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Biological activity of a novel rationally designed lipophilic thymidylate synthase inhibitor

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Abstract. AG-331 {N[6][4-(*N*-morpholinosulfonyl)benzyl]-*N*[6]-methyl-2,6-diamino-benz[*cd*]indole glucuronate} is a novel lipophilic thymidylate synthase (TS) inhibitor. The properties of this compound were investigated in H35 rat hepatoma cells and in three variant cell lines resistant to antifolates by differing mechanisms. There was no evidence for any intracellular effect of AG-331 on dihydrofolate reductase (DHFR); however, the low degree of cross-resistance found for the H35FF line, which has elevated TS levels, suggested that TS may not be the sole locus of action of AG-331 in hepatoma cells. TS-directed effects of AG-331 were suggested by the pattern of its inhibition of deoxyuridine incorporation into DNA and the lesser effects on purine incorporation. In addition, H35 cells treated with 10 μ*M* AG-331 were shown to accumulate in the S phase of the cell cycle, and this effect could be reversed by coadministration of thymidine. However, when treatments were conducted at a 5-fold higher concentration of AG-331, no S-phase block was apparent, suggesting the loss of a TS-directed effect at high inhibitor concentrations. Thymidine and folinic acid also failed to protect cells against AG-331 cytotoxicity, suggesting an alternate mode of action. Similar results were also obtained in protection experiments with a human hepatoma cell line, HEPG2, although previous results obtained in colon- and breast-cancer cell lines have suggested TS specific effects for AG-331. The possibility that biotransformation of AG-331 to other toxic species may occur in liver-derived cell lines has yet to be investigated.

Introduction

The crucial role of thymidylate synthase (TS, EC2.1.1.45) in the de novo synthesis of thymidylate (dTMP), a nucleotide exclusively incorporated into DNA, makes it an appropriate target for anticancer agents. TS catalyzes the reductive transfer of a methyl group from its cofactor 5,10-methylenetetrahydrofolate (5,10-CH_2FH_4) to deoxyuridylate (dUMP) to form dihydrofolate and dTMP. Inhibitors of TS that bind either at the substrate binding site (antipyrimidines) or at the cofactor binding side (antifolates) have elicited antitumor activities in the clinic [2, 29]. 5-Fluorouracil (5-FU) is converted to the potent mechanism-based TS inhibitor 5-fluorodeoxyuridylate (5-FdUMP) and has been used for several decades in the treatment of gastrointestinal tumors [29]. The mechanism of action of 5-FU is complicated by the observation that its metabolites may be incorporated into RNA and/or DNA, resulting in unfavorable toxicities [29]. The classic folate-based TS inhibitor N^{10}-propargyl-5,8-dideazafolate (CB3717) showed activity against breast, ovarian, liver, and lung cancer [1, 5, 6, 33, 35]. However, it did possess some unacceptable kidney toxicities that resulted in its withdrawal from clinical study. The potent 2-desamino-2-methyl analog DMPDDF was much more water-soluble, transported by the reduced folate/methotrexate transport system, readily polyglutamylated, and hence more cytotoxic [16, 19]. The water-soluble N^{10}-methyl thiophene ring analog ICI D1694 has subsequently entered clinical trial and looks promising [20, 23]. These antifolate TS inhibitors rely upon an active transport mechanism to enter cells; thus, their activity can be hindered by a mutation in this transport process [21]. Another characteristic of classic antifolates is their ability to be polyglutamylated by the enzyme folylpolyglutamyl synthetase (FPGS, EC 6.3.2.17). CB3717, DMPDDF, and ICI D1694 are approximately 100 times more potent as TS inhibitors when polyglutamylated [18, 32] and may even be dependent upon polyglutamylation to elicit their cytotoxic effect. Hence, mutations in FPGS can be another mechanism of resistance to classic antifolates [21]. In addition, levels of the enzyme that cleaves the polyglutamates can be elevated, resulting in acquired resistance to antifolylglutamates [32]. Furthermore, the TS-inhibiting antifolates can also inhibit dihydrofolate reductase (DHFR, EC 1.5.1.3), which complicates the interpretation of their in vivo activity [17].

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The development of a folate-based lipophilic TS inhibitor has been attempted by several investigators in recent years as a means of overcoming some of the problems outlined above. Encouraged by the success seen with the lipophilic DHFR inhibitor trimetrexate (TMTX, reviewed in [9]), Bisset and co-workers [3] replaced the CO-glutamic acid moiety of DMPDDF with lipophilic substituents. The NO_2 substituent was found to be the most potent but was nonetheless 30 times less active than CB3717 as a TS inhibitor [3]. McNamara et al. [26] examined similar substitutents in the 2-amino and 2-desamino series and concluded that the CO-glutamic acid moiety was not absolutely necessary for potent inhibition of TS and cell growth.

AG-331, N^6[4-(*N*-morpholinosulfonyl)benzyl]-N^6-methyl-2,6-diamino-benz[*cd*]indole glucuronate (see Fig. 1), a rationally designed lipophilic TS inhibitor, was synthesized on the basis of knowledge of the X-ray crystallographic threedimensional structure of the active site of *Escherichia coli* TS [34]. AG-331 binds at the folate-binding site of TS despite showing limited structural resemblance to the cofactor 5,10-CH_2FH_4. This compound was found to be a potent inhibitor of purified recombinant human TS with a K_i value (0.012 μ*M*) of the same order of magnitude as that of DMPDDF [27]. AG-331 has shown significant cytotoxicity against a number of murine and human tumor cell lines [34] as well as against leukemia and colon tumors in mice [36] and has recently entered phase I clinical study. Its effect on solid tumors will be investigated in subsequent phase II trials.

We studied the biological properties of AG-331 in the H35 rat hepatoma cell line and compared them with those of established antifolates: the DHFR inhibitors MTX and TMTX and the TS inhibitor DMPDDF.

Materials and methods

Folate analogs were kindly provided as follows: MTX, by Lederle Laboratories (Pearl River, N. Y.); TMTX, by Dr. D. Fry, Warner Lambert Laboratories (Ann Arbor, Mich.); and DMPDDF, by Dr. M. G. Nair, University of South Alabama. [6-^{3}H]-deoxyuridine and [2-^{14}C]-glycine were purchased from Moravek Biochemicals (Brea, Calif.) and DuPont-NEN Research Products (Boston, Mass.), respectively. All other chemicals were of the highest purity available and were obtained from commercial sources.

Cell culture. Mycoplasma-free H35 rat hepatoma cells were maintained in monolayer culture as previously described [10]. H35RO.3 cells with acquired resistance to MTX (100-fold) were maintained in 0.3 μ*M* MTX and were resistant by virtue of a defect in transport [10]. H35R10 cells with acquired resistance to MTX (1,000-fold) were maintained in 10 μ*M* MTX and displayed both a defect in transport and an increase in DHFR activity [11]. H35FF cells were maintained in 0.1 μ*M* 5-fluorodeoxyuridine (5-FdUrd) and 25 μ*M* folinic acid and had a 100-fold increase in TS activity [30]. H35RO.3, H35R10, and H35FF cells were cultured in the absence of the selective agent for 1 week prior to their use in experiments. Growth inhibition was measured in 96-well dishes at a plating density of 1×10^4 cells/well (area, 0.69 mm^2) in the presence or absence of inhibitor for 72 h to estimate the concentrations required to produce a 50% reduction in the numbers of treated cells relative to controls (IC_{50} values). Cells were counted by direct counting and by a spectrophotometric assay that utilizes methylene blue staining as described by Finlay et al. [8].

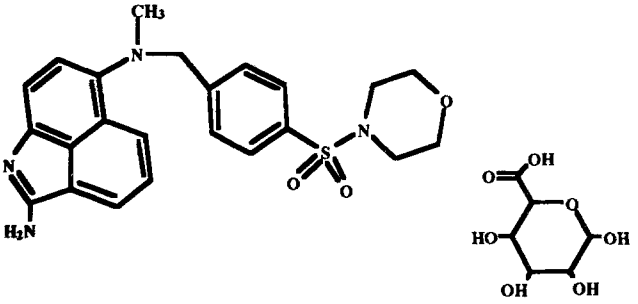

Fig. 1. Structure of AG-331, N^6-[4-(*N*-morpholinosulfonyl)benzyl]-N^6-methyl-2,6-diamino-benz[*cd*]indole glucuronate

Protection studies were conducted with folinic acid (100 μ*M*), hypoxanthine (50 μ*M*), and/or thymidine (20 μ*M*) present throughout the culture period.

De novo thymidylate and purine biosynthesis. Cells were plated at 2×10^5 cells/60-mm dish and cultured for 72 h, with various drug concentrations being present for the last 4 h. De novo thymidylate biosynthesis was estimated by measuring [6-^{3}H]-deoxyuridine incorporation into DNA according to the method of Duch et al. [7], which was modified as previously described [12], with [6-^{3}H]-deoxyuridine being present for the last 30 min of the 72-h culture period. Measurement of de novo purine biosynthesis was accomplished by the incorporation of [2-^{14}C]-glycine into DNA according to the method of Cadman et al. [4], with [2-^{14}C]-glycine being present for the last 2 h of culture.

Cell cycle analysis. After 72 h of culture, cells were trypsinized, fixed with methanol, treated with RNAase, and stained with propidium iodide and the DNA content was analyzed on EPICS-753 as previously described [12].

Results

AG-331, a rationally designed lipophilic TS inhibitor (Fig. 1), was found to inhibit the growth of H35 rat hepatoma cells in vitro with an IC_{50} value of 5 μ*M* (Table 1). The H35RO.3 cell line, which is resistant to MTX due to a transport defect, was not cross-resistant to AG-331 (Table 1). This result was expected since the lipophilic nature of AG-331 would allow it to enter cells independently of the reduced-folate transporter. The H35R10 cell line, which is resistant to MTX by virtue of both a transport defect and an increase in DHFR activity, was only 2.8-fold cross-resistant to AG-331, whereas substantial cross-resistance (107-fold) was seen with the lipophilic DHFR inhibitor TMTX. Thus, DHFR does not appear to be a target for AG-331. Both of these cell lines were cross-resistant to DMPDDF since this TS inhibitor utilizes the same mode of transport as MTX and reduced folates [22, 28]. The H35FF cell line, which is resistant to 5-FdUrd by virtue of a 100-fold increase in TS activity, was 132-fold resistant to DMPDDF but only 6-fold cross-resistant to AG-331. This result suggests that the inhibition of TS by AG-331 may not be the only mode of action in this cell system.

AG-331 inhibited the incorporation of [6-^{3}H]-dUrd into H35 cellular DNA with an IC_{50} value of 2 μ*M* (Table 2) and was less effective in inhibiting the incorporation of [^{14}C]-glycine (IC_{50}, 23 μ*M*). In this regard AG-331 was similar to

Table 1. Cytotoxicity of AG-331 in cell lines resistant to MTX or 5-FdUrd

Inhibitor	IC_{50} (μM)[a]			
	H35	H35R0.3	H35R10	H35FF
MTX	0.01	1 (100)	100 (10,000)	0.01 (1)
TMTX	0.0075	0.009 (1.2)	0.8 (107)	0.002 (0.27)
DMPDDF	0.055	2.24 (41)	6.3 (115)	7.28 (132)
AG-331	5	6 (1.2)	14 (2.8)	30 (6)

[a] Mean of ≥3 separate experiments consisting of ≥2 estimations each, with a standard deviation of not greater than 15% being obtained in any experiment. IC_{50} is the concentration of inhibitor required to cause a 50% reduction in cell number relative to cultures grown in the absence of inhibitor. Numbers given in parentheses represent the order of magnitude of resistance

Table 2. Inhibition of de novo thymidylate and purine biosynthesis in H35 cells

Inhibitor[a]	IC_{50} (μM)[b]	
	Deoxyuridine incorporation	Glycine incorporation
MTX	0.22 ±0.03	0.24±0.04
TMTX	0.024±0.008	0.08±0.02
DMPDDF	0.18 ±0.02	10 ±3.6
AG-331	2 ±0.5	23 ±3.88

Data represent mean values ±SD (n = 3)

[a] The inhibitor was present for the last 4 h of a 72-h culture period

[b] IC_{50} is the concentration of inhibitor required to cause a 50% reduction in the incorporation of [6-^{3}H]-dUrd or [2-^{14}C]-glycine into DNA. [6-^{3}H]-dUrd and [2-^{14}C]-glycine incorporation for controls lacking inhibitors amounted to 2.55×10^5 and 1.95×10^5 dpm/mg protein, respectively

DMPDDF (Table 2), but the difference between its effects on deoxyuridine and glycine incorporation was 5 times lower than that for DMPDDF.

Protection studies were employed to attempt to determine further the role of inhibition of TS by AG-331 in its cytotoxicity. As expected, thymidine (Tdr) alone completely protected cells from DMPDDF cytotoxicity, whereas the addition of hypoxanthine (HPX) was also required to protect then against the DHFR inhibitors MTX and TMTX (Table 3). Neither folinic acid nor HPX with Tdr protected cells against the cytotoxic effects of AG-331. Thus, AG-331 did not appear to be a potent antifolate in this system at a concentration that exceeded the IC_{50} value by approximately 1 order of magnitude. There was a small amount of protection with thymidine that was not greater than that observed with MTX and TMTX, which are primarily DHFR inhibitors.

The cell-cycle-phase specificity of AG-331 was also examined in H35 cells. A 20-h period of exposure to 10 μM AG-331 produced a 2-fold increase in the number of S-phase H35 cells with a concomitant 5- and 2-fold decrease in the numbers of G_0/G_1- and G_2/M-phase cells, respectively (Table 4). This effect of AG-331 was prevented when excess Tdr was included in the culture. This finding implicates an interaction of AG-331 with TS and is consistent with the idea that S-phase cells are more susceptible to the growth-inhibitory effects of antifolates [14]. At a 5-fold higher AG-331 concentration, however, no significant change in S-phase or G_2/M-phase cells was observed. It is likely that at high concentrations AG-331 may cause inhibition of another as yet unidentified target. It is interesting to compare these data with those reported for TMTX, which at 3 nM caused a marked accumulation of cells in the S phase but at 30 nM caused an accumulation of cells in the late G_1 phase [15].

Table 3. Protection against AG-331 cytotoxicity in the H35 cell line

Inhibitor	% of Control			
	Inhibitor alone	+Tdr	+Tdr+HPX	+Folinic acid
MTX (0.1 μM)	5	20	100	91
TMTX (0.075 μM)	9	57	97	83
DMPDDF (55 μM)	12	98	100	99
AG-331 (50 μM)	20	35	42	15
AG-331 (100 μM)	5	4	4	6

Thymidine (Tdr, 20 μM), hypoxanthine (HPX, 50 μM), or folinic acid (100 μM) was added with the inhibitor at the beginning of a 72-h culture period. The results are expressed as a percentage of the respective values obtained in controls lacking the inhibitor. The concentration of inhibitor used was 10 times the IC_{50} value and is indicated in parentheses; AG-331 was also examined at 20 times the IC_{50} value

Table 4. Cell-cycle analysis of AG-331-treated H35 cells

Treatment[a]	G_0/G_1 (%)	S (%)	G_2/M (%)
None	45.2±3.5	45.2±4.1	9.5±1.5
AG-331 (10 μM)	8.7±3.1	85.8±5.1	5.6±2.3
AG-331 (50 μM)	43.6±3.5	50.7±3.7	5.7±0.6
Thymidine (20 μM)	44.9±1.5	44.2±0.1	10.9±1.1
AG-331 (10 μM) +Thymidine (20 μM)	46.2±0.6	42.3±1.1	11.5±1.6
AG-331 (50 μM) +Thymidine (20 μM)	41.2±0.3	49.5±0.2	9.4±1.1

Data represent mean values ±SD (n = 3)

[a] Cells were incubated in the presence of AG-331 for the last 20 h of a 72-h culture period

Discussion

It has previously been established that AG-331 is a potent inhibitor of purified TS that has cytotoxic properties in vitro [34]. In general, its growth-inhibitory effects (IC_{50}) occur at concentrations below 1 μM. AG-331 was somewhat less active against the H35 hepatoma cells employed in the current studies (IC_{50}, 5 μM). We utilized this cell line, for which the molecular responses to antifolates have been well characterized [10–12, 30, 31], to investigate whether inhibition of TS is the site through which AG-331-induced growth inhibition occurs. Several experimental

results suggested an interaction of AG-331 with TS in H35 cells. Deoxyuridine incorporation was inhibited by AG-331 and was more sensitive than purine incorporation. Moreover, at an inhibitory concentration, AG-331 caused H35 cells to accumulate in the S phase, and this effect was reversed by thymidine. At higher concentrations of AG-331, however, very little change in cell-cycle distribution occurred, suggesting that growth inhibition can also occur in the absence of blockage of thymidine biosynthesis. Further support for a non-TS site of action for AG-331 came from the observation that neither thymidine nor folinic acid protected cells against AG-331 growth inhibition. In addition, a 100-fold TS-amplified cell line was only 6-fold resistant to AG-331. Taken together, these results suggest that AG-331 may exert an inhibitory effect on TS in H35 cells but that growth inhibition, at least at high inhibitor concentrations, is caused by effects at another site. Since relatively high concentrations of AG-331 are required for cell growth inhibition relative to other antifolates and the structure of AG-331 is novel, a rationale exists for alternate site inhibition.

The current results, achieved with H35 hepatoma cells are interesting in comparison with data obtained in cell lines of different origin. AG-331 inhibits cell growth and DNA synthesis in colon and breast cancer cells by a mechanism that is reversible by thymidine [24, 25]. The H35 system is a liver-derived cell line that contains a wide battery of detoxification systems that could cause biotransformation of AG-331 to other toxic species. It would be interesting to determine the metabolic profile following incubation of these cells with AG-331 and to relate any metabolites to the mode of action of AG-331. Preliminary experiments in a human hepatoma cell line (HEPG2) have also lent support to the idea that the activity of AG-331 in hepatoma cells may differ from that in other cell types. AG-331 exhibited an IC_{50} value of 15 μM against HEPG2 cells, which are highly sensitive to MTX [13]. When HEPG2 cells were exposed to 100 μM AG-331 under the protection conditions described in Table 3, none of the agents used had any effect in restoring cell growth.

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