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SYNTHESIS AND EVALUATION OF 2,4-DIAMINOQUINAZOLINE ANTIFOLATES WITH ACTIVITY AGAINST METHOTREXATE-RESISTANT HUMAN TUMOR CELLS*

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Abstract—In an attempt to find potent antifolates with selectivity against tumor cells with intrinsic or acquired resistance to methotrexate, eleven nonclassical 2,4-diaminoquinazoline antifolates were synthesized and tested as inhibitors of dihydrofolate reductase from L5178Y cells. Several compounds appeared to be good enzyme inhibitors, with I_{50} values around 1 nM. Two of the compounds were also good inhibitors of cell growth *in vitro*. One of these (PKC-32, 9-(2,4-diamino-5-methylquinazoline-6-methylene)aminophenanthrene) appeared to be 100-fold more potent than methotrexate as an inhibitor of growth of a methotrexate-resistant cell line with impaired transport for methotrexate. PKC-32 and PKC-155 were also tested against mouse tumors *in vivo*. PKC-32 was modestly active *in vivo* as compared with methotrexate. This drug may be a useful agent in the treatment of methotrexate-resistant tumors.

In recent years, several non-classical 2,4-diaminoquinazoline antifolates have been described with different spectra of antitumor activity compared with methotrexate [1–3]. Some of these compounds appeared to be good inhibitors of an altered dihydrofolate reductase from L5178Y leukemia cells with low affinity to methotrexate [3], giving rise to the hope that such drugs may be effective against selected tumors with intrinsic or acquired resistance to methotrexate. In an attempt to find potent antifolates with selectivity against such tumors, a series of 2,4-diaminoquinazoline antifolates with hydrophobic substituents (Table 1) were synthesized and evaluated as inhibitors of dihydrofolate reductase (DHFR) and as inhibitors of growth of mouse and human tumor cells.

The synthesis of some of these compounds is described and the evaluation of growth inhibitory properties is presented. The data indicate that one of the compounds has good activity against human leukemic cells resistant to methotrexate by virtue of impaired transport.

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§ J. B. Hynes, personal communication (1981).

‖ Abbreviations: DMF, *N,N*-dimethyl formamide; DMSO, dimethyl sulfoxide; MTX, methotrexate, 2,4-diamino-10-methylpteroylglutamic acid; DHFR, dihydrofolate reductase; FH_2, dihydrofolate; and PCA, perchloric acid.

MATERIALS AND METHODS

Chemical synthesis

Aromatic amines and aldehydes were purchased from the Aldrich Chemical Co., Milwaukee, WI. Elemental analyses were performed by the Baron Consulting Co., Orange, CT. As examples, syntheses of PKC-32 and PKC-155 are described below:

2,4-Diamino-5-methyl-6-cyanoquinazoline. 2,4,6-Triamino-5-methylquinazoline (6.62 g, 35 mmoles) was converted to the 6-cyanoquinazoline (2.96 g, 43% yield) by the procedure of Davoll and Johnson [4] with the modification that the crude product was extracted with 25% acetic acid five times (1 × 100 ml, 4 × 50 ml).

2,4-Diamino-5-methylquinazoline-6-carboxaldehyde. 2,4-Diamino-5-methyl-6-cyanoquinazoline was converted to the 6-carboxaldehyde by the method used for 2,4-diaminoquinazoline-6-carboxaldehyde according to Hynes.§ A stirred mixture of the cyanoquinazoline (2.7 g, 13 mmoles) in 98–100% formic acid (40 ml) was warmed to 65–70° under a N_2 purge. Moist T-1 Raney nickel catalyst [5] (*ca.* 6 g) was added in several portions and the stirring continued under N_2 purge for 5.5 hr at 65–70°. The mixture was diluted with DMF‖ (12 ml), treated with charcoal, and filtered hot. The catalyst was washed with additional DMF (3 × 12 ml), and the washings were added to the filtrate. The formate salt crystallized partially on cooling but redissolved upon stepwise addition of triethylamine (45 ml). The mixture was basified with 25% triethylamine (63 ml), diluted with H_2O (140 ml), and kept at 4° for overnight. The product, precipitated as the free base, was collected by filtration, washed with H_2O, CH_3OH, and CH_3COCH_3, and dried *in vacuo* at

100° for 1 hr, 1.57 g (60% yield), m.p. 308–310° (dec). It is suitable for condensation with aromatic amines without further purification.

9-(2,4-*Diamino-5-methylquinazoline-6-methylene*)-*aminophenanthrene* (*PKC*-32). A stirred mixture of 2,4-diamino-5-methylquinazoline-6-carboxaldehyde (789 mg, 3.9 mmoles), 9-aminophenanthrene (756 mg, 3.9 mmoles) in CH_3OH (25 ml) was evaporated at 70° until most of the CH_3OH was removed. The residue, after drying *in vacuo* to remove the last trace of CH_3OH, was kept at 140–147° for 35 min and then cooled slowly to 125°. After addition of acetic acid (5 ml), the mixture was cooled with stirring to 25°. The crude Schiff base was collected by filtration and washed with acetic acid (3 × 1 ml) and ethylacetate (2 × 5 ml). A suspension of the solid in ethylacetate (50 ml) was heated to boiling and then cooled to 4°. After 20 min, it was filtered, washed with ethylacetate and diethylether, and air-dried, 945 mg (64% yield).

A solution of the Schiff base (590 mg, 1.42 mmoles) in warm DMF (30 ml) was hydrogenated over 10% Pd-C (180 mg) at 40 psi for 2 hr. After removal of the catalyst by filtration, the filtrate, on standing at −16°, gave the first batch of PKC-32 (90 mg). An additional 230 mg of the product was obtained by concentration of the mother liquor *in vacuo* at 50° to a volume of 5 ml (60% yield). The analytical sample was recrystallized from CH_3OH–CH_2Cl_2, m.p. 286–289° (dec). Anal. Calc. for $C_{24}H_{21}N_5 \cdot 0.75\ H_2O$: C, 73.49; H, 5.59; N, 17.86. Found: C, 73.44; H, 5.83; N, 18.28.

2,4-Diamino-5-methyl-6-(phenanthrene-9-methylene)aminoquinazoline (PKC-155). The Schiff base of 2,4,6-triamino-5-methylquinazoline [1] and phenanthrene-9-carboxaldehyde was obtained in 71% yield in the same manner as described in the procedure of PKC-32, except that the reactants were kept at 136–140° for 50 min. Catalytic hydrogenation of the intermediate gave PKC-155 in 57% yield. The analytical sample was recrystallized from CH_3OH–CH_2Cl_2, m.p. 234–235° (dec). Anal. Calc. for $C_{24}H_{21}N_5 \cdot 1.5\ H_2O$: C, 70.92; H, 5.95; N, 17.23. Found: C, 71.23; H, 5.83; N, 17.11.

The absorption spectra of these two compounds were determined on a Cary spectrophotometer. Dissolved in 1% DMSO in water, PKC-32 had a λ_{max} of 242 nm and an extinction coefficient ε_{max} of 34.0. For PKC-155, a λ_{max} of 252 nm and an ε_{max} of 27.0 (estimated from graph) were found. Fluorescence excitation and emission spectra were determined on a Farrand model 801 spectrofluorometer equipped with a corrected excitation module and differential component. Fluorescence was seen only at millimolar concentrations (data not shown).

Chemicals

NADPH was obtained from the Sigma Chemical Co. (St. Louis, MO). Dihydrofolate (FH_2) was prepared by dithionite reduction of folic acid, as described by Blakley [6]. [^{3}H]dUrd was purchased from Amersham, Arlington Heights, IL. Methotrexate (MTX) was obtained from the National Cancer Institute, Bethesda, MD. All other chemicals were of the highest grade purity and obtained commercially.

Enzyme source

Dihydrofolate reductases (DHFR), purified from a methotrexate resistant subline of L5178Y mouse leukemia, from human K562 leukemia cells and from a highly MTX-resistant mouse fibroblast cell line, 3T6R, were used for enzyme inhibition studies [7–9].

Enzyme assay

DHFR was assayed spectrophotometrically on a Gilford model 2000 as described previously [10]. I_{50} values were obtained by inhibition assays in which the compound was added at various concentrations to a mix containing in a final volume of 1.0 ml: Tris–HCl buffer, pH 7.5, 100 μmoles; KCl, 150 μmol; NADPH, 0.1 μmole; enzyme and water. After a 2-min incubation at 37°, 0.02 μmole dihydrofolate and 0.2 μmole 2-mercaptoethanol in 0.01 M Tris–HCl, pH 8.5, were added to start the reaction. To compare the I_{50} values, care was taken to use the same amount of enzyme activity for each assay (ΔA 0.030/min at 37°). The drugs were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mM and then diluted in water to give the final concentration desired.

Cell culture studies

Logarithmically growing cells (L1210, K562, CCRF-CEM, HCT-8) in Fischer's medium with 10% horse serum, at a concentration of approximately 3×10^5 cells/ml, were diluted with medium plus serum to approximately 5×10^4 cells/ml. The cells were then distributed to 15 ml tissue culture tubes, in duplicate, containing either no inhibitor or various concentrations of inhibitor and incubated at 37°. Cells were counted at 48 hr (L1210, CCRF-CEM) or 72 hr (K562, HCT-8) with a model B Coulter Counter. The concentration of drug required to decrease the cell count to 50% of control (ED_{50}) was determined by plotting the cell number versus the drug concentration.

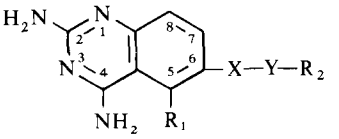
[^{3}H]dUrd incorporation into DNA

The ability of two of the compounds to supress dUrd incorporation into DNA was tested as described [11]. Two different lines of CCRF-CEM, one with transport resistance to methotrexate (provided by Dr. H. Lazarus from the Dana-Farber Cancer Center, Boston, MA), were incubated in a shaking water bath at 37° with either no inhibitor or various concentrations of inhibitor. After 1 hr of incubation, [^{3}H]dUrd (100 μCi) was added to 12 ml of cell suspension (2×10^6 cells/ml) to give a final concentration of 0.9 μM dUrd. The reaction was then stopped by adding 1 ml of cell suspension to 9 ml of ice-cold 5% PCA at different time points. The precipitates were subsequently washed in 5% PCA (10 ml), lysed by heating and counted in a liquid scintillation counter.

In vivo *studies*

Toxicity and antitumor effects of PKC-32 and -155 were tested in normal and tumor-bearing CDFl mice (females) by Dr. William C. Rose of the Pharmaceutical Research and Development Division of the Bristol-Myers Co. at Syracuse, NY. The drugs were dissolved either in DMSO and water or Tween-

Table 1. Structures and *in vitro* inhibitory effects of PKC compounds against purified dihydrofolate reductase from a methotrexate-resistant subline of L5178Y murine leukemia



Compound	X-Y	R_1	R_2	I_{50} (nM)
PKC-32	CH_2-NH	CH_3	9-Phenanthrene	1.4
PKC-153	CH_2-NH	H	2-Fluorene	2.2
PKC-154	CH_2-NH	H	9-Hydroxy-2-fluorene	43
PKC-158	CH_2-NH	CH_3	2-Fluorene	10
PKC-31	NH-CH_2	CH_3	1-Pyrene	15
PKC-151	NH-CH_2	H	9-Phenanthrene	94
PKC-152	NH-CH_2	H	2-Fluorene	150
PKC-155	NH-CH_2	CH_3	9-Phenanthrene	1.5
PKC-156	NH-CH_2	CH_3	2-Fluorene	1.7
PKC-157	NH-CH_2	CH_3	9-Anthracene	0.9
PKC-159	NH-CH_2*	CH_3	9-Phenanthrene	21
MTX				0.6

* The NH-CH_2 linkage is at the 8-position of the quinazoline ring.

80 and water. Drugs were injected i.p. either daily × 5 or on days 1 and 5. A plasma decay curve was generated for PKC-32 in BDF1 mice.

RESULTS

Two compounds, PKC-32 and PKC-155, were superior to the others, both as inhibitors of the DHFR enzyme as well as in growth inhibitory effects in cultures of various mouse and human tumor cell lines.

Enzyme inhibition studies

With the exceptions of PKC-151 and -152, all compounds were good inhibitors of the L5178Y DHFR. The best inhibitors were PKC-32, -153, -155, -156 and -157, with I_{50} values ranging from 0.9 nM to 2.2 nM (Table 1). Thus, these compounds were nearly as potent as the reference drug methotrexate (MTX) (I_{50} = 0.6 nM).

Two compounds (PKC-32 and -155) were tested further as inhibitors of DHFR of the human leukemia K562 cells, and of 3T6R cells, a mouse fibroblast cell line with an altered DHFR, highly resistant to MTX [9]. The results are shown in Table 2. MTX appears to be a superior inhibitor of both L5178Y- and K562-DHFR, but PKC-32 and -155 were both potent inhibitors as well. Interestingly, PKC-32 was some 7-fold more potent than MTX against the altered DHFR from 3T6R.

Tissue culture studies

All compounds were tested as growth inhibitors in various cell lines in tissue culture (outgrowth method). Two of the eleven compounds (PKC-32 and -155) were almost as potent as MTX, as shown in Table 3. It is of interest that PKC-32, although 20-fold less active than MTX against the human K562 DHFR, was equally effective as MTX in tissue culture against K562 cells. Poor enzyme inhibitors were invariably poor inhibitors of cell growth.

In view of the results in enzyme and tissue culture studies, PKC-32 and -155 were selected for further evaluation. PKC-32 and MTX were both good inhibitors of the growth of CCRF-CEM cells with EC_{50} values of 0.01 to 0.05 μM. However, PKC-32 was more than 100-fold more potent than MTX against CCRF-CEM/R, a variant cell line with resistance to MTX by virtue of impaired transport, as shown in Fig. 1. The EC_{50} values were 32 nM and 4.0 μM for PKC-32 and MTX respectively.

PKC-32 and -155 and MTX were potent inhibitors of [^{3}H]dUrd incorporation into DNA of CCRF-CEM cells. PKC-32 (0.5 μM) caused almost total inhibition of [^{3}H]dUrd incorporation into DNA in CCRF-CEM/R, but MTX was totally inactive in this cell

Table 2. Inhibitory effects of methotrexate, PKC-32 and PKC-155 expressed as the concentration of drug (nM) required for 50% inhibition (I_{50}) of dihydrofolate reductase of various cell lines

	I_{50} (nM)		
	Enzyme		
Compound	L5178Y-DHFR	K562-DHFR	3T6R-DHFR
MTX	0.6	1.5	6000
PKC-32	1.4	30	800
PKC-155	1.5	6.5	1300

Table 3. Growth inhibitory effects of PKC compounds expressed as the concentration of drug (nM) required for inhibition of cell growth by 50% (EC_{50})

Compound	EC_{50} (nM)			
	L1210	K562	CCRF-CEM	HCT-8
PKC-31	42	250	50	190
PKC-32	16	20	18	85
PKC-151	>1000		>1000	770
PKC-152	>1000	650		
PKC-153	150	370	>1000	
PKC-154		>1000	>1000	
PKC-155	37	52	32	58
PKC-156	600	650	160	
PKC-157	>1000	>1000	>1000	
PKC-158		340	120	370
PKC-159	130	>1000		>1000
MTX	6	40	12	19

line, even at a 2 μM drug concentration (Fig. 2). In all these tests, PKC-155 (data not shown) was somewhat less active than PKC-32.

In vivo *studies*

The maximum tolerated dose of PKC-32 was 16 mg/kg daily × 5 or 32 mg/kg on days 1 and 5. PKC-155 appeared to be both nontoxic and ineffective in tumor-bearing mice at dose levels up to 128 mg/kg Q × 4D × 2. The *in vivo* antitumor effects of the PKC-compounds compared with those of MTX are summarized in Table 4. From these

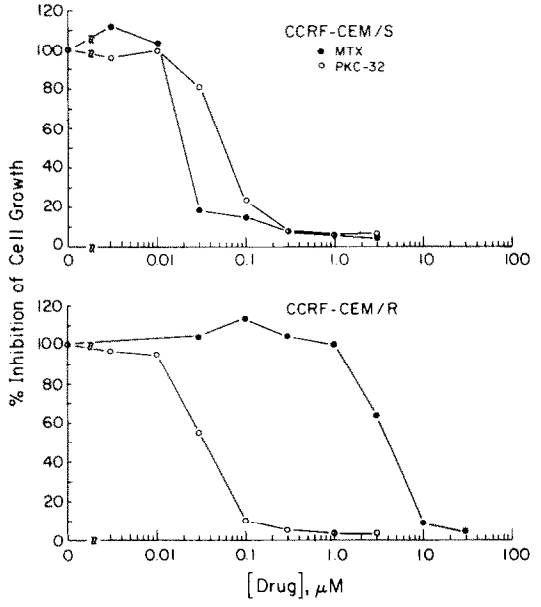


Fig. 1. Comparison of growth inhibitory effects of MTX (●) and PKC-32 (○) in sensitive CCRF-CEM cells (top) and in CCRF-CEM cells resistant to MTX by virtue of impaired membrane transport (bottom). To be sure that the observed difference was not partly related to the fact that PKC-32 had been dissolved in dimethyl sulfoxide (DMSO) and water, a separate experiment was done comparing the EC_{50}'s values of MTX dissolved in DMSO and water and of MTX in water alone; these curves were superimposable.

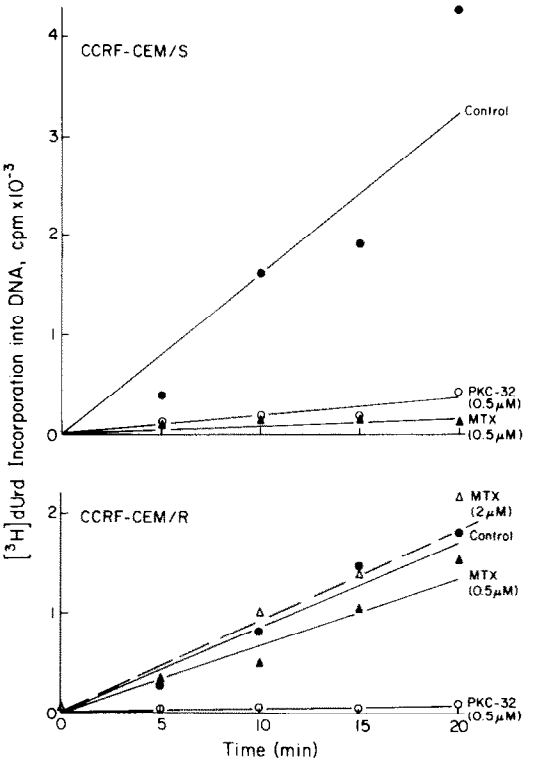


Fig. 2. Inhibition of [^{3}H]dUrd incorporation into DNA by MTX and PKC-32 in normal CCRF-CEM cells (top) and in transport-resistant CCRF-CEM cells (bottom). Symbols indicate: control (●), MTX, 0.5 μM (▲), MTX, 2 μM (△) and PKC-32, 0.5 μM (○).

data it appears that PKC-32 had modest but definite activity against murine leukemia cell lines P388 and L1210 *in vivo*. PKC-155 failed to cause any improved survival compared with controls (data not shown).

After a single i.p. injection of PKC-32 (30 mg/kg), an initial plasma $T_{1/2}$ of *ca.* 100 min, was found with a slow final phase of the plasma decay curve.

DISCUSSION

PKC-32 and PKC-155 are potent inhibitors of DHFR and of the growth of several mouse and human tumor cell lines. Particularly interesting was the finding that PKC-32 was very effective in tissue culture against CCRF-CEM/R, the cell line with transport resistance to MTX, suggesting that PKC-32 enters cells by a different membrane transport mechanism than does MTX.

In methotrexate sensitive tumors, PKC-32 was somewhat less active than MTX, both *in vitro* and *in vivo*. PKC-155, which differs from PKC-32 only in reversal of the carbon-nitrogen bridge (see Table 1), was completely inactive and also nontoxic in the mouse studies, suggesting that this compound is either rapidly catabolized *in vivo*, or markedly differs from the other compound in pharmacokinetic behaviour.

On a molar basis, PKC-32 was less toxic *in vivo* than MTX, suggesting either a different mechanism of action or metabolism. PKC compounds cannot be

Table 4. Comparison of methotrexate and PKC-32 against murine leukemias P388 and L1210 *in vivo*

Drug	Dose (mg/kg)	Schedule	P388 %T/C*	L1210 %T/C*
MTX	64	i.p., Q × 4D × 2		123 (5/6)
	32			131 (6/6)
	16			192 (6/6)
	8			169 (5/5)
	8	i.p., Q × 1D × 5		TOX (1/6)
	4		89 (4/6)	131 (6/6)
	2		111 (6/6)	169 (6/6)
	1		172 (6/6)	185 (6/6)
	0.5		167 (6/6)	
PKC-32	128	i.p., Q × 4D × 2		TOX (1/6)
	64			100 (6/6)
	32			138 (6/6)
	16			115 (6/6)
	8			108 (6/6)
	4			108 (6/6)
	32	i.p., Q × 1D × 5		TOX (3/6)
	16		144 (6/6)	123 (5/6)
	8		128 (6/6)	123 (6/6)
	4		122 (6/6)	108 (5/6)
	2		106 (6/6)	108 (5/6)
	1		100 (6/6)	108 (6/6)
	0.5		100 (6/6)	

* T/C, ratio of test (T) evaluation to control (C) evaluation expressed as a percentage.

polyglutamylated; therefore, a different schedule of administration may be required to obtain maximum antitumor effects (e.g. a low dose continuous infusion). Recently, other quinazoline antifolates have been described [3] that are significantly more potent (up to 100-fold) than MTX as inhibitors of an altered, methotrexate-insensitive DHFR. These studies indicate that it may be possible to synthesize antifolates which have specificity against tumors with intrinsic or acquired resistance to MTX.

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