Irreversible Enzyme Inhibitors. CXXXIII.[1-3] Studies on Differential Irreversible Inhibition of Dihydrofolic Reductase from Three Strains of L1210 Leukemia, Liver, Spleen, and Intestine of the Mouse

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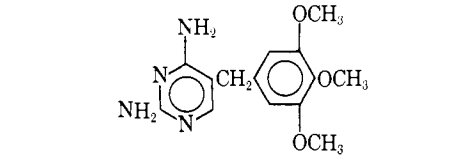
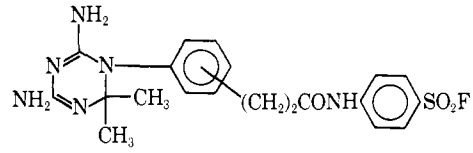
By use of selected irreversible inhibitors it appears that the dihydrofolic reductase from L1210/FR8, L1210/DF8, and the parent L1210/0 strain are not appreciably different. Contrariwise the dihydrofolic reductase from mouse liver, spleen, and intestine could be shown to be structurally different from each tissue as well as different from the enzyme from the three L1210 strains. These structural differences are most probably outside of the active site. It has been further demonstrated that of two sulfonyl fluoride type inhibitors with almost equal reversible inhibition power, only the one that showed irreversible inhibition of L1210/0 dihydrofolic reductase was effective in giving a life extension of mice bearing L1210/0; from these data, minimum criteria for enzyme activity have been set to determine whether a candidate irreversible inhibitor of dihydrofolic reductase is worthy of *in vivo* assay against L1210 mouse leukemia.

It is relatively simple to design an enzyme inhibitor by proper isosteric replacement of one or more groups on a substrate;[5] such isosteric-type inhibitors show little if any specificity toward the enzyme[6] from different sources, since only complexing with the enzymic active site is employed where little difference can be expected to occur in the enzyme from different sources.[8] By utilization of binding areas just adjacent to the active site it is possible to inhibit selectively the same enzyme from different species or even different tissues in the same animal.[10,11]

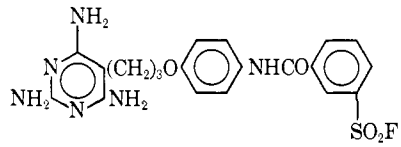
A study of the binding to the hydrophobic bonding region of dihydrofolic reductase from Walker 256 tumor and the liver of the rat by 45 selected heterocycles showed only a maximum of 100-fold difference in binding; smaller differences of two- to tenfold were fairly frequent.[12] Thus it is unlikely that utilization of only the active site and the adjacent hydrophobic binding area of dihydrofolic reductase would give sufficient difference for chemotherapy of cancer. Therefore, two additional parameters for possible tissue selectivity were introduced: (a) a covalent-forming group such as bromoacetamido[13] or SO_2F[14-17] was introduced



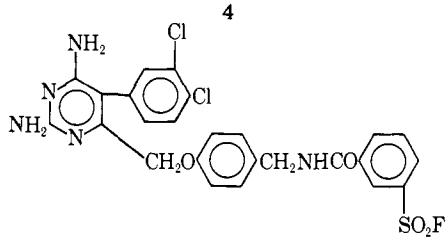
1



2, *meta*
3, *para*



4



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on the hydrophobic bonding group of the inhibitor where the leaving group could now reside in a polar region of the enzyme at the end of the hydrophobic bonding region[18] and (b) with the SO_2F leaving group two reactions involving the enzyme–inhibitor complex could occur, namely, the desired covalent bond formation

(1) This work was generously supported by Grant CA-08695 from the National Cancer Institute, U. S. Public Health Service.

(2) For the previous paper of this series see B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **11**, 1054 (1968).

(3) For the previous paper on dihydrofolic reductase see B. R. Baker and G. J. Lourens, *ibid.*, 677 (1968), paper CXXIX of this series.

(4) G. J. L. wishes to thank the Council for Scientific and Industrial Research, Republic of South Africa, for a tuition fellowship.

(5) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors. The Organic Chemistry of the Enzymic Active-Site," John Wiley and Sons, Inc., New York, N. Y., 1967, Chapters I and VI.

(6) An enzyme from two sources is considered to have the same function if each converts an identical substrate to the identical product. That evolutionary changes in the linear sequence of an enzyme could have occurred between species has been documented and discussed.[7]

(7) (a) See ref 5, Chapter IX; (b) V. Bryson and H. J. Vogel, "Evolving Genes and Proteins," Academic Press Inc., New York, N. Y., 1965.

(8) Evolutionary changes within the active site would be expected to lead to a nonfunctional enzyme in most cases.[7,9]

(9) J. K. Hardman and C. Yanofsky, *Science*, **156**, 1369 (1967).

(10) (a) G. H. Hitchings and J. J. Burchall, *Advan. Enzymol.*, **27**, 417 (1965); J. J. Burchall and G. H. Hitchings, *Mol. Pharmacol.*, **1**, 126 (1965).

(11) B. R. Baker, *J. Med. Chem.*, **10**, 912 (1967), paper XCVII of this series.

(12) (a) B. R. Baker, *ibid.*, **11**, 483 (1968), paper CXVII of this series; (b) B. R. Baker and M. A. Johnson, *ibid.*, **11**, 486 (1968), paper CXVIII of this series.

(13) (a) B. R. Baker and G. J. Lourens, *ibid.*, **11**, 26 (1968), paper CIX of this series; (b) B. R. Baker and G. J. Lourens, *ibid.*, **11**, 34 (1968), paper CX of this series; (c) B. R. Baker and M. A. Johnson, *J. Heterocycl. Chem.*, **4**, 507 (1967), paper CXI of this series.

(14) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **10**, 1113 (1967), paper CV of this series.

(15) B. R. Baker and G. J. Lourens, *ibid.*, **11**, 39 (1968), paper CXII of this series.

(16) B. R Baker and R. B. Meyer, Jr., *ibid.*, **11**, 489 (1968), paper CXIX of this series.

(17) B. R. Baker and P. C. Huang, *ibid.*, **11**, 495 (1968), paper CXX of this series.

(18) (a) B. R. Baker and G. J. Lourens, *J. Pharm. Sci.*, **56**, 737 (1967), paper LXXXVI of this series; (b) see ref 5, pp 248–252.

or enzyme-catalyzed hydrolysis of the sulfonyl fluoride or both;[19,20] which reaction took place was extremely sensitive to positioning of the SO_2F group in the complex, a matter of 3 Å being sufficient to change from all covalent bonding to all hydrolysis or *vice versa*.[19] Thus a small difference in the hydrophobic region of the dihydrofolic reductase from two sources could have a dramatic effect on the positioning and action of the SO_2F group.[21]

The first differential irreversible inhibition between two vertebrate enzymes was seen with **2**,[14] which could rapidly inactivate the enzyme from pigeon liver, but not the enzyme from Walker 256 rat tumor or L1210/FR8 mouse leukemia; when the side chain was moved to the *para* position (**3**), specificity was lost and all three enzymes were rapidly inactivated.[14] Even though there was little difference in reversible binding of **2** to the three enzymes, it is uniquivocal that the SO_2F group does not reside in the same way in the complex with the pigeon liver enzyme as in the other two enzyme complexes.

The first differential irreversible inhibition of a tumor *vs.* liver enzymes, a tissue specificity, was seen with **4**[16] and **5**.[17] The dihydrofolic reductase from Walker 256 rat tumor or L1210/FR8 mouse leukemia was rapidly inactivated by **4**, but irreversible inhibition of the rat liver or mouse liver enzymes was barely detectable by a much higher concentration of **4**.[16] Similar results were seen with **5** with the L1210/FR8 and mouse liver enzymes.

A study has now been conducted on the enzyme from three strains of L1210: (a) the parent L1210/0 strain regularly used in the CCNSC screen; (b) L1210/FR8,[22] a strain selected for resistance to amethopterin, the resistance being due to a 60-fold increase in the level of dihydrofolic reductase;[22b] and (c) L1210/DF8,[23] another strain selected for resistance to amethopterin with a 50-fold increased level of dihydrofolic reductase. All of the sulfonyl fluoride type of candidate irreversible inhibitors previously published[3,14–17,24,25] were investigated on L1210/0; further, many of the compounds were also evaluated on L1210/DF8, particularly those showing irreversible inhibition of L1210/FR8, but not mouse liver. Some of the key results with the enzyme from the three strains of L1210 are reported here (Table I), including selected compounds from the accompanying papers. Furthermore, a comparison of differential irreversible inhibition of dihydrofolic reductase from mouse spleen, intestine, and liver are presented in Table I.

Enzyme Testing Methodology.—Considerably more difficulty with variable results was initially encountered with the dihydrofolic reductase from the 45–90% ammonium sulfate fraction from L1210/0 than encountered from this enzyme from L1210/FR8 and L1210/DF8. The main difficulty was due to the higher ratio of extraneous protein to dihydrofolic reductase in the L1210/0 enzyme preparation since the L1210/FR8 and L1210/DF8 had about 50 times as much dihydrofolic reductase.[22,23] The L1210/0 preparation showed considerable variation in the assay base lines for the irreversible inhibition studies, due to precipitation of protein and an extraneous TPNH oxidase; this difficulty was not encountered in the preparations from L1210/DF8 and L1210/FR8 since they could be diluted 50-fold for assay. This difficulty was partially surmounted by use of 0.15 *M* KCl in the assays which stimulated the L1210/0 dihydrofolic reductase threefold,[26] thus allowing a threefold dilution of the enzyme preparation which diluted the total protein and extraneous TPNH oxidase a corresponding amount and mainly alleviated the base-line difficulty. The use of 0.15 *M* KCl[26] is now used in all assays of dihydrofolic reductase, regardless of source.

A second set of difficulties was occasionally encountered with some compounds. If the compound was an extremely fast irreversible inhibitor (<2 min), difficulty was encountered in obtaining a suitable zero-point concentration of enzyme due to irreversible inhibition in the assay cuvette. The presence of this difficulty can usually be detected (a) by the zero point of per cent enzyme with the inhibitor being considerably lower than the zero point of the enzyme control, and (b) by curvature in the rate of conversion of TPNH to TPN due to the decreasing amount of enzyme in the cuvette because of the irreversible inhibition taking place. The first difficulty is avoided by decreasing the contact time between irreversible inhibitor and enzyme before starting the enzyme assay by adding the inhibitor to the cuvette, then adding the enzyme aliquot last. The second difficulty of rate curvature is not avoidable, but an accurate zero point can be obtained by using a tangent to zero time in the rate curve providing care is taken to observe the presence of curvature (see Experimental Section). With a fast, but good irreversible inhibitor that can give >80% inactivation, or with a slower irreversible inhibitor, reliable results can be obtained without the above precautions; however, with a poor, fast irreversible inhibitor that gives <40% inactivation, the irreversible inhibition could be missed completely. For example, the irreversible inhibition by **7** was variable between 0 and 20% unless the above precautions were taken when 30–50% irreversible inhibition could be observed with consistency; thus the earlier report[24] that **7** did not inactivate the dihydrofolic reductase from L1210/FR8 should be tempered by the observation that the inhibitor incubation zero point was low.

Enzyme Results.—The inhibitors (**3**–**13**) in Table I probe for differences outside the active site where evolutionary changes still allowing a functional enzyme would have been more apt to have occurred than inside the active site.[8] With the irreversible inhibition data in Table I it can be concluded that the dihydrofolic reductase from the mouse liver, spleen, and intestine is different from the enzyme of the three strains of

(19) B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **11**, 233 (1968), paper CXIII of this series.

(20) B. R. Baker and E. H. Erickson, *ibid.*, **11**, 245 (1968), paper CXV of this series.

(21) For a diagramatic presentation of this mode of selectivity see ref 16 or ref 5, p 249, Figure 10-6.

(22) (a) E. R. Kashket, E. J. Crawford, M. Friedkin, J. R. Humphreys, and A. Goldin, *Biochemistry*, **3**, 1928 (1964); (b) M. Friedkin, E. Crawford, S. R. Humphreys, and A. Goldin, *Cancer Res.*, **22**, 600 (1962).

(23) L1210/DF8 was selected in the laboratory of Dr. A. Goldin for resistance to amethopterin.

(24) B. R. Baker and G. J. Lourens, *J. Med. Chem.*, **11**, 666 (1968), paper CXXVII of this series.

(25) B. R. Baker and G. J. Lourens, *ibid.*, **11**, 672 (1968), paper CXXVIII of this series.

(26) J. R. Bertino, J. P. Perkins, and D. G. Johns, *Biochemistry*, **4**, 839 (1965).

L1210. Smaller differences occur between the L1210/0 and L1210/DF8 enzymes. However, all these differences are a lower amount of inactivation of the L1210/0 enzyme. If the enzyme from these two sources were different, then cases should be seen where less inactivation of the L1210/DF8 enzyme occurs; since no such case has yet been observed, the differences between L1210/0 and L1210/DF8 can be accounted for by the earlier described difficulties in assay. The earlier preliminary report[16] that **4** was a poor irreversible inhibitor of L1210/0 proved to be erroneous when proper precautions were taken (Table I). Thus the data obtained to date supports the conclusion of Friedkin, *et al.*,[22] that the dihydrofolic reductase from L1210/FR8 and L1210/0 are identical; however, it is still possible that compounds will be found that can show that the enzymes from two sources are not identical.

Three classes of compounds that can inactivate dihydrofolic reductase by the active-site-directed mechanism have been found: (a) 1-phenyl-1,2-dihydro-*s*-triazines such as **2**, **3**, and **6**–**8**;[14,15] (b) 5-phenoxypropylpyrimidines such as **4**,[16] **9**, and **10**; and (c) 5-phenylpyrimidines with a leaving group on the 6 position such as **5**,[17] **12**, and **13**.

In the first class, **3** can irreversibly inhibit all three L1210 enzymes, but is somewhat less effective on the mouse liver enzyme. When the side chain of the 1-phenyl group of **3** moved to the *meta* position (**2**), irreversible inhibition of both L1210/FR8[14] and L1210/0 was lost. When the propionamide bridge of **3** was substituted with a β-CH_3 group, the resultant **6** showed little change in I_{50} other than the liver enzyme where the change was about fivefold. The enzyme from all three strains of L1210 could be partially inactivated by 0.16 μM of **6**, but the liver enzyme showed no inactivation; thus the ratio of the rate of covalent linkage to the rate of enzyme-catalyzed hydrolysis[19,20] is less favorable with the L1210 enzyme than in the case of the parent **3**.

When the α-methylene group of **3** was replaced by NH (**7**), both the L1210/0 and L1210/DF8 enzymes could still be partially inactivated by 0.16 μM of **7**, which is sufficient to reversibly complex 95–98% of the enzyme. This NH substitution had less effect on the liver enzyme, since **3** and **7** were about equally effective as irreversible inhibitors of the liver enzyme. Insertion of a 3-chloro atom (**8**) on the benzene ring of **7** improved reversible binding about fourfold; furthermore, **8** was more effective than **7** and the L1210 enzyme, but about as effective as **7** on the liver and spleen enzymes.

The prototype of the second class of irreversible inhibition was the triaminopyrimidine (**4**).[16] Note that reversible inhibition only varied about twofold among the four sources of dihydrofolic reductase; however, the order of effectiveness of irreversible inhibition was more variable. The L1210/DF8, L1210/FR8, and L1210/0 enzymes were still inactivated at near K_i concentration of **4**, but the liver enzyme was not inactivated at a $40K_i$ concentration. Although this compound (**4**) showed excellent specificity of inactivation of dihydrofolic reductase from L1210 with no inactivation of the enzyme from liver, its K_i was too high for the compound to be effective *in vivo*, as will be discussed later. Insertion of an *o*-Cl on the phenoxy group of **4** enhanced reversible binding of the resultant **9** by two- to fourfold; although the irreversible inhibition pattern of **9** also showed good specificity, the K_i was still too high.

Replacement of the 6-NH_2 group of **4** by CH_3 (**10**) could be expected to enhance reversible binding 15–100-fold;[27] however, such a structural change could shift the positioning of the SO_2F moiety within the enzyme–inhibitor complex sufficiently to destroy either irreversible inhibition or the specificity pattern. In fact, the specificity pattern of **10** was the same as the parent **4**, namely, good irreversible inhibition of the enzyme from L1210/0 and L1210/DF8 at 0.05 μM ($18K_i$) of **10** with no significant irreversible inhibition of the liver enzyme with a $200K_i$ concentration of **10**; furthermore, **10** had K_i = 0.003 μM, about a 100-fold increment in reversible binding over **4**. However, at a $6K_i$ concentration (0.016 μM), **10** showed only about 60% and at a K_i concentration about 10% inactivation of dihydrofolic reductase before the inhibitor was destroyed; in contrast, a K_i concentration of **4** (0.4 μM) could give 85–97% inactivation. The lower effectiveness of **10** than **4** at a K_i concentration is primarily due to the fact that the enzyme concentration in the incubation is higher than 0.016 μM, as reported in the last accompanying paper.

The third class of compound is represented by **5**, which at near $2K_i$ concentration was an effective irreversible inhibitor of the enzyme from L1210/FR8 and L1210/DF8 but was less effective on the enzyme from L1210/0; a $70K_i$ concentration shows only slight irreversible inhibition of the liver enzyme. When the sulfonyl fluoride group of **5** was moved to *para* position (**11**), reversible binding was enhanced 5–16-fold. The irreversible patterns with **5** and **11** were similar with the L1210 enzymes except **11** could operate at a lower concentration due to its lower I_{50}, but **11** was not as effective as **5** at a $6K_i$ concentration or lower; unfortunately, **13** was a fairly good irreversible inhibitor of the mouse liver enzyme, specificity being lost. When the methylene group of **5** bridged to the amide was removed to give the lower homolog (**12**), reversible binding changed less than twofold; the specificity pattern was also unchanged compared to **5**.

Insertion of a chloro (**13**) on **12** did not change the specificity pattern with L1210 or liver, but did change the specificity toward the spleen and intestine enzymes; the latter two enzymes were inactivated poorly by **13** and not at all by **12**.

Liver serves as an admirable source of normal tissue for comparison of its dihydrofolic reductase with that from L1210. However, there is no *a priori* reason to expect that compounds showing irreversible inhibition of the L1210 enzyme and no irreversible inhibition of the liver enzyme would also show no inactivation of the dihydrofolic reductase from other normal tissues. Therefore, the inactivation of the dihydrofolic reductase from the spleens and intestines of normal mice were investigated with the compounds in Table I. The spleen enzyme was as stable as the liver enzyme, showing little thermal inactivation in 1 hr at 37°. In contrast, the intestine enzyme was completely denatured under these conditions, but was sufficiently stable at 25° for 20 min to give some useful information. Since most of the inactivation takes place in the first 10 min,

(27) B. R. Baker, B.-T. Ho, and D. V. Santi, *J. Pharm. Sci.*, **54**, 1415 (1965).

TABLE I

INHIBITION[a] OF DIHYDROFOLIC REDUCTASE FROM THREE STRAINS OF L1210 LEUKEMIA AND THREE NORMAL TISSUES OF THE MOUSE

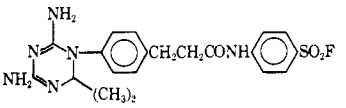
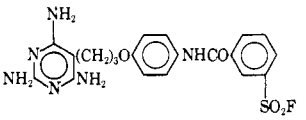
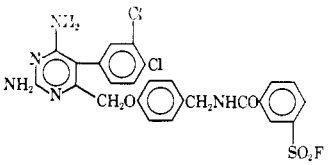
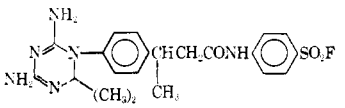
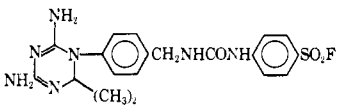
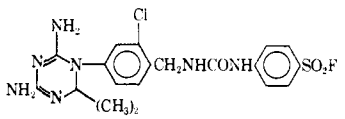
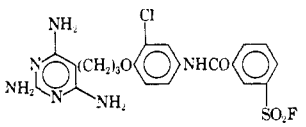
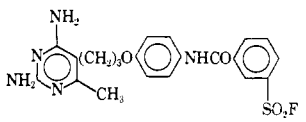
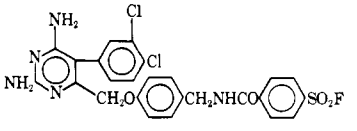
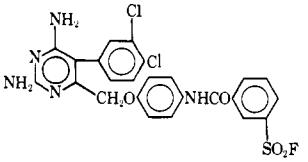
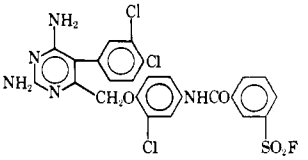
No.	Compound	Enzyme source	Reversible[b] I_{50}[d] μM	Reversible[b] Est'd $K_i \times 10^8$ M	Irreversible[c] Inhib. μM	Irreversible[c] % EI[f]	Irreversible[c] Time, min	Irreversible[c] % inactive
3		L1210/FR8[g]	0.080	0.013	0.070	86	<2, 10	77[h,i]
		L1210/0	0.012	0.0020	0.070	98	60	53[h]
					0.035	94	60	30[h]
					0.012	87	60	0
		L1210/DF8	0.025	0.0041	0.14	97	60	92[h]
					0.070	94	60	73[h]
					0.010	71	60	0
		Liver	0.015	0.0025	0.40	99	<2, 60	59, 59[i,j]
					0.070	96	<2, 10	38, 38[i,j]
		Spleen			0.10		60	73[h]
		Intestine			0.10		20	85[h]
4		L1210/FR8[k]	6.5	1.1	1.3	55	2, 60	50, 96[i]
		L1210/0	2.7	0.45	1.3	71	60	93[h]
					0.4	48	60	87[h]
		L1210/DF8	3.1	0.51	1.3	71	60	100
					0.4	44	60	97
		Liver[k]	3.6	0.60	25	98	60	20
					1.3	67	60	0
5		L1210/FR8[l]	0.70	0.12	0.70	87	2, 60	50, 97[i]
					0.12	50	8, 60	50, 90[i]
		L1210/0	0.24	0.040	1.1	97	60	88[h]
					0.24	87	60	83[h]
					0.040	50	60	23[h]
		L1210/DF8	0.37	0.062	1.4	96	60	101
					0.70	88	60	88
					0.12	66	60	75
		Liver[k]	0.29	0.048	3.5	99	2, 60	12, 12[i]
					0.70	93	60	0[i]
		Spleen			0.70		60	0[h]
		Intestine			0.70		20	23[h]
6		L1210/FR8[m]	0.081	0.013	0.081	87	2, 60	55, 55[i]
		L1210/0	0.044	0.0073	0.16	97	60	25[h]
		L1210/DF8			0.16		60	40[h]
		Liver	0.080	0.0013	0.40	98	60	0[h]
		Spleen			0.16		60	38[h]
		Intestine			0.16		20	35[h]
7		L1210/0	0.052	0.0087	0.16	95	60	51[h]
		L1210/DF8	0.045	0.0075	0.16	95	60	42[h]
		Liver	0.023	0.0039	0.16	98	60	31[h]
		Spleen			0.16		60	45[h]
		Intestine			0.16		20	33[h]
8		L1210/0	0.012	0.0020	0.060	97	60	76[h]
					0.010	87	60	0[h]
		L1210/DF8			0.060		60	85[h]
					0.012			0[h]
		Liver			0.060		60	30[h]
		Spleen			0.060		60	68[h]
9		L1210/FR8	1.2	0.20	0.20	50	4, 60	50, 90[i]
		L1210/0	1.4	0.23	2.4	91	60	98[h]
					0.23	50	60	76[h]
		L1210/DF8	1.2	0.20	0.20	50	60	96[h]
		Liver	1.5	0.25	6.0	95	60	6[h]
		Spleen			2.0		60	0[h]
		Intestine			2.0		20	5[h]
10		L1210/DF8	0.016	0.0027	0.050	95	60	100[h]
					0.016	87	60	65[h]
					0.005	67	60	10[h]
		L1210/0	0.016	0.0027	0.050	95	60	94[h]
					0.016	87	60	50[h]
					0.005	67	60	18[h]
		Liver	0.019	0.0032	0.60	99	60	6[h]
					0.20	98	60	0
		Spleen			0.20		60	0[h]
		Intestine			0.20		20	12[h]

TABLE I (*Continued*)

No.	Compound	Enzyme source	Reversible[b] I_{50},[d] μM	Reversible[b] Estd $K_i \times 10^6$ M[e]	Irreversible[c] Inhib, μM	Irreversible[c] % EI[f]	Irreversible[c] Time, min	Irreversible[c] % inactvn
11		L1210/FR8	0.062	0.010	0.32	97	60	97
					0.062	87	4, 60	67, 67[i]
		L1210/0	0.055	0.0092	0.12	93	60	82
					0.055	87	2, 60	57, 57[i]
		L1210/DF8	0.035	0.0058	0.12	95	60	96
					0.060	91	60	65
		Liver	0.018	0.0030	0.12	98	60	56
		Spleen			0.12		60	56[h]
		Intestine			0.12		20	35[h]
12		L1210/FR8[l]	0.98	0.16	1.0	87	16, 60	50, 78[i]
		L1210/0			2.0		60	79[h]
					0.5		60	43[h]
		L1210/DF8	0.82	0.13	0.50	78	60	94
					0.13	50	60	43
		Liver[l]			5.0		60	0[i]
		Spleen			1.0		60	0[h]
		Intestine			1.0		20	0[h]
13		L1210/DF8	0.53	0.090	1.1	92	60	92
					0.53	85	60	76[h]
					0.10	52	60	57[h]
		L1210/0			1.1		60	89[h]
					0.16		60	71[h]
		Liver			1.1		60	0[h]
		Spleen			1.1		60	38[h]
		Intestine			1.1		20	16[h]

[a] The technical assistance of Sharon Lafler, Diane Shea, and Carolyn Wade is acknowledged. [b] Assayed with 6 μM dihydrofolate and 30 μM TPNH in pH 7.4 Tris buffer containing 0.15 M KCl as previously described;[14] see Experimental Section. [c] Incubated at 37° in pH 7.4 Tris buffer in the presence of 60 μM TPNH, then assayed as previously described[14] in the presence of 0.15 M KCl unless otherwise indicated; see Experimental Section. [d] I_{50} = concentration for 50% inhibition. [e] Estimated from $K_i = K_m[I_{50}]/[S]$ which is valid since $[S] = 6K_m = 6\mu M$ dihydrofolate; see ref 5, p 202. [f] Estimated from $[EI] = [E_t]/(1 + K_i/[I])$ where [EI] is the amount of total enzyme (E_t) reversibly complexed; see ref 5, Chapter VIII. [g] Data from ref 14. [h] Zero point obtained by adding inhibitor to assay cuvette prior to addition of enzyme aliquot;[14] see Experimental Section. [i] From six-point time study; see ref 14. [j] From ref 24. [k] Data from ref 16. [l] Data from ref 17. [m] Data from ref 25.

comparison of inactivation of the enzyme from the three sources is fairly valid if the percentages are grouped. For practical chemotherapy, 0–15% inhibition can be considered negative (−), 16–30% as positive (+), and greater than 30% as detrimental (++).

Four compounds (**3**, **7**, **8**, **11**) showing >30% inactivation (++) of the liver enzyme also showed >30% inactivation of the enzyme from spleen and intestine. Of six compounds (**5**, **6**, **9**, **10**, **12**, and **13**) showing a (−) rating on liver, **9**, **10**, and **12** were also (−) on the enzyme from spleen and intestine. Of the remaining three, **5** was (−) on spleen and (+) on intestine, whereas **13** was (+) and **6** was (++) on spleen and intestine.

From these data it is clear that the dihydrofolic reductase is different in each of three normal tissues of the mouse, namely, liver, spleen, and intestine. The liver and spleen enzymes show differences toward **6** and **13** as irreversible inhibitors but these two appear more closely related to each other than to the intestine enzyme.

Intact Cell Assays.—Three questions can be asked with intact cell systems: (a) does the compound show cytotoxicity at an appropriate level, thus showing cell-wall penetration; (b) if the compound is cytotoxic does it show blockade of the expected enzyme system; (c) if a compound shows selective irreversible inhibition on isolated enzyme systems, would it show selectivity of action on a tumor in an animal?

For cytotoxicity of L1210 in cell culture, amethopterin makes a convenient base line. Amethopterin has an ED_{50} of 0.01 μM to L1210 cells in culture[28] and has an $I_{50} \sim 0.001$ μM on dihydrofolic reductase;[29] thus the concentration gradient between ED_{50} and I_{50} for amethopterin is about tenfold. The phenoxypropylpyrimidine irreversible inhibitor (**4**) has an ED_{50} of 45 μM on L1210/0 cells in culture[28] and an I_{50} of 2.7 μM (Table I); thus the concentration gradient between ED_{50} and I_{50} is 18-fold clearly showing that **6** can penetrate the cell wall, presumably by passive diffusion.[16]

The measure of DNA synthesis in cells in tissue culture from ^{3}H-deoxyuridine involves as one of the steps the coupled enzyme system thymidylate synthetase–dihydrofolic reductase for introduction of the 5-methyl group; thus a compound blocking dihydrofolic reductase will block DNA synthesis from ^{3}H-deoxyuridine. At 10^{-7} M, **3** showed complete inhibition of DNA synthesis from ^{3}H-deoxyuridine.[30] The concentration gradient between inhibition of DNA and I_{50} is about 2, showing the cell wall penetration of **3**, but also showing that **3** can block DNA synthesis as it was designed to do.[14]

Preliminary assays of the compounds in Table I against L1210 in the mouse have been performed with only **2–4**, **9**, and **10**.[28] The results to date with **2** and

(28) We wish to thank Dr. Florence White of the CCNSC, National Cancer Institute, for these data and for her aid in the experimental design.

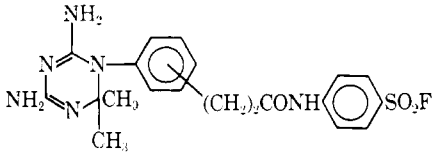
(29) (a) Reference 5, pp 197–198; (b) B. R. Baker and J. H. Jordaan, *J. Heterocycl. Chem.*, **2**, 162 (1965).

(30) We wish to thank Professor J. R. Bertino, Yale University School of Medicine, for this information.

3 on L1210/0 *in vivo* are presented in Table II. Note that **2** and **3** are reversible inhibitors of the same magnitude of the dihydrofolic reductase from L1210/0, but only **3** is an irreversible inhibitor. No significant life extension of the mice bearing L1210/0 was seen with the reversible inhibitor **2** at 50–400 mg/kg daily. In contrast, the irreversible inhibitor **3** showed significant life extension at 50–400 mg/kg daily, the effect peaking at 300 mg with a life extension of 71% beyond the controls. It should be pointed out that **3** is far from an optimum irreversible inhibitor of dihydrofolic reductase since it shows little selectivity between L1210, liver, spleen, and intestine enzymes; thus the chemotherapeutic index would be expected to be small as was seen in Table I.

TABLE II

In Vivo INHIBITION[a] OF L1210/0 BY THE *meta* (**2**) AND *para* (**3**) ISOMERS OF



Compd	I_{50}, μM	Irrev inhib	Dose, mg/kg/day	Survival, days	% T/C
2	0.011	No	0	8.5	
			500	Toxic	
			400	9.2	108
			300	9.0	105
			200	9.0	105
			100	8.7	102
			50	8.5	100
3	0.012	Yes	0	8.5	
			500	8.0	95
			400	10.5	123
			300	14.6	171
			200	10.5	123
			100	12.3	144
			50	11.7	137

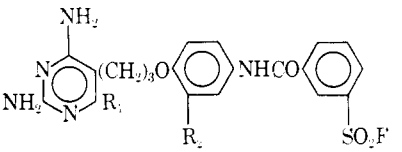
[a] Standard CCNSC screen; all assays were performed simultaneously with the same control group.

Again amethopterin as a "pseudo" irreversible inhibitor of dihydrofolic reductase can be taken as a base line. The optimum dosage of 1 mg/kg/day will extend survival of mice bearing L1210/0 about 100% beyond the controls.[31] The optimum level of 300 mg/kg/day of **3** is 300-fold greater than that of amethopterin, but the difference in concentration for inhibition of dihydrofolic reductase may be as large as 70-fold. Since this discrepancy might have been due in part to the poor solubility of **3**, a search for a more soluble salt was conducted. Less than a twofold difference in solubility was observed between the ethanesulfonate of **3** assayed in Table II and the hydrochloride and hydroxyethanesulfonate. Less soluble salts were the acid phosphate (1:1) and sulfoacetate. Thus, solubility of salts of **3** is influenced little by the anion, probably because of the high molecular weight and high hydrocarbon content of **3**.

Although greater selectivity in inactivation of dihydrofolic reductase is seen with **4** than **3**, the effective concentration requirement for **4** is much higher than **3** *in vitro*. When **4** as the insoluble sulfate salt was assayed against L1210/0 or L1210/DFS *in vivo*, considerable difficulty was encountered with variable toxicity due to the difficulty of homogenizing this salt; toxicity varied between 100 and 500 mg in different runs with no beneficial effect on life extension (Table III).[28] This toxicity occurs at a lower dose than would be expected by comparing **3** and **4** as inhibitors of dihydrofolic reductase. That **4** had toxicity for other reasons was indicated by little protection by 5-formyltetrahydrofolic acid (CF) at a dose that would protect against amethopterin toxicity.[28] Similarly, the triaminopyrimidine (**9**) at nontoxic doses showed no life extension of mice bearing L1210/DFS (Table III).

TABLE III

In Vivo INHIBITION[a] OF L1210 BY



No.	R_1	R_2	I_{50}, μM	Strain	Dose, mg/kg/day	Survival, days	% T/C
1	NH_2	H	3.1	L1210/DFS	0	9.5	
					500	Toxic	
					200	10.5	111
					100	10.5	111
					20	9.5	100
4	NH_2	H	2.7	L1210/0	0	9.5	
					500	Toxic	
					100	10.5	111
					20	9.0	95
9	NH_2	Cl	1.2	L1210/DFS	0	14	
					500	Toxic	
					250	8.5	61
					125	12.5	89
					62	15	107
10	CH_3	H	0.016	L1210/0	0	8.5	
					200	8.8	103
					100	9.0	105
					50	8.8	103
					25	8.8	103
					12.5	8.8	103

[a] Standard CCNSC screen.

Based only on enzyme data, it would be predicted that the pyrimidine **10** would be a superior compound to **3** *in vivo* since the I_{50}'s are comparable and **10** shows more specificity; however, this prediction could be negated by other important factors in an *in vivo* assay. The *in vivo* results with **10** are shown in Table III; neither toxicity, as seen with the 6-amino analog (**4**), nor activity, as seen with **3**, was observed in the range of 12–200 mg/kg/day of **10**. Although disappointing, at this early stage of correlation of enzyme data with *in vivo* data these results are not too surprising. It is obvious that **10** did not reach the target enzyme in sufficient concentration. Although one or more factors could cause these negative results, the lack of *in vivo* activity of **10** has been traced to poor cell-wall penetration, as presented in an accompanying paper. Thus, at best these broken cell enzyme assays can only sort out compounds that are worthy of *in vivo* assay, but at this stage give no assurance that chemotherapeutic efficacy will be achieved.

(31) A. Goldin, S. R. Humphreys, J. M. Venditti, and N. Mantel, *J. Natl. Cancer Inst.*, **22**, 811 (1959).

Based on the *in vitro* and *in vivo* data in Tables I–III, the following criteria have been set to establish whether or not a candidate irreversible inhibitor is worthy of studies with tumor-bearing animals.

(1) The compound should have an I_{50} of 10^{-7} M or less, which is equivalent to $K_i \leq 2 \times 10^{-8}$ M, on the tumor dihydrofolic reductase. This low an I_{50} is needed for a reasonable degree of reversible specificity for dihydrofolic reductase compared to other enzymes in the animal.[32]

(2) At a K_i concentration, the inhibitor should show >70% inactivation of the tumor dihydrofolic reductase in 60 min at 37°. At this concentration, 50% of the total enzyme will be in the form of the reversible E···I complex, the rate-determining species for active-site-directed irreversible inhibition.[33] If a concentration of inhibitor at $6K_i$ or $30K_i$ is needed to inactivate the enzyme, due to enzyme-catalyzed hydrolysis of the SO_2F group,[19,20] then reversible inhibition of dihydrofolic reductase from other tissues becomes a serious problem and the specificity achievable with an irreversible inhibitor will be lost.

(3) Less than 20% inactivation of liver dihydrofolic reductase should occur with a $6–12K_i$ concentration of candidate irreversible inhibitor; this is possible to achieve if the liver enzyme is either not inactivated or can rapidly catalyze hydrolysis of the SO_2F group.[19,20]

The search for compounds meeting the above criteria for *in vivo* assay, as well as studies on cell-wall penetration, are continuing.

Chemistry.—The synthesis of compounds **2**, **3**,[14] **4**,[16] **5**,[17] **6**,[25] and **7**[34] have been previously described. The synthesis of **8**,[34] **9**,[35] **10**,[36] **11**,[37] **12**,[38] and **13**[38] are described in the papers that follow.

(32) (a) Reference 5, pp 246–252; (b) B. R. Baker and J. H. Jordaan *J. Heterocycl. Chem.*, **4**, 31 (1967), paper LXXXIII of this series.

(33) For the kinetics of irreversible inhibition see (a) ref 5, Chapter 8; (b) B. R. Baker, W. W. Lee, and E. Tong, *J. Theoret. Biol.*, **3**, 459 (1962).

(34) B. R. Baker and G. J Lourens, *J. Med. Chem.*, **12**, 101 (1969), paper CXLI of this series.

(35) B. R. Baker and R. B. Meyer, Jr., *ibid.*, **12**, 104 (1969), paper CXLII of this series.

(36) B. R. Baker and R. B. Meyer, Jr., *ibid.*, **12**, 108 (1969), paper CXLIII of this series.

(37) B. R. Baker and N. M. J. Vermeulen, *ibid.*, **12**, 86 (1969), paper CXXXVII of this series.

(38) B. R. Baker and N. M. J. Vermeulen, *ibid.*, **12**, 82 (1969), paper CXXXVI of this series.

Experimental Section

Enzyme Preparations.—Dr. Florence White of the CCNSC, National Cancer Institute, kindly provided L1210/0 grown subcutaneously as a solid tumor and L1210/DF8 in infiltrated spleens; liver, spleen, and intestine were from normal BDF_1 mice. The preparation of extracts of L1210/FR8 or mouse liver containing dihydrofolic has been previously described.[14,15] The dihydrofolic reductase from spleen, intestine, and L1210/0 was a 45–90% ammonium sulfate fraction prepared as described for mouse liver.[14,15] The mouse intestine enzyme was unstable at 5° with a half-life of about 4 days; this enzyme was therefore stored at −15° in aliquots sufficient for 1 day's assays. The L1210/DF8-infiltrated spleens were extracted with 10 ml/g of 0.05 M Tris buffer (pH 7.4) in a Waring Blendor, then centrifuged at 20,000 rpm for 20 min. A final volume of 1.5 ml/g of L1210/0 gave an OD change of 0.0073 unit/min when 25 μl was assayed in 1 ml of solution without KCl.[14] The L1210/DF8 extract (9 ml/g) showed 0.055 OD unit/min under similar conditions; thus the infiltrated spleens contained 50-fold more enzyme than the subcutaneous L1210/0 per gram of tissue.

Enzyme Assays.—A number of improvements in assay conditions have been incorporated since the last description.[14]

(1) By use of a 0.15 M cuvette concentration of KCl (0.17 M in the buffer), the rate of the enzyme reaction was enhanced[26] three-, two-, and fourfold, respectively, with the enzyme from L1210/0, L1210/DF8, and liver; KCl was not used in the incubations, but only in the aliquot assay.

(2) In determination of the reversible I_{50} of these sulfonyl fluorides, the shape of the rate line should be noted. If irreversible inhibition with a compound is slow, then the rate line will be linear until almost all the dihydrofolate has been consumed. With a fast irreversible inhibitor such as **3** or **6**, the enzyme rate will show curvature; in such cases, single cuvettes are run with fast mixing and the initial rate is taken by drawing a tangent to zero time.

(3) The same caution on curvature must be used for determination of a zero point in the incubation with an inhibitor.

A good fast irreversible inhibitor showing >70% irreversible inhibition was readily measured by adding the inhibitor to the zero-point aliquot, but a poor, fast irreversible inhibitor showing <40% irreversible inhibition could be missed. In order to avoid missing a fast, but low total amount of irreversible inhibition, the presence of curvature in the zero-point assay or a low zero point requires a considerable number of runs to obtain a reasonable average number for the amount of irreversible inhibition. For example, a wrong zero point for **7** will be obtained if these precautions are not observed. The procedure for fast irreversible inhibitors is now used in all assays, that is, the inhibitor is added to the assay cuvette in the appropriate concentration rather than adding the inhibitor to the zero-time aliquot cooled in an ice bath,[14] and any curvature is corrected by a tangent to zero time.