Biochemical Pharmacology, Vol. 34, No. 12, pp. 2163–2167, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

INHIBITION OF DIHYDROFOLATE REDUCTASE, METHOTREXATE TRANSPORT, AND GROWTH OF METHOTREXATE-SENSITIVE AND -RESISTANT L1210 LEUKEMIA CELLS *IN VITRO* BY 5-SUBSTITUTED 2,4-DIAMINOQUINAZOLINES

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(*Received* 1 *August* 1984; *accepted* 24 *October* 1984)

Abstract—A series of eighteen 2,4-diaminoquinazoline analogues of folic, isofolic, pteroic and isopteroic acids having various substituents at position 5 was studied. Each compound was evaluated as an inhibitor of L1210 dihydrofolate reductase, methotrexate influx into L1210 leukemia cells, and growth of methotrexate-sensitive and -resistant L1210 cells *in vitro*. Bridge reversal at positions 9 and 10 reduced the effectiveness of the classical analogues only with regard to the inhibition of the drug-sensitive cells as compared to methotrexate (MTX). Absence of the glutamate moiety adversely affected the potency of the compounds, particularly when coupled with reversal of the 9,10-bridge. However, the presence of –Cl at position 5 restored significantly the potency of these compounds. The pteroate and isopteroate analogue ethyl esters were generally more effective inhibitors of cell growth than their non-esterified counterparts. Regarding the effects of substituents at position 5, the data suggest that $-Cl > -CH_3 > -H$ for inhibition of methotrexate transport and growth of methotrexate-sensitive L1210 cells. The 5-Cl pteroate analogue and its corresponding ethyl ester were highly effective as growth inhibitors of methotrexate-resistant, transport-defective, L1210 cells *in vitro*.

Methotrexate (MTX)§, an antagonist of folic acid metabolism, is widely used as a chemotherapeutic agent acting as an inhibitor of the enzyme dihydrofolate reductase (DHFR). Some neoplastic disorders are naturally resistant to the action of MTX, while others develop an acquired resistance to the drug despite initial sensitivity. Resistance may be due to increased cellular production of DHFR [1], production of a methotrexate-insensitive DHFR [2, 3], or reduced transport of MTX into cells [4, 5].

Effectiveness as a potential chemotherapeutic agent requires efficient transport of the compound into cells as well as potent inhibition of the target enzyme. Some structural determinants for folate antagonists which influence transport into cells and inhibition of DHFR have been identified [6]. The structure of the primary ring system for inhibition of DHFR is in the order quinazoline > pteridine ≃ pyrimidine ⋙ purine, while for transport into cells the order is pteridine > quinazoline ⋙ pyrimidine ≃ purine. It has been further suggested that positions 1, 3, 4, 5, 8 and the α-carboxyl group of glutamic acid are important for binding to DHFR, but that position 10 and the γ-carboxyl group are less important. For transport into cells, positions 4, 5, 8, 10 and both α- and γ-carboxyl groups have been proposed to be important [6].

It has been observed that 2,4-diaminoquinazolines are more powerful inhibitors of DHFR than their 4-oxo counterparts, and that small hydrophobic groups located in position 5 significantly increase inhibitory potency [7–10]. In view of these considerations, a homologous series of 2,4-diaminoquinazoline analogues of folic, isofolic, pteroic and isopteroic acids with $-CH_3$ or –Cl substituents at position 5 was studied. Each compound was evaluated as an inhibitor of DHFR from L1210 murine leukemia cells and of [^{3}H]MTX influx into L1210 cells. In addition, each compound was examined as a growth inhibitor of MTX-sensitive and -resistant, transport defective, L1210 cells *in vitro*.

MATERIALS AND METHODS

Reagents. Cell culture products were purchased from KC Biologicals (Lenexa, KS). MTX was a gift from Lederle Laboratories (Pearl River, NY). [3′,5′,7-^{3}H]MTX was obtained from Amersham (Arlington Heights, IL) and was purified before use as described previously [5]. Dihydrofolic acid was prepared from commercial folic acid by dithionite reduction [11] and stored at −20° as a suspension in 10^{-3} N HCl. NADPH was purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade or of the highest quality available.

Cell lines. MTX-sensitive L1210 cells (L1210/S) were grown in suspension culture as described previously [5]. MTX-resistant L1210/R81 cells with a 35-fold elevation of DHFR and completely defective

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§ Abbreviations: MTX, methotrexate (4-amino-4-deoxy-10-methylpteroylglutamate); DHFR, dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3); and DMSO, dimethyl sulfoxide.

MTX transport [5] were maintained in culture medium containing 10 μM MTX. Resistant cells were cultured in the absence of MTX for at least eight cell doublings before use in order to eliminate bound MTX. Both cell lines have population doubling times of 12–14 hr.

Quinazoline solutions. The quinazoline compounds were freshly dissolved in dimethyl sulfoxide (DMSO). Aliquots of the DMSO solutions were slowly added to the appropriate aqueous buffers. The DMSO concentration in working solutions was ≤0.5% (v/v) in order to avoid interference with the DHFR assay and intact cell studies.

Standard enzyme assay. Dihydrofolate reductase from L1210 cells was purified by affinity chromatography as previously described [12], and the enzyme activity was assayed spectrophotometrically. Initial rates were derived from the change in absorbance continuously recorded with a Cary model 219 spectrophotometer. The assay mixture, in a total volume of 1 ml, consisted of 0.05 M Tris–chloride buffer (pH 7.2), 150 μM NADPH, and 113 μM dihydrofolate. The standard unit of enzyme activity was determined from the change in absorbance at 340 nm using a $\Delta\varepsilon$ for the reaction of 12,300 M^{-1} at 22° [13]. Inhibition studies were carried out by preincubating the enzyme, NADPH and inhibitor in the assay buffer for 2 min at 22°, and residual enzyme activity was determined after the addition of dihydrofolate. Remaining activity was expressed as a percentage of activity compared to the activity of the enzyme obtained in the absence of inhibitor.

[^{3}H]MTX uptake determinations. Uptake of [^{3}H]-MTX into L1210/S cells was measured in RPMI 1640 without folic acid following the procedures previously described [5]. Uptake at 37° was determined 10 min after the simultaneous addition of inhibitor and [^{3}H]MTX (to a final concentration of 1 μM [^{3}H]-MTX). I_{50} represents the amount of compound required for 50% inhibition of [^{3}H]MTX uptake into drug-sensitive cells.

Cell growth inhibition. Cells at an initial concentration of 5×10^4 cells/ml were grown in medium containing various concentrations of quinazoline compound in 24-well cluster dishes and incubated at 37° in a 5% CO_2 atmosphere. After 48 hr, cells were counted using a model Z_F Coulter counter (Coulter Electronics, Hialeah, FL). The drug concentration which reduces the number of cells in 48 hr to 50% of the untreated control value is expressed as IC_{50}.

Synthesis. Melting points were determined on a Mel-Temp apparatus. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. All analytical samples were dried under vacuum at 100° and gave combustion values for C, H, and N within ±0.4% of the theoretical values unless indicated otherwise. All intermediates were free of significant impurities on TLC using silica gel sheets (Baker 1B2-F). Target compounds were analyzed by high performance liquid chromatography (HPLC) using a Micromeritics model 7000B liquid chromatograph with a u.v. (254 nm) detector. The pteroic acid analogues were run on a Microsil C_{18} (7.5 μm) reverse phase column (4.6 × 250 mm i.d.) and eluted with a 15-min linear gradient (0–25%, v/v) of acetonitrile in water containing 0.007 M triethylamine and 0.017 M acetic acid, pH 6.5, at a flow rate of 1.5 ml/min. Samples for HPLC were dissolved in dimethyl sulfoxide just prior to injection. The u.v. spectra were determined using a Cary 219 spectrophotometer in 0.1 M phosphate buffer, pH 7.0. The ^{1}H NMR spectra were determined with a Varian EM 390 spectrometer operating at 90 MHz and were consistent with the assigned structures. Fast atom bombardment mass spectra (FAB/MS) were obtained on a Finnigan MAT 212 Spectrometer using Argon bombardment.

The compounds employed in this study (see Table 1) were prepared as described elsewhere [7–10] with the exception of 6-[(4-carboxybenzyl)amino]-2,4-diaminoquinazoline, **10**, and its 5-methyl analogue, **11**. In earlier attempts to prepare **10** and **11**, the 4-amino group was removed concurrently with the hydrolysis of the ester function [9]. Therefore, alternative synthetic methods were used. Compound **11** was produced by the direct alkylation of 5-methyl-2,4,6-triaminoquinazoline [14] with 4-bromomethylbenzoic acid. An equimolar mixture (5 mmoles) of 5-methyl-2,4,6-triaminoquinazoline and 4-bromomethylbenzoic acid in *N*,*N*-dimethylacetamide (30 ml) was stirred at 55–60° for 120 hr while being monitored by TLC (*n*-butanol–acetic acid–water, 8:1:1). The solvent was removed under vacuum and the residual solid was triturated with water (2 × 20 ml), filtered, and washed with chloroform (3 × 20 ml) and acetone (2 × 10 ml). The product was dissolved in 0.5 N NaOH (10 ml) and filtered to remove insoluble impurities. The solution was acidified to pH 4 with 0.5 N HCl in a cold bath. The yellowish-green precipitate was separated by filtration, and washed with water (2 × 20 ml), ether (2 × 20 ml) and acetone (10 ml). After drying under vacuum at 100°, there was obtained 0.9 g (51%) of yellow solid; m.p. >360°; dec. TLC (*n*-butanol–acetic acid–water, 6:2:2); FAB/MS, $m/e = 324$ $(M + 1)^+$; UVλ_{max} 252 nm (ε 34.4 × 10^3). Anal.: ($C_{17}H_{17}N_5O_2 \cdot 0.5\ H_2O \cdot 0.5$ HCl) C, H; N calcd. 19.98, found 18.41.

Compound **10** was synthesized by reductive condensation of 2,4,6-triaminoquinazoline [14] with ethyl 4-formylbenzoate followed by careful saponification of the resulting ester, **16** [9]. To a stirred suspension of **16** (2.6 mmoles) in 80% ethanol (20 ml), 0.2 N NaOH (80 ml) was added and the resulting mixture allowed to stir at room temperature for 12 hr. The solution was filtered, concentrated under vacuum, and then diluted with 80 ml of cold water. Next, it was acidified with 0.5 N HCl to pH 4 while cooling and stirring. The greenish yellow precipitate thus obtained was separated by filtration, washed with water (4 × 20 ml), acetone (2 × 20 ml), ether (10 ml), and dried under vacuum at 100° to yield 0.65 g (67%); m.p. >360°; TLC (*n*-butanol–acetic acid–water, 6:2:2); FAB/MS, $m/e = 310$ $(M + 1)^+$; UVλ_{max} 244 (ε 37.6 × 10^3) Anal.: ($C_{16}H_{15}N_5O_2 \cdot 1.5\ H_2O \cdot$ HCl). C, H, N.

An alternate preparation of **10** was conducted in the following manner. Compound 4 (90 mg) was added to 10 ml of 0.1 M Tris buffer, and the pH was adjusted to 9.5 with 1 N KOH. When the compound was completely dissolved, the pH was lowered to 7.5 with 1 N HCl. Carboxypeptidase G_1 [15] (48 units)

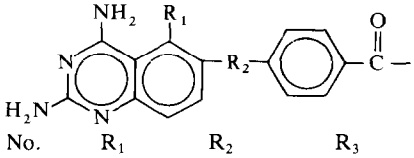
was added, and the reaction continued overnight at room temperature with stirring. The product, present as a precipitate, was washed twice with 5-ml aliquots of water. It was then dissolved in ethanol–benzene (1:1), evaporated to dryness on a rotary evaporator, and purified by HPLC [16]. This material had an identical retention time to that prepared chemically, and the mass spectra and ultraviolet spectra were also the same.

RESULTS

The DHFR inhibitory activities of compounds **1–18** were compared with that of MTX against purified enzyme from L1210 murine leukemia cells (Table 1). Compounds **1–3**, which have a normal folate configuration at positions 9 and 10 and a terminal L-glutamate moiety with $-H$ (1), $-CH_3$ (2) or $-Cl$ (3) substituents at position 5, showed the same potency of inhibition as MTX. The corresponding isofolate analogues (**4–6**) in which the 9 and 10 positions are reversed showed only a slight decrease (4,5) or no change in potency (6). The pteroate analogues (**7–9**), lacking a terminal L-glutamate, were also reasonably good inhibitors of L1210 DHFR, compounds **8** and **9** being nearly as potent as MTX. For the isopteroate analogues (**10–12**), the reversal of the configuration at positions 9 and 10 had a deleterious effect on enzyme binding. This was especially true for compound **10** which was profoundly less inhibitory than its isomer **7**. Samples of **10** prepared synthetically or by removal of the glutamate moiety of **4** by carboxypeptidase G_1 treatment were shown to be identical chemically and gave virtually identical enzyme inhibition results. By contrast, the addition of $-CH_3$ (**11**) or $-Cl$ (**12**) at position 5 of the isopteroate analogue dramatically restored reasonable potency, with **12** being only slightly less inhibitory than its isomer 9. The ethyl ester pteroate analogues (**13–15**) were somewhat less potent as DHFR inhibitors compared to the corresponding free acids (**7–9**). The ethyl ester isopteroate analogue, **16**, was 20-fold more potent as a DHFR inhibitor than its corresponding free acid, **10**. Compound **17** was 2.3-fold more inhibitory than **11**, while **18** was *ca.* 3.5-fold less potent than **12**.

Each of the eighteen compounds was evaluated for inhibition of the influx of [^{3}H]MTX into drug-sensitive L1210 cells. Compounds **1** through **6** were all effective inhibitors of [^{3}H]MTX influx. The presence of $-CH_3$ or $-Cl$ substituents at position 5 (**2**, **3** or **5**, **6**) or the reversal of the 9,10-bridge (**4–6**) had virtually no effect on transport competition with [^{3}H]-MTX. Removal of the glutamate moiety resulted in a drastic reduction in the competition with most of the compounds examined. Pteroate analogues **7** and **8** exhibited *ca.* a 20-fold reduction in their affinity for the MTX transport system, whereas compound **9**, bearing a 5-Cl substituent, was only 3.7-fold less inhibitory in this regard. Reversal of positions 9 and 10 resulted in a further reduction of transport competition. Compounds **10** and **11** showed an additional 5-fold reduction in terms of inhibition of [^{3}H]MTX transport as compared with **7** and **8**,

Table 1. Effects of 5-substituted 2,4-diaminoquinazolines on cell growth, influx of methotrexate and dihydrofolate reductase inhibition in L1210 leukemia cells



No.	R_1	R_2	R_3	L1210 DHFR I_{50}* (μM)	[^{3}H]MTX influx I_{50}† (μM)	Growth inhibition IC_{50}‡ (μM) L1210/S	L1210/R81
			(MTX)	(0.045)	(1.0)	(0.012)	(205)
1	H	CH_2NH	Glu	0.048	2.1	0.021	48
2	CH_3	CH_2NH	Glu	0.044	1.2	0.018	25
3	Cl	CH_2NH	Glu	0.046	1.1	0.0025	38
4	H	$NHCH_2$	Glu	0.060	1.6	1.5	42
5	CH_3	$NHCH_2$	Glu	0.063	2.0	0.46	54
6	Cl	$NHCH_2$	Glu	0.045	1.4	0.13	15
7	H	CH_2NH	OH	0.083	45	3.9	100
8	CH_3	CH_2NH	OH	0.051	24	0.14	18
9	Cl	CH_2NH	OH	0.048	4.1	0.019	0.29
10	H	$NHCH_2$	OH	10.0	200	74	120
11	CH_3	$NHCH_2$	OH	0.123	125	3.8	120
12	Cl	$NHCH_2$	OH	0.063	26	0.42	33
13	H	CH_2NH	OC_2H_5	0.125	100	0.093	1.45
14	CH_3	CH_2NH	OC_2H_5	0.063	35	0.14	5.5
15	Cl	CH_2NH	OC_2H_5	0.190	0.69	0.012	0.25
16	H	$NHCH_2$	OC_2H_5	0.50	190	1.5	9.0
17	CH_3	$NHCH_2$	OC_2H_5	0.054	13	1.5	3.7
18	Cl	$NHCH_2$	OC_2H_5	0.22	30	0.047	2.2

* Assayed spectrophotometrically at 340 nm. Conditions: dihydrofolate, 113 μM; NADPH, 150 μM; DHFR, 9×10^{-8} M; in 0.05 M Tris–chloride buffer, pH 7.2.

† Amount required for 50% inhibition of influx of 1.0 μM [^{3}H]MTX into L1210/S cells at 37° for 10 min.

‡ Amount required for 50% inhibition of cell growth in 48 hr, relative to untreated controls.

respectively, while **12** exhibited a 6-fold reduction as compared with **9**. The pteroate ethyl ester analogues **13** and **14** showed at most a 2-fold reduction in affinity for the MTX transport system as compared with **7** and **8** respectively. By contrast, **15**, bearing a 5-Cl substituent, was 6-fold more potent than **9**. The order of effectiveness of 5-substituents for compounds **7** through **15** was clearly $-Cl > -CH_3 > -H$ in terms of transport competition for MTX. In fact, the 5-Cl compound (**15**) was 51-fold more inhibitory than the corresponding 5-CH_3 compound (**14**). The presence of an ethyl ester group on the 5-Cl pteroate analogue (**15**) contributed *ca.* a 6-fold greater potency of transport inhibition as compared with the corresponding free acid (**9**). This pattern did not hold for the ethyl ester isopteroate analogues (**16–18**), none of which were effective inhibitors of MTX influx. However, the 5-CH_3 isopteroate ethyl ester analogue (**17**) was 2.3-fold more potent than the corresponding 5-Cl analogue (**18**).

The compounds indicated in Table 1 were also evaluated as growth inhibitors of MTX-sensitive L1210 cells. As expected, the folic acid analogues (**1–3**) were effective growth inhibitors of L1210/S cells; in fact, compound **3**, bearing a 5-Cl substituent, was nearly 5-fold more potent than MTX against this cell line. Reversal of the groups at positions 9 and 10 (**4–6**) reduced the potency of the compounds for growth inhibition of L1210/S cells. However, it is apparent that the compounds possessing either a $-CH_3$ or $-Cl$ at position 5 (**5** and **6** respectively) were more effective growth inhibitors than the unsubstituted compound **4**.

The pteroate analogues **7–9**, lacking the glutamate moiety of the folic acid analogues, were 8- to 186-fold less potent as growth inhibitors of L1210/S than the corresponding classical analogues, **1–3**. However, the growth inhibitory effectiveness of the 5-chloro derivative, **9**, was comparable to that of MTX. Again, reversal of the groups at positions 9 and 10 further reduced the potency of the isopteroate analogues (**10–12**) beyond that of the pteroate counterparts (**7–9**). The presence of the ethyl ester (**13**) on the pteroate analogue enhanced its growth inhibitory effect in L1210/S cells compared to **7**, but the 5-CH_3 analogue (**14**) showed no change in growth inhibitory effectiveness (**14** vs **8**). The effectiveness of the 5-Cl ester (**15**) was similar to that of its corresponding free acid, **9**, and MTX against L1210/S cells. The isopteroate ethyl ester analogues (**16–18**) were more effective growth inhibitors of L1210/S cells than the corresponding free acids (**10–12**), but they were not as potent as the corresponding normal-bridged ethyl esters (**13–15**).

A MTX-resistant L1210 cell subline (L1210/R81) was also utilized in these studies. This subline exhibits a double mutational defect in that it has impaired MTX transport and also contains a 35-fold elevation of DHFR [5]. Although the L1210/R81 cell line was highly resistant to MTX, it did not appear to be completely cross-resistant to the 2,4-diaminoquinazoline compounds. Most of the compounds evaluated were more effective growth inhibitors than MTX in L1210/R81 cells. The classical analogues of MTX (**1–3**) were 5- to 8-fold more potent than MTX for growth inhibition of L1210/R81 cells. The potency of the bridge-reversed analogues (**4–6**) against the MTX-resistant cell line was similar to that of compounds **1–3**, even though this modification had an adverse effect upon activity against L1210/S cells.

Removal of the glutamate moiety reduced the potency of compound **7** against L1210/R81 cells but had little effect on the activity of **8**. The 5-Cl pteroate analogue (**9**), however, was *ca.* 700-fold more effective than MTX in growth inhibition of L1210/R81 cells. As is the case against L1210/S cells, the isopteroate analogues (**10–12**) were poorer inhibitors of L1210/R81 growth than the corresponding pteroate analogues (**7–9**). The ethyl ester derivatives of the pteroate and isopteroate analogues (**13–18**) were 23- to 800-fold more effective than MTX in inhibiting the growth of L1210/R81 cells. With the exception of compound **15**, the ethyl ester derivatives were also more effective than their corresponding free acids (**7–12**). Clearly, compounds **9** and **15** show the most dramatic effects in this series on the growth inhibition of these MTX-resistant cells.

DISCUSSION

The inhibitory potency of classical folate analogues with $-CH_3$ or $-Cl$ substituents at position 5 against DHFR from mammalian sources has been reported previously [10, 17, 18]. These compounds were found to effectively inhibit the influx of [^{3}H]MTX into L1210/S cells and have been shown to have K_m values for influx similar to that of MTX in L1210 cells [18]. Growing L1210/S cells were inhibited by low concentrations of the folate analogues, while MTX-resistant L1210/R81 cells, which have elevated levels of DHFR and defective MTX transport, were not completely cross-resistant to the quinazoline compounds. Increased sensitivity to the growth inhibitory effects of these folate analogues could be due to lower rates of efflux of the quinazoline compounds from the cells [18], perhaps governed by differences in rate and/or extent of polyglutamate formation. A similar pattern of increased sensitivity to the folate analogues has been observed in other L1210 cell lines [19]. Although the isofolate analogues are potent inhibitors of DHFR [8, 10] and of [^{3}H]MTX influx into L1210/S cells, the compounds are considerably less effective than the folate analogues as growth inhibitors of L1210/S cells. The compounds may not be transported into the cell as readily as the folate analogues even though they effectively bind to the surface receptor to prevent uptake of [^{3}H]MTX. Alternatively, these compounds may be less efficiently polyglutamated than are those with a normal bridge region. Reversal of the groups at positions 9 and 10 did not significantly alter the potency of the analogues in L1210/R81 cells.

The effectiveness of $-CH_3$ or $-Cl$ substituents at position 5 of the pteroate analogues clearly indicates that $-Cl > -CH_3 > -H$ for each of the parameters studied. Although the unsubsituted pteroate analogue, **7**, was a relatively poor inhibitor of L1210/S growth, the 5-Cl compound, **9**, was as potent as MTX. In addition, **9** effectively inhibited the growth of MTX-resistant L1210/R81 cells. Previous work

has indicated that [^{3}H]MTX apparently binds weakly to the surface of L1210/R81 cells, but is not transported across the cell membrane [5]. Since compound **9** is more hydrophobic than MTX, it may enter L1210/R81 cells by an altered transport system which is incapable of translocating MTX or by an alternative pathway. Studies are in progress to determine whether or not the compound is actively transported into L1210/R81 cells and if its mode of action within the cells differs from that of MTX. Compound **9** has been shown to be an effective inhibitor of a methotrexate-insensitive DHFR produced by an MTX-resistant subline of L5178Y mouse leukemia cells [20]; however, no evidence of a methotrexate-insensitive DHFR has been found in the L1210/R81 cells.

The isopteroate analogues were less effective inhibitors for all parameters examined than the corresponding pteroate analogues, but again demonstrated an enhanced potency provided by the introduction of the nonpolar substituents at position 5. The unsubstituted isopteroate analogue, **10**, was the least effective inhibitor evaluated. This compound was synthesized by a chemical as well as an enzymatic route in order to confirm its structure.

The ethyl ester derivatives of the pteroate and isopteroate analogues were generally more effective inhibitors of cell growth than their free acid counterparts, perhaps due to the more lipophilic nature of these compounds which facilitates entry into cells. Lipophilic 2,4-diamino-6-[(3-bromobenzylamino)-methyl] quinazoline compounds with $-H$, $-CH_3$, or $-Cl$ substituents at position 5 are potent inhibitors of L1210 cell growth *in vitro* [21]. Growth of a transport-defective, MTX-resistant, human leukemic cell line has been shown to be inhibited significantly *in vitro* by γ-mono- and di-*n*-butyl esters of MTX [22]. Although **15** was a less potent inhibitor of DHFR than the corresponding free acid **9**, it had enhanced ability to compete with [^{3}H]MTX for uptake, and it remains an effective inhibitor of resistant L1210 cell growth. It is possible that **15** may be hydrolyzed to **9** intracellularly and, therefore, its mechanism of action may be similar to that of **9**. Further studies with these compounds against other types of MTX- and quinazoline-resistant cell lines appears warranted.

Acknowledgements—This work was supported by Grants CA 11666, National Cancer Institute, NIH, PHS (to J. H. F.) and CA 25014 (to J. B. H.). One of us (A. K.) was the recipient of a Postdoctoral Fellowship from the College of Graduate Studies, Medical University of South Carolina, 1983–1984. The authors thank Eric Adolph, Kelvin Wade and Regina McStraw for technical assistance at various stages in the completion of this work. We also wish to thank Mr. Kevin Ballard and Mr. James Vrbanac of the Mass Spectrometry Laboratory, Department of Pharmacology, Medical University of South Carolina for the FAB/MS spectra.

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