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# COMBINATION STUDIES WITH 3′-AZIDO-3′-DEOXYTHYMIDINE (AZT) PLUS ICI D1694

## CYTOTOXIC AND BIOCHEMICAL EFFECTS

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**Abstract**—The cytotoxicity of 3′-azido-3′-deoxythymidine (AZT), a thymidine analogue, and ICI D1694, a folate-based thymidylate synthase (TS) inhibitor, was examined individually and in combination in two human tumor cell lines, MGH-U1 bladder cancer and HCT-8 colon cancer cells, grown as a monolayer culture with and without thymidine (TdR). In addition, TS inhibition, [$^3$H]AZT incorporation into DNA, [$^3$H]AZT-MP (monophosphate) production, and DNA double-strand breaks were measured. Twenty-four hour exposure of AZT at 0.5, 5, 50 and 500 μM was not cytotoxic to MGH-U1 or HCT-8 cells in a colony-forming assay. ICI D1694 cytotoxicity increased with drug concentration, and the $IC_{50}$ and $IC_{90}$, respectively, were 0.0064 and 0.01 μM in MGH-U1 cells and 0.009 and 0.018 μM in HCT-8 cells. TdR in concentrations of 0.1 to 1.0 μM did not affect ICI D1694 cytotoxicity in either cell line. AZT at 5, 50 or 500 μM increased ICI D1694 cell kill. The $IC_{50}$ and $IC_{90}$ for MGH-U1 were 0.0037 and 0.0075 μM for 50 μM AZT combined with ICI D1694. The $IC_{50}$ and $IC_{90}$ for HCT-8 were 0.0075 and 0.015 μM for 50 μM AZT plus ICI D1694. The incorporation of [$^3$H]AZT into DNA increased with increasing concentrations of ICI D1694. Concentrations producing an $IC_{50}$ and $IC_{90}$ of ICI D1694, respectively, increased incorporation of [$^3$H]AZT into DNA by 319 and 569% in MHG-U1, and 243 and 400% in HCT-8 cells. The formation of [$^3$H]AZT-MP paralleled the increase in [$^3$H]AZT incorporated into DNA. AZT, ICI D1694 and the combination of AZT and ICI D1694 caused DNA double-strand breaks, with the combination of these agents being additive. CFU-GM survival, exposed to drug concentrations, as those used in the tumor cell lines, revealed that the therapeutic index was greater for AZT plus ICI D1694 than for ICI D1694 alone. These findings suggest that AZT plus ICI D1694 may increase antitumor effect with minimal myelosuppression. We conclude that AZT increases the cytotoxicity of ICI D1694 with increasing AZT incorporation into DNA.

3′-Azido-3′-deoxythymidine (AZT)¶ (Fig. 1) is a thymidine (TdR) analogue which is used clinically in the treatment of AIDS and AIDS-related complex. AZT was originally developed as an anticancer agent [1]; however, early assessment of its cytotoxic properties did not show promise and further development of drugs with these properties was abandoned [2]. Interest in AZT in combination with other antimetabolites has been renewed recently [2–4]. Investigators have shown significant cytotoxic activity both *in vivo* and *in vitro* in a human colon tumor model when AZT was combined with either 5-fluorouracil (5-FU) [3, 4] or methotrexate (MTX) [2]. Based upon these preclinical studies, Phase I and II clinical and pharmacological evaluations of high-dose AZT in combination with 5-FU and folinic acid have been initiated [5].

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¶ Abbreviations: AZT, 3′-azido-3′-deoxythymidine; TdR, thymidine; 5-FU, 5-fluorouracil; TS, thymidylate synthase; AZT-MP, AZT monophosphate; TK, thymidine kinase; TI, therapeutic index; and PBS, phosphate-buffered saline.

ICI D1694 (Fig. 1) is a water-soluble, folate-based thymidylate synthase (TS) inhibitor which is transported rapidly into cells by the reduced folate carrier system and then metabolized extensively to polyglutamate derivatives [6, 7]. Polyglutamation increases the affinity of ICI D1694 binding to TS and enhances its potency *in vitro* and *in vivo* [6]. Whereas the monoglutamate of ICI D1694 has similar inhibitory activities against TS and dihydrofolate reductase (DHFR), the polyglutamates have increasing specificity of TS over DHFR [7]. In addition, polyglutamated forms of ICI D1694 are retained intracellularly, so that prolonged TS inhibition occurs [7–9].

AZT permeates the cell membrane primarily by nonfacilitated diffusion [10] and is firstly phosphorylated to AZT monophosphate (AZT-MP) by thymidine kinase (TK) and ultimately to the triphosphate form, AZT-TP. The exact biochemical mechanism(s) of action of AZT has not been resolved. Suggested mechanisms by which AZT may inhibit DNA synthesis include imbalance of deoxyribonucleotide pools by AZT [11], incorporation of AZT into DNA [2, 12], and an inability of the cells to repair AZT-induced DNA damage [13]. AZT-TP competes with deoxythymidine triphosphate (dTTP) for incorporation into DNA with subsequent termination of chain elongation

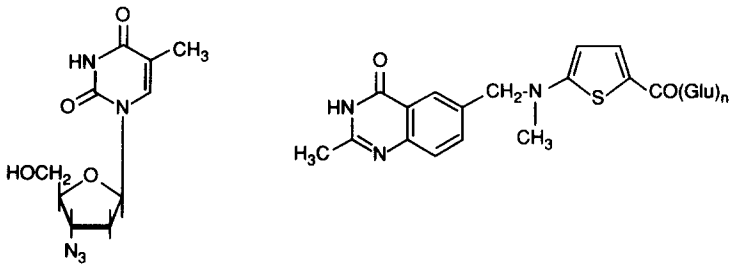


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Fig. 1. Chemical structures of (A) AZT and (B) ICI D1694.

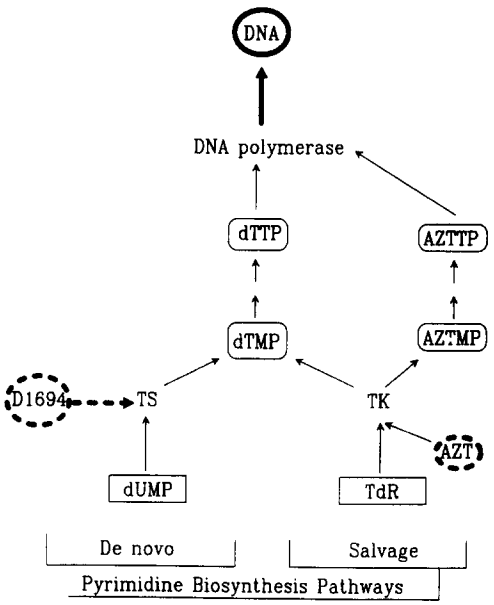


Fig. 2. Pathways of thymidylate synthesis: sites of action for ICI D1694 and AZT.

[2, 12]. Thus, we hypothesized that inhibition of TS by ICI D1694 with depletion of dTTP pools should increase AZT incorporation into DNA and be associated with increased cytotoxicity of the combination (Fig. 2).

In this study we have determined whether inhibition of TS by ICI D1694 can increase the effectiveness of AZT, defined the biochemical mechanism of the increased cytotoxicity, and related this cytotoxicity to the biochemical endpoints involved. It was our intention to identify a potential novel drug combination. To determine the effects of these drugs on normal tissues, we assessed the cytotoxicity of the combination on CFU-GM cells as a surrogate for *in vivo* myelosuppression. These findings will be discussed in light of their biochemical implications.

## MATERIALS AND METHODS

*Chemicals.* ICI D1694, a gift from ICI Pharmaceuticals (Alderly Park, Macclesfield, Cheshire, U.K.) was dissolved in 0.4 M sodium bicarbonate. AZT was purchased from the Sigma Chemical Co. (St. Louis, MO). AZT-MP was a gift from R. Ferone of the Burroughs Wellcome Co. (Research Triangle Park, NC). Drugs were protected from light, and dilutions were made in phosphate-buffered saline (PBS). [5-$^3$H]2′-Deoxycytidine (dCyd) (17 Ci/mmol) and [$^3$H]AZT (14 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Diphenylamine reagent (DPA) was purchased from BDH Ltd. (Poole, U.K.). ACS scintillant was obtained from Amersham. Media, PBS, antibiotics and trypsin were purchased from Gibco (Grand Island, NY). CytoScint liquid scintillation fluid was purchased from ICN Biomedicals (Costa Mesa, CA). Plasticware was purchased from Falcon (Bedford, MA). All other chemicals were reagent grade and were obtained from Sigma.

*Cells.* A human bladder cancer cell line, MGH-U1, and a human colon cancer cell line, HCT-8, were maintained as a monolayer in Alpha Minimum Essential Medium (MEM) supplemented with 0.1% streptomycin, 0.1% penicillin and 10% fetal bovine serum (Whittaker, Walker Wille & Bockneck, Toronto) at 37° in a 5% $CO_2$ humidified atmosphere [14]; the cells were subcultured twice weekly and were used until passage 20. Under these conditions, the doubling time of the cells growing exponentially was approximately 24 hr, and plating efficiency was $>80$ and $>60\%$ for MGH-U1 and HCT-8 cells, respectively. Exponentially growing asynchronous cultures were used in all experiments.

*Cytotoxicity assay.* The clonogenic survival of drug-treated cells was performed as described previously [14, 15]. Briefly, $1 \times 10^6$ cells were seeded in a 75-cm$^2$ flask in 10% dialyzed fetal bovine serum (DFBS) and nucleoside-free MEM with or without thymidine (TdR) at concentrations present in human plasma (0.1 to 1.0 $\mu$M). After 24 hr, the exponentially growing cells were exposed to various drug concentrations and combinations for 24 hr. Cells were then washed three times in calcium and magnesium-free PBS, trypsinized, syringed to obtain a single cell suspension, counted in a cell counter (Ontario Cancer Institute), and plated in serial dilutions in replicates of 6 to 12. Two weeks after plating, the plates were stained with a methylene

blue solution and the colonies were counted. Survival was expressed as a fraction relative to control.

*Thymidylate synthase assay.* Exponentially growing MGH-U1 and HCT-8 cells were incubated with various concentrations of AZT, ICI D1694 or combinations of AZT plus ICI D1694 for a period of 24 hr. Following drug incubation, cells were washed in PBS, and TS activity was determined in intact cells by Yalowich and Kalman [16]. All values were corrected for background counts (the amount of tritiated water production as a result of the spontaneous breakdown of the radioisotope). Concentration–response curves were obtained by expressing the amount of tritium released from each treated culture as a percentage of the radioactivity measured in untreated controls.

*DNA double-strand breaks.* The cells were exposed to drug and drug combinations as described above in the cytotoxicity assay. Quantitation of DNA double-strand breaks in unlabeled cells was determined by a method described previously [17]. This method enables separation of fragmented DNA from intact chromatin by centrifugation. The intact and fragmented DNA was quantified using a DPA colorimetric assay [18].

*Incorporation of* [$^3$H]*AZT into DNA.* The incorporation of [$^3$H]AZT into DNA was determined *in vitro* in both MGH-U1 and HCT-8 cells by a modification of methods described previously [19]. Briefly, to assess the effect of ICI D1694 on the incorporation of [$^3$H]AZT into nucleic acids, exponentially growing cells were exposed to [$^3$H]-AZT (2.5 $\mu$M, 35 $\mu$Ci/well) alone or in combination with different concentrations of ICI D1694 in nucleoside-free 10% DFBS MEM. After 24 hr, cell number was determined, and the cells were washed once with PBS and pelleted. The cells were then extracted twice with 100 $\mu$L of 0.2 N perchloric acid (PCA). The supernatant was set aside for analysis of AZT-MP. The insoluble material was incubated at 37° for 20 min with 100 $\mu$L of RNase solution (7.5 mg DNase-free RNase plus 50 mL of 50 $\mu$M Tris–EDTA, pH 7.4). The reaction was stopped by the addition of 200 $\mu$L of 0.2 N PCA, centrifuged at 14,000 *g* for 10 min. The pellet was then dissolved in 5 mL of CytoScint liquid scintillation fluid and counted in an LS-330 Beckman Scintillation Counter.

*HPLC analysis of* [$^3$H]*AZT-MP.* The formation of [$^3$H]AZT-MP from [$^3$H]AZT was determined in supernatants from cells extracted for [$^3$H]AZT incorporation into DNA. Modification of a previously described method [11] was used. Briefly, the extract was filtered and injected onto a Waters Associates HPLC (model 440). The AZT-MP peak was separated using a Partisil 10-SAX column (Whatman, Clifton, NJ) using a linear gradient from 0.007 M $KH_2PO_4$, pH 4.0, to 0.25 M $KH_2PO_4$ plus 0.50 M KCl, pH 5.0, over 15 min at a flow rate of 1.5 mL/min. AZT-MP was analyzed simultaneously by a Waters Associates U.V. detector (model 440) set at 254 nm and a Radioactive Instruments radioactive flow detector (model IC). Elution of the AZT-MP peak was at about 10 min, and peak areas were digitized and integrated by computer (Shimadzu 510C, Japan) and corrected for radioactive detector efficiency.

*Culture of CFU-GM human bone marrow and cytotoxic studies.* Cells were obtained and separated by H. A. Messner and N. Jamal from The Ontario Cancer Institute, Toronto, Canada, and grown as described previously [20]. Briefly, $1 \times 10^6$ CFU-GM cells were exposed to the same drug treatments as in the cytotoxicity assay. After a 24-hr drug treatment, $5 \times 10^4$ and $1 \times 10^5$ cells were cultured in Iscove's Modified Dulbecco's Minimum Essential Medium, 2-mercaptoethanol ($5 \times 10^{-5}$ M), 10% fetal bovine serum, 5% phytohemagglutinin (PHA-LCM) and 0.9% methylcellulose. These cultures were incubated for 14 days at 37° in a humidified atmosphere supplemented with 5% $CO_2$. Colonies were detected and counted under an inverted microscope (Carl Zeiss Ltd., Don Mills, Canada). Each drug condition was repeated at least three times.

*Statistical analysis.* Differences between drug treatment alone and in combination were determined by ANOVA and Student's *t*-test using Epistat Software (Tracey L. Gustafson, Round Rock, TX).

## RESULTS

*MGH-U1 and HCT-8 cytotoxicity.* Clonogenic survival was determined in both MGH-U1 and HCT-8 cells exposed to AZT or ICI D1694 for 24 hr. ICI D1694 was cytotoxic in both cell lines, although HCT-8 cells appeared to be more resistant than MGH-U1 cells. The $IC_{50}$ and $IC_{90}$ values of ICI D1694 for MGH-U1 cells were 0.0064 and 0.01 $\mu$M, respectively, and for HCT-8 cells, were 0.009 and 0.018 $\mu$M, respectively (Fig. 3). AZT was not cytotoxic in either cell line at 5, 50 and 500 $\mu$M for 24 hr. When AZT was combined with ICI D1694 the cytotoxicity of ICI D1694 was enhanced. Combinations of 50 $\mu$M AZT with various concentrations of ICI D1694 produced a 2-fold increase in cytotoxicity in both cell lines (Fig. 3). Table 1 summarizes the $IC_{50}$ and $IC_{90}$ values for ICI D1694 alone and combined with 5, 50 and 500 $\mu$M AZT in each cell line. Combinations of 5, 50 and 500 $\mu$M AZT and ICI D1694 at cytotoxic $IC_{50}$ and $IC_{90}$ concentrations in MGH-U1 and HCT-8 cells revealed that increasing concentrations of AZT caused a greater enhanced cytotoxic effect of ICI D1694 (Fig. 4). Cytotoxicity experiments were repeated with TdR present at concentrations of 0.1, 0.3 and 1.0 $\mu$M. No significant difference ($P > 0.1$) in cytotoxicity was observed when compared with treatment in the absence of TdR (data not shown).

*Inhibition of thymidylate synthase activity.* Inhibition of TS by ICI D1694 was observed in both human tumor cell lines. The $IC_{50}$ values for ICI D1694 inhibition of TS activity in MGH-U1 and HCT-8 cells were 0.0024 and 0.007 $\mu$M, respectively. AZT at 5, 50 and 500 $\mu$M showed no evidence of TS inhibition in either cell line. In addition, 50 $\mu$M AZT had no effect on ICI D1694 inhibition of TS activity.

[$^3$H]*AZT incorporation into DNA and AZT-MP formation.* Studies to assess whether ICI D1694 could affect the incorporation of [$^3$H]AZT into the nucleic acid fraction of both MGH-U1 and HCT-8 cells revealed that in the presence of ICI D1694 the

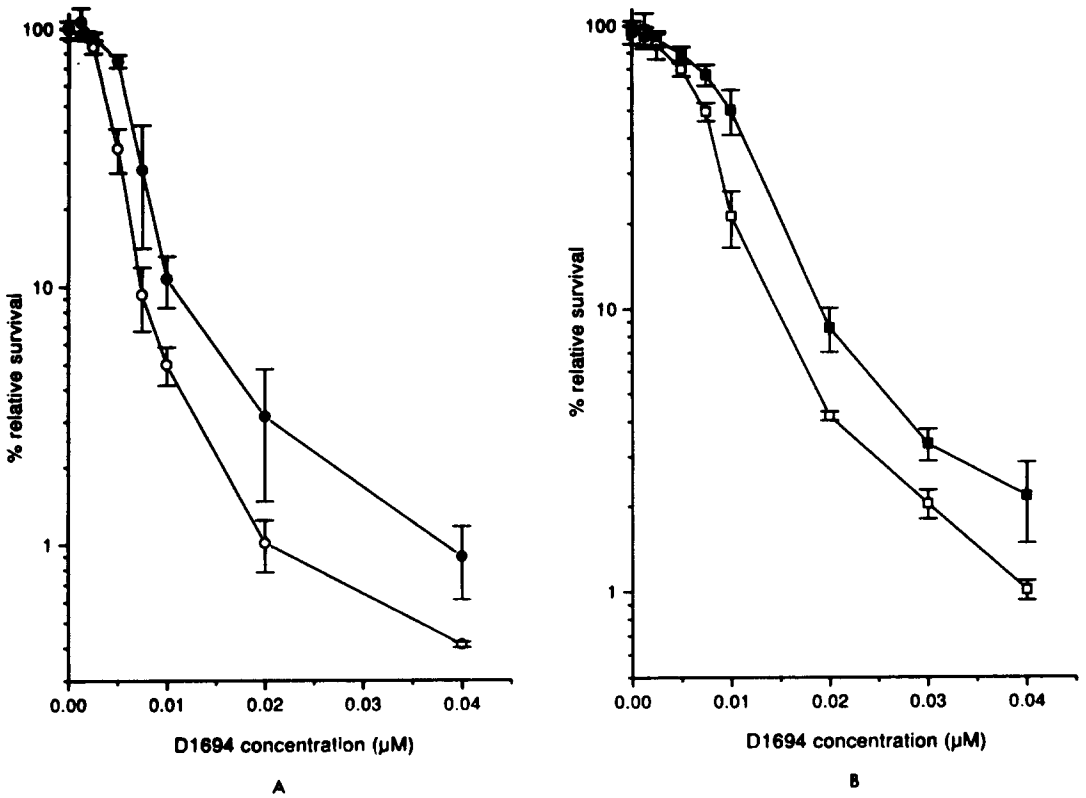


Fig. 3. Clonogenic survival of ICI D1694 (●, ○) alone or with 50 μM AZT (■, □) in (A) MGH-U1 and (B) HCT-8 cells. Points are the means ± SD of three or more assays. The $IC_{50}$ value of ICI D1694 plus AZT was significantly different from that of ICI D1694 alone in both MGH-U1 ($P = 0.008$) and HCT-8 ($P = 0.006$) cells.

Table 1. Cytotoxic $IC_{50}$ and $IC_{90}$ values for ICI D1694* alone or combined with AZT in MGH-U1 and HCT-8 cells

| Sample | MGH-U1 cells | | HCT-8 cells | |
|---|---|---|---|---|
| | $IC_{50}$ (μM) | $IC_{90}$ (μM) | $IC_{50}$ (μM) | $IC_{90}$ (μM) |
| D1694 | 0.0064 | 0.0100 | 0.0090 | 0.0180 |
| 500 μM AZT | Not cytotoxic | | Not cytotoxic | |
| D1694 + 5 μM AZT | 0.0064 | 0.0090 | 0.0090 | 0.0175 |
| D1694 + 50 μM AZT | 0.0037 | 0.0075 | 0.0075 | 0.0150 |
| D1694 + 500 μM AZT | 0.0015 | 0.0060 | 0.0030 | 0.0100 |

* Referred to as D1694 throughout this table.

percentage of [$^3$H]AZT incorporated into DNA and the amount of AZT-MP formed, relative to control cells treated with AZT alone, were increased. In addition, there appeared to be an ICI D1694-related concentration dependency on AZT incorporation into DNA (Fig. 5) and on AZT-MP formation. Exposure to either 2.5 μM AZT alone, or in combination with cytotoxic $IC_{50}$ or $IC_{90}$ ICI D1694 concentrations, respectively, yielded 2.24, 6.61, and 13.83 pmol AZT-MP/$10^6$ MGH-U1 cells and 3.68, 9.11, and 15.99 pmol AZT-MP/$10^6$ HCT-8 cells.

*DNA double-strand breaks.* The background double-strand breaks of control cells was 2%, and all values reported were corrected for background. In MGH-U1 cells, 500 μM AZT produced 0.795 ± 0.007% double-strand breaks and 0.01 μM ICI D1694 (cytotoxic $IC_{90}$) produced 3.67 ± 0.40%. These agents in combination, at the same concentrations, produced 7.31 ± 1.20% double-strand breaks (Fig. 6). Similar results were observed with HCT-8 cells. AZT at 500 μM produced 3.11 ± 0.55%, 0.018 μM ICI D1694 (cytotoxic $IC_{90}$) produced

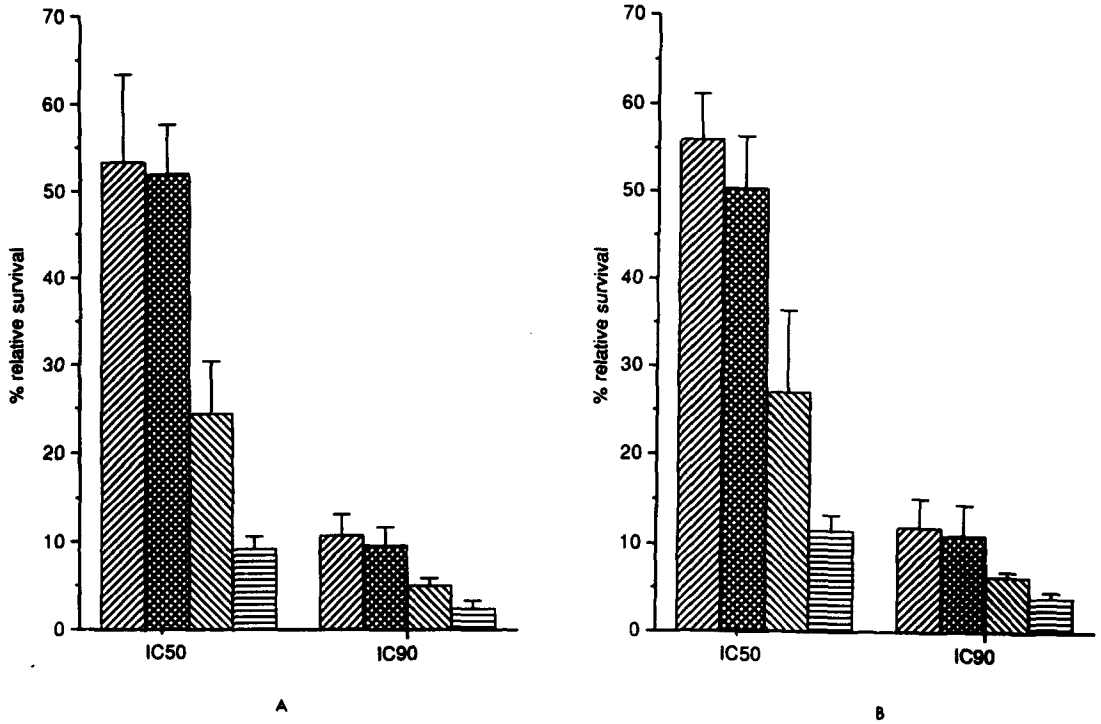


Fig. 4. Survival of cells treated with $IC_{50}$ and $IC_{90}$ concentrations of ICI D1694 alone (▨) or combined with AZT at 5 (■), 50 (▩) and 500 μM (▤) in (A) MGH-U1 and (B) HCT-8 cells. Each bar represents the mean ± SD of three or more assays. The mean value of ICI D1694 alone was not significantly different ($P > 0.1$) from that of ICI D1694 + 5 μM AZT in either tumor cell line. However, the addition of either 50 or 500 μM AZT to ICI D1694 produced a significant difference ($P < 0.01$).

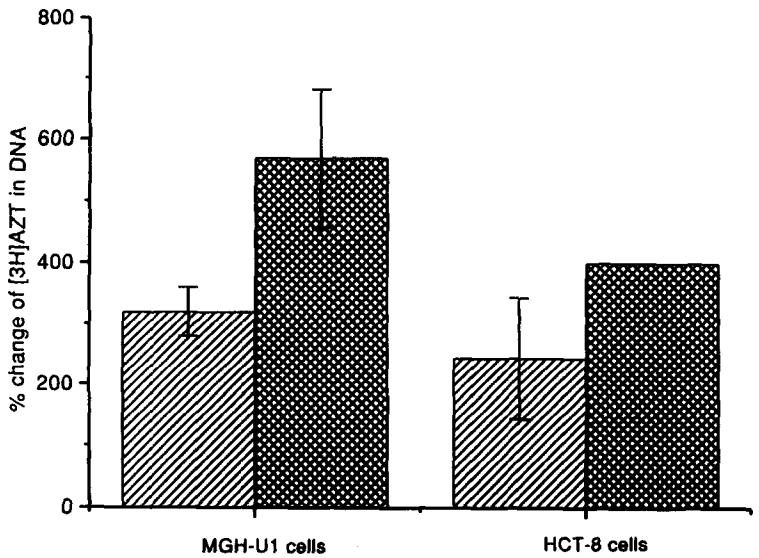


Fig. 5. Increase in DNA incorporation of AZT in the presence of $IC_{50}$ (▨) and $IC_{90}$ (■) ICI D1694 in MGH-U1 and HCT-8 cells. Each bar represents the mean of two determinations with the range indicated.

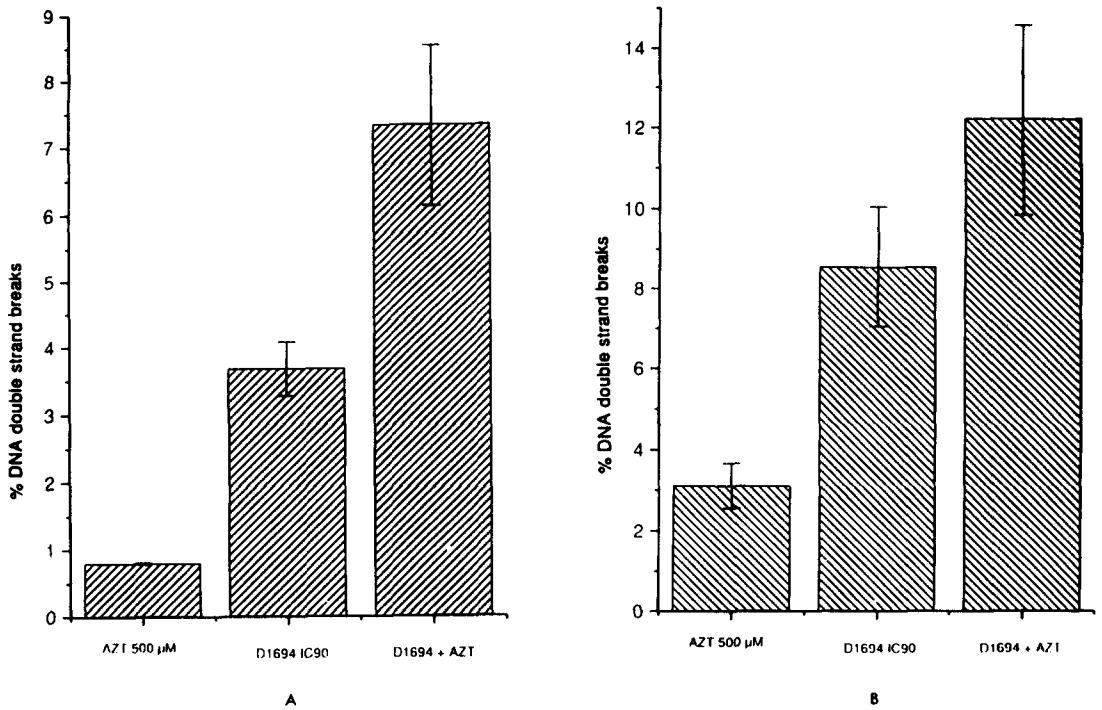


Fig. 6. Percent DNA double-strand breaks caused by 500 $\mu$M AZT, $IC_{90}$ ICI D1694, and AZT + ICI D1694 in (A) MGH-U1 and (B) HCT-8 cells. Each bar represents the mean ± SD of three separate assays. There was a significant difference ($P < 0.05$) between the means of a single agent and the combination treatment in both tumor cell lines.

8.55 ± 1.50%, and the combination of AZT and ICI D1694 produced 12.23 ± 2.37% in HCT-8 cells (Fig. 6).

*Cytotoxic effects of AZT and ICI D1694 on normal human bone marrow CFU-GM.* To assess whether this combination of AZT and ICI D1694 results in an improved therapeutic index (TI), clonogenic survival of CFU-GM was determined with the same drug concentrations and exposure time used for the tumor cells. Exposure to either agent alone or in combination resulted in minimal cytotoxic consequences (Figs. 7 and 8); AZT showed no significant cytotoxic effect at 5, 50, and 500 $\mu$M (data not shown). From these data, the TI as defined by $IC_{50}$ (CFU-GM)/$IC_{50}$ (MGH-U1 or HCT-8) was calculated for ICI D1694 (Fig. 7) and the combination of 50 $\mu$M AZT plus ICI D1694 (Fig. 8). The TI values for ICI D1694 for MGH-U1 and HCT-8 cells were 3.8 and 2.4, respectively. When ICI D1694 was combined with 50 $\mu$M AZT, the TI values were 5.3 and 3.5 for MGH-U1 and HCT-8 cells, respectively.

## DISCUSSION

TS inhibition is a target for fluoropyrimidine-based chemotherapy [21], but efficacy has been limited. One possible reason for the failure of TS-targeted anticancer therapy is that cells can salvage preformed TdR in plasma and synthesize thymidylate (dTMP) [22]. Some investigators have attempted to exploit differences in pyrimidine salvage between normal and tumor tissue by combining 5-FU with AZT [3, 4]. However, 5-FU can be incorporated into RNA and DNA, in addition to inhibiting TS [23]. Hence, the multiple mechanisms of action of 5-FU can confound the interpretation of the mechanism of cytotoxicity of 5-FU plus AZT. We attempted to overcome this limitation of 5-FU by using a specific TS inhibitor, ICI D1694.

The aim of the present study was to determine whether combining AZT with ICI D1694 would increase ICI D1694 cytotoxicity in tumor cells, to determine the biochemical effects of these drugs on TS and DNA, and to measure the effects on CFU-GM as a surrogate for myelosuppression. We demonstrated that AZT enhanced the cytotoxicity of ICI D1694 in an AZT-related concentration-dependent manner. Physiologic concentrations of TdR did not adversely affect the cytotoxicity observed, indicating that at concentrations of 0.1, 0.3, and 1.0 $\mu$M TdR did not circumvent dTMP depletion caused by TS inhibition nor did it decrease the amount of AZT-TP incorporated into DNA by competition at TK with AZT. Weber *et al.* [3, 24] reported that MTX plus AZT leads to increased cytotoxicity. Their results indicate that AZT competitively inhibits TK phosphorylation of TdR. In our studies, in nucleoside-free media, inhibition of TK is unlikely to be a contributing factor. However, the lack of protection by TdR at physiologic concentrations raises the possibility that TK inhibition may occur.

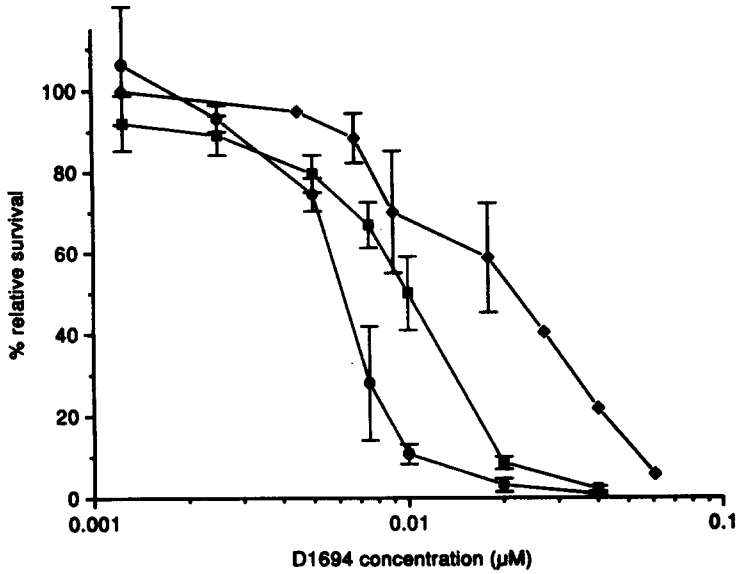


Fig. 7. Cytotoxic effects of ICI D1694 in MGH-U1 (●), HCT-8 (■), and CFU-GM (◆) cells. Each point represents the mean ± SD of at least three experiments. Those points lacking an error bar were not repeated. There was a significant difference between the $IC_{50}$ values of MGH-U1 ($P = 0.002$) or HCT-8 ($P = 0.004$) and that of CFU-GM cells.

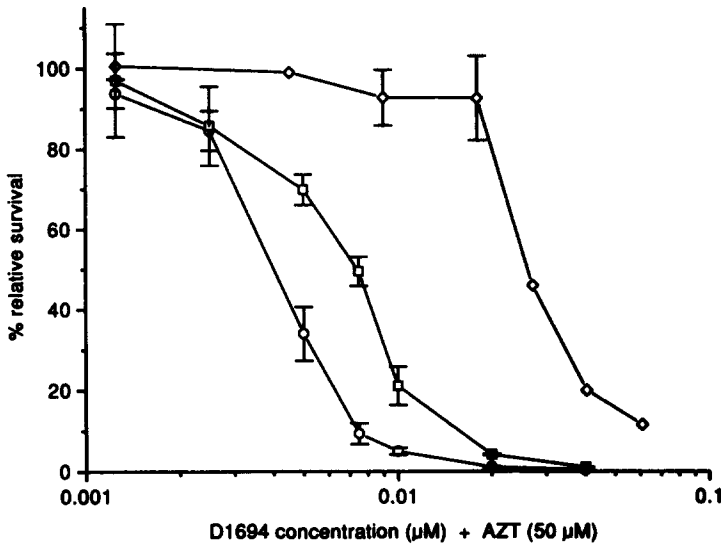


Fig. 8. Cytotoxic effects of 50 μM AZT combined with various ICI D1694 concentrations in MGH-U1 (○), HCT-8 (□), and CFU-GM (◇) cells. Each point represents the mean ± SD of at least three separate experiments. Those points lacking an error bar were not repeated. There was a significant difference between the $IC_{50}$ values of MGH-U1 ($P = 3 \times 10^{-6}$) or HCT-8 ($P = 6 \times 10^{-6}$) and that of CFU-GM cells.

The increased cytotoxicity of ICI D1694 observed when it was combined with AZT was associated with an increased AZT incorporation into DNA and an increased formation of AZT-MP. This was related to the ICI D1694 concentration used, suggesting that increased TS inhibition increases AZT phosphorylation by TK, resulting in increased DNA incorporation of AZT and increased DNA damage.

DNA double-strand breaks were determined in both MGH-U1 and HCT-8 cells exposed to either AZT, ICI D1694, or the combination of AZT and ICI D1694. The percentage of DNA double-strand

breaks caused by the combination of AZT plus ICI D1694 appeared to be additive.

ICI D1694 was determined to be a potent TS inhibitor, consistent with previous results [6, 8]. We found that ICI D1694 cytotoxicity paralleled TS inhibition. HCT-8 cells appeared more resistant than MGH-U1 cells to the cytotoxic effects of ICI D1694. This increased resistance was associated with a higher $IC_{50}$ for TS inhibition by ICI D1694 in HCT-8 cells than in MGH-U1 cells. This difference in sensitivity may be related to differences in folylpolyglutamate synthetase (FPGS) activity or differences in dTTP pools in the two cell lines.

AZT alone did not inhibit TS activity in either MGH-U1 or HCT-8 cells, nor did it increase TS inhibition of ICI D1694. This finding differs from that reported previously [4]. This difference may be due to the use of [$^3$H]deoxyuridine (dUrd) in the study of Brunetti *et al.* [4] and the use of [$^3$H]dCyd in ours. The [$^3$H]dUrd nucleoside must be phosphorylated by TK prior to being biotransformed by TS. AZT is also phosphorylated by TK, so that it will compete with [$^3$H]dUrd. Furthermore, AZT-MP may inhibit TK activity. Both these effects could result in an apparent inhibition of TS. This lack of effect on TS by AZT alone or in combination with ICI D1694 indicates that the enhanced cytotoxicity of AZT plus ICI D1694 was not mediated by a direct TS effect of AZT.

In both MGH-U1 and HCT-8 cells the increase in cytotoxicity observed with the combination treatment paralleled the increase in both AZT incorporation into DNA and AZT-MP formed. The comparable enhancement of cytotoxicity and AZT anabolism is consistent with our hypothesis that ICI D1694 will decrease dTTP formation, increase AZT activation and lead to enhanced cytotoxicity. Recently, Darnowski and Goulette [25] reported that TK activity is also increased in the presence of 5-FU. The increased formation of AZT-MP in our studies is consistent with this report.

AZT, ICI D1694 and their combinations were evaluated in normal human bone marrow CFU-GM cells as a surrogate for myelosuppression *in vivo*. No added toxicity was generated by this drug combination over ICI D1694 cytotoxicity alone. In fact, the TI was greater for AZT and ICI D1694 as compared with ICI D1694 alone for both cell lines. The difference in cytotoxicity produced by these agents alone and in combination between normal and tumor cells could be due to differences in the proportion of cells cycling, doubling time, or enzymatic profiles. It has been reported that tumor cells possess elevated amounts of TK, which results in an increased activity of this enzyme [26]. Hence, this would predict greater phosphorylation of AZT by tumor cells, leading to greater incorporation into DNA and greater cell death, as compared with normal cells. From our *in vitro* studies we would predict that *in vivo* this combination of AZT plus ICI D1694 will have a wider TI than ICI D1694 alone. In our studies we observed increased cytotoxicity when AZT and ICI D1694 were added simultaneously; however, other drug treatment schedules may show differing results. Further studies in drug scheduling with this combination are warranted.

In summary, biochemical and therapeutic exploitation of differences in TdR salvage in tumor cells may be possible when *de novo* thymidylate synthesis is inhibited, as with ICI D1694. The favorable therapeutic index of ICI D1694 and AZT in our studies indicates that this combination should be evaluated *in vivo*.

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