Irreversible Enzyme Inhibitors. CXLIII.[1,2] Active-Site-Directed Irreversible Inhibitors of Dihydrofolic Reductase Derived from 5-(*p*-Aminophenoxypropyl)-2,4-diamino-6-methylpyrimidine with a Terminal Sulfonyl Fluoride

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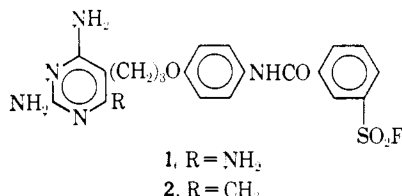
2,4-Diamino-5-[*p*-(*m*-fluorosulfonylbenzamido)phenoxypropyl]-6-methylpyrimidine (**2**) and three variants in the benzamido moiety have been synthesized *via* the intermediate 2-amino-6-methyl-5-(*p*-nitrophenoxypropyl)-4-pyrimidinol and 5-(*p*-aminophenoxypropyl)-2,4-diamino-6-methylpyrimidine (**6**); the key reaction was azide displacement of the chloro atom of 2-acetamido-4-chloro-6-methyl-5-(*p*-nitrophenoxypropyl)pyrimidine (**5**) followed by reduction of the azidopyrimidine (**7**) to the 4-aminopyrimidine since the usual ammonia displacement caused cleavage of the nitrophenoxy side chain. Evaluated as an irreversible inhibitor of dihydrofolic reductase, **2** met all the criteria needed for *in vivo* evaluation; **2** had K_i = 0.003 μM, showed good irreversible inhibition of L1210 dihydrofolic reductase at a $2K_i$ concentration, and showed no significant irreversible inhibition of dihydrofolic reductase from normal mouse liver, spleen, or intestine at a concentration of $>70K_i$. However, **2** showed poor penetration of the L1210 cell wall in culture and hence was inactive *in vivo*. In contrast, N-[*p*-(4,6-diamino-2,2-dimethyl-1,2-dihydro-*s*-triazin-1-yl)hydrocinnamoyl]sulfanilyl fluoride (**12**) showed good penetration of the L1210 cell wall in culture and good *in vivo* activity, but **12** was not a selective irreversible inhibitor of L1210 dihydrofolic reductase since it also inactivated the enzyme from mouse liver, spleen, and intestine. Future studies to combine the selectivity of **2** with the *in vivo* effectiveness of **12** are discussed.

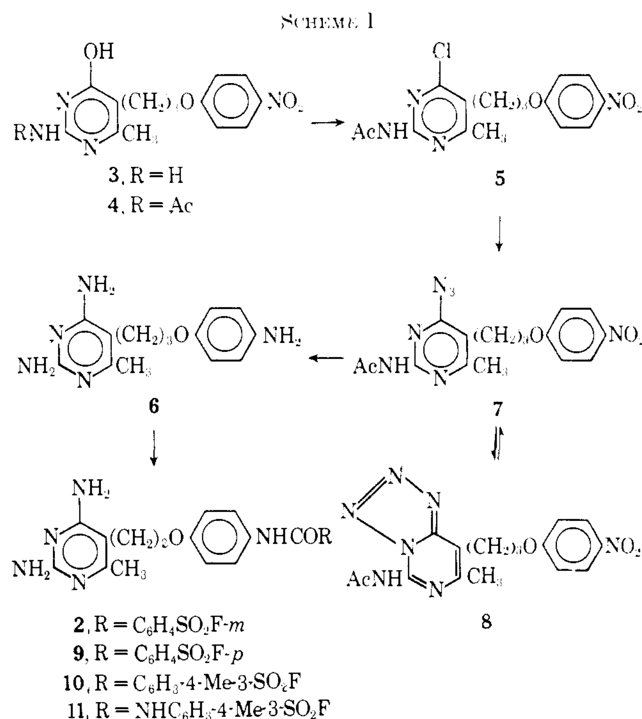
The triaminopyrimidine bearing a terminal sulfonyl fluoride (**1**) was the first active-site-directed irreversible inhibitor[3] of dihydrofolic reductase[4] that could inactivate the enzyme from L1210 mouse leukemia with



little inactivation of the enzyme from mouse liver, spleen, or intestine.[5] Since the failure of **1** to show *in vivo* activity[5] may have been due in part to its poor solubility or its relatively high K_i ($\simeq$0.5 μM), two approaches were investigated to obtain irreversible inhibitors related to **1** with better reversible binding. The first approach was the study of the effects of substitution on the two benzene rings of **1** to increase binding, which was described in the preceding paper.[2] The second approach was to replace the 6-amino group of **1** by a 6-methyl (**2**) which could be expected to enhance binding 10–150-fold.[6] The synthesis and enzymic evaluation of **2**, as well as some of its congeners, is the subject of this paper.

A two-step synthesis of the 4-pyrimidinol (**3**) has been previously described.[7] The 2-amino group of **3** was then acetylated in 78% yield to **4** in order to aid in the replacement of the 4-hydroxyl of the pyrimidinol by 4-chloro (**5**)[8] (Scheme I); this was accomplished with $POCl_3$ at 75° in 49% yield of pure material. Treatment of **5** with methanolic ammonia at 150° under the usual conditions[6,9] led to cleavage of the side chain to *p*-nitroaniline, a not unexpected result with a substituted 4-nitroanisole system; under conditions sufficiently mild to avoid cleavage of the nitrophenyl group, the 4-Cl group failed to displace.

Scheme I



It was previously observed that ammonia and amines rapidly deacetylated 2-acetamido-4-chloropyrimidines and the resultant 2-amino-4-chloropyrimidines were

(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 R. B. Meyer, Jr., *J. Med. Chem.*, **12**, 104 (1969).

(3) 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.

(4) B. R. Baker and R. B. Meyer, Jr., *J. Med. Chem.*, **11**, 489 (1968), paper CXIX of this series.

(5) B. R. Baker, G. J. Lourens, R. B. Meyer, Jr., and N. M. J. Vermeulen, *ibid.*, **12**, 67 (1969), paper CXXXIII of this series.

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

(7) B. R. Baker and D. V. Santi, *ibid.*, **56**, 380 (1967), paper LXIX of this series.

(8) B. R. Baker, C. E. Morreal, and B.-T. Ho, *J. Med. Chem.*, **6**, 658 (1963).

(9) B. R. Baker and B.-T. Ho, *J. Pharm. Sci.*, **53**, 1457 (1964).

then much less reactive toward a neutral nucleophile such as thiourea.[8] Therefore it was essential that a neutral nitrogen nucleophile be used with **5** under aprotic conditions so that the N-acetyl group would not be cleaved with resultant deactivation of the 4-chloro group; such a nucleophile is azide ion. When **5** was heated with NaN_3 in DMSO at 85–90°, reaction was complete in 2 hr as shown by tlc. A crystalline azide (**7**) was isolated in 83% yield, but showed two spots on tlc that had identical uv spectra; whether or not these two spots were due to equilibration with the isomeric tetrazole (**8**)[10] was not ascertained, since both **7** and **8** would be reducible to the same amine (**6**). The crude azide (**7**) was catalytically reduced with a Raney Ni catalyst to 2,4-diaminopyrimidine (**6**) which was isolated as its sulfate in 61% yield.

Due to its insolubility, attempts to convert the sulfate salt of **6** to **2** with *m*-fluorosulfonylbenzoyl chloride in DMF or DMSO in the presence of Et_3N were unsuccessful. The sulfate salt of **6** was converted to the noncrystalline base with aqueous NaOH, which was isolated by $CHCl_3$ extraction. Acylation then proceeded in DMF when the 2,4-diaminopyrimidine moiety of **6** was allowed to act as the acid acceptor; the yield of pure **2** from the sulfate salt was only 14%. It was found more convenient to treat the more soluble hydrochloride salt of **6** with acid chlorides where 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) was used as the acid acceptor; this method was used for preparation of **9** and **10**. The urea derivative (**11**) was prepared similarly from **6**·HCl by reaction with *p*-nitrophenyl N-(3-fluorosulfonyl-4-methylphenyl)carbamate.[11]

Experimental Section

Melting points were taken in capillary tubes on a Mel-Temp block and are uncorrected. Each analytical sample moved as a single spot on tlc on Brinkmann silica gel GF, and had ir and uv spectra in agreement with their assigned structures; each gave combustion values for C, H, and N or F within 0.4% of theoretical.

2-Acetamido-6-methyl-5-(*p*-nitrophenoxypropyl)-4-pyrimidinol (4).—A stirred mixture of 3.50 g (11.5 mmoles) of **3**[7] and 40 ml of Ac_2O was heated at 80–85° for 4 hr, solution not taking place. The mixture was spin evaporated *in vacuo*. The residue was suspended in 30 ml of EtOH and the evaporation was repeated; the evaporation was repeated with two 30-ml portions of EtOH. Recrystallization from MeOEtOH gave 3.10 g (78%) of product, mp 232–233°. *Anal.* ($C_{16}H_{18}N_4O_5$) C, H, N.

2-Acetamido-4-chloro-6-methyl-5-(*p*-nitrophenoxypropyl)pyrimidine (5).—A stirred mixture of 5.0 g (14.5 mmoles) of **4** and 25 ml of $POCl_3$ was protected from moisture in a bath at 75° for 1 hr, solution being complete in 15 min. The cooled solution was poured into 200 ml of petroleum ether (bp 60–110°), then allowed to stand until the solution was no longer turbid (1 hr). The petroleum ether was decanted from the gum; the latter was washed with an additional 100 ml of petroleum ether (bp 60–110°). To the residual gum was added a mixture of 50 g of ice and 250 ml of 10% aqueous NaOAc. The mixture was stirred for 30 min when the gum had changed to a solid. The latter was collected on a filter and washed with H_2O. Recrystallization from MeOEtOH–H_2O gave 2.7 g (49%) of off-white crystals, mp 173°. *Anal.* ($C_{16}H_{17}ClN_4O_4$) C, H, N.

2-Acetamido-4-azido-6-methyl-5-(*p*-nitrophenoxypropyl)pyrimidine (7).—A stirred mixture of 5.0 g (13.7 mmoles) of **5**, 3.5 g (54 mmoles) of NaN_3, and 30 ml of reagent grade DMSO was heated in a bath at 85–90° for 2 hr when tlc showed **5** had been consumed. The cooled reaction mixture was diluted with 100 ml of H_2O and 200 ml of $CHCl_3$. The separated $CHCl_3$ layer was washed with 100 ml of H_2O, then dried with $MgSO_4$ and evaporated *in vacuo* to about 20 ml. Petroleum ether was added to turbidity, when the mixture was chilled at 5°. The crystalline product was collected on a filter and washed with petroleum ether; yield 4.1 g (83%); mp 141–144° dec; ν_{max}^{KBr} 1680 (amide I), 2100 (N_3), 1520, 1350 cm^{-1} (NO_2); this was suitable for the next step. Tlc in EtOAc showed two major spots and one minor spot. A small-scale tlc separation of two major spots showed they had identical uv spectra: λ_{max} 256, 314 mμ (pH 1); 300 mμ (pH 13). Since the minor product may have resulted from deacetylation, but is also convertible to **6**, no further purification was considered necessary.

5-(*p*-Aminophenoxypropyl)-2,4-diamino-6-methylpyrimidine (6) Sesquisulfate.—A solution of 1.3 g (3.5 mmoles) of crude **7** in 200 ml of MeOEtOH was shaken with H_2 at 2–3 atm in the presence of 1 g of Raney Ni for 1 hr. To the filtered solution was added 20 ml of 6 *N* HCl, then the solution was heated on a steam bath for 30 min to remove the N-acetyl group, which was shown by the shift in λ_{max} at pH 1 from 264 to 273 mμ. Evaporation to dryness *in vacuo* gave a glassy hydrochloride that could be precipitated as a white powder from a small volume of hot H_2O by addition of *i*-PrOH to turbidity; yield 87% (calculated as 3HCl), mp >200° dec, sufficiently pure for further transformation. For analysis a sample was dissolved in 20 ml of hot 2 *N* H_2SO_4, then the solution was clarified with carbon. The hot solution was diluted with *i*-PrOH to turbidity, then cooled; yield 61%, mp >250° dec. *Anal.* ($C_{24}H_{21}N_5O \cdot 1.5H_2SO_4$) C, H, N. Preparatively, it was more convenient to use the HCl salt of **6** for further transformation.

2,4-Diamino-5-[*p*-(*m*-fluorosulfonylbenzamido)phenoxypropyl]-6-methylpyrimidine (2) Ethanesulfonate.—A mixture of 0.90 g (2.1 mmoles) of **6** sulfate, 30 ml of $CHCl_3$, and 100 ml of 1 *N* NaOH was vigorously shaken until solution was complete. The separated H_2O layer was extracted with three 30-ml portions of $CHCl_3$. The combined extracts were dried with $MgSO_4$, then evaporated *in vacuo* leaving 0.50 g (85%) of **6** base as a gum. The latter (1.8 mmoles) was dissolved in a mixture of 3 ml of DMF, 5 ml of $CHCl_3$, and 200 mg (2 mmoles) of Et_3N. To this solution cooled in an ice–salt bath and protected from moisture was added 0.33 g (1.5 mmoles) of *m*-fluorosulfonylbenzoyl chloride. After being stirred 15 min, the solution was treated with 0.60 g (5 mmoles) of $EtSO_3H$, then the $CHCl_3$ was removed by evaporation *in vacuo*. The evaporation was repeated with three 20-ml portions of *o*-PrOH. The residual DMF solution was diluted with about 30 ml of 50% aqueous *i*-PrOH, then cooled. The product that separated on standing was collected on a filter and washed with 50% aqueous *i*-PrOH; yield 0.12 g (14% based on the acid chloride) of white needles, which gradually softened over 145°, but showed no definite melting point. *Anal.* ($C_{21}H_{22}FN_5O_4S \cdot C_2H_5SO_3H$) C, H, F.

2,4-Diamino-5-[*p*-(*p*-fluorosulfonylbenzamido)phenoxypropyl]-6-methylpyrimidine (9).—To a stirred mixture of 250 mg (0.65 mmole) of **6**·3HCl and 145 mg (0.65 mmole) of *p*-fluorosulfonylbenzoyl chloride in 2 ml of DMF protected from moisture and cooled in an ice bath was added 200 mg (1.6 mmoles) of 1,5-diazabicyclo[4.3.0]non-5-ene (DBN). After 30 min the solution was poured into 50 ml of saturated aqueous $NaHCO_3$. The product was collected on a filter and washed with H_2O. Recrystallization from EtOH–H_2O gave 100 mg (33%) of white crystals, mp 229–231°, that moved as a single spot on tlc in 1:4 EtOH–$CHCl_3$. *Anal.* ($C_{21}H_{22}FN_5O_4S$) C, H, F.

2,4-Diamino-5-[*p*-(3-fluorosulfonyl-4-methylbenzamido)phenoxypropyl]-6-methylpyrimidine (10) Hemisulfate.—Reaction of 400 mg (1.04 mmoles) of **6**·3HCl with the acid chloride from 240 mg (1 mmole) of 3-fluorosulfonyl-4-methylbenzoic acid[2] for 1 hr as described for **9** gave a solution that was poured into 20 ml of 1 *N* H_2SO_4. The product was collected on a filter, washed with H_2O, then recrystallized three times from EtOH–H_2O when it moved as one spot on tlc; yield 60 mg (11%) of white needles, mp >180° dec. *Anal.* ($C_{22}H_{24}FN_5O_4S \cdot 0.5H_2SO_4 \cdot H_2O$) C, H, F.

2,4-Diamino-5-[*p*-(3-fluorosulfonyl-4-methylphenylureido)-phenoxypropyl]-6-methylpyrimidine (11) Hemisulfate.—Reaction of 250 mg (0.65 mmole) of **6**·3HCl with 220 mg (0.62 mmole) of *p*-nitrophenyl N-(3-fluorosulfonyl-4-methylphenyl)carbamate[11] for 1 hr as described for **9** gave a solution that was poured into a stirred mixture of 20 ml of 1 *N* H_2SO_4 and 20 ml of $CHCl_3$.

(10) (a) C. Temple, Jr., and J. A. Montgomery, *J. Org. Chem.*, **30**, 826 (1965); (b) C. Temple, Jr., R. L. McKee, and J. A. Montgomery, *ibid.*, **30**, 829 (1965).

(11) B. R. Baker and N. M. J. Vermeulen, *J. Med. Chem.*, **12**, 74 (1969), paper CXXXIV of this series.

The product was collected on a filter and recrystallized three times from MeOEtOH–H_2O when it moved as single spot on tlc; yield 90 mg (26%), mp >194° dec. *Anal.* ($C_{22}H_{25}FN_6O_4S \cdot 0.5H_2SO_4 \cdot H_2O$) C, H, F.

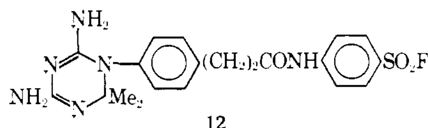
Enzyme Results and Discussion

The following arbitrary criteria had been chosen to determine whether a candidate irreversible inhibitor of dihydrofolic reductase is worthy of *in vivo* assay: the compound (a) should have $I_{50} \simeq 6K_i \leq 0.1\ \mu M$, (b) should give >70% inactivation of the tumor enzyme at a K_i concentration, and (c) should give <20% inactivation of liver enzyme at $>12K_i$ concentration.[5] Compound **2** meets two of the three criteria but shows only 50–65% inactivation of the tumor enzyme at a concentration of 0.016 μM (about $6K_i$) and 10% inactivation at 0.005 μM (about $2K_i$) concentration.

When the SO_2F group of **2** was moved to the *para* position, the resultant **9** was not as good an irreversible inhibitor as **2**; a concentration of $3I_{50}$ of **9** showed only 28% inactivation of the L1210/DF8 enzyme compared to a $3I_{50}$ concentration of **2** which gave 100% inactivation. This difference can be rationalized on the basis that the relative rate of enzyme-catalyzed hydrolysis of the SO_2F group[12] to rate of inactivation by **9** is higher than with **2**. Slight, but perhaps insignificant, irreversible inhibition of the liver enzyme was noted with **9**.

Insertion of a Me *ortho* to the SO_2F of **2** gave **10** that showed little change in reversible binding or specificity of irreversible inhibition. When the CONH bridge of **10** was lengthened to NHCONH (**11**),[13] reversible inhibition of dihydrofolic reductase did not change; however, **11** was both a less effective and less selective irreversible inhibitor than **10**, since **11** showed some irreversible inhibition of the mouse liver enzyme.

The best compounds in Table I are **2** and **10**. However, **2** still had two faults, the second of which was major: (a) **2** failed to give good irreversible inhibition of dihydrofolic at a K_i concentration, and (b) **2** failed to show *in vivo* activity or toxicity in mice bearing L1210/0, as reported previously in a summary paper.[5] The failure of **2** to show *in vivo* activity could be accounted for by the fact that **2** showed poor penetration of the L1210/0 cell wall, as shown by tissue culture studies. The ED_{50} of **2** against L1210/0 in cell culture was 2.2 μM;[14] since 0.05 μM of **2** shows complete inactivation of the L1210/0 enzyme in a broken cell system, the increment between ED_{50} and effective intracellular concentration is at least 40-fold. These results with **2** should be contrasted with those of the dihydrotriazine (**12**). The dihydrotriazine (**12**)[15] showed a 70% life extension of mice with L1210/0 leukemia when **12** was assayed at the optimum once daily dose of 300 mg/kg even though **12** was a nonspecific irreversible inhibitor of dihydrofolic reductase that showed good inactivation of the L1210/0, mouse liver, spleen, and intestine enzymes at a concentration of 0.07 μM;[5] **12** was not as good an irreversible inhibitor of the L1210/0 enzyme as **2** when assayed at $6I_{50}$. Nevertheless, **12** had an ED_{50} of 0.002 μM against L1210/0 in cell culture.[14] Thus the increment for **12** between ED_{50} and effective intracellular concentration is 0.03.



12

The difference between **2** and **12** in ability to penetrate the L1210/0 cell wall is surprising. *A priori* the opposite was anticipated since **12** would have a pK_a near 11[16] and would be fully protonated at physiological pH, whereas **2** would have a pK_a near 7[17] and would be about half-protonated at physiological pH. A charged species such as **12** would not be expected to penetrate a cell wall by passive diffusion as readily as a partially neutral species such as **2**. Extensive studies are now being performed to compare effective concentration for irreversible inhibition of dihydrofolic reductase with the ED_{50} using a wide variety of previously published and unpublished candidate irreversible inhibitors of the SO_2F type. Such studies are important to determine what structural types of selective irreversible inhibitors of dihydrofolic reductase can readily penetrate the L1210/0 cell wall.

The failure of **2** at a $6K_i$ concentration (0.016 μM) to give complete irreversible inhibition of dihydrofolic reductase could be due to the enzyme-catalyzed hydrolysis of the SO_2F group,[12] or due to the possibility that the enzyme was present in the irreversible inhibition incubation at higher concentration than the inhibitor (**2**),[18] or both.

The concentration of dihydrofolic reductase was determined by "titration" with aminopterin at pH 5.9 as described for amethopterin by Werkheiser[21] and as modified by Bertino, *et al.*[22] In the time study of irreversible inhibition by 0.016 μM and 0.05 μM **2** the enzyme concentration was 0.024 μM. Thus 0.016 μM **2** can only inactivate maximally 67% of the total enzyme when the latter is present at 0.024 μM and 48% was observed (Table I). Similarly, 0.005 μM **2** could only inactivate maximally 19% when incubated with 0.027 μM enzyme and 10% was observed. The enzyme concentration can be decreased by tenfold if the total aliquot is assayed rather than quenching an aliquot in ice and diluting tenfold in the assay.[23] Under

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

(13) B. R. Baker and G. J. Lourens, *ibid.*, **11**, 677 (1968), paper CXXIX of this series.

(14) These assays were performed by solution of the compounds in propylene glycol, then addition to the cell culture. We wish to thank Dr. Florence White of the CCNSC for these results.

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

(16) 4,6-Diamino-1-(4-chlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine has pK_a = 11; see E. J. Modest, *J. Org. Chem.*, **21**, 1 (1956).

(17) The pK_a of 7.7 for 2,4-diamino-6-methylpyrimidine should differ little from that of **2**; see J. C. Gage, *J. Chem. Soc.*, 469 (1949).

(18) We wish to thank Professor Howard J. Schaeffer for pointing out this difficulty with some of the experiments from this laboratory on chymotrypsin[12,19] and trypsin.[20]

(19) B. R. Baker and J. A. Hurlbut, *J. Med. Chem.*, **11**, 241 (1968), paper CXIV of this series.

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

(21) W. C. Werkheiser, *J. Biol. Chem.*, **236**, 888 (1961).

(22) J. R. Bertino, B. A. Booth, A. L. Bieber, A. Cashmore, and A. C. Sartorelli, *ibid.*, **239**, 479 (1964).

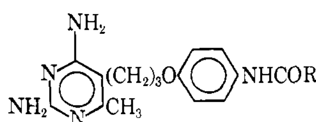
(23) This incubation technique without aliquot dilution was used in earlier work on irreversible inhibition;[24] this was replaced by the aliquot dilution technique since irreversible inhibition is much better quenched by ice-cooling and dilution and, secondly, higher concentrations of inhibitor such as $5I_{50}$ can be studied when the aliquot is diluted tenfold for assay.[15,25]

(24) B. R. Baker, W. W. Lee, and E. Tong, *J. Theoret. Biol.*, **3**, 459 (1962).

(25) B. R. Baker, *Biochem. Pharmacol.*, **11**, 1155 (1962).

TABLE I

INHIBITION[a] OF DIHYDROFOLIC REDUCTASE BY



No.	R	Enzyme source	Reversible[b] I50,[d] μM	Reversible[b] Ki,[e] μM	Irreversible[c] Inhib, μM	Irreversible[c] Enzyme, μM	Irreversible[c] Time, min	Irreversible[c] % inactvn
2[f]	$C_6H_4SO_2F$-*m*	L1210/DF8	0.016	0.0027	0.050	0.024	2, 8, 60	50, 84, 100[g,h]
				0.0031[i]	0.016	0.024	2, 8, 60	24, 48, 48[g,h]
					0.0050	0.027	60	10
					0.0050	0.0020[g]	60	52[g,j]
		L1210/0	0.016	0.0027	0.050	0.023	60	94
					0.016	0.023	60	50
		Liver	0.019	0.0032	0.60	0.024	60	6
		Spleen			0.20	0.025	60	0
		Intestine			0.20	0.023	60	0
9	$C_6H_4SO_2F$-*p*	L1210/DF8	0.023	0.0038	0.069	0.027	8, 15, 30	17, 28, 28[h]
		Liver			0.10	0.024	60	9
10	C_6H_3-4-Me-3-SO_2F	L1210/0	0.020	0.0033	0.040	0.023	60	52
		L1210/DF8			0.040	0.021	60	82
		Liver			0.10	0.024	60	0
11	NHC_6H_3-4-Me-3-SO_2F	L1210/DF8	0.015	0.0025	0.045	0.021	60	44
		Liver			0.10	0.029	60	19

[a] The technical assistance of Sharon Lafler and Diane Shea with these assays is acknowledged. [b] Assayed with 6 μ*M* dihydrofolate and 30 μ*M* TPNH in pH 7.4 Tris buffer containing 0.15 *M* KCl as previously described.[5] [c] Unless otherwise indicated, the incubation was performed at 37° in pH 7.4 Tris buffer in the presence of 60 μ*M* TPNH, then the remaining enzyme was determined by dilution of an aliquot 1:10 with buffer containing 0.17 *M* KCl and assayed with 12 μ*M* dihydrofolate and 30 μ*M* TPNH;[5] the zero point was determined by adding the inhibitor to the assay cuvette. [d] I_{50} = concentration for 50% inhibition. [e] Unless otherwise indicated, the K_i was estimated from $K_i = K_m[I_{50}]/[S]$ which is valid since $[S] = 6K_m$ = 6 μ*M* dihydrofolate; see ref 3, Chapter 10. [f] Data from ref 5 unless otherwise indicated. [g] New data. [h] From six-point time study.[15] [i] Determined by Dixon plot of $1/V$ *vs.* [I] using 6 and 12 μ*M* dihydrofolate, which showed competitive kinetics. [j] Incubated at 25° with one-tenth the usual amount of enzyme by assaying the aliquot without dilution.

these conditions with 0.005 μ*M* **2** and 0.002 μ*M* enzyme, 52% inactivation was seen even though the ratio of excess inhibitor to enzyme could theoretically inactivate all the enzyme. Enzyme-catalyzed hydrolysis of the SO_2F moiety[12,20] is still a pertinent factor; this is further supported by the fact that the *para* isomer (**9**) in 2.6-fold excess over the enzyme gave only 28% total inactivation in 15 min, then no further inactivation occurred.

A posteriori, the irreversible inhibition data reported in the accompanying papers and in previous papers were, in general, run with an enzyme concentration of 0.02–0.04 μ*M*. In only a few cases was the inhibitor concentration lower than the enzyme concentration; these cases are now subject to reinvestigation. For example, 0.01 μ*M* **12** gave only 10% inactivation of 0.023 μ*M* enzyme, considerably below the theoretically achievable 43%; however, when 0.01 μ*M* **12** was incubated with 0.002 μ*M* enzyme, 68% inactivation occurred, which is comparable to the 73% inactivation observed with 0.07 μ*M* **12** and about 0.020 μ*M* enzyme.

From the above discussion it is clear that care must be taken with the first of the three criteria for *in vivo* evaluation; with irreversible inhibitors that have a $K_i < 0.03$ μ*M*, the irreversible inhibition experiment must take into consideration the enzyme concentration. Thus, if the proper ratio of 0.003 μ*M* = K_i concentration of **2** to enzyme is used, >70% inactivation of the enzyme should be achievable. It follows that **2** can meet all three criteria for *in vivo* evaluation. It also follows that a fourth criterion should also be met before whole animal testing is performed; the compound should show good transport through the L1210 cell wall as determined in cell culture. It can be stated that **2** meets only the first three of these four criteria, that is, **2** penetrates the L1210 cell wall poorly. The dihydrotriazine (**12**) meets a different three of these four criteria; **12** can penetrate the L1210 cell wall effectively, but fails the first criterion in that it does not show selective inhibition of the L1210 dihydrofolic reductase with no inactivation of this enzyme in normal cells. By further manipulation of the structure of **2**, **12**, or 6-substituted 5-aryl-2,4-diaminopyrimidines[5] it is reasonably certain that a compound will emerge that meets all four criteria; such a compound should therefore be more effective than **12** against L1210/0 in mice. Studies on further modification of **2**, **12**, and 6-substituted 2,4-diamino-5-(3,4-dichlorophenyl)pyrimidines[5] with the four criteria follow-up are being vigorously pursued.