Cancer Chemother Pharmacol (1995) 35: 387–390

© Springer-Verlag 1995

ORIGINAL ARTICLE

Josie Pressacco · Biserka Mitrovski · Charles Erlichman

Cytotoxic and biochemical implications of combining AZT and AG-331

Received: 20 May 1994/Accepted: 22 September 1994

Abstract We have reported that noncytotoxic concentrations of 3′-azido-3′-deoxythymidine (AZT) increase the cytotoxicity of ICI D1694, a folate-based thymidylate synthase (TS) inhibitor, with increasing AZT incorporation into DNA. We postulated that the inhibition of TS by ICI D1694 would decrease 5′-deoxythymidine triphosphate (dTTP) pools, which compete with AZT triphosphate (AZT-TP) as a substrate for DNA polymerase. Furthermore, the inhibition of TS would increase the activity of both thymidine kinase (TK) and thymidylate kinase (TdK). Each of these consequences of TS inhibition would favor more incorporation of AZT into DNA. Thus, we reasoned that other TS inhibitors should also result in increased AZT incorporation into DNA and, perhaps, in increased cytotoxicity. N^6-[4-(Morpholinosulfonyl)benzyl]-N^6-methyl-2,6-diaminobenz[*cd*]indole glucuronate (AG-331) differs from ICI D1694 in that it is a de novo designed lipophilic TS inhibitor, it does not require a specific carrier for cellular uptake, and it does not undergo intracellular polyglutamation. This potent TS inhibitor causes minimal cytotoxicity in MGH-U1 human bladder cancer cells. A 24-h exposure to 5 μ*M* AG-331 causes almost complete TS inhibition but only 35% cell kill. The combination of AZT and AG-331 in MGH-U1 cells resulted in an enhanced antitumor effect relative to that of each agent alone; 50 μM AZT, noncytotoxic alone, increased the cell kill of induced by AG-331 from 35% to 50%. Biochemical studies of this combination revealed that simultaneous treatment with 5 μ*M* AG-331 plus 1.8 μ*M* [^{3}H]-AZT produced as much as a 68% ± 7% increase in AZT incorporation into DNA. This observation was associated with an increase in DNA single-strand breaks, measured as comet tail moment, of up to 6.6-fold. These studies support our original premise that TS inhibition favors increased incorporation of AZT into DNA and that the combination causes more cell kill than either drug alone in MGH-U1 cells.

Key words: AZT · AG-331 · Thymidylate synthesis

Abbreviations: *AZT*, 3′-Azido-3′-deoxythymidine · *TS*, thymidylate synthase · *dTTP*, 5′-deoxythymidine triphosphate · *AZT-TP*, AZT triphosphate · *TK*, thymidine kinase · *TdK* thymidylate kinase · *AG-331*, N^6-[4-(morpholinosulfonyl)benzyl]-N^6-methyl-2,6-diaminobenz[*cd*]indole glucuronate · *dTMP* thymidylate · *TdR*, thymidine

Supported by a grant from the NCI (Canada)

J. Pressacco · B. Mitrovski · C. Erlichman
Division of Experimental Therapeutics, The Ontario Cancer Institute, Toronto, Canada

J. Pressacco · C. Erlichman
Department of Pharmacology, University of Toronto, Toronto, Canada

C. Erlichman
Department of Medical Biophysics, University of Toronto, Toronto, Canada

C. Erlichman
Department of Medicine, University of Toronto, Toronto, Canada

C. Erlichman (✉)
Department of Oncology, Mayo Clinic E72B, 200 First Street, Southwest, Rochester, MN 55905, USA

Introduction

The inhibition of thymidylate synthase (TS), the key enzyme in the de novo synthesis of thymidylate (dTMP), can lead to thymineless cell death [1, 2]. Thymidylate pools can be partially replenished through the salvage of preformed thymidine (TdR) in plasma. Hence, the salvage pathway for pyrimidine nucleotide biosynthesis can decrease the therapeutic utility of agents that act by inhibiting de novo synthesis. However, previous studies have shown that

tumor cells possess elevated amounts of thymidine kinase (TK), the key enzyme in thymidylate salvage, resulting in increased activity of this enzyme in tumor versus normal cells [3]. In this regard, biochemical exploitation of differences in TdR salvage in tumor cells may be possible when TS inhibitors are combined with thymidine analogues (Fig. 1).

We have previously reported that non cytotoxic concentrations of 3′-azido-3′-deoxythymidine (AZT), a TdR analogue that passively diffuses into cells, increase the cytotoxicity of ICI D1694, a folate-based TS inhibitor, and is associated with increased AZT incorporation into DNA [4]. We had postulated that inhibition of TS by ICI D1694 would deplete dTMP levels and lead subsequently to decreased dTTP pools. Another consequence of TS inhibition is the increase in TK and thymidylate kinase (TdK) activity [5]. It follows that AZT phosphorylation would be increased, producing elevated amounts of its active metabolite, AZT triphosphate (AZT-TP) [4, 5]. Hence, increased levels of AZT-TP would be competing with previously depleted pools of 5′-deoxythymidine triphosphate (dTTP) at DNA polymerase for incorporation into cellular DNA.

In the present study we combined noncytotoxic concentrations of AZT with N^6-[4-(morpholinosulfonyl)benzyl]-N^6-methyl-2,6-diaminobenz[*cd*]indole glucuronate (AG-331), a lipophilic TS inhibitor [6]. AG-331 differs from the folate-based TS inhibitors in that it does not require the folate transporter to enter cells and it is not polyglutamated intracellularly [6, 7].

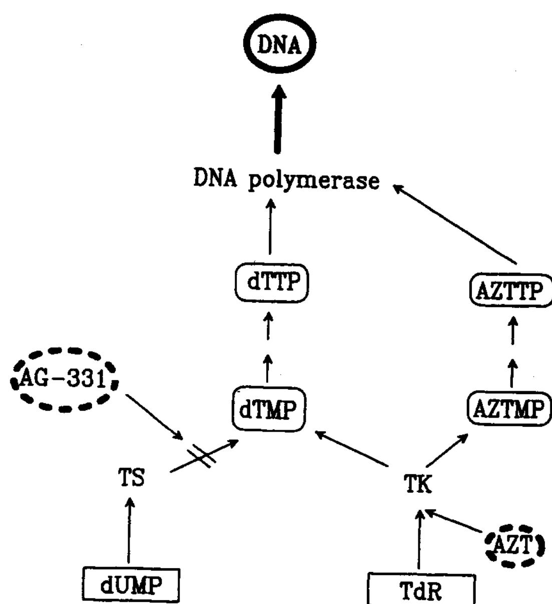

Fig. 1. Cessation of dTMP formation due to inhibition of TS by AG-331 in the de novo synthesis pathway, leading to increased uptake of AZT and AZT-TP formation with increasing incorporation of AZT-TP into DNA

In this report we provide cytotoxic and biochemical evidence to support our hypothesis that TS inhibition can increase the effectiveness of AZT by increasing its incorporation into cellular DNA.

Materials and methods

Chemicals

AG-331 was a gift from R. Jackson, Agouron Pharmaceuticals Inc. (La Jolla, Calif.), and AZT was purchased from Sigma (St. Louis, Mo). AZT was protected from light and drug dilutions were made in phosphate-buffered saline (PBS). [^{3}H]-AZT (14 Ci/mmol) was purchased from Moravek Biochemicals (Brea, Calif.). Media, PBS, antibiotics, and trypsin were purchased from Gibco (Grand Island, N.Y.). CytoScint liquid scintillation cocktail was purchased from ICN Biomedicals (Costa Mesa, Calif.). Plasticware was purchased from Falcon (Bedford, Mass). All other chemicals were of reagent grade and were obtained from Sigma.

Cell culture

The human bladder-cancer cell line MGH-U1 was maintained as a monolayer in alpha minimum essential medium (MEM) supplemented with 0.1% streptomycin, 0.1% penicillin, and 10% fetal calf serum (Whittaker, Walkersville, Md., and P.A. Biologicals, Sidney, Australia) at 37°C in a humidified atmosphere contining 5% CO_2 [8, 9] and was subcultured twice weekly until passage 20. Under these conditions, the doubling time of the cells growing exponentially was approximately 24 h and the plating efficiency was > 80%. Exponentially growing asynchronous cultures were used in all experiments.

Cytotoxicity assay

The clonogenic survival of drug-treated cells was performed as described previously [4, 8, 9]. Briefly, 1×10^6 cells were seeded in a 75-cm^2 flask in 10% dialyzed fetal calf serum (DFCS) and nucleoside-free MEM. After 24 h, the exponentially growing cells were exposed to various drug concentrations and combinations for an additional 24 h. Cells were then washed three times in calcium and magnesium-free PBS, trypsinized, syringed to obtain a single-cell suspension, counted, and plated in serial dilutions in replicates of six. At 2 weeks after plating, colonies were stained with methylene blue solution and then counted. Survival was expressed as a fraction relative to untreated control values

Incorporation of [^{3}H]-AZT into DNA

The incorporation of [^{3}H]-AZT into DNA was determined in vitro in MGH-U1 cells by a modification of the methods previously described [10]. Briefly, to assess the effect of AG-331 on the incorporation of [^{3}H]-AZT into nucleic acids, cells were seeded 5×10^4 in a 24-well plate with 1 ml of nucleoside-free 10% DFCS MEM for 48 h. Subsequently, [^{3}H]-AZT (1.8 μM, 25 μCi/well) was added either alone or in combination with different concentrations of AG-331. After 24 h, the cell number was determined and the cells were washed once with PBS and pelleted. The cells were then extracted twice with 100 µl of 0.2 N perchloric acid (PCA). The insoluble material was incubated at 37°C for 20 min with 100 µl of RNase solution [7.5 mg DNAse-free RNAse plus 50 ml of 50 μM TRIS-ethylenediaminetetraacetic acid (EDTA), pH 7.4]. The

reaction was stopped by the addition of 200 μl of 0.2 *N* PCA and the solution was centrifuged at 14,000 *g* for 10 min. The pellet was then dissolved in 5 ml of CytoScint liquid scintillation cocktail and the radioactivity was counted in a LS-330 Beckman Scintillation Counter.

DNA damage

The alkaline comet assay was used to detect DNA single-strand breaks, with some modifications to the methods described previously [11]. Following 24 h of drug exposure, cells were embedded in 1% agarose gel, lysed in a 1-*M* NaCl/0.03 *M* NaOH solution for 20 min, and subjected to electrophoresis (1 V/cm) for 10 min. After staining with 2.5 μg propidium iodide/ml for 30 min, a characteristic comet appeared with a tail consisting of DNA fragments. The samples were examined using a 25X objective with an Olympus epifluorescence microscope fitted with an intensified charge-coupled device (CCD) camera interfaced to a SAMBA 4000 image-analysis system (IPI, Chantilly, Va). The tail moment was defined [12] as the product of the relative amount of DNA in the comet tail multiplied by a measurement of the tail length. The tail moment represents the DNA single-strand breaks caused by AZT and AG-331 either alone or in combination.

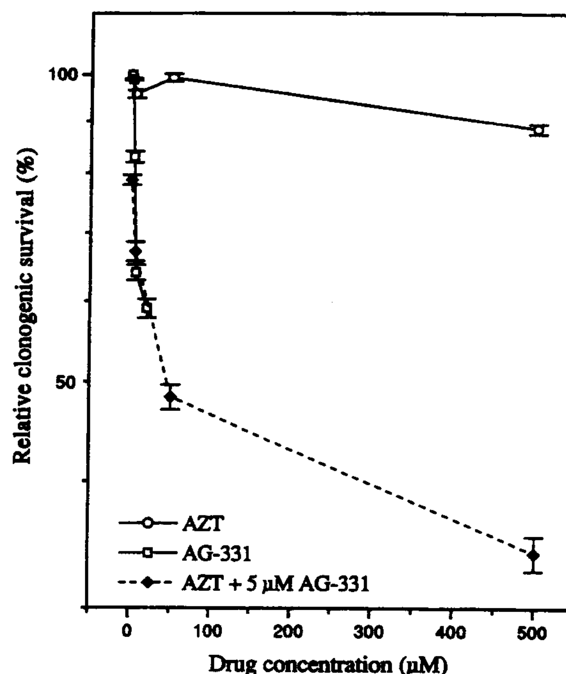

Fig. 2 Clonogenic survival of MGH-U1 cells treated with AG-331, AZT, and the combination of 5 μ*M* AG-331 plus AZT. Each point represents the mean value for at least three experiments ± SD

Results and discussion

The exact mechanism of action of AZT is not yet understood; however, proposed mechanisms of action for this agent have been cited: AZT could inhibit DNA synthesis through an imbalance of deoxyribonucleotide pools [13], or by incorporating itself into DNA in the place of dTTP [14, 15], or due to an inability of the cells to repair AZT-induced DNA damage [16]. Exposure of MGH-U1 cells to AZT at concentrations ranging from 5 to 500 μ*M* for 24 h resulted in less than 10% cytotoxicity (Fig. 2). The negligible cytotoxicity observed with AZT in MGH-U1 cells could be attributed to inadequate incorporation of AZT into DNA or to rapid excision repair in this particular cell line. Biochemical analysis confirmed that there were very low levels (0.0014 ± 0.0003 pmol/10^6 cells) of AZT incorporated into DNA. This may have been due to inhibition of TdK by AZT-MP [17], resulting in decreased AZT incorporation into DNA. Alternately, excision of AZT from DNA may be rapid, leading to few DNA single-strand breaks. Our studies with idoxuridine indicate that this TdR analogue is incorporated to a greater degree than AZT in this cell line and this is reflected in increased formation of DNA single-strand breaks [18]. Further studies to address the possibility of an increased rate of excision repair in AZT-induced DNA lesions will be necessary to assess the importance of DNA repair in the resistance to AZT.

AG-331 was also shown to cause minimal cytotoxicity in MGH-U1 cells at concentrations of 1–10 μ*M* for 24 h exposures [19]. Interestingly, 5 μ*M* AG-331 produces almost complete TS inhibition [19] but less than 35% cytotoxicity (Fig. 2). Perhaps the lack of polyglutamation and AG-331's lipophilic nature allows this agent to move freely in and out of cells. Thus, even though TS inhibition occurs, it may not be retained intracellularly for sufficiently long periods to deplete dTTP pools and cause inhibition of DNA elongation, which would be manifested by decreases in clonogenic survival.

When these agents are combined simultaneously, an enhanced cytotoxic effect occurs. In MGH-U1 cells exposed to the combination for 24 h, non-cytotoxic concentrations of AZT produced greater than 50% clonogenic inhibition with the addition of 5 μ*M* AG-331, which by itself produced less than 35% cytotoxicity. With AG-331 inhibition of TS activity, intracellular dTTP pools should decrease and both TK and TdK activity would increase. Hence, an increased amount of AZT-TP would be competing with previously depleted dTTP pools for incorporation into cellular DNA. Under these conditions, it is likely that more AZT-TP would be incorporated into DNA, which could lead to increased DNA damage and cytotoxicity.

Biochemical investigation of the incorporation of [^{3}H]-AZT into DNA and DNA single-strand breaks in the presence and absence of AG-331 supported our hypothesis. AG-331 increased the incorporation of [^{3}H]-AZT into DNA. In fact, 5 μ*M* AG-331 increased the incorporation of 1.8 μ*M* [^{3}H]-AZT by 68% ± 7% from 0.0014 ± 0.0003 pmol/10^6 cells in cells treated with AZT alone to 0.0024 ± 0.0004 pmol/10^6 cells when AZT and AG-331 were combined. In association with this increased incorporation of AZT into DNA,

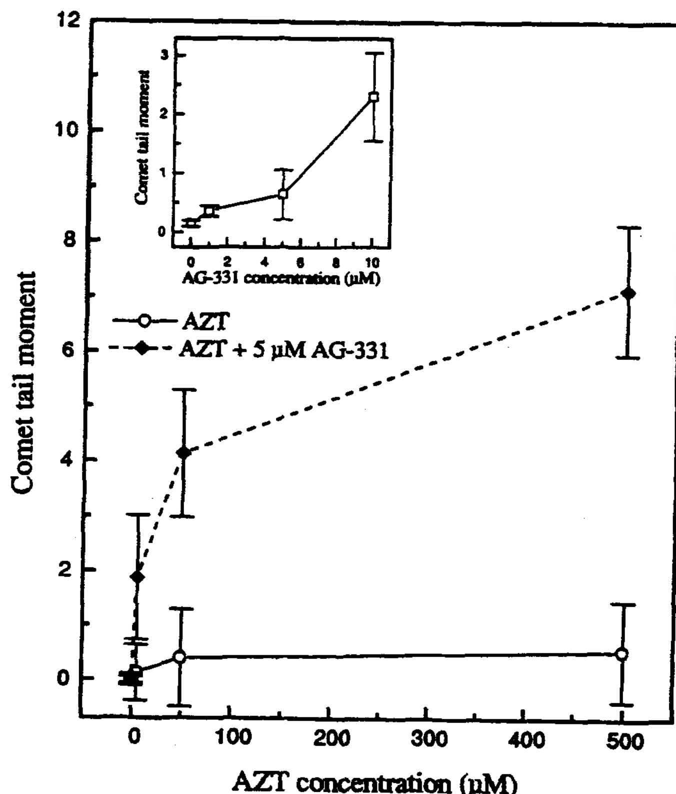

Fig. 3 DNA single-strand breaks caused by AG-331, AZT, and the combination of 5 μM AG-331 plus AZT as determined by comet tail. Each point represents the mean tail-moment value for 30 images from three separate experiments. Error bars represent the SD

increased numbers of DNA single-strand breaks were observed with the combination as compared with each agent alone. The combination of 5 μM AG-331, which produced a comet tail moment of less than 1.0, enhanced the damage induced by 5, 50 and 500 μM AZT by 1.7-, 3.7-, and 6.6-fold in comet tail moment, respectively (Fig. 3).

We conclude from our studies that exploitation of the TdR salvage pathway should be targeted for future development of improved chemotherapeutic combinations in the treatment of cancer.

References

1. Danenberg PV (1977) Thymidylate synthetase: a target enzyme in cancer chemotherapy. Biochim Biophys Acta 473: 73
2. Houghton JA, Houghton PJ, Woonten RS (1979) Mechanism of induction of gastrointestinal toxicity in the mouse by 5-fluorouracil, 5-fluorouridine and 5-fluoro-2′-deoxyuridine. Cancer Res 39: 2406
3. Weber G (1983) Biochemical strategy of cancer cells and the design of chemotherapy: G.H.A. Clowes Memorial Lecture. Cancer Res 43: 3466
4. Pressacco J, Erlichman C (1993) Combination studies with 3′-azido-3′-deoxythymidine (AZT) plus ICI D1694: cytotoxic and biochemical effects. Biochem Pharmacol 46: 1989
5. Darnowski JW, Goulette FA (1993) Increased azidodeoxythymidine metabolism in the presence of fluorouracil reflects increased thymidine kinase activity. Proc Am Assoc Cancer Res 34: 302
6. Jones TR, Varney MD, Webber SE, Welsh KM, Webber S, Matthews DA, Appelt K, Smith WS, Janson C, Bacquet R, Lewis KK, Marzoni GP, Kathardekar V, Howland E, Booth C, Herrmann S, Ward R, Sharp J, Moomaw E, Bartlett C, Morse C (1990) New lipophilic thymidylate synthase inhibitors designed from the X-ray structure of the *E. coli* enzyme. Proc Am Assoc Cancer Res 31: 340
7. Nicander B, Reichard P (1983) Dynamics of pyrimidine deoxynucleoside triphosphate pools in relationship to DNA synthesis in 3T6 mouse fibroblasts. Proc Natl Acad Sci, USA 80:1347
8. Erlichman C, Vidgen D (1984) Cytotoxicity of adriamycin in MGH-U1 cells grown as monolayer cultures, spheroids and xenografts in immune-deprived mice. Cancer Res 44: 5369
9. Mackillop WJ, Stewart SS, Buick RN (1982) Density/volume analysis in the study of cellular heterogeneity in human ovarian carcinoma. Br J Cancer 45: 812
10. Chu M-Y, Fischer GA (1968) The incorporation of ^{3}H-cytosine arabinoside and its effect on murine leukemic cells (L5178Y). Biochem Pharmacol 17: 553
11. Olive PL, Banath JP, Evans HH (1993) Cell killing and DNA damage by etoposide in Chinese hamster V79 monolayers and spheroids: influence of growth kinetics, growth environment and DNA packaging. Br J Cancer 67: 522
12. Olive PL, Banath JP, Durand RE (1990) Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. Radiat Res 122: 86
13. Tosi P, Calabresi P, Goulette FA, Renaud CA, Darnowski JW (1992) Azidothymidine-induced cytotoxicity and incorporation into DNA in the human colon tumor cell line HCT-8 is enhanced by methotrexate in vitro and in vivo. Cancer Res 52: 4069
14. Frick LW, Nelson DJ, St Clair MH, Furman PA, Krenitsky TA (1988) Effects of 3′-azido-3′-deoxythymidine on the deoxynucleotide triphosphate pools of cultured human cells. Biochem Biophys Res Commun 154: 124
15. Sommadossi J-P, Carlisle R, Zhou Z (1989) Cellular pharmacology of 3′-azido-3′-deoxythymidine with evidence of incorporation into DNA of human bone marrow cells. Mol Pharmacol 36: 9
16. Vazquez-Padua MA, Starnes MC, Cheng Y-C (1990) Incorporation of 3′-azido-3′-deoxythymidine into cellular DNA and its removal in a human leukemic cell line. Cancer Commun 2: 55
17. Furman PA, Fyfe JA, St Clair MH, Weinhold K, Rideout JL, Freeman GA, Lehrman SN, Bolognesi DP, Broder S, Mitsuya H, Barry DW (1986) Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc Natl Acad Sci USA 83: 8333
18. Pressacco J, Hedley DW, Hu Q, Chow S, Newcombe D, Erlichman C (1994) D1694 biochemical modulation of idoxuridine (IUDR) causes synergistic cytotoxicity. Proc Am Assoc Cancer Res 35: 326
19. Mitrovski B, Johnston PG, Erlichman C (1994) Cytotoxic and biochemical effects of a lipophilic (AG-331) and a non-lipophilic (D1694) thymidylate synthase inhibitor in MGH-U1 cells. Proc Am Assoc Cancer Res 35: 300