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Novel pyrrolo[2,3-*d*]pyrimidine antifolate TNP-351: cytotoxic effect on methotrexate-resistant CCRF-CEM cells and inhibition of transformylases of de novo purine biosynthesis

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Abstract. *N*-{4-[3-(2,4-Diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]benzoyl}-L-glutamic acid (TNP-351), characterized by a pyrrolo[2,3-*d*]pyrimidine ring, is a novel antifolate that exhibits potent antitumor activities against mammalian solid tumors. The mechanism of action of TNP-351 was evaluated using some methotrexate-resistant CCRF-CEM human lymphoblastic leukemia cell lines as well as partially purified enzymes folylpolyglutamate synthetase (FPGS), aminoimidazolecarboxamide ribonucleotide transformylase (AICARTFase), and glycinamide ribonucleotide transformylase (GARTFase) from parent CCRF-CEM cells. TNP-351 was found to inhibit the growth of L1210 and CCRF-CEM cells in culture, with the doses effective against 50% of the cells (ED_{50} values) being 0.79 and 2.7 n*M*, respectively. The growth inhibition caused by TNP-351 was reversed by leucovorin or a combination of hypoxanthine and thymidine. The methotrexate-resistant CCRF-CEM cell line, which has an impaired methotrexate transport, showed less resistance to TNP-351 than to methotrexate. TNP-351 was also found to be an excellent substrate for FPGS with a Michaelis constant (K_m) of 1.45 μ*M* and a maximum of velocity (V_{max}) of 1,925 pmol h^{-1} mg^{-1}. Inhibitory activities of TNP-351-G_n ($n = 1-6$) for AICARTFase were found to be significantly enhanced with increasing glutamyl chain length [inhibition constants (K_i): G_1, 52 μ*M*; G_6, 0.07 μ*M*]. Neither TNP-351 nor its polyglutamates were very strong inhibitors of GARTFase. These findings have significant implications regarding the mechanism of action of TNP-351.

Key words: TNP-351 – CCRF-CEM cells – Aminoimidazolecarboxamide ribonucleotide transformylase

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Introduction

Methotrexate (MTX), a classic antifolate, is an important drug in the treatment of acute lymphocytic leukemia; however, its clinical use is limited because of its toxicity to patients, the development of resistance, and its lack of efficacy against most human solid tumors. The mechanism of action of MTX is related to its potent inhibition of the enzyme dihydrofolate reductase (DHFR, EC.1.5.1.3).

Recently, some novel antifolates that inhibit DHFR [29] or other folate-requiring enzymes such as thymidylate synthetase (TS, EC.2.1.2.45) [15, 31], glycinamide ribonucleotide transformylase (GARTFase, EC.2.1.2.2) [5], and aminoimidazolecarboxamide ribonucleotide transformylase (AICARTFase, EC.2.1.2.3) [11, 24] have been reported. For antifolates, polyglutamylation plays an important role in their efficacy since the inhibitory activity of most antifolates is enhanced with an increasing number of glutamyl moieties and, therefore, their cytotoxicity against tumor cells, due to higher affinity for the enzymes and longer retention of the drug in the cell, is correspondingly increased [2–4, 11, 13, 24, 28].

We have previously reported that *N*-{4-[3-(2,4-diamino-7*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)propyl]benzoyl}-L-glutamic acid (TNP-351), a novel DHFR inhibitor characterized by a pyrrolo[2,3-*d*]pyrimidine ring (Fig. 1), has potent antitumor activity against not only leukemia cells but also solid tumor cells in vitro and in vivo [1, 19]. The primary mode of action of TNP-351 was thought to be inhibition of DHFR; however, the 50% inhibitory concentration (IC_{50}) for DHFR was 100 times the dose effective against 50% of the tumor cells (ED_{50}) in vitro (10^{-7} and 10^{-9} *M*, respectively) [1]. Therefore, it seemed possible that TNP-351 might have other important modes of action or that uptake and polyglutamylation might play important roles in its potent antitumor activities.

In this study, the importance of polyglutamates for this drug was investigated. TNP-351 was found to be an excellent substrate for folylpolyglutamate synthetase (FPGS, EC.6.3.2.17). TNP-351 polyglutamates (TNP-351-G_n; $n = 2-6$; see Fig. 1), were also found to be potent inhibitors

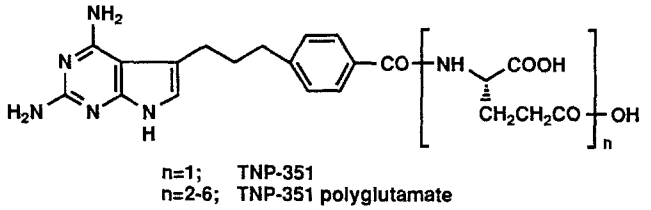

Fig. 1. Structure of TNP-351 and its polyglutamates

of AICARTFase. These findings point to another mode of action of this drug.

Materials and methods

Chemicals. TNP-351 and TNP-351 polyglutamates were chemically synthesized in our laboratories [1, 19]. These drugs were dissolved in 1 *N* NaOH, and the pH was adjusted to 7.0. L-[3,4-^{3}H]-glutamic acid (1 mCi/ml) was purchased from DuPont. (–)-10-Formyl-tetrahydrofolate was prepared by the procedure of Uyeda and Rabinowitz [32]. 10-Formyl-5,8-dideazafolate was synthesized according to the procedure of Hynes et al. [14]. AICA and AICAR were purchased from Sigma Chemical Co. GAR was synthesized according to the procedure of Chettur and Benkovic [9]. AICAR-Sepharose was prepared according to the method of Smith et al. [30]. 10-Formyl-5,8-dideazafolate-Sepharose was prepared according to the procedure of Rode et al. [26, 33]. Sepharose 4B was purchased from Pharmacia Fine Chemicals. Diethylaminoethyl (DEAE)-cellulose (DE52) was purchased from Whatman Ltd., England. The Bio-Gel HPHT column was purchased from Bio-Rad Laboratories. All other chemicals were of the highest grade commercially available.

Cell lines. The parent CCRF-CEM human lymphoblastic leukemia cell line [12] and the MTX-resistant sublines CCRF-CEM R_1, characterized by increased DHFR activity [17, 18], CCRF-CEM R_2, which has normal DHFR levels but impaired MTX transport [27], and CCRF-CEM $R_{30/6}$, characterized by impaired polyglutamylation of MTX [23], were routinely cultured in RPMI 1640 medium supplemented with 10% horse serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37° C in an atmosphere containing 5% CO_2. The L1210 cell line was also cultured in the same manner.

Growth-inhibition studies. Exponentially growing cells were prepared at a density of $3-5\times10^4$ cells/ml and distributed in duplicate 5-ml portions into tissue-culture tubes to which 0.05 ml of drug solutions at various concentrations was added. The cells were incubated at 37° C. After the indicated time, cell density was determined with a model B Coulter counter (Coulter Electronics, Hialeah, Fla.). For short-term exposure, cells were incubated for 4 h in the presence of drug, washed, resuspended in drug-free medium, and counted after the indicated time. Additives for rescue experiments were added at the same time as the drug. ED_{50} values were determined by plotting cell number versus drug concentration and interpolating to 50% inhibition.

Enzyme purification. All steps were carried out at 4° C unless otherwise specified. AICARTFase was purified from CCRF-CEM cells by a modification of the method described by Mueller and Benkovic [22]. In brief, $3-5\times10^9$ cells were disrupted by sonication in a solution of 10 m*M* potassium phosphate (pH 7.5) ethylenediaminetetraacetic acid (EDTA) at 4° C. The homogenate was centrifuged at 23,000 *g* for 45 min, and the supernatant was adjusted to pH 7.5 with 1 *N* NH_4OH after the solution had been made to 10 m*M* in 2-mercaptoethanol (2ME). A solution of 2% protamine sulfate in 10 m*M* potassium phosphate (pH 7.5) was slowly added to the stirred supernatant and the pH was maintained at pH 7.5 with 1 *N* NH_4OH. After 20 min, the suspension was centrifuged at 23,000 *g* for 10 min. The supernatant was brought to 40% saturation by addition of $(NH_4)_2SO_4$ while the pH was maintained at pH 7.5 with 1 *N* NH_4OH. After 50 min, the suspension was centrifuged at 23,000 *g* for 10 min and the supernatant was brought to 60% saturation with $(NH_4)_2SO_4$. After 50 min, the pellet was collected by centrifugation and dissolved in homogenization buffer that had been made to 10 m*M* in 2ME. The solution was dialyzed against 10 m*M* potassium phosphate (pH 7.5) containing 10 m*M* 2ME for 15 h. The solution was then concentrated with a Centriprep 30 concentrator (Amicon) to 1 ml and injected into a high-performance liquid chromatography (HPLC) system using a Bio-Gel HPHT column (100×7.8 mm, Bio-Rad) for further purification.

Elution was carried out with a linear gradient (from 4% to 70%) of 250 m*M* potassium phosphate buffer containing 10 m*M* 2ME (pH 7.5) in an aqueous solution of 10% glycerol in 90 min. The flow rate was 0.5 ml/min. AICARTFase was obtained with approximately 45% potassium phosphate buffer. The AICARTFase-containing fractions (1 ml each) were combined and dialyzed twice against 7.5 m*M* potassium phosphate (pH 7.5). The dialyzed protein was applied to an AICAR-Sepharose column prepared as described by Smith et al. [30]. The column was equilibrated with 7.5 m*M* potassium phosphate (pH 7.5), washed with 7.5 m*M* TRIS-HCl (pH 7.5), and then eluted with 7.5 m*M* TRIS-HCl (pH 7.5) containing 10 m*M* AICAR. The AICARTFase-containing fractions were dialyzed against 7.5 m*M* TRIS-HCl (pH 7.5) for 4 h.

GARTFase was purified from CCRF-CEM cells as described by Daubner et al. [10]. In brief, $3-4\times10^9$ cells were disrupted by sonication at 4° C in 37.5 m*M* potassium phosphate (pH 7.5)/20% glycerol. Phenylmethylsulfonylfluoride (PMSF) was added at a ratio of 1 mg/10^9 cells. After centrifugation at 23,000 *g* for 45 min, the supernatant was applied to a column of 10-formyl-5,8-dideazafolate-Sepharose [33] (1×5 cm) and washed first with 5 vols. of 37.5 m*M* potassium phosphate (pH 7.5)/20% glycerol and then with 1 *M* KCl dissolved in the same buffer. Elution of the enzyme was effected with 2 *M* urea, 37.5 m*M* potassium phosphate (pH 7.5), and 20% glycerol. The GARTFase-containing fractions were dialyzed against 37.5 m*M* potassium phosphate (pH 7.5)/20% glycerol for 6 h.

Enzyme assay. FPGS was assayed using the method of McGuire et al. [16] with slight modification: L-[3,4-^{3}H]-glutamic acid was used instead of L[2,3-^{3}H]-glutamic acid, and potassium glutamate (50 m*M*) in addition to 25 m*M* 2ME was used to stop the reaction at the end of the incubation period. These changes minimized the background counts to 75 cpm/ml of column eluent. Standard assay mixtures that contained TRIS-HCl (0.1 *M*, pH 8.4), adenosine triphosphate (ATP, 5 m*M*), $MgCl_2$ (10 m*M*), KCl (20 m*M*), 2ME (100 m*M*), L-[^{3}H]-glutamate (4 m*M*, 4.1 cpm/pmol), drug, and enzyme in a total volume of 0.25 ml were incubated at 37° C for 1 h under gentle mixing every 15 min. At the end of the incubation period, the reaction mixture was diluted with 1 ml of ice-cold 50 m*M* potassium glutamate (pH 8.4) containing 25 m*M* 2ME and then transferred to an ice-water bath. Each reaction mixture was transferred to the respective DE52 column. The assay tube was rinsed with 1 ml of 10 m*M* TRIS-HCl (pH 7.5), 110 m*M* NaCl containing 25 m*M* 2ME (column buffer), and this was then transferred to the column. The columns were washed with column buffer until a total volume of 15 ml was collected. All polyglutamates were eluted with 3 ml of 0.1 *N* HCl following the washing procedure, and 1 ml of eluent was counted in 10 ml of scintillation cocktail (Optifluor, Packard Instrument Co., Downers Grove, Ill.).

AICARTFase was assayed by measuring the increase in absorbance at 298 nm due to the formation of tetrahydrofolate according to the procedure of Black et al. [6]. The reaction was initiated by adding 0.05 ml of 1 m*M* AICAR to a cuvette containing 32.5 µ*M* of TRIS-HCl (pH 7.4), 5 µ*M* of 2ME, 25 µ*M* of KCl, 0.1 µ*M* of (–)-10-formyl-tetrahydrofolate, and enzyme to make 0.950 ml at 25° C. All solutions were argon-saturated before their use.

GARTFase was assayed spectrophotometrically with 10-formyl-5,8-dideazafolate as the formyl donor according to the procedure of Daubner et al. [10]. The reaction was initiated by the addition of enzyme to a cuvette containing 75 µ*M* of HEPES (pH 7.5), 20% glycerol, 0.24 µ*M* of GAR, and 0.01 µ*M* of 10-formyl-5,8-dideazafo-

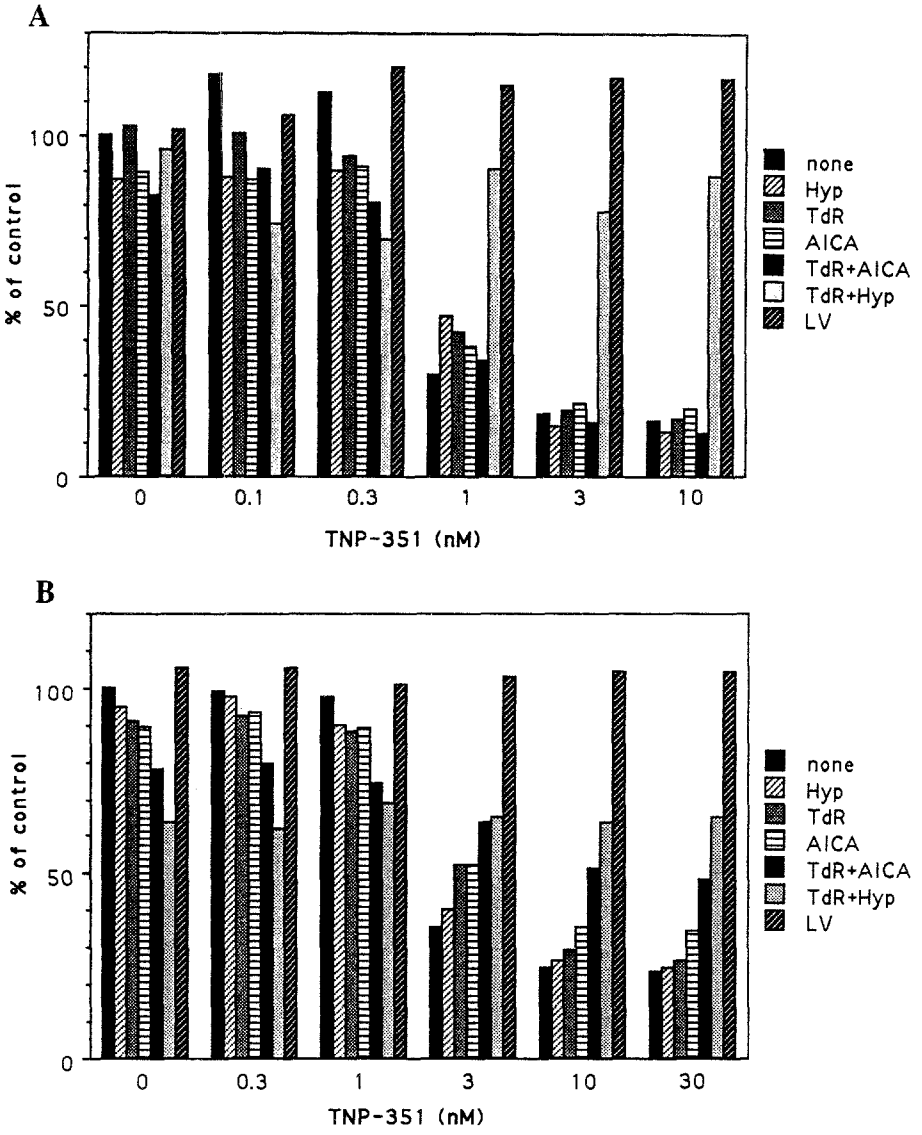

Fig. 2A, B. Protection of cultured L1210 or CCRF-CEM cells from growth inhibition by TNP-351 with thymidine, hypoxanthine, 5-aminoimidazole-4-carboxamide, and leucovorin. Protection is expressed as a percentage of the growth of control cultures. **A** L1210 cells (3×10^4/ml) were incubated for 48 h in the presence of 10 μ*M* thymidine (*TdR*), 100 μ*M* hypoxanthine (*Hyp*), 320 μ*M* 5-amino-imidazole-4-carboxamide (*AICA*), 10 μ*M* TdR plus 320 μ*M* AICA, 10 μ*M* TdR plus 100 μ*M* Hyp, or 1 μ*M* leucovorin (*LV*). **B** CCRF-CEM cells (5×10^4/ml) were incubated for 72 h in the same manner except that 3 μ*M* TdR plus 100 μ*M* Hyp or 3 μ*M* TdR plus 320 μ*M* AICA were used. TNP-351 inhibited L1210 and CCRF-CEM cells with ED_{50} values of 0.79 and 2.7 n*M*, respectively

late in a total volume of 1 ml at 25° C, and the reaction was monitored at 295 nm.

Protein determination. Protein concentration was determined either by the method of Bradford [7] using bovine serum albumin as the standard with a Bio-Rad protein assay or by assuming an extinction value of 1.0 at 280 nm for a 1-mg/ml solution.

Results

Growth inhibition of leukemia cells by TNP-351

The growth inhibition of leukemia cells caused by TNP-351 was investigated. TNP-351 inhibited the proliferation of mouse L1210 leukemia cells and human CCRF-CEM lymphoblastic leukemia cells with ED_{50} values of 0.79 and 2.7 m*M*, respectively (Fig. 2, Table 1). The cells were rescued from TNP-351-induced growth inhibition by leucovorin or a combination of hypoxanthine and thymidine. Thymidine, hypoxanthine, AICA, or a combination of AICA and thymidine had no effect on the growth inhibition caused by TNP-351 (Fig. 2). The patterns of reversal of TNP-351-induced growth inhibition seemed to be similar to those noted for MTX. This would indicate that the primary mode of action of TNP-351 is inhibition of DHFR. The combination of hypoxanthine and thymidine protected the leukemia cells, but the combination of AICA and thymidine did not. This suggests that TNP-351 inhibits other steps in de novo purine synthesis, presumably those involving AICARTFase.

We examined TNP-351-induced growth inhibition using three MTX-resistant CCRF-CEM sublines, namely, the

Table 1. Inhibitory effects of TNP-351 and MTX on the growth of CCRF-CEM parent and MTX-resistant cell lines[a]

	ED_{50} (n*M*)			
	TNP-351	(Resistant[b])	MTX	(Resistant[b])
CCRF-CEM	2.7		17	
CCRF-CEM R_1	94	(35)	580	(34)
CCRF-CEM R_2	76	(28)	2,300	(135)
CCRF-CEM $R_{30/6}$	5.8	(2)	29	(1.7)

[a] The MTX-resistant sublines CCRF-CEM R_1, characterized by increased DHFR activity, CCRF-CEM R_2, which has normal DHFR levels but impaired MTX transport, and CCRF-CEM $R_{30/6}$, characterized by impaired polyglutamylation of MTX, were used. Cells (5×10^4/ml) were incubated in the presence of each drug at various concentrations. The number of cells was counted after 72 h of incubation

[b] ED_{50} value for resistant cell line / ED_{50} value for parent cell line

Table 2. Comparative sensitivities of parent CCRF-CEM cells and FPGS-deficient subline CCRF-CEM $R_{30/6}$ cells to TNP-351 and MTX with varying exposure times[a]

	ED_{50} (n*M*)			
	TNP-351		MTX	
	4 h	72 h	4 h	72 h
CCRF-CEM	70	2.7	85	17
CCRF-CEM $R_{30/6}$	8,500	5.8	26,000	29

[a] Cells (5×10^4/ml) were incubated for 4 or 72 h with the indicated drug and counted as described in Materials and methods

CCRF-CEM R_1 [17, 18], CCRF-CEM R_2 [27], and CCRF-CEM $R_{30/6}$ cell lines [23], which are resistant to MTX because of increased levels of DHFR, impaired drug transport, and decreased FPGS activities, respectively. As shown in Table 1, the ED_{50} value determined for the CCRF-CEM $R_{30/6}$ subline (5.8 m*M*) was only slightly different from that found for the parent cell line CCRF-CEM (2.7 m*M*). On the other hand, the ED_{50} values determined for CCRF-CEM R_1 and CCRF-CEM R_2 were higher (94 and 76 m*M*, respectively). These findings are similar to those previously reported for MTX-induced growth inhibition in our laboratories [23]; however, CCRF-CEM R_2 was less resistant to TNP-351 than to MTX (i. e., it was 28 and 135 times as resistant as the parent cell line), probably suggesting a different affinity for the transport system.

We further examined short-term (4 h) exposure in an experiment using the parent CCRF-CEM cell line and subline CCRF-CEM $R_{30/6}$, since CCRF-CEM $R_{30/6}$ cells displayed their characteristics on briefer exposure [25]. The CCRF-CEM $R_{30/6}$ cell line showed 120-fold resistance as compared with the parent cell line on short-term exposure to TNP-351. In contrast, there was a 300-fold decrease in the sensitivity to growth inhibition of this cell line as compared with the parent line for a 4-h MTX exposure (Table 2). This suggests that TNP-351 might be more extensively converted to its polyglutamates, TNP-351-G_n ($n > 1$), by FPGS than is MTX.

Activities of TNP-351 and TNP-351-G_2 as substrates for human CCRF-CEM FPGS

FPGS substrate activities were displayed as $k_{rel.}$ (V_{max}/K_m) values related to aminopterin. TNP-351 was found to be an excellent substrate for human CCRF-CEM folylpolyglutamate synthetase with a Michaelis constant (K_m) of 1.45 μ*M* and a maximum of velocity (V_{max}) of 1,925 pmol h^{-1} mg^{-1} and showed a substrate affinity for FPGS that was 3 times that of aminopterin (Table 3). On the other hand, TNP-351-G_2 showed weaker FPGS substrate activity than TNP-351 with decreasing V_{max} value; however, the FPGS substrate activity for TNP-351-G_2 was kept at half of that for aminopterin.

Inhibition of transformylases purified from CCRF-CEM cells

We first determined the kinetic properties of AICARTFase purified from CCRF-CEM human lymphoblastic leukemia cells. The K_m was 61 μ*M* when (–)-10-formyl-tetrahydrofolate served as the substrate, with a V_{max} of 0.5 μmol of formyl-AICAR being formed per milligram per minute (data not shown). The effects of TNP-351 and TNP-351-G_n ($n = 2-6$) as direct inhibitors of AICARTFase were studied. Inhibition constants of TNP-351 and TNP-351-G_n were determined at a constant 10-formyl-tetrahydrofolate concentration of 1×10^{-5} *M* and at variable concentrations of TNP-351 or its polyglutamates (Table 4 [8]). Although TNP-351 was not a very strong inhibitor of AICARTFase [inhibition constant (K_i), 52 μ*M*], the addition of one glutamyl residue led to a 16-fold increase in the inhibitory capacity of the drug (TNP-351-G_2: K_i, 3.3 μ*M*). Further additions of glutamyl residues (TNP-351-G_{3-6}) led to further decreases in the observed K_i values. TNP-351-G_6 showed the most potent inhibition of AICARTFase with a K_i of 0.07 μ*M*, i. e., it was 700 times as potent as TNP-351. To determine the type of inhibition involved, TNP-351-G_5-induced AICARTFase inhibitions were determined at variable 10-formyl-tetrahydrofolate concentrations and a double reciprocal plot of its AICARTFase inhibition was drawn (Fig. 3). TNP-351-G_5 was found to be a competitive inhibitor of AICARTFase when 10-formyl-tetrahydrofolate served as the co-substrate.

The effectiveness of TNP-351 and TNP-351-G_n ($n = 2-6$) as inhibitors of GARTFase purified from CCRF-CEM cells was also evaluated. TNP-351-G_5 was 6.5-fold more potent than TNP-351 as an inhibitor of GARTFase; however, neither TNP-351 nor TNP-351-G_n were found to be very strong inhibitors of GARTFase (K_i, 7–47 μ*M*; Table 4).

Discussion

The present growth-inhibition studies demonstrate that TNP-351 is a potent inhibitor of both L1210 and CCRF-CEM cell lines in vitro. Complete rescue by leucovorin supports the assumption that the tumor growth inhibition induced by TNP-351 is a result of depletion of the tetra-

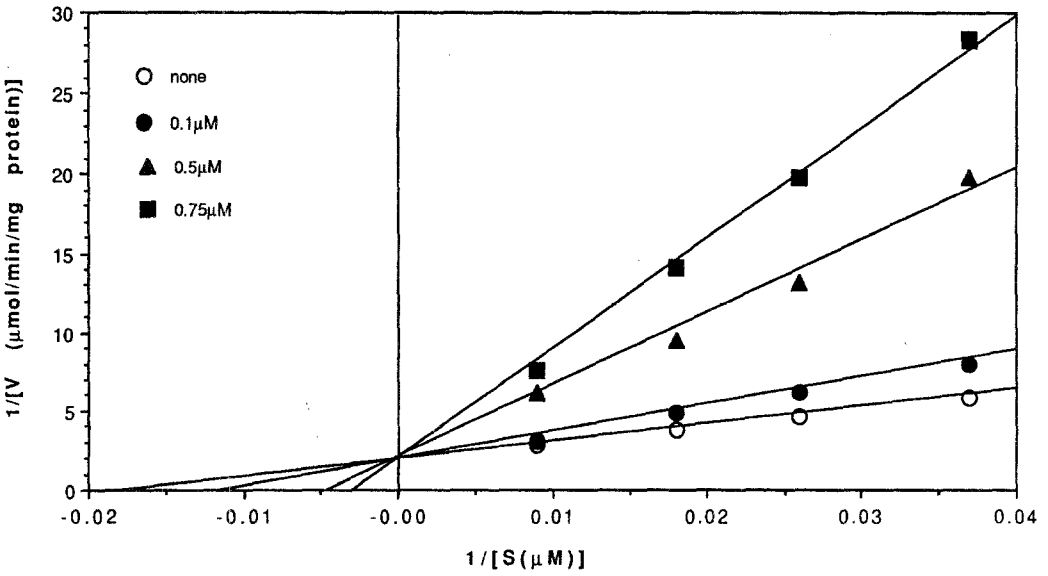

Fig. 3. A double reciprocal plot of AICARTFase inhibition by TNP-351-G_5. AICARTFase activity was assayed in the presence of variable 10-formyl-tetrahydrofolate concentrations with no inhibitor (○) and with 0.1 (●), 0.5 (▲), and 0.75 (■) μ*M* of TNP-351-G_5. *V*, Moles of formyl-AICAR formed per minute × 10^6; *S*, concentration of 10-formyl-tetrahydrofolate × 10^6

Table 3. Activity of TNP-351 and TNP-351-G_2 as substrates for CCRF-CEM FPGS[a]

	K_m (μ*M*)	V_{max} (pmol h^{-1} mg^{-1})	k_{rel} [b](V_{max}/K_m)
TNP-351	1.45	1,925	339
TNP-351-G_2	1.07	244	60
Aminopterin	3.20	1,250	100

[a] After the FPGS assay mixture containing 0.3 mg protein/ml of CCRF-CEM enzyme, 4 m*M* [^{3}H]-glutamic acid, 5 m*M* ATP, and the indicated substrate has been incubated for 1 h, the produced polyglutamates were separated by a short column and counted for radioactivity. Data represent average values for two separate experiments carried out in duplicate

[b] This value is related to aminopterin being used as the internal control in each experiment

Table 4. Inhibition of transformylases from CCRF-CEM by TNP-351-G_n

	K_i (μ*M*)	
	AICARTFase	GARTFase
TNP-351	52	47
TNP-351-G_2	3	24
TNP-351-G_3	2	24
TNP-351-G_4	0.2	13
TNP-351-G_5	0.14	7
TNP-351-G_6	0.07	14

The inhibition of AICARTFase and GARTFase purified from parent CCRF-CEM cells was spectrophotometrically measured as described in Materials and methods

hydrofolate pool due to the inhibition of folate-requiring enzymes. The patterns of reversal of growth inhibition obtained with TNP-351 were very similar to those noted for MTX. In addition, the CCRF-CEM R_1 cell line, which has increased DHFR levels as compared with the parent cell line, showed a similar level of resistance to TNP-351 as to MTX (35-fold more resistant). These results indicate that the primary mode of action of TNP-351 is inhibition of DHFR, as is the case for MTX. Our previous studies, however, showed that the IC_{50} for DHFR inhibition was 100-fold greater than the ED_{50} for growth inhibition of tumor cells in vitro, that the inhibitory activity of TNP-351 pentaglutamate for DHFR was only twice as strong as that of the monoglutamate, that TNP-351-G_ns (n = 1–6) were poor inhibitors of thymidylate synthetase, and that TNP-351 was active against not only leukemia cells but also some solid tumors [13, 34]. Therefore, TNP-351 might have another important mode of action in addition to that of DHFR inhibition.

It is well known that transport and polyglutamylation play an important role in the toxicity of antifolate against tumor cells. The CCRF-CEM R_2 cell line with impaired MTX transport was much less resistant to TNP-351 than to MTX. This suggests that the uptake of TNP-351 in this cell line might be much better than that of MTX. In fact, our previous studies showed that TNP-351 was taken up into Meth A murine fibrosarcoma cells more than 10 times better than MTX [1]. On short-term exposure (4 h), the CCRF-CEM $R_{30/6}$ cell line with decreased activities of FPGS showed less resistance to TNP-351 than to MTX. In addition, TNP-351 showed excellent substrate activity for FPGS from CCRF-CEM cells in vitro, exhibiting more than 3-fold better substrate activity than aminopterin, which is one of the best-known FPGS substrates [20, 21]. In fact, polyglutamate forms of TNP-351 (mainly TNP-351-G_2 and TNP-351-G_3) were formed intracellularly in Meth A cells

after only 30 min of exposure to 0.15 μ*M* of [^{14}C]-TNP-351 (K. Yukishige et al., manuscript in preparation). The excellent uptake and FPGS substrate activity of TNP-351 might play important roles in its potent antitumor activities.

Cells were rescued from TNP-351-induced growth inhibition by a combination of hypoxanthine and thymidine but not by a combination of thymidine and AICA. These results suggest that TNP-351 or TNP-351-G_n might strongly inhibit AICARTFase. In fact, TNP-351-G_ns ($n > 3$) were found to be potent competitive inhibitors of AICARTFase purified from CCRF-CEM cells. These findings indicate another site of action of TNP-351. Therefore, TNP-351 might be efficiently taken up into these cells and very quickly converted to polyglutamates by FPGS in tumor cells. Polyglutamates are not easily discharged from tumor cells and might strongly inhibit AICARTFase as well as DHFR.

In conclusion, our results demonstrate that TNP-351 potently inhibits the growth of murine and human tumor cells in vitro and is an excellent substrate for FPGS. TNP-351 polyglutamates were found to be potent competitive inhibitors of AICARTFase. These findings have significant implications regarding the mechanism of action of this drug.

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