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Effects of Thymidylate Synthase Inhibition on Thymidine Kinase Activity and Nucleoside Transporter Expression

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ABSTRACT

The effects of *de novo* dTMP inhibition by *N*-{5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl}-L-glutamic acid (D1694) or N^6-[4-(morpholinosulfonyl)benz]-N^6-diaminobenz[*cd*]indole glucuronate (AG-331) on clonogenic survival, thymidylate synthase (TS) and thymidine kinase (TK) activity, and expression of *S*-(*p*-nitrobenzyl)-6-thioinosine-sensitive nucleoside transporter (NT) sites were addressed in the human bladder cancer cell line, MGH-U1. These two TS inhibitors are structurally diverse. D1694 is a folate-based TS inhibitor, whereas AG-331 is a novel agent that inhibits the cofactor binding site of the enzyme. They also differ with respect to their cytotoxic effects in this cell line; D1694 cytotoxic 50% inhibitory concentration (IC_{50}) and IC_{90} were 6.0 and 9.0 nM, respectively, and IC_{50} and IC_{90} for TS inhibition were 2.5 and 4.8 nM, respectively. In contrast, AG-331 cytotoxic IC_{50} could not be achieved even at concentrations of up to 20 μM for 24-h exposures, and IC_{50} and IC_{90} for TS inhibition were 0.7 and 3.0 μM, respectively. Similar effects for D1694 and AG-331 were observed in their modulation of TK activity and NT expression. 5-(SAENTA-x_8)-Fluorescein, a highly modified form of adenosine incorporating a fluorescein molecule which binds with a 1:1 stoichiometry to *S*-(*p*-nitrobenzyl)-6-thioinosine-sensitive NT sites, was used to investigate the expression of NT following exposure of cells to D1694 and AG-331. TK activity was addressed by the metabolism of [^{3}H]thymidine to [^{3}H]TMP by cellular extracted protein and by an alternative flow cytometric method using a modified form of thymidine incorporating a fluorescent molecule, dansyl-5-amino-2-deoxyuridine. Results obtained by both methods were comparable. At concentrations of 5 and 10 nM, D1694 increased TK activity 2.3–4.5-fold and NT expression 34–39-fold. AG-331, at concentrations of 5 and 10 μM, increased TK activity 1.8–2.5-fold and NT expression 22–31-fold, respectively.

These data suggest that TK activity and NT expression have a common regulatory mechanism which is sensitive to endogenous dTTP pools and that the salvage pathway is a complex system of kinases coordinated with transport of nucleosides.

INTRODUCTION

dTMP is synthesized by two pathways, the *de novo* and the salvage. The *de novo* pathway can be inhibited at TS[2] by agents such as 5-FUra (1), D1694 (2), and AG-331 (3). Repletion of decreased intracellular dTMP levels relies on the transport of preformed dThd from the extracellular fluid and phosphorylation by thymidine kinase in the salvage pathway. Although the substrates and products of TS and TK are similar, they have been reported to have different regulatory mechanisms in cells (4). The regulation of TS is proposed to be coupled to folate metabolism (5), whereas TK is dependent on the cellular dTTP levels (6, 7).

Nucleoside salvage involves transport and phosphorylation to nucleotide forms. In mammalian cells initial uptake is by facilitated diffusion via the ubiquitous, NBMPR-sensitive, equilibrative nucleoside transporter (*es*) (9–11). In some cell types, *es* is coexpressed with other NT processes, such as the NBMPR-insensitive equilibrative NT (*ei*) (12, 13), or the Na^+-linked concentrative NT process (*cif*) (13–15). Nucleoside transport appears to be an important step in the salvage of preformed nucleoside because kinetic and computer analysis show that the transport of nucleosides is rate limiting at low concentrations (~1 μM) of nucleosides (16). Thymidine concentrations of this order have been reported in human serum (17).

D1694 is a folate-based TS inhibitor (2, 18) that is extensively metabolized intracellularly to its polyglutamate derivatives. Polyglutamation increases its affinity for TS, both *in vitro* and *in vivo* (2, 19). Whereas the monoglutamate of D1694 has similar inhibitory activity against TS and dihydrofolate reductase, the polyglutamates have increasing specificity for TS (19). In addition, polyglutamated forms of this agent are retained intracellularly so that prolonged TS inhibition occurs (19–21). In contrast, AG-331 is a lipophilic TS inhibitor that differs from the folate-based TS inhibitors because it does not utilize a folate transporter and it is not polyglutamated (3).

Cells can be rescued from the effects of TS inhibition *in vitro* by the addition of thymidine to the growth medium (20). Because it has been reported previously that TS inhibition results in an increase in TK activity (8), we postulated that there may also be increased initial dThd uptake by NT. We therefore measured expression of NT by flow cytometry using the specific ligand 5-(SAENTA-x_8)-fluorescein recently described by Jamieson *et al.* (22). This compound binds with high affinity and specificity to NBMPR-sensitive nucleoside transport sites on the plasma membrane. The effects of TS inhibitors on TK activity were analyzed by two different methods. Cellular protein extracts were analyzed using a standard biochemical assay which measures the conversion of [^{3}H]dThd to [^{3}H]dTMP (23). In addition, *in situ* enzyme activity in viable cells was measured using a fluorescent dThd analogue, AUdR/DANS (24), which is taken up and phosphorylated by cells as the naturally occurring dThd. Analysis of the uptake of this compound into the intracellular pools in single cells by flow cytometry gives a measure of relative TK activity in control *versus* drug-treated cells (24).

In this paper we report the effects of the TS inhibitors D1694 and AG-331 on clonogenic survival, TS and TK activity, and NT expression in the human bladder cancer cell line, MGH-U1.

MATERIALS AND METHODS

Chemicals. 5-(SAENTA-x_8)-Fluorescein was the gift of Drs. G. Jamieson and J. Wiley, Melbourne, Australia, and was synthesized as described previously (22). AUdR/DANS was the gift of Dr. E. Wawra, University of Vienna, Vienna, Austria, and was synthesized as described by Hengstschläger and Wawra (24). ICI D1694, a gift from ICI Pharmaceuticals (Alderly Park, Macclesfield, Cheshire, United Kingdom) was dissolved in 0.4 M sodium bicarbonate. AG-331 was a gift from R. Jackson, Agouron Pharmaceuticals, Inc. (La Jolla, CA), and was dissolved in water. [^{3}H]dThd (6.7 Ci/mmol) was

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[2] The abbreviations used are: TS, thymidylate synthase; 5-FUra, 5-fluorouracil; D1694, *N*-{5-[-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl}-L-glutamic acid; AG-331, N^6-[4-(morpholinosulfonyl)benz]-N^6-diaminobenz[*cd*]indole glucuronate; dThd, thymidine; TK, thymidine kinase; NT, nucleoside transporter; NBMPR, *S*-(*p*-nitrobenzyl)-6-thioinosine; RT, room temperature; IdUrd, 5-iodo-2′-deoxyuridine; AZT, 3′-azido-3′-deoxythymidine.

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purchased from Dupont NEN (Boston, MA). Media, PBS, antibiotics, and trypsin were purchased from GIBCO (Grand Island, NY). Plasticware was purchased from Falcon (Bedford, MA). NBMPR and all other chemicals were reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. The human bladder cancer cell line, MGH-U1, was maintained as a monolayer in α-MEM supplemented with 0.1% streptomycin, 0.1% penicillin, and 10% FCS (Whittaker, Toronto, Ontario, Canada) at 37°C in a 5% CO_2 humidified atmosphere (25, 26) and subcultured twice weekly until passage 20. Under these conditions, the doubling time of the cells growing exponentially was approximately 24 h and 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 (26) by seeding a single cell suspension of 1×10^6 cells in a 75-cm^2 flask in 10% dialyzed FCS and nucleoside-free α-MEM. After 24 h, the exponentially growing cells were exposed to various drug concentrations for an additional 24 h. Cells were then washed three times in calcium- and magnesium-free PBS prior to trypsinization. The cells were then dispersed with a syringe to obtain a single-cell suspension, counted, and plated in serial dilutions in replicates of six. Two weeks after plating, the colonies were stained with methylene blue and counted. Survival was expressed as a percentage relative to control cells.

Thymidylate Synthase Assay. As previously reported (27), 5×10^4 cells were seeded in a 24-well plate. Following a 48-h incubation, the cells were drug treated for an additional 24 h. The reaction was then initiated by the addition of [^{3}H]deoxyuridine (3 μCi/well; 17 Ci/mmol) and terminated by transferring 100-μliter aliquots into 1-ml centrifuge tubes containing 100 μliter of a 15% activated charcoal suspension in 4% aqueous perchloric acid. The tubes were then vortexed and centrifuged at 14,000 $\times$ *g* for 10 min at 4°C. The radioactivity of a 100-μliter aliquot of supernatant fraction from each sample was counted in a Beckman LS 330 liquid scintillation spectrometer. Each value obtained was corrected for background counts.

Thymidine Kinase Assay. The radioactive assay for analyzing TK activity is based on the conversion of [^{3}H]dThd to [^{3}H]dTMP and their separation on anion exchange filters. As described previously (23), with some minor modifications, MGH-U1 cells were washed twice with cold PBS following drug treatment. The cells were then scraped off the plates and centrifuged at 1000 $\times$ *g* for 10 min at 4°C. The pellet was then resuspended in 100 μliter of PBS ($\sim 5 \times 10^6$ cells/ml) and freeze-thawed three times by alternating the tubes from a dry ice:methanol to a lukewarm water bath. The samples were then centrifuged at 14,000 $\times$ *g* at 4°C for 60 min. The supernatant was then assayed for TK activity. The reaction mixture, with a final volume of 100 μliter, contained 0.19 M Tris-HCl (pH 7.5), 12.5 mM NaF, 2.5 mM $MgCl_2$, 2 mM DTT, 1% BSA, 4.5 mM phosphocreatine, 2.5 mM ATP, 6 units/ml creatine kinase, 6.7 Ci/mmol [^{3}H]dThd, and an appropriate volume of the enzyme mixture which contained the protein extracted (supernatant) in 0.05 M Tris-HCl (pH 7.5), 2 mM DTT, and 10% glycerol. Incubation was performed at 37°C in a shaking water bath for various time points (0–60 min). Following incubation, 50 μliter of the reaction mixture were spotted onto DEAE-paper (anion exchange) filters which were immediately washed three times with 95% ethanol (10 ml/filter/10 min). The filters were placed at RT to dry and counted for ^{3}H with 7.5 ml of aqueous counting scintillant in an LS 330 Beckman scintillation counter.

The flow cytometric TK assay utilizes the compound AUdR/DANS, which was synthesized by the methods of Hengstschläger and Wawra (24), a fluorescent nucleoside which is taken up and phosphorylated by cells as the naturally occurring dThd nucleoside. Analysis of the uptake of this compound into the intracellular pools in single cells by flow cytometry gives an indication of relative TK activity in control *versus* drug-treated cells. According to the method of Hengstschläger and Wawra (24), cells were incubated with 1.5 μM/liter AUdR/DANS in PBS for 1 h at 37°C, trypsinized, and then resuspended in cold PBS. Fluorescence was measured using an Epics Elite flow cytometer (Coulter, Hialeah, FL) using a 325 nm helium-cadium laser to excite AUdR/DANS and measuring emission at 450 nm. Forward and right angle scatter signals were used to gate viable cells.

5-(SAENTA-x_8)-Fluorescein-binding Assay. Binding of 5-(SAENTA-x_8)-fluorescein was measured by flow cytometry as described previously (22). Cells were preincubated for 10 min at RT with or without 2.5 μM NBMPR in phenol red-free medium and then with the addition of 5 nM 5-(SAENTA-x_8)-fluorescein, which saturates NBMPR-sensitive NT sites, for an additional 10 min at RT. Specific binding of 5-(SAENTA-x_8)-fluorescein was calculated from the difference between mean fluorescence intensities obtained with and without NBMPR.

RESULTS

Drug Cytotoxicity. Clonogenic survival was determined in MGH-U1 cells exposed to D1694 or AG-331 for 24 h in nucleoside-free media. D1694 had greater cytotoxic effects compared to AG-331 (Fig. 1). The IC_{50} and IC_{90} for D1694 was determined as 6.0 and 9.0 nM. An IC_{50} for AG-331 could not be achieved even at concentrations up to 20 μM with 24-h exposures.

Inhibition of Thymidylate Synthase Activity. Inhibition of TS activity in MGH-U1 cells was observed with both D1694 and AG-331 at 24-h exposures, although D1694 appeared to be more potent than AG-331 (Fig. 2). The IC_{50} and IC_{90} for D1694 were 2.5 and 4.8 nM, respectively, and for AG-331 they were 0.7 and 3.0 μM, respectively.

Thymidine Kinase Activity. The activity of TK was investigated with two different experimental techniques, [^{3}H]dThd kinetic assay and AUdR/DANS detection by flow cytometry. With both assays, there was a consistent increase in TK activity following 24-h exposure to either D1694 or AG-331, as compared to control untreated cells (Table 1). Analysis of the results obtained by the two different methods were comparable.

Modulation of Nucleoside Transporter. Treatment of MGH-U1 cells with D1694, at 5.0 and 10 nM for 24 h increased the expression of NT sites in the range of 34–39-fold as compared to control untreated MGH-U1 cells (Fig. 3). This increase in NT expression was also evident with AG-331, at concentrations of 5.0 and 10 μM exposed for 24 h in MGH-U1, in the range of 22–31-fold. Specificity of 5-(SAENTA-x_8)-fluorescein-binding was confirmed by coincubating the cells with a 500-fold excess of the nonfluorescent compound NBMPR, which reduced cell fluorescence essentially to that of background autofluorescence.

DISCUSSION

Thymidylate synthase inhibitors, such as D1694 and AG-331, block the formation of dTMP and deplete dTTP pools so that proliferating

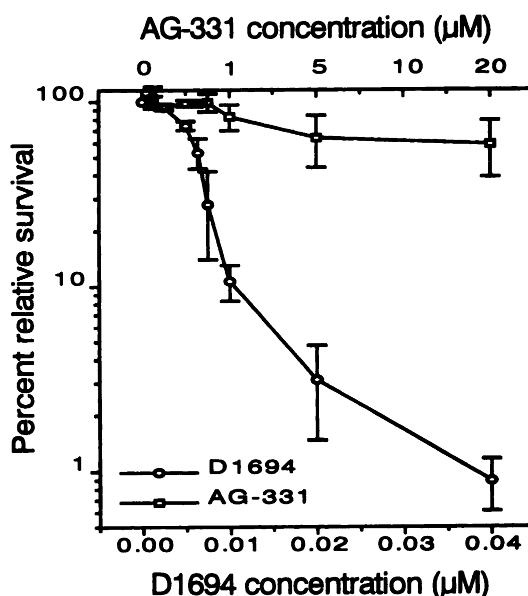

Fig. 1. Clonogenic survival of MGH-U1 cells exposed to either D1694 or AG-331 for 24 h. *Points*, mean of at least three separate experiments; *bars*, SD.

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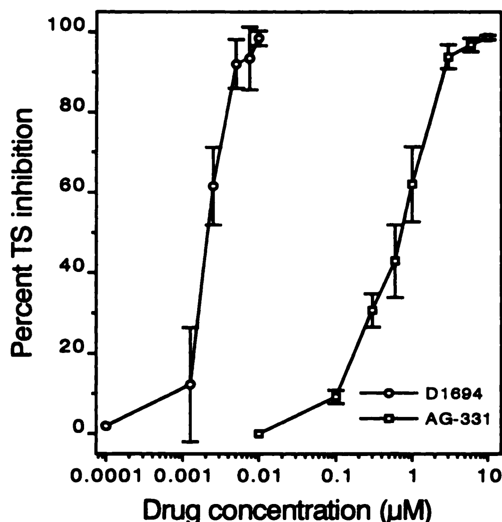

Fig. 2. Percentage of TS inhibition in MGH-U1 cells exposed to either D1694 or AG-331 for 24 h. *Points,* mean of at least three separate experiments; *bars,* SD.

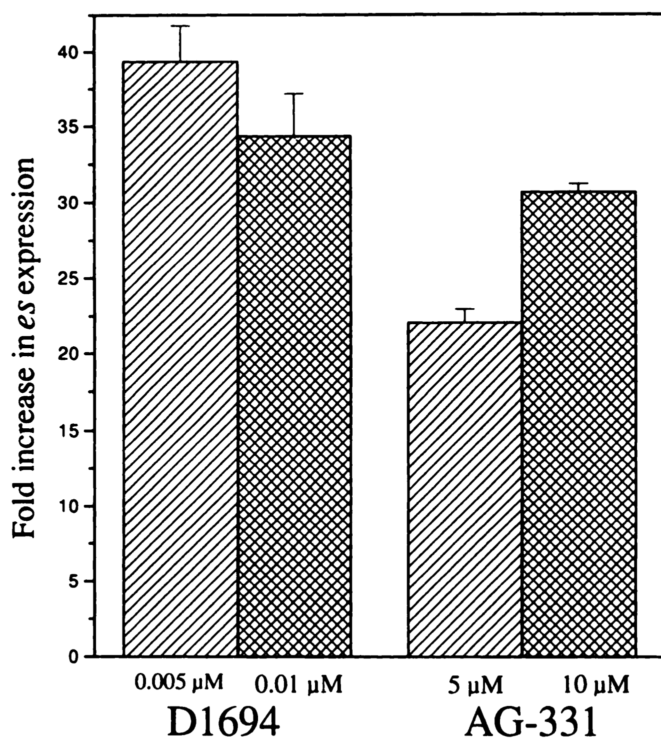

Fig. 3. Effect of TS inhibition by D1694 and AG-331, exposed for 24 h, on NT expression on the cellular membrane of MGH-U1 cells. *Columns,* mean of at least three separate experiments; *bars,* SD.

cells then depend on the salvage of preformed dThd from the extracellular fluid. Uptake of preformed nucleosides from the extracellular fluid occurs via specific transport elements that mediate both entry and exit of nucleosides (28, 29). Substrate specificity for the ubiquitous equilibrative, NBMPR-sensitive nucleoside transporter is broad and includes such structurally diverse molecules as purine and pyrimidine ribonucleosides and deoxyribonucleosides, synthetic nucleosides, and nucleoside analogues. Once inside the cells, nucleosides are phosphorylated by specific kinases, such as TK, thus trapping them intracellularly. TK activity is commonly determined with the [^{3}H]dThd assay utilizing extracted cellular protein containing the TK enzyme (23). As an alternative, we used flow cytometric analysis of AUdR/DANS uptake as described by Hengstschläger and Wawra (24). This is a viable cell assay that does not disrupt the cell membrane or other biochemical processes taking place in the cell, whereas the [^{3}H]dThd assay requires the cellular extraction of protein. Results for the two TK assays were comparable in this study. A previous study inhibiting thymidylate synthesis with 5-FUra reported a 1.4-fold increase in activity of TK in the human colon cancer cell line HCT-8 (8). Although the enzyme assay method differed from those used in the present study (30), the results are similar to those reported here using the TS inhibitors D1694 or AG-331. Although we have not measured dTTP our results are consistent with TK activity being sensitive to TS inhibition and to depletion of dTTP. An additional element which may contribute to the increase in TK activity is increased TK gene transcription and/or mRNA translation. Measurement of TK protein and mRNA will be necessary to elucidate the contribution of this factor.

We have also shown that NT increases with TK, although the increase in NT expression is much more dramatic. D1694 and AG-331 increased TK activity by up to 3.7- and 2.5-fold, respectively, and NT expression up to 39- and 31-fold, respectively. Although D1694 and AG-331 differ with respect to their cytotoxic potency in MGH-U1 cells, these TS inhibitors have similar effects on TK activity and NT expression at concentrations that cause equivalent degrees of TS inhibition, suggesting that NT expression and TK activity are coregulated. Although deoxynucleotide depletion has been shown to increase nucleoside analogue uptake, this has been attributed to effects in kinases (31).

Both D1694 and AG-331 increased NT expression to a greater extent than they affected TK activity. There are a number of possible explanations for this. The assays for TK measure enzyme activity, whereas the NT assay measures the amount of protein on the cell surface. It is possible that some of the NT measured by this assay is inactive. Another explanation may be that the rate of transport of nucleoside is slower than the rate of phosphorylation by TK. Thus, levels of NT must be increased to a greater degree to facilitate the supply of nucleoside substrate for the relatively small increase in TK activity. Further studies of nucleoside influx and efflux in the absence or presence of TS inhibitors will be necessary to elucidate this further.

The relevance of NT to cancer treatment has two different aspects. First, reduced expression might be associated with resistance to treatment using modified nucleosides that enter cells via NT, such as 1-β-D-arabinofuranosylcytosine (16). On the other hand, increased capacity to salvage preformed nucleosides from extracellular fluid is believed to confer resistance to antimetabolite drugs, such as methotrexate (32, 33) and 5-FUra (34), that inhibit the synthesis of DNA precursors in the *de novo* pathway. This later aspect can be exploited by combining TS inhibitors with dThd analogues, and we have reported previously that D1694 increases IdUrd uptake and incorporation into DNA (35), whereas the combination of AZT and D1694 (26) resulted in a smaller increase in nucleoside analogue incorporation into DNA. AZT differs from IdUrd in that it passively diffuses into

Table 1 *Fold increase in TK activity in MGH-U1 following 24-h exposure to either D1694 or AG-331*

Two different methods of analysis were utilized: (a) AUdR/DANS detection by flow cytometry in viable cells; and (b) metabolism of [^{3}H]dThd to [^{3}H]dTMP by cellular extracted TK. Each value represents the mean of three separate experiments ± SD.

Method	D1694 0.005 μM	D1694 0.01 μM	AG-331 5.0 μM	AG-331 10 μM
AUdR/DANS	3.31 ± 0.46	2.99 ± 0.99	1.81 ± 0.81	2.46 ± 1.05
[^{3}H]dThd	NA[a]	4.52 ± 0.66	NA	2.35 ± 0.17

[a] NA, not assayed.

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and out of cells, whereas IdUrd requires NT for cell entry. We now report that D1694 increases both NT expression and TK activity, suggesting that the relatively small increase in AZT incorporation into DNA may have been due to increased TK activity only, whereas with D1694 plus IdUrd both increased NT expression and TK activity might contribute to the increased incorporation of IdUrd into DNA. Further studies addressing advantages of utilizing agents to increase NT expression and their application to clinical investigations are warranted.

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Effects of Thymidylate Synthase Inhibition on Thymidine Kinase Activity and Nucleoside Transporter Expression

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