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New folate analogs of the 10-deaza-aminopterin series

Further evidence for markedly increased antitumor efficacy compared with methotrexate in ascitic and solid murine tumor models

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Summary. *A group of folate analogs of the 10-deaza-aminopterin series, which were designed on the basis of the results of an intensive biochemical and pharmacokinetic program, have been examined in therapy experiments utilizing a group of murine tumor models. These new analogs were found to be markedly superior to methotrexate against four of five ascites tumors (L1210 leukemia, Sarcoma 180, Ehrlich carcinoma and Tapper carcinosarcoma) and against four of five solid tumors (S180, Tapper carcinosarcoma, E0771 mammary adenocarcinoma, Lewis lung carcinoma, and T241 sarcoma). Analogs alkylated (methyl or ethyl) at the 10 position of 10-deaza-aminopterin were found to be the most effective of the group. These analogs achieved log_{10} reduction in tumor burden several-fold greater in magnitude than methotrexate against L1210 and S180 ascites tumors and there were also long-term survivors. 10-Deaza-aminopterin itself gave a result intermediate between those obtained with the 10-alkyl derivatives and methotrexate. Against the solid forms of the Tapper tumor some partial regressions were obtained with methotrexate and 10-deaza-aminopterin, but a far greater number, extending over a longer period were obtained with the 10-ethyl derivative of 10-deaza-aminopterin. Against the E0771 tumor, 10-deaza-aminopterin was 2-fold and the ethyl derivative of 10-deaza-aminopterin was > 5-fold more effective than methotrexate in retarding tumor growth. Evidence for partial regressions and marked effects against metastatic disease were also obtained in the case of the 10-alkyl derivative. Similar results were also obtained with the T241 sarcoma. For Lewis lung carcinoma the relative potency was about the same but overall antitumor effects were more modest.*

Introduction

A rationale for the design of new folate analogs with greater antitumor selectivity which we have been pursuing in our laboratory exploits [8, 13, 14] differences between tumor and normal proliferative tissue at the level of membrane transport of these agents. These differences have been documented by both biochemical and pharmacologic studies reported [8, 13, 14] from our laboratory over the last several years. These studies extended previous work done elsewhere [4, 5], which provided evidence for a correlation between the extent of cellular penetration of methotrexate in vitro and the responsiveness of a group of murine and human leukemias. In additional studies we have derived evidence [9–11] for differences in specific kinetic parameters for transport, primarily the Michaelis constant for influx, among various murine tumors with variable responsiveness. More importantly, similar differences in the Michaelis constant for influx of these analogs between responsive tumor cells and normal proliferative tissues appear to account [2, 9, 10], at least to a large measure, for the selectivity of the antitumor effects observed in these murine models. In an effort to develop new folate analogs which might be more effective than methotrexate against a broader range of human cancers, we have sought information on the structural basis for these differences in mediated membrane transport of these folate compounds. Our initial studies showed that selectivity was associated [2, 8, 13, 14] with a difference in tumor versus drug-limiting normal proliferative tissue (small intestine) in the specificity for transport involving at least one site, the N^{10} position.

Although factors other than transport may also be involved, this differential specificity for mediated entry, which is not manifested at the level of target enzyme inhibition [8, 13, 14], appears to favor higher levels of accumulation and longer persistence of drug in tumor tissue. In initial studies of a newly synthesized N^{10} analog, 10-deaza-aminopterin, superior antitumor properties were demonstrated [12] against a group of murine tumors. Since this improvement in response compared with methotrexate was predicted by the results [8, 13, 14, 16] of our biochemical and pharmacologic studies, additional N^{10} analogs were synthesized [3] and examined in both ascitic and solid murine tumor models. Our results, which we now report here, document further substantial improvement in antitumor efficacy for some of these new analogs. Attendant biochemical and pharmacologic studies which appear to explain this improvement will be presented in a companion report [17].

Materials and methods

Since very detailed descriptions of our experimental procedures have been reported [8, 12] earlier, only a brief summary of these will be given here. Harvesting of tumor cells from the peritoneal cavity and maintenance of the various tumor cell lines in $BD2F_1$ ($C57BL/6 \times DBA/2F_1$) mice, obtained from Sprague/Dawley (Madison, Wisconsin), have been described [8, 12]. All folate compounds were prepared as the sodium salt in neutral aqueous solution. The synthesis of these compounds, which are shown in Fig. 1, have been reported elsewhere [3]. Samples of methotrexate used during these studies were provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. All compounds

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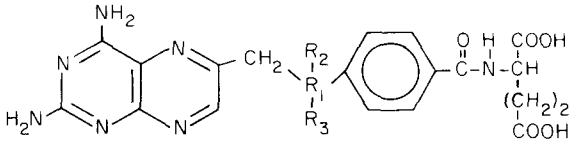
used were > 95% pure as determined [3] by UV spectrophotometry and analytical high-performance liquid chromatography (HPLC).

For therapy experiments, L1210, S180, Ehrlich, Tapper, and 1498c cells were maintained as ascites tumors in $BD2F_1$ mice and initiated from a bank of frozen cells prepared earlier from a line determined to have characteristic malignancy, and we maintained only five transplant generations before discarding. The E0771 mammary adenocarcinoma and T241 fibrosarcoma were transplanted SC as a cell suspension prepared from the solid tumor. Therapy was initiated SC 24 h after IP transplantation of L1210, S180, Ehrlich, and Tapper (ascites tumor experiments), ID transplantation (S180 and Tapper solid tumor experiments), or SC transplantation (E0771 mammary adenocarcinoma solid tumor experiments). After transplantation animals were randomly selected for control and treated groups (av. wt. 20 ± 0.5 g). Antitumor effects against ascite tumors were expressed as mean survival time (MST) in days and increased lifespan (ILS) as a percentage for treated versus control groups. Estimations of $\log_{10}$ tumor cell kill were made by the method of Schabel et al. [7]. This procedure estimates the tumor cell number at the end of therapy from the mean survival time (MST ± SE-10) and includes long-term survivors. Antitumor effects against solid tumors were expressed as average tumor volume (ATV) and percent treated/control (T/C), and in the case of the E0771 tumor, MST and %ILS. Toxicity was monitored by weight loss and drug-induced deaths in which animals were tumor-free and showed a > 20% weight loss and intestinal pathology [17].



R_1	R_2	R_3	analog
N	CH_3	–	methotrexate
C	H	H	10-deaza-aminopterin
C	CH_3	H	10-methyl, 10-deaza-aminopterin
C	C_2H_5	H	10-ethyl, 10-deaza-aminopterin
C	CH_3	CH_3	10, 10-dimethyl, 10-deaza-aminopterin

Fig. 1. Structures of the folate analogs used in the present study

Results and discussion

The antitumor activity of SC administered 10-deaza-aminopterin, some 10-alkyl analogs of 10-deaza-aminopterin, and methotrexate against three murine ascites tumors is summarized in Table 1. The activity against L1210 leukemia with a schedule of one dose every other day for five doses was, for all but one (10,10-dimethyl analog) of the new agents, consistently greater than that of methotrexate itself. Results derived from dosages in the approximate LD_{10} range in four separate experiments show increased therapeutic efficacy for the new analogs in the ascending order of 10-deaza-aminopterin, 10,10-dimethyl, 10-ethyl, and 10-methyl derivatives. When expressed as $\log_{10}$ cell kill [7], log reductions in viable tumor cells were 0.8 for methotrexate but 2- to 5-fold greater for the new analogs. Also, a few long-term survivors were obtained with both the 10-methyl and 10-ethyl derivatives. LD_{10}s for this group of compounds were in the ascending order 10-deaza-aminopterin, methotrexate and 10-methyl, 10-ethyl, and 10,10-dimethyl derivatives. The greater activity of the 10-methyl and, particularly, of the 10-ethyl derivative may be at least partially attributable to the somewhat higher tolerance to these agents than to 10-deaza-aminopterin and methotrex-

Table 1. Activity of folate analogs against some murine ascites tumors

Tumor	R_x	Optimum dosage[a] (mg/kg)	MST ± SE[b] (days)	ILS[c] (%)	Cell kill[d] ($\log_{10}$)	60-day survivors
L1210	–	–	6.8 ± 0.3	–	–	0/20
	Methotrexate	12– 18	16.6 ± 0.5	+ 151	0.8	0/20
	10-Deaza-aminopterin	9– 15	18.3 ± 1.3	+ 178	1.8	0/20
	10-CH_3,10-deaza-AM	15– 24	22.1 ± 1.7	> + 235	3.9	2/20
	10-C_2H_5,10-deaza-AM	18– 24	21.4 ± 1.2	> + 217	3.4	1/20
	10-$(CH_3)_2$,10-deaza-AM	144–288	20.8 ± 1.4	+ 182	1.9	0/20
S180	–	–	11.1 ± 1.5	–	–	0/20
	Methotrexate	12– 15	19.1 ± 2.4	+ 74	0	0/20
	10-Deaza-aminopterin	9– 12	30.3 ± 3.4	> + 172	2.8	1/20
	10-CH_3,10-deaza-AM	15– 24	35.5 ± 4.2	> + 221	4.8	3/20
	10-C_2H_5,10-deaza-AM	18– 24	38.8 ± 3.9	> + 242	5.7	6/20
1498c	–	–	8.3 ± 1.3	–	–	0/20
	Methotrexate	12– 18	16.3 ± 2.1	+ 96	0	0/20
	10-Deaza-aminopterin	9– 15	15.9 ± 3.2	+ 92	0	0/20
	10-CH_3,10-deaza-AM	15– 24	19.2 ± 2.4	+ 131	0.4	0/20
	10-C_2H_5,10-deaza-AM	18– 24	18.2 ± 2.7	+ 118	0.2	0/20

[a] LD_{10}: methotrexate, 13.6 mg/kg; 10-deaza-aminopterin, 10.7 mg/kg; 10-CH_3,10-deaza-AM, 14.1 mg/kg; 10-C_2H_5,10-deaza-AM, 19.3 mg, given SC every 2 days for five doses. AM, aminopterin

[b] Four experiments done on separate days with five mice per group (mean survival time ± SE)

[c] Increase in lifespan (initial tumor burden was 10^6 cells)

[d] Calculated by the method of Schabel et al. [7]

ate. The extremely low toxic potency of the 10,10-dimethyl derivative, but not the other two 10-alkyl derivatives, was to a large extent related to more rapid plasma clearance [17].

Against S180, the new analogs were markedly superior to methotrexate. With the same schedule and route of administration increased activity was seen in the ascending order 10-deaza-aminopterin, and 10-methyl, and 10-ethyl derivatives. No reduction in tumor cell burden was obtained with methotrexate, while log reductions were approximately 3 for 10-deaza-aminopterin and 5–6 in the case of the 10-alkyl derivatives. Long-term survivors were obtained only with the new analogs and varied from 5% (10-deaza-aminopterin) through 15% (10-methyl derivative) to 30% (10-ethyl derivative). Similar experiments carried out with the 1498c myloid leukemia show antitumor activity for the new analogs which was similar or only marginally better than that of methotrexate.

The results of related experiments in which mice were treated after implantation of 10^7 cells, i.e., one log higher than the standard inoculum employed in the other ascites tumor experiments, are shown in Table 2. Only methotrexate and 10-ethyl aminopterin were compared in this experiment. Against S180, the new analog was again substantially more effective. Values for ILS were 6-fold greater and a 3-log reduction in cell number was obtained, compared with none for methotrexate. Against Ehrlich carcinoma and the Tapper liver tumor the new analog was also superior, although the increased activity was not as marked.

The activity of methotrexate, 10-deaza-aminopterin, and its 10-ethyl derivative against solid (interdermal) forms of S180 and Tapper tumors are compared in Table 3. The schedule of administration employed for these experiments was one dose daily SC for 5 days, giving a total of five individual doses. Three doses of drug (3, 6, and 9 mg/g) were employed to define the LD_{10} range on this schedule. One day after therapy was stopped the value for T/C (average tumor volume) against S180 obtained at the approximate LD_{10} dosage was 79% for methotrexate (3 mg/kg), 39% for 10-deaza-aminopterin (3 mg/kg), and 19% for 10-ethyl-10-deaza-aminopterin (6 mg/kg). In parallel experiments, the value for T/C against

Table 2. Antitumor activity of methotrexate and 10-ethyl,10-deaza-aminopterin in mice following implantation of a high tumor burden

Tumor[a]	R_x	Optimum dosage[b] (mg/kg)	MST ± SE[c] (days)	ILS[d] (%)	Cell kill[e] (log_{10})
S180	–	–	8.9 ± 1.2	–	–
	Methotrexate	15–18	11.9 ± 1.6	+ 33	0
	10-C_2H_5,10-deaza-AM	18–24	25.6 ± 3.2	+ 187	3.1
Ehrlich	–	–	15.9 ± 1.9	–	–
	Methotrexate	12–18	19.2 ± 2.3	+ 18	0
	10-C_2H_5,10-deaza-AM	15–24	28.6 ± 3.8	+ 79	0
Tapper	–	–	10.6 ± 1.2	–	–
	Methotrexate	12–15	17.3 ± 2.4	+ 63	0
	10-C_2H_5,10-deaza-AM	15–24	21.6 ± 3.1	+ 104	0

[a] Inoculum was 10^7 cells IP (R_x every 2 days for 7 doses)
[b] See footnote to Table 1 for calculated LD_{10} dosages. Drug was given SC every 2 days for 5 doses. AM, aminopterin
[c] Mean survival time (3 experiments with 5 mice per group)
[d] Increase in lifespan
[e] Calculated by the method of Schabel et al. [7]

Table 3. Activity of folate analogs against solid sarcoma 180 and Tapper sarcocarcinoma in mice

R_x	mg/kg	S180		Tapper		Toxic deaths[d, e] No./total
		Ave tumor (mg)	Vol.[a, b, c] (%)	Ave tumor (mg)	Vol.[a, b, c] (%)	
Control	–	292 ± 42	100	542 ± 78	100	–
Methotrexate	3	228 ± 38	79	56 ± 6	10	3/40
	6	145 ± 26	53	22 ± 3	4	13/40
	9	46 ± 8	15	<1	<1	29/40
10-Deaza-aminopterin	3	114 ± 19	39	15 ± 2	3	2/20
	6	62 ± 14	21	<1	<1	5/20
	9	38 ± 5	13	<1	<1	8/20
10-C_2H_5,10-deaza-AM	3	128 ± 32	40	11 ± 2	4	1/40
	6	59 ± 17	19	<1	<1	5/40
	9	44 ± 12	14	<1	<1	21/40

[a] V (mg) ± SE = $4/3\pi\, r^3$ (diameter = 2 mm initially)
[b] $\% = T(V_t - V_0)/C(V - V_0) \times 100$
[c] Measurement of tumor volume and net loss done on day 7; two to eight separate expts; five mice/group
[d] Animals held for 14 days after implant
[e] LD_{10}: methotrexate, 3 mg/kg;10-deaza-aminopterin, 2.9 mg/kg; 10-CH_3,10-deaza-AM, 5.6 mg/kg; (R_x daily SC for 5 days)

the interdermal Tapper tumor was 10% for methotrexate and < 1% for 10-deaza-aminopterin and the 10-ethyl derivative. A large percentage of these animals did not show palpable tumors at the end of therapy. Since the average tumor diameter at the start of therapy was 3 mm, absence of palpable tumors can be considered partial regression in these animals. In view of this greater sensitivity to therapy of the interdermal tumor than of the ascites tumor (Table 2), additional experiments were run at a lower dosage range, and the number of animals in each group that had palpable tumors was also recorded. These results are shown in Table 4. In the group treated with the approximate LD_{10} dosage for methotrexate (3 mg/kg) the percentages of animals showing palpable tumors were 55, 94, and 100 on days 1, 4, and 8 after therapy was stopped. For 10-deaza-aminopterin (3 mg/kg) the percentages were 20, 40, and 100 on the same days. However, for the 10-ethyl derivative (6 mg/kg) the corresponding percentages were 0, 9, and 68 on the same days. It is also of interest to note that this difference in palpable tumors after therapy with the three agents was also obtained at dosages which were one-half to one-fourth below the approximate LD_{10}.

In a separate group of experiments, the activity of these analogs against the E0771 mammary adenocarcinoma was determined. The results obtained following therapy according to a schedule of one SC dose daily for 5 days are given in Table 5. Values for T/C at the approximate LD_{10} for methotrexate were 51% and 88% on days 1 and 8 after therapy. The corresponding values for 10-deaza-aminopterin were 39% and 68% (the LD_{10} for this analog in this experiment was higher, i.e., approximately 6 mg/kg). The corresponding values for the 10-ethyl derivative were < 1% and 11%, and some of these animals did not show palpable tumors 1 day after therapy.

The same relative efficacy of these agents was also expressed in terms of survival time of the animals which die of metastatic disease. Animals treated with methotrexate did not survive any longer than controls. Animals treated with 10-deaza-aminopterin showed a modest increase in survival.

Table 4. Antitumor activity of folate analogs against the solid form of the Tapper live tumor

R_x	mg/kg	Day 7			Day 10			Day 14			Toxic deaths No./total
		Tumors[a] (%)	ATV[b] (mg)	T/C[c] (%)	Tumors[a] (%)	ATV[b] (mg)	T/C[c] (%)	Tumors[a] (%)	ATV[b] (mg)	T/C[c] (%)	
Control	–	100	226	100	100	410	100	100	630	100	–
Methotrexate	1.5	80	38	17	100	179	29	100	435	69	0/10
	3.0	55	22	10	94	46	11	100	344	54	3/20
	6.0	10	<1	4	50	36	6	100	248	39	7/20
10-Deaza-aminopterin	1.5	40	17	8	100	101	26	100	345	55	0/10
	3.0	24	<1	<1	68	45	11	100	210	33	2/15
	6.0	12	<1	<1	60	17	4	100	182	29	5/15
10-Ethyl,10-deaza-AM	1.5	25	11	4	60	71	17	100	264	42	0/10
	3.0	9	<1	<1	40	17	4	100	210	33	1/20
	6.0	0	<1	<1	9	15	3	68	139	22	3/20

[a] Number of mice palpable tumors (no./total × 100 = %)
[b] Average tumor volume ± SE = V (mg) = $4/3\,\pi\,r^3$ (initial dia. = 2 mm)
[c] % = $T(V_t - V_0)/C(V_t - V_0) \times 100$: three to four separate experiments: five mice per group. R_x daily SC for 5 days

Table 5. Antitumor activity of folate analogs against the E0771 mammary adenocarcinoma

R_x	mg/kg	Day 7		Day 14		MST (days)	ILS (%)	Toxic deaths No./total
		Ave tumor (mg)	Vol.[a] (%)	Ave tumor (mg)	Vol.[a] (%)			
Control	–	710	–	4,116	–	20.6 ± 3	–	–
Methotrexate	3	371	52	3,006	75	24.1 ± 2	+ 16	1/22
	6	78	11	2,620	64	22.7 ± 4	+ 10	3/14
	9	57	8	1,680	45	Toxic	–	5/14
10-Deaza-aminopterin	3	441	62	3,380	80	23.9 ± 3	+ 10	1/14
	6	199	28	2,815	69	26.4 ± 5	+ 28	1/14
	9	142	20	2,660	65	Toxic	–	5/14
10-Ethyl,10-deaza-AM	3	70	10	2,220	53	28.6 ± 4	+ 38	0/14
	6	7[b]	<1	354	9	41.6 ± 4	+ 102	2/22
	9	4[b]	<1	39	1	Toxic	–	5/14

[a] Average tumor volume V (mg) ± SE = $4/3\pi\,r^3$ (initial dia. = 1.5 mm). R_x daily for 5 days; % = $T(V_t - V_0)/(V_t - V_0) \times 100$ (2–3 separate experiments: 7–8 mice/group)
[b] Absence of palpable tumors in some animals (6/22 at 6 mg/kg)

However, in animals treated with the 10-ethyl derivative the survival times doubled.

The results of other solid tumor experiments using the Lewis lung carcinoma and T241 sarcoma models are shown in Table 6. Against Lewis lung carcinoma 10-deaza-aminopterin was only slightly more active than methotrexate and 10-ethyl, 10-deaza-aminopterin was about 3-fold more active. Against the T241 sarcoma, 10-deaza-aminopterin was less active than methotrexate. However, 10-ethyl,10-deaza-aminopterin was markedly more active than the other two analogs. Values for T/C for this analog were 18- to 24-fold lower than for methotrexate and 10-deaza-aminopterin. Also, approximately 30% of the animals treated with 10-ethyl,10-deaza-aminopterin did not show palpable tumors at the end of therapy.

The results presented here, which extend our earlier [12] studies with 10-deaza-aminopterin, would appear to further document the superior antitumor properties of this group of new antifolates in these murine models. The 10-alkyl derivatives of 10-deaza-aminopterin, in particular, seem to possess markedly greater antitumor properties against ascitic and solid forms of these murine tumors, and at least in the case of one mammary adenocarcinoma (E0771), substantially greater effects against metastatic disease.

The extent to which the superior antitumor activity of 10-deaza-aminopterin and its analogs compared with methotrexate is related to improved membrane transport or other biochemical properties in tumor versus normal proliferative tissue is evaluated in the companion report [17]. In contrast to the results obtained with the N^{10} analogs, these related studies appear to show that further improvement in selective antitumor action of this class of analog is obtained largely by maintaining the same potential for preferential accumulation in tumor cells of 10-deaza-aminopterin itself, while reducing by 10-alkylation the accumulation in drug-limiting normal proliferative compartments in the small intestine. In view of the similarity in biochemical and pharmacologic behavior [for reviews see 1, 8] of this category of antitumor agents in rodents and in man, the results described here suggest substantial clinical potential for this new family of analogs. With this goal in mind, studies are continuing in our laboratory.

Table 6. Antitumor activity of folate analogs against Lewis lung tumor and T241 sarcoma

Tumor	R_x	LD_{10} (mg/kg)	ATV[a] (mg)	T/C (%)
Lewis lung	–	–	741 ± 35	–
	Methotrexate	3–6	481 ± 16	66
	10-Deaza-AM	3–6	355 ± 8	48
	10-Ethyl,10-deaza-AM	3–6	179 ± 6	24
T241 sarcoma	–	–	852 ± 92	–
	Methotrexate	3	293 ± 26	35
	10-Deaza-AM	3	410 ± 53	48
	10-Ethyl,10-deaza-AM	6	17 ± 3[b]	2

[a] Average tumor volume on day 7 ± SE ($n = 2$, Lewis lung; $n = 3$, T241 sarcoma): R_x = daily for 5 days beginning 1 day after implantation

[b] Some animals had no palpable tumors (6/21)

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