paclitaxel, to identify synergistic combinations for future clinical evaluation.

MATERIALS AND METHODS

Chemicals and Reagents

Paclitaxel was a gift from the Bristol-Myers Squibb Co. (Wallingford, CT) and the National Cancer Institute (Bethesda, MD). AZT was supplied by the National Cancer Institute (Bethesda, MD). Gentamicin was purchased from Solo Pak Laboratories (Franklin Park, IL), and other cell culture supplies from GIBCO Laboratories (Grand Island, NY).

Cell Culture

FaDu cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in minimum essential medium. Culture medium was supplemented with 9% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 90 μg/ml gentamicin, and 90 μg/ml sodium cefotaxamine. Cells were incubated with complete medium at 37°C in a humidified atmosphere of 5% CO_2 in air. For experiments, cells were harvested from preconfluent cultures after two rinses with versene and a 10-min incubation with trypsin. The harvested cells were resuspended in fresh medium. Cells were seeded in 96-well microtiter plates and allowed to attach for 20 to 24 h.

Drug Treatment

Stock solutions of paclitaxel were prepared in 100% ethanol at a concentration of 1 mM and stored at −70°C.

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ABBREVIATIONS: AZT, 3′-azido-3′-deoxythymidine; AZTTP, AZT triphosphate; CI, combination index; IC, inhibitory drug concentration; SRB, sulforhodamine B.

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Stock solutions of AZT were prepared in double-distilled sterile water at a concentration of 10 mM.

Drug treatment was initiated after cells were allowed to attach to the growth surface. On the day of experiments, the culture medium was removed and replaced with drug-containing medium. Treatment with AZT was initiated 24 h before paclitaxel treatment to allow for the conversion of AZT to AZTTP, which is the active metabolite that inhibits telomerase (10). Cells were then treated with paclitaxel, with or without AZT, for an additional 48 h. Control cells were processed similarly but without drugs.

Drug Activity Evaluation

The remaining cell number after drug treatment was measured using the sulforhodamine B (SRB) assay, which stains for cellular protein and represents the overall drug effect, i.e., the combination of cytostatic and cytotoxic effects (11). In brief, cells (2,000 cells per well) were seeded onto 96-well plates. At the end of drug treatment, cells were fixed with 0.2 ml 10% trichloroacetic acid at 4°C for 1 h, rinsed with distilled water, allowed to air dry, stained with SRB (0.05 ml of 0.4%), rinsed with 1% glacial acetic acid, and again allowed to air dry. Tris-HCl (0.2 ml, 10 mM) was then added to each well to dissolve the SRB, which was measured by absorbance at 490 nm using an EL 340 microplate biokinetics reader (Bio-Tek Instruments, Inc., Winooski, VT).

Evaluation of Interaction

The nature of the drug interaction was evaluated using two methods. The first method used fixed concentrations of AZT together with increasing concentrations of paclitaxel, i.e., the fixed-concentration method. The advantage of this method is that it yields the conventional sigmoidal concentration–effect curves showing increases in effect as a function of increasing paclitaxel concentration and provides a measure of the enhancement of the paclitaxel activity at a fixed AZT concentration. The latter facilitates the selection of the AZT dose to be used during *in vivo* studies. However, the experimental design of the fixed concentration ratio is such that only limited AZT concentrations can be studied.

The second method used fixed concentration ratios of paclitaxel and the telomerase inhibitor, i.e., the fixed-ratio method. The advantage of this method is that it enables the measurement of the nature of the interaction between paclitaxel and AZT at much broader concentration ranges as compared to the first method. An additional advantage is that it allows the identification of the optimal ratios of the two drugs that give the maximal synergy. For this method, cells were treated with solutions containing both drugs at 5 to 320% of their respective initial concentrations. The initial concentrations were 7 nM for paclitaxel and 5 μM for AZT, which were approximately their respective IC_{50} values. The concentration ratio of paclitaxel to AZT was kept at four fixed ratios (80:20, 60:40, 40:60, and 20:80).

Synergy Determination

The nature of the interaction between paclitaxel and AZT was analyzed using the combination index method (12). Concentration–effect curves for single agents and their combinations were used to determine the amount of each agent, either alone or in combination, needed to achieve a given level of effect. The combination index (CI) was calculated as follows.

$$CI = \frac{IC_{A,B}}{IC_A} + \frac{IC_{B,A}}{IC_B}$$

IC_A and IC_B are the concentrations of agents A and B needed to produce a given level of cytotoxicity when used alone, whereas $IC_{A,B}$ and $IC_{B,A}$ are the concentrations needed to produce the same effect when used in combination. A CI value of 1 indicates additive interaction, values less than 1 indicate synergistic action, and values greater than 1 indicate antagonistic interaction (13).

RESULTS

Cytotoxicity of Paclitaxel and AZT: Results of Fixed-Concentration Method

Cells were treated with paclitaxel, with or without AZT, for 48 h. For the combination treatment, cells were also pretreated with AZT for 24 h. Table I summarizes the IC_{50} values.

Treatment with single agent paclitaxel for 48 h resulted in a maximum cell number reduction of about 60% (Fig. 1). This effect was reached at 100 nM paclitaxel and was not increased by increasing the drug concentration another 10-fold. The remaining cell numbers after treatment with 1, 5, 10, 50, 100, and 500 nM paclitaxel were 122 ± 1, 102 ± 4, 93 ± 2, 71 ± 3, 66 ± 8, and 66 ± 3% of the pretreatment cell number, respectively (mean ± SD of three experiments). Hence, the antitumor activity reflected inhibition of proliferation at ≤10 nM paclitaxel and cell kill at higher concentrations. Because

Table I. Synergy between Paclitaxel and AZT: Results of Fixed-Amount Method

AZT (μM)[a]	Paclitaxel IC_{50} or IC_{70} (nM)	Combination index	Synergy, fold
50% effect			
0.0	7.34 ± 3.18	Not applicable	Not applicable
1.0	4.92 ± 2.77	0.54 ± 0.15[b]	1.94 ± 0.53
5.0	2.23 ± 1.48	0.73 ± 0.02[b]	1.37 ± 0.04
10.0	0.78 ± 0.43	ND[c]	ND[c]
70% effect			
0.0	>1,000	Not applicable	Not applicable
1.0	160 ± 10.4	0.11 ± 0.02[b]	9.14 ± 1.50
5.0	10.5 ± 5.9	0.39 ± 0.01[b]	2.34 ± 0.36
10.0	5.10 ± 3.0	0.78 ± 0.01[b]	1.50 ± 0.39

Note: Synergy between paclitaxel and AZT was determined using the fixed-concentration method. Cells were treated with paclitaxel solutions (0–1,000 nM) and fixed concentrations of AZT (0, 1, 5, and 10 μM). Drug activity was measured by the SRB assay. The results were analyzed for the nature of interaction between paclitaxel and AZT as described in Materials and Methods. A combination index of less than 1 indicates synergy, and its inverse value indicates the extent of synergy. Results at two effect levels, i.e., 50% and 70%, are shown. Mean ± SD of three experiments, with six replicates per experiment.

[a] IC_{50} and IC_{70} values of single agent AZT were 5.82 ± 1.10 μM and 11.22 ± 1.50 μM.

[b] $p < 0.05$ compared to 1.

[c] Cannot be determined because single-agent AZT produced greater than 50% cytotoxicity.

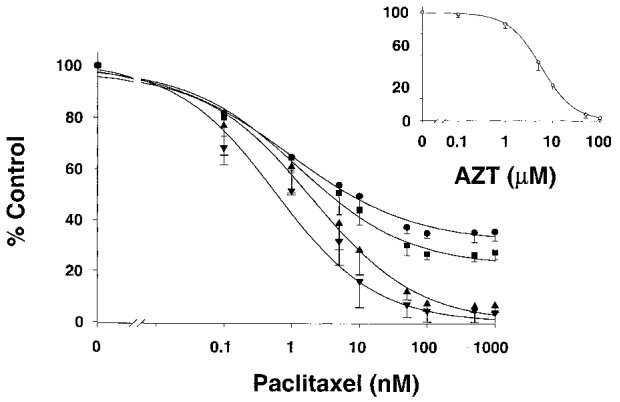

Fig. 1. Cytotoxicity of paclitaxel and/or AZT: results of fixed-concentration method. Cells were treated with paclitaxel, with or without AZT, for 48 h. For the combination treatment, cells were also pretreated with AZT for 24 h. Drug effect was measured as reduction of number of FaDu cells attached to growth surface. Note that the results have been normalized for the 10, 50, and 70% cytotoxicity derived from single agent AZT at 1, 5, and 10 μM (i.e., the effect of the combination was expressed as a percentage of the value of AZT-treated sample). Results of a representative experiment are shown (six replicates per experiment). The IC_{50} values are shown in Table I. Symbols: ●, paclitaxel alone; ○, AZT alone (inset); ■, paclitaxel plus 1 μM AZT; ▲, paclitaxel plus 5 μM AZT; ▼, paclitaxel plus 10 μM AZT.

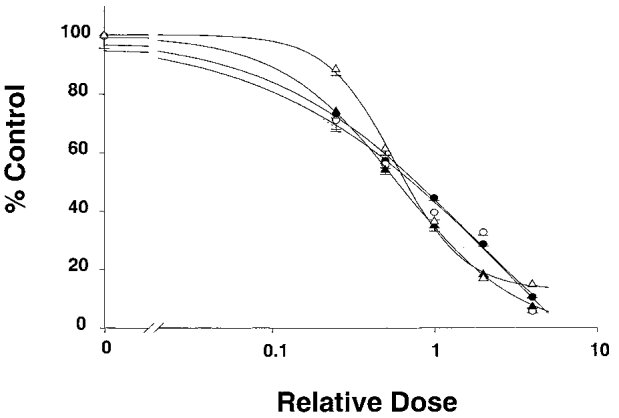

Fig. 2. Cytotoxicity of paclitaxel and/or AZT: results of fixed-ratio method. Cells were treated with solutions containing both drugs at 5 to 320% of their respective initial concentrations (7 nM for paclitaxel and 5 μM for AZT), which were approximately their respective IC_{50} values. The concentrations of paclitaxel to AZT were kept at four fixed ratios: ●, 80:20; ○, 60:40; ▲, 40:60; △, 20:80.

the IC_{50} of paclitaxel was about 10 nM, the major drug effect at concentrations below its IC_{50} was antiproliferation, whereas cell kill became more prominent at higher concentrations.

Treatment with single agent AZT for 72 h resulted in a 97% maximum cell number reduction at 100 μM AZT (Fig. 1, inset). The synergy studies used AZT concentrations ranging from 0.25 to 100 μM. At these AZT concentrations, the cell number reduction ranged from <1 to 97%.

Figure 1 also shows the results of the paclitaxel and AZT combinations using the fixed-concentration method. Note that the results have been normalized for the cytotoxicity derived from single agent AZT (i.e., the effect of the combination was expressed as a percentage of the value of the AZT-treated sample). Cotreatment with AZT significantly increased paclitaxel activity. First, there was an increase in the maximum effect, resulting in nearly complete reduction of cell number in the combinations containing 5 or 10 μM AZT. Second, the concentration–effect curve of paclitaxel (after corrected for AZT activity) was shifted to the left, and the IC_{50} of paclitaxel was decreased by 1.5- to 9.4-fold (Table I) by 1 to 10 μM AZT ($p < 0.05$, Student's *t* test).

Cytotoxicity of Paclitaxel and AZT: Results of Fixed-Ratio Method

Cells were treated as described for the fixed-concentration method, except that paclitaxel and AZT were applied at preselected ratios of concentrations (see Methods). The results of single agent AZT or paclitaxel, used at the same concentrations as in the combinations, are shown for comparison (Fig. 2). Consistent with the results of the fixed-concentration method, AZT enhanced paclitaxel cytotoxicity such that the concentration–effect curves were shifted to the left. Furthermore, there was an increase in the maximum effect, resulting in nearly complete reduction of cell number in all paclitaxel/AZT combinations.

Synergy Determination

Figure 3 shows the combination index analysis of the results of the fixed concentration method at the three AZT concentrations (1, 5, or 10 μM). Table I summarizes the CI values and extents of synergy. The CI values were below 1.0, indicating synergy between paclitaxel and AZT. The extent of synergy (equal to the inverse value of combination index) was inversely related to the AZT concentrations and ranged from 9.0-fold at 1 μM to 1.3-fold at 10 μM AZT. The results further show greater extents of synergy at higher drug effect levels. For example, for combinations containing 1 μM AZT, the extent of synergy was between 1.3- and 1.8-fold at 10 to 50% drug effect levels and much greater at higher drug effect levels (9.0-fold at the 70% effect level). Note that because 10 μM AZT resulted in greater than 60% cell growth inhibition and because paclitaxel, at the highest concentration of 1,000 nM, produced ≤70% maximum inhibition, the only CI that could

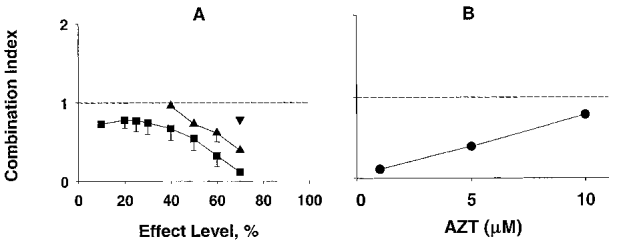

Fig. 3. Analysis of synergy between paclitaxel and AZT: results of fixed-concentration method. Results depicted in Fig.1 were analyzed by the combination index method as described in Methods. A combination index of <1 indicates synergy. Symbols: ■, paclitaxel plus 1 μM AZT; ▲, paclitaxel plus 5 μM AZT; ▼, paclitaxel plus 10 μM AZT. Data represent the average of the means from three experiments. Bar, 1 SD. (A) Relationship between combination index and drug effect level. A 50% drug effect level equals to 50% reduction in cell number compared to the untreated control. (B) Relationship between combination index and AZT concentration, at 70% drug effect level.

be calculated was at the 70% effect level for combinations containing 10 μM AZT.

Figure 3B shows a plot of CI values vs. AZT levels obtained from the fixed concentration method. This result indicates an inverse relationship between the extent of synergy and AZT concentration, with a 9.1-fold synergy at 1 μM AZT and 1.5-fold synergy at 10 μM AZT (Table I).

Figure 4 and Table II show the results of the combination index analysis for the fixed-ratio studies. Consistent with the results of the fixed-concentration method, the extent of synergy was greater at higher drug effect levels (Fig. 4A). For example, the combination indices were indistinguishable from 1.0 at effect levels ≤40% but were significantly less than 1.0 at higher effect levels for each of the four ratios examined. Also consistent with the fixed-concentration method, the extent of synergy increased with the paclitaxel-to-AZT ratios in the combinations. The maximal synergy of about 3.3-fold was achieved at the 60% effect level with a paclitaxel:AZT ratio of 80:20 (Fig. 4B and Table II).

Table II. Synergy between Paclitaxel and AZT: Results of Fixed Ratio Method

Paclitaxel: AZT ratio	Paclitaxel concentration (nM)[a]	AZT concentration (μM)[b]	Combination index	Synergy, fold
20% effect				
80:20	0.84 ± 0.30	1.18 ± 0.98	0.84 ± 0.30	1.19 ± 0.39
60:40	0.94 ± 0.40	1.02 ± 0.82	0.94 ± 0.40	1.07 ± 0.69
40:60	1.12 ± 0.27	0.88 ± 0.47	1.12 ± 0.27	0.89 ± 0.26
20:80	1.20 ± 0.18	0.53 ± 0.14	1.20 ± 0.18	0.85 ± 0.12
50% effect				
80:20	5.10 ± 1.67	0.91 ± 0.30	0.60 ± 0.12[c]	1.70 ± 0.30
60:40	3.84 ± 1.30	1.83 ± 0.62	0.65 ± 0.12[c]	1.57 ± 0.28
40:60	2.35 ± 0.78	2.51 ± 0.84	0.64 ± 0.09[c]	1.59 ± 0.20
20:80	1.27 ± 0.45	3.62 ± 1.29	0.73 ± 0.15[c]	1.41 ± 0.31

Note: Synergy between paclitaxel and AZT in human FaDu cells was determined using the fixed-ratio method. The paclitaxel-to-AZT concentration ratios were kept constant for each concentration–response curve. Cells were treated with solutions containing both drugs at 5 to 320% of their respective initial concentrations (7 nM for paclitaxel and 5 μM for AZT). Drug activity was measured by the SRB assay. The results were analyzed for the nature of interaction between paclitaxel and AZT as described in Materials and Methods. A combination index less than 1 indicates synergy, and its inverse value indicates the extent of synergy. Results at two effect levels, i.e., 20% and 50%, are shown. Mean ± SD of three experiments, with four replicates per experiment.

[a] IC_{20} and IC_{50} values of paclitaxel were 1.46 ± 0.78 nM and 11.6 ± 1.89 nM, respectively.

[b] IC_{20} and IC_{50} values of AZT were 2.22 ± 0.92 μM and 5.95 ± 1.89 μM, respectively.

[c] $p < 0.05$ compared to 1.0 (additivity).

DISCUSSION

Results of the present study indicate a concentration-dependent synergy between paclitaxel and AZT. The extent of synergy was greatest for combinations containing higher paclitaxel concentrations and/or greater paclitaxel-to-AZT concentration ratios and increased with the level of drug effect. For example, in combinations containing 1 μM AZT, synergy was 1.3-fold at the 20% effect level and 3.1-fold at the 60% effect level. These data, because the major antitumor activity of paclitaxel was antiproliferation at the 20% effect level and cell kill at the 60% effect levels, suggest that AZT mainly enhances the cell kill effect of paclitaxel. This is consistent with our previous finding, established using a different method to measure apoptosis (i.e., release of DNA–histone complex from the nucleus), that AZT enhances the paclitaxel-induced apoptosis, presumably by increasing the cell fraction with damaged telomere (1). There are multiple studies suggesting a relationship between telomere instability/erosion and apoptosis (8,14–20). For example, apoptosis in human HeLa 293 and MW451 cells induced by hydroxyl radicals showed telomere erosion without caspase activation (21), and elongation of telomeres in human fibroblast IDH4 and prostate DU145 cells resulted in higher resistance to apoptosis induced by serum depletion (17). Telomere erosion by paclitaxel is associated with cell death (21). Further studies are needed to determine the mechanisms of telomere erosion by paclitaxel and the role of telomere erosion in paclitaxel-induced apoptosis.

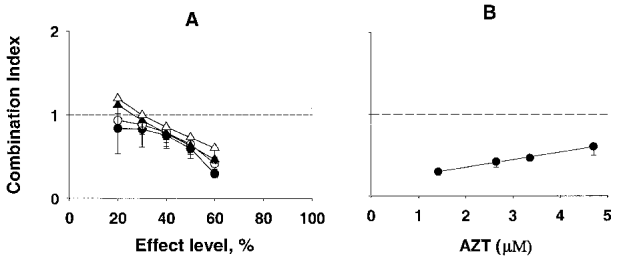

Fig. 4. Analysis of synergy between paclitaxel and AZT: results of fixed-ratio method. Results depicted in Fig. 1 were analyzed by the combination index method as described in Methods. A combination index of <1 indicates synergy. Symbols for the different paclitaxel-to-AZT concentration ratios are: ●, 80:20; ○, 60:40; ▲, 40:60; △, 20:80. Data represent the average of the means from three experiments. Bar, 1 SD. (A) Relationship between combination index and drug effect level. A 50% drug effect level equals 50% reduction in cell number compared to the untreated control. (B) Relationship between combination index and the paclitaxel-to-AZT ratios at the 60% drug effect level.

The mechanism of the lessened synergy between paclitaxel and AZT at higher AZT concentrations (i.e., >5 μM) is not clear. AZT has multiple pharmacologic actions including inhibition of reverse transcriptase, the reverse transcriptase component of human telomerase, integrase, DNA polymerase γ, and thymidine kinase and is preferentially incorporated into telomeric DNA and Z-DNA-containing regions of Chinese hamster ovary cells (22–27). AZT at concentrations above 3 μM increased the percentage of cells in S-phase and prolonged the cell doubling time (28–30). We previously showed, using fluorescence *in situ* hybridization analysis, that paclitaxel selectively shortened telomeres in M-phase cells but not interphase cells (1). Hence, blockade of cells in the S-phase at high AZT concentrations may diminish the telomere erosion effect of paclitaxel and diminishes the synergy. Further studies are needed to test this hypothesis.

In summary, the present study demonstrates a synergistic interaction between paclitaxel and AZT and supports the use of a combination of a low and nontoxic AZT dose and a therapeutically active dose of paclitaxel.

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