Structure-Based Design of Lipophilic Quinazoline Inhibitors of Thymidylate Synthase

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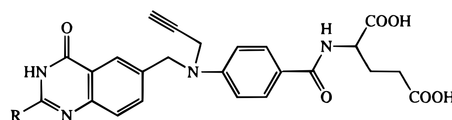
To develop novel lipophilic thymidylate synthase (TS) inhibitors, the X-ray structure of *Escherichia coli* TS in ternary complex with FdUMP and the inhibitor 10-propargyl-5,8-dideazafolic acid (CB3717) was used as a basis for structure-based design. A total of 31 novel lipophilic TS inhibitors, lacking a glutamate residue, were synthesized; 26 of them had in common a *N*-((3,4-dihydro-2-methyl-6-quinazolinyl)methyl)-*N*-prop-2-ynylaniline structure in which the aniline was appropriately substituted with simple lipophilic substituents either in position 3 or 4, or in both. Compounds were tested for their inhibition of *E. coli* TS and human TS and also for their inhibition of the growth in tissue culture of a murine leukemia, a human leukemia, and a thymidine kinase-deficient human adenocarcinoma. The crystal structures of five inhibitors complexed with *E. coli* TS were determined. Five main conclusions are drawn from this study. (i) A 3-substituent such as CF_3, iodo, or ethynyl enhances binding by up to 1 order of magnitude and in the case of CF_3 was proven to fill a nearby pocket in the enzyme. (ii) A simple strongly electron-withdrawing substituent such as NO_2 or CF_3SO_2 in the 4-position enhances binding by 2 orders of magnitude; it is hypothesized that the transannular dipole so induced interacts favorably with the protein. (iii) Attempts to combine the enhancements of i and ii in the same molecule were generally unsuccessful. (iv) A 4-$C_6H_5SO_2$ substituent provided both electron withdrawal and a van der Waal's interaction of the phenyl group with a hydrophobic surface at the mouth of the active site. The inhibition (K_{is} = 12 nM) of human TS by this compound, **7n**, showed that $C_6H_5SO_2$ provided virtually as much binding affinity as the CO-glutamate which it had replaced. (v) The series of compounds were poorly water soluble, and also the potent TS inhibition shown by several of them did not translate into good cytotoxicity. Compounds with large cyclic groups linked to position 4 by an SO or SO_2 group did, however, have IC_{50}'s in the range 1−5 μM. Of these, 4-(*N*-((3,4-dihydro-2-methyl-6-quinazolinyl)methyl)-*N*-prop-2-ynylamino)phenyl phenyl sulfone, **7n**, had IC_{50}'s of about 1 μM and was chosen for further elaboration.

Introduction

In the past decade there have been several developments which have stimulated and refocused antifolate cancer chemotherapy.[1] One has been the discovery,[2] development,[3] and clinical trial[4] of 10-propargyl-5,8-dideazafolic acid (**1**), a specific inhibitor of thymidylate synthase (TS). Clinically active but renally toxic,[5] the drug was withdrawn. Development of the more soluble[6] and nontoxic[7] desamino analog **2** led to the 2-methyl-2-desamino compound **3** with improved pharmacological properties.[8,9] Active cellular uptake of classical TS inhibitors followed by enzymic polyglutamation[10] to tighter binding, noneffluxable forms[9,11,12] is an important aspect of their pharmacology, full recognition of which was given in the further development of **3** to the second-generation thiophene-containing D1694[13,14] and also in the development of 5,8-dideazaisofolate[15] and benzoquinazoline compounds.[16]

However, a drug requiring both active transport and polyglutamation begins to look less favorable when thought is given to drug resistance. Acquired resistance



to antifolates owing to defective transport is well described,[17] and reduced capacity for polyglutamation has been seen with methotrexate,[18,19] edatrexate,[20] and compound **3** itself.[21] It is important therefore to develop a potent lipophilic TS-inhibiting drug. This type of agent would pass into cells by diffusion and efflux similarly. The duration of enzyme blockade would thus be dependent upon plasma levels which in turn, provided the drug had a reasonably long half-life, would be under clinical control. The full rationale for this work is stated in a preliminary communication.[22] Briefly, the task was to enhance the potency of the CO-glutamate-lacking lipophilic analog **7a** (Scheme 1) of **3** by substituting lipophilic groups into the phenyl ring. Three series of compounds were synthesized, the first of which was 4-substituted. Since in the crystal structure of **1** with TS there was apparent a small unfilled pocket of the enzyme in the position *meta* to the N^{10} nitrogen, a

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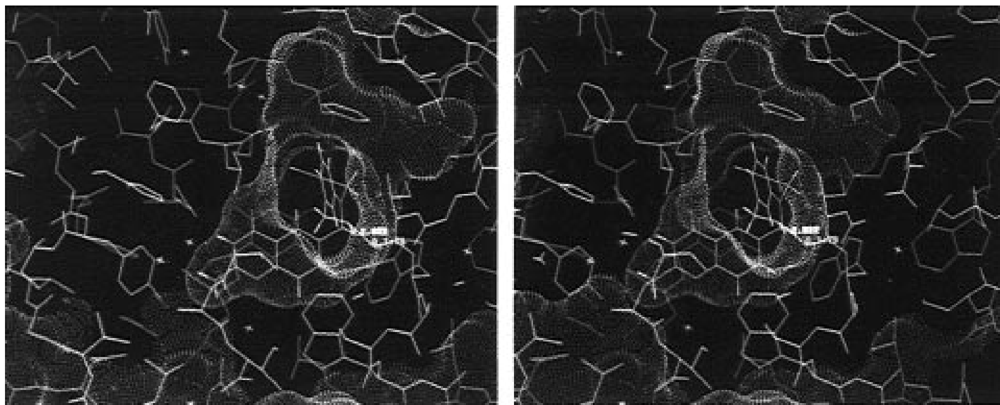


Figure 1. Stereoview showing opening of the *E. coli* active site. The active site is outlined with a Connolly surface in yellow, and the protein is shown in purple. The modeled ligand **7d** is shown in green for carbon, red for oxygen, and blue for nitrogen with the hydroxy OH hydrogen bonded to the Ile79 carbonyl oxygen.

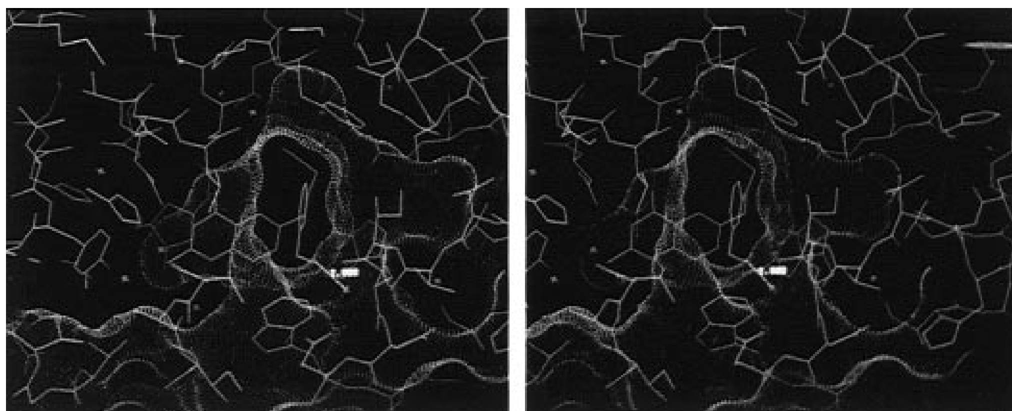


Figure 2. Stereoview showing the experimentally determined structure of compound **7d** complexed to *E. coli* TS. The active site is outlined with a Connolly surface in yellow, and both the protein and the ligand are shown in green for carbon, red for oxygen, and blue for nitrogen. The ligand hydroxy group points toward bulk solvent and makes a hydrogen bond to a bound water molecule.

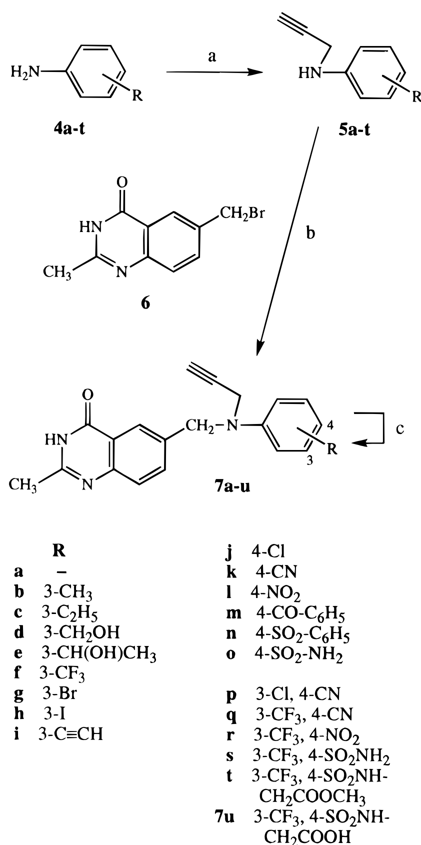
second series of compounds with 3-substituents was designed to exploit it; the third series comprised 3,4-disubstituted compounds. During the course of these studies, and indeed central to our approach, we examined five X-ray structures of the *Escherichia coli* TS complexed with FdUMP and compounds newly synthesized. This ability to "see" the newly synthesized drug candidate bound to the targeted receptor, either to confirm the mode of binding hypothesized for it or to show otherwise, is a powerful alternative to more traditional methods of inhibitor design. In this early, relatively conservative work, three out of four hypotheses were confirmed.

Inhibitor Design, Results, and Discussion

The structure of the ternary complex of *E. coli* TS, FdUMP, and **1**[23] contains *meta* to the nitrogen in the *p*-aminobenzoyl (pAB) ring a predominantly hydrophobic pocket formed by residues Leu172 and Val262 (found as Met in human TS), the edge of Trp80, and the backbone carbonyl of Ile79 (all residue numbers refer to the *E. coli* enzyme). Our first attempts to fill this were with methyl and ethyl groups as in compounds[24] **7b**,**c**. However, these when made and tested offered no improvement of inhibition for either enzyme. Next it was noticed that the carbonyl oxygen of Ile79 was appropriately positioned to accept a hydrogen bond from a hydroxyl group, and when the hydroxymethyl and hydroxyethyl compounds **7d**,**e** were modeled to place the hydroxyl group facing into the pAB-binding cavity, hydrogen bonds could be made. The model for compound **7d** is shown in Figure 1 with 2.82 Å separating the oxygens. But again when the compounds were made and tested, there was no improvement in inhibition for either compound against either enzyme. When the crystal structure of the complex of **7d** in *E. coli* TS was solved, it became apparent that the hydroxyl group preferred to point out of the cavity and make a hydrogen bond to a water molecule that also interacted with Glu82 seen beneath (Figure 2). In the human enzyme there is Ala in place of this Glu82. Thus the K_i's measured for compound **7d** against *E. coli* and human TS, while similar, could reflect different modes of binding, and it is possible that the model of Figure 1 is correct for the human enzyme. The trifluoromethyl compound **7f**, the first significantly better TS inhibitor, was next developed and confirmed to bind to the enzyme as predicted.[22] Three further substituents were used to probe the *meta* pocket—bromine, iodine, and ethynyl, as found in compounds **7g**–**i**. For these it was found that bromine and ethynyl functioned similarly to CF_3, while, relative to hydrogen, iodine clearly conferred tighter binding by 1 order of magnitude for both enzymes.

At the *para* position, chlorine (compound **7j**), tried first, gave a modest enhancement of inhibition. Next, on the hypothesis[25] that a transannular dipole in the pAB ring would increase binding to TS, compounds **7k**,**l** containing the strongly electron-withdrawing cyano and nitro groups were synthesized. The cyano group in **7k**

Scheme 1[a]



[a] (a) Propargyl bromide/K_2CO_3/ΔH (propargyl tosylate for **5d**,**e**) (method not used for **5j**,**l**); (b) **6**/$CaCO_3$/DMF or DMA/ΔH; (c) LiOH to convert **7t** into **7u**.

enhanced binding for both enzymes by 30-fold, and the nitro group in **7l** caused 60- and 40-fold enhancements for bacterial and human TS, respectively. Since on another front sulfones had become of interest (see below), two further compounds with sulfonyl-containing substituents were synthesized, the sulfanilamide **7o** and the trifluoromethyl sulfone **17** (Scheme 2). The former was about 40-fold more active than **7a** and the latter 100-fold more active, having a K_i of 21 nM for the human enzyme. Electron withdrawal by the 4-substituent clearly conferred inhibitory potency. Since the CF_3-SO_2 substituent is the most powerful substituent in this category commonly found in drugs, further exploration on this front alone was considered unnecessary. Figure 3 shows the relationship between the experimental dipole[26] of the corresponding substituted aniline and the TS inhibition for each of the enzymes.

A stabilizing edge to face interaction[27,28] between the pAB ring of **1** and Phe176 was a noticeable feature in the working structure;[23] this gave the idea of setting up an additional aromatic residue, with its face to the outer edge of Phe176, by appending a phenyl group at position 4 through a strongly electron-withdrawing linker. A carbonyl group was first considered as the linker. AM1 calculations on a 4-aminobenzophenone model fragment showed a stable conformation with 24° and 38° angles, respectively, between the substituted and unsubstituted rings and the carbonyl plane; the angle between the ring planes was 56°. This was not 90° as required, but nonetheless compound **7m** was synthesized, and it showed a 20-fold stronger affinity for the bacterial enzyme and a 40-fold stronger affinity

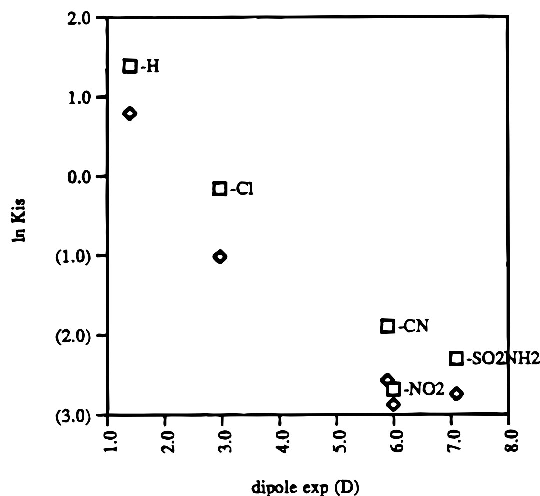


Figure 3. Correlation of experimental dipoles of *para*-substituted anilines and TS inhibition. Compounds with increased dipoles have increased TS inhibition. *E. coli* TS inhibition is represented by a square, and the human TS inhibition is represented by a diamond.

for the human enzyme relative to **7a**. The crystal structure for this compound was eventually solved, but the density for the distal phenyl group was disordered, and the group could not be positioned. It was doubtful that the desired interaction of this group with Phe176 was taking place, and consideration was given to a different linking group—sulfonyl. The sulfone **7n** was thus modeled, synthesized, and found to potently inhibit TS, to which it bound as predicted.[22] In that stereographic depiction, it is apparent that the uppermost of the sulfone oxygens in **7n** is facing a hydrophobic wall constituted by the side chain of Leu172 and has likely in the process of binding undergone desolvation at cost of binding energy. The corresponding sulfoxide would not have to pay this penalty, but there would be an opposing cost of less electron withdrawal. When the sulfoxide **21** was prepared and tested, it was found to be 5-fold less inhibitory to either enzyme. The compound[22] **18** (Scheme 2) combining the 3-CF_3 and 4-$SO_2C_6H_5$ substituents was found no better an inhibitor than **7n**. Four other compounds (**7q**–**s**,**u** combining the *m*-CF_3 substituent respectively with cyano, nitro, sulfamoyl, and SO_2-glycine substituents at the *para* position were synthesized. Compound **7r** was modeled as the 5-amino-2-nitrobenzotrifluoride fragment to assess conformational effects. AM1 calculations showed that the nitro group needed to turn 13.5° out of the ring plane in relief of steric interaction with the trifluoromethyl group; this conformation was accommodated by the enzyme. When the enzyme inhibition results for three of these disubstituted compounds are analyzed to assess the additional effect of the CF_3 group on the inhibition of the corresponding 4-monosubstituted compound, there is seen for the nitrile **7q** a 10-fold enhancement for *E. coli* and no effect on human; for the nitro compound **7r**, a 2-fold enhancement for *E. coli* and no effect on human; and for the sulfanilamide **7s**, a 4-fold enhancement for *E. coli* and 2-fold enhancement for human. It is possible that the attenuation of the inhibitory effect of the nitro group relative to that of cyano is due to a lessening of through resonance by the rotation of the nitro group since the modern view that an aromatic nitro group withdraws inductively may not apply for polar solvents.[29] It is interesting that the

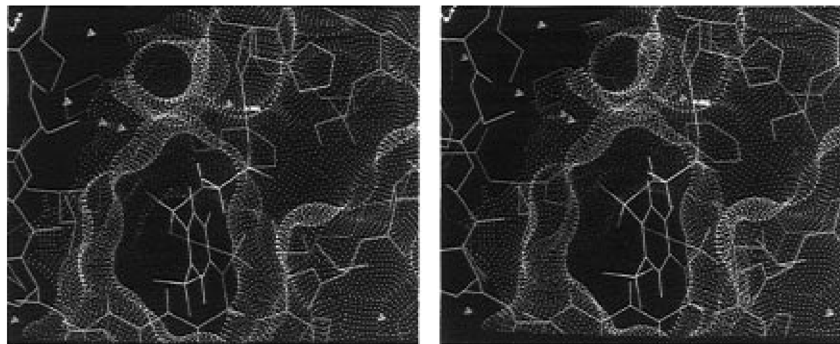
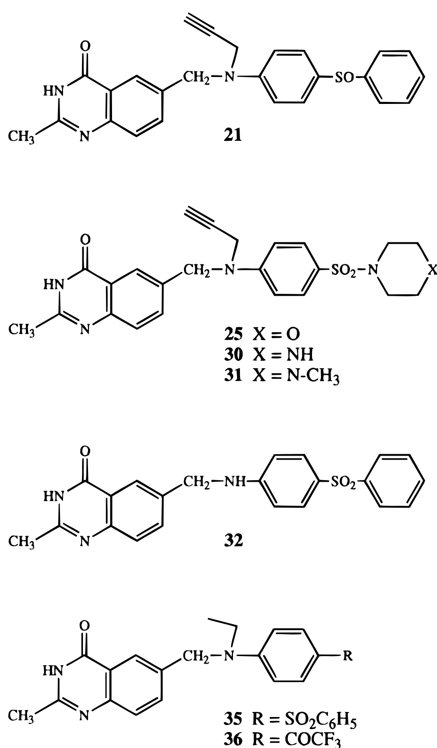


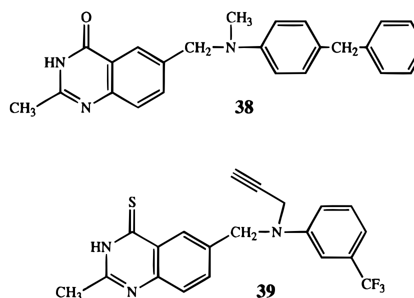
Figure 4. Stereoview showing the experimentally determined structure of compound **7u** complexed to *E. coli* TS. The active site is outlined with a Connolly surface in white, and the protein is in blue. The ligand is shown in pale green for carbon, red for oxygen, blue for nitrogen, green for fluorine, and yellow for sulfur. The side chain of His51 is shown to have moved from its original position in magenta to make a hydrogen bond with one of the carboxylate oxygens of the inhibitor.

compound **7q** selects for inhibition of the bacterial enzyme, presumably due to its CF_3 group. In exploration of this, the 3-chloro analog **7p** was synthesized, but the test results for it showed an ablation of the selective effect. Considered in the round, in disubstituted compounds the 3-CF_3 group moderately favors binding to the bacterial enzyme.

The glycine **7u** was a potent inhibitor of enzyme from both species. The crystal structure of this compound was solved, and the binding of the glycine unit is shown in Figure 4. A notable feature is that the side chain of His51 has shifted markedly from its normal position, by rotation on χ_1 from −174° to −84°, so as to donate a hydrogen bond (3.02 Å) to a carboxylate oxygen of **7u**; there is probably a charge interaction as well. In the human enzyme there is a phenylalanine in place of this histidine so the interaction observed in Figure 4 cannot take place. Other conformations of **7u** are possible in the human enzyme, and without structural illumination, no explanation of the species similarity of the K_i's is possible.



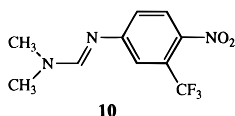
The activity conferred by the 4-phenylsulfonyl substituent prompted the synthesis of the three related compounds **25**, **30**, and **31**, wherein the distal phenyl ring was replaced by saturated cyclic amines attached via nitrogen in a sulfonamide linkage. These amine residues were expected to bind hydrophobically to the Ile79 side chain and also increase aqueous solubility. The trio **25**, **30**, and **31** showed slightly less inhibition than the sulfone **7n** with no discrimination between the enzymes. The N^{10}-unsubstituted compound **32** was required as a synthetic intermediate; a comparison of its TS inhibitions with those of compound **7n** shows that the propargyl group tightens binding by 80-fold in this series. The analog of compound **1** containing the N^{10} ethyl group in place of propargyl was known to be only 3-fold less potent as an inhibitor of murine TS.[3] The ethyl group was thus exemplified in two compounds, **35** and **36**, the latter containing the hitherto untried electron-withdrawing group trifluoroacetyl. Comparison of the inhibitions of **35** with those of **7n** show also a 3-fold difference between the N^{10} substituents in conferring inhibitory potency against human TS. AM1 calculations on diphenylmethane revealed many available conformations, but that mimicking the conformation of compound **7n** seen in ref 22 was of the lowest energy. Compound **38**, however, with a methyl group at N^{10} was thus synthesized. The methyl group is known[6] to give about 10-fold less inhibition of TS than the propargyl; nonetheless, compound **38** failed to give any inhibition when measured at 1 μM, the limit of its solubility. This loss of activity was attributed to the absence of electron withdrawal by the 4-benzyl substituent. Lastly, since sulfur was well accommodated when modeled in place of oxygen in the heterocyclic ring, the 4-thioquinazoline derivative **39** was synthesized. This compound had slight changes in TS inhibitions compared to compound **7f** that together gave a small selective inhibition of *E. coli* TS.



Finally, attention is drawn to position 2 of the quinazoline ring where for all compounds tested a

methyl substituent was present in place of the amino substituent found in compound **1**; this change of substituent causes only a slight change (**1** vs **3**, Table 2) in inhibition. It is thus interesting to observe that in five crystal structures the arrangement of the protein at this locus was the same for all the 2-methylquinazoline ligands seen accommodated and was identical with that seen for **1**.

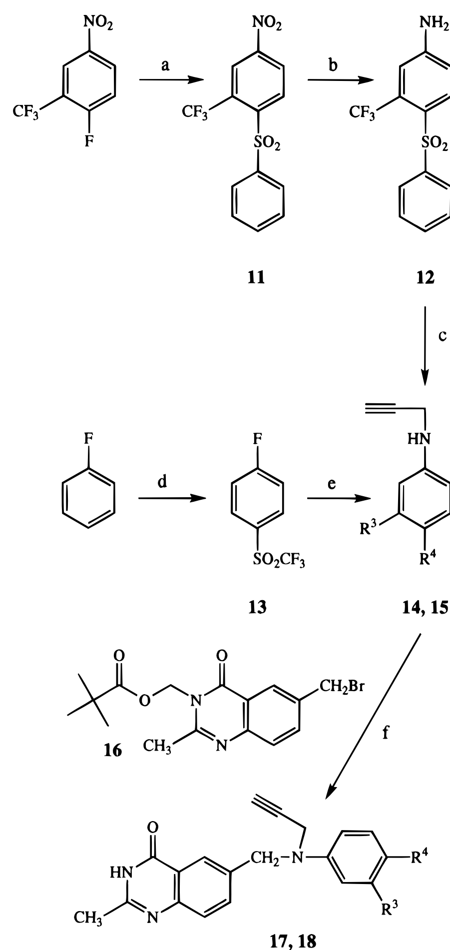
Chemistry. Twenty lipophilic antifolates, **7a**–**s**,**u**, were prepared from the corresponding propargylanilines **5** (**5t** for **7u**) by N-alkylation (Scheme 1). Eighteen of the propargylanilines **5** were prepared from the primary anilines **4**, all of which were commercially available with four exceptions. Thus, **4n** was prepared as described,[30] **4q** was prepared via a von Braun reaction,[31] and **4s**,**t**, containing sulfonamide moieties, were prepared from 2-amino-5-nitrobenzotrifluoride by diazotization, sulfonyl chloride formation, amination, and reduction. Propargylation of the anilines **4a**–**c**,**f**–**i**,**k**,**m**–**t** utilized propargyl bromide and a metal carbonate as auxiliary base (Scheme 1), and details of these reactions are collected in the Supporting Information. In general, and as expected, the conditions and yields (ranging from 73% for **5a** to 16% for **5r**) varied with the electron withdrawal capability of the substituent(s). In the purification of **5r**, a strange byproduct eluted last that was proven to be the amidine **10**. A repeat of the experiment but

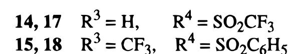


omitting propargyl bromide left the amine **4r** unchanged, even after 16 h. The propargylamine **5r** when heated in DMF/K_2CO_3 gave no **10**. These two control experiments implicated the involvement of propargyl bromide. A repeat of the original experiment with 4-nitroaniline gave no amidine formation. We hypothesize that the sufficiently acidic aniline **4r** forms an anion with K_2CO_3 which attacks the complex formed from propargyl bromide and DMF to give the amidine **10** with expulsion of propargyl alcohol. Two propargylanilines with hydroxyl-containing substituents (**5d**,**e**) were prepared using propargyl tosylate.[32] Use was made of the trifluoroacetyl activating/protecting group[33] to give, in one pot, the chloro derivative **5j**. The propargylaniline **5l** was prepared from 4-fluoronitrobenzene,[34] and this method was used for a convenient alternative preparation of **5n**.

The quinazoline derivatives **7a**–**t** were prepared by N-alkylation of the corresponding propargylanilines **5** with the (bromomethyl)quinazoline **6**, prepared as published,[8] and details of these reactions are collected in Table 1. As in the first set of alkylations, yields upon introducing the quinazoline moiety reflected electron withdrawal by the aniline ring substituents but were now, in certain cases, **7q**,**r**, very low. The realization that the reduced nucleophilicity of the nitrogen in **5r**, occasioned by three electron-withdrawing groups allowing a yield of only 2.3% of **7r**, together with the likely genesis of the amidine **10** suggested an alternative approach wherein the hydrogen of the aniline could be ionized off to give a more reactive anionic nitrogen. This approach is evident in Scheme 2.

Scheme 2[a]





[a] Conditions: (a) $C_6H_5SO_2Na$/135 °C; (b) SnCl2/HCl; (c) propargyl bromide/DMF/K_2CO_3/110 °C; (d) $(CF_3SO_2)_2O$/$AlCl_3$/25 °C; (e) propargylamine/DMSO/K_2CO_3/25 °C; (f) 1. NaH/DMF, 2. **16**, 3. LiOH.

In Scheme 2, the synthetic routes to the 4-sulfonyl-derivatized propargylanilines **14** and **15** incorporated increasingly useful aromatic nucleophilic reactions. Thus, the diphenyl sulfone **11** was prepared by displacement of fluoride ion using benzenesulfinate, and this product was taken on by reduction to give the aniline **12**, propargylation of which gave **15**. Nucleophilic displacement of fluorine from 4-fluorophenyl trifluoromethyl sulfone (**13**) is known,[35] but rather than embarking on the difficult chemistry involved in the first preparation of this compound,[36] a newer method was found.[37] Thus **13** was prepared in low yield, although conveniently, from fluorobenzene in a Friedel–Crafts sulfonation reaction. The product was proven by NMR spectroscopy to be a mixture of *para* and *meta* isomers in 88/12 ratio, and they proved in our hands impossible to separate. The mixture was therefore committted to a nucleophilic displacement reaction with propargylamine, in which only the activated *para* isomer **13** participated and from which the desired product **14** was easily and cleanly obtained. Alkylation of the anions of **14** and **15**, generated with sodium hydride in DMF, was conducted using the quinazoline **16**,[38] protected at its lactam nitrogen with a (pivaloyloxy)methyl group,

Table 1. Preparation of Quinazoline Antifolates **7**

compd	quantity of reactant (mmol) aniline	quinazoline	$CaCO_3$	solvent	mL	reactn temp (°C)	reactn time (h)	purif	yield (%)	mp (°C)	formula	anal.
7a	7.9	7.9	7.9	DMF	30	95	2	a	33	242.5–243.5	$C_{19}H_{17}N_3O$	C,H,N
7b	10	10	20	DMF	30	95	2	b	44	229–230.5 dec	$C_{20}H_{19}N_3O$	C,H,N
7c	10	10	20	DMF	20	105	1.8	c	33	195.5–197	$C_{21}H_{21}N_3O$	C,H,N
7d	4.65	4.65	4.65[d]	DMF	25	90	2	e	58	215–217	$C_{20}H_{19}N_3O_2$	C,H,N
7e	5.71	5.71	5.71[d]	DMF	25	105	2	f	46	171–180	$C_{21}H_{21}N_3O_2$	C,H,N
7f	10	10	20	DMF	30	100	1.8	g	32	228–229.5	$C_{20}H_{16}F_3N_3O$	C,H,N,F
7g	9.5	9.5	19	DMF	30	110	1.8	h	32	227.5–229 dec	$C_{19}H_{16}BrN_3O$	C,H,N,Br
7h	5	5	10	DMF	30	100	2	i	34	249.5–250.5	$C_{19}H_{16}IN_3O$	C,H,N,I
7i	3.22	3.22	6.44	DMA	15	80	3	j	29	234–236 dec	$C_{21}H_{17}N_3O$	C,H,N
7j	20	20	40	DMA	40	95	2	k	65	240–240.5	$C_{19}H_{16}ClN_3O$	C,H,N,Cl
7k	10	10	20	DMF	30	110	3	l	13[m]	266–267.5 dec	$C_{20}H_{16}N_4O$	C,H,N
7l	10	10	20	DMF	30	110	3.3	n	20	278–279 dec	$C_{19}H_{16}N_4O_3$	C,H,N
7m	10	10	20	DMA	35	115	3.5	o	23	213–214.5 dec	$C_{26}H_{21}N_3O_2$	C,H,N
7n	10	10	20	DMA	30	115	4	p	13	191–193	$C_{25}H_{21}N_3O_3S$	C,H,N,S
7o	10	10	20	DMA	30	105	2	q	35	>278 dec	$C_{19}H_{18}N_4O_3S$	C,H,N,S
7p	10	10	20	DMF	30	110	3.6	r	18	267–268	$C_{20}H_{15}ClN_4O$	C,H,N,Cl
7q	10	10	20	DMA	30	115	6	s	5	270.5–271.5	$C_{21}H_{15}F_3N_4O$	C,H,N,F
7r	3.85	3.85	7.7	DMF	50	115	24	t	2.3	261–262 dec	$C_{20}H_{15}F_3N_4O_3$	*u*
7s	10	10	20	DMA	30	90	3.5	v	21	>286 dec	$C_{20}H_{17}F_3N_4O_3S$	C,H,N,F,S
7t[w,y]	16	32	32	DMA	25	110	9	x	19	247.5–248.5 dec	$C_{22}H_{19}F_3N_4O_5S$	C,H,N,S

[a] 1.87 g recrystallized from acetone to give off-white microneedles. [b] 2.62 g twice recrystallized from EtOAc to give off-white microneedles. [c] 2.63 g twice recrystallized from EtOAc to give white microneedles. [d] K_2CO_3 was used in place of $CaCO_3$ and the Celite filtration omitted. [e] 1.09 g taken up in hot MeOH (150 mL); the filtered, cooled solution was diluted with H_2O (250 mL) to precipitate the pure product as a beige crystalline solid. [f] 1.37 g taken up in hot MeOH (100 mL); the filtered, cooled solution was diluted with H_2O (250 mL) to precipitate the pure product as a beige crystalline solid. [g] 2.82 g twice recrystallized from EtOAc to give off-white microneedles. [h] 3.00 g twice recrystallized from EtOAc to give off-white microneedles. [i] 2.07 g twice recrystallized from EtOAc to give white crystals. [j] 0.42 g flash chromatographed eluting sequentially with 90% CH_2Cl_2 in hexane, CH_2Cl_2, and 1.5% CH_3CN in CH_2Cl_2 to provide white flakes. [k] 6.01 g twice recrystallized from 1,2-dichloroethane to give white microneedles. [l] 2.03 g flash chromatographed with 40% CH_3CN in CH_2Cl_2 to give a white solid; the sample was applied in the eluant with the aid of a little DMF. [m] Not optimized. [n] 2.78 g coated onto SiO_2 from $CHCl_3$ and flash chromatographed eluting sequentially with CH_3CN, and 15% MeOH in CH_3CN to give a yellow solid. [o] 3.81 g twice recrystallized from 1,2-dichloroethane to give off-white microneedles. [p] 3.83 g coated onto SiO_2 from 1,2-dichloroethane and flash chromatographed using 10% MeOH in benzene to give 2.19 g of impure material which was rechromatographed using 5% MeOH in CH_2Cl_2 to give 0.56 g which was recrystallized from benzene/hexane to give off-white crystals. [q] 2.94 g coated onto SiO_2 from DMF and flash chromatographed using 5% MeOH/EtOAc to give a white solid. [r] Extracted with $CHCl_3$ to give 3.03 g which was coated onto SiO_2 from $CHCl_3$ and flash chromatographed using 40% CH_3CN in CH_2Cl_2 as eluant to give a white solid. [s] Extracted with EtOAc to give 1.96 g which was coated onto SiO_2 from CH_3CN and flash chromatographed using CH_3CN as eluant to give a white solid. The analytical sample was recrystallized from CH_3CN. [t] Extracted with EtOAc to give 0.65 g which was flash chromatographed using 40% CH_3CN in CH_2Cl_2 to give 0.1 g of material 84% pure by HPLC. This was coated onto SiO_2 from CH_3CN and rechromatographed using 30% ether in EtOAc to give a yellow solid 98% pure by HPLC. [u] This compound failed to microanalyze well. C: calcd, 57.70; found, 58.83, 58.62. H: calcd, 3.63; found, 4.17, 4.18. N: calcd, 13.46; found, 12.46, 12.54. High-resolution mass spectrometry proved the structure: calcd for $C_{20}H_{15}F_3N_4O_3$, 416.1096; found, 416.1100. [v] 3.05 g coated onto SiO_2 from DMF and flash chromatographed using 80% CH_3CN in CH_2Cl_2 to give a white solid. [w] The saponification of this methyl glycinate ester to give the carboxylic acid **7u** is given in footnote y. [x] 0.40 g coated onto SiO_2 from DMF and flash chromatographed using 80% CH_3CN in CCl_4 to give a white solid. [y] N-(N^4-((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)-N^4-prop-2-ynyl-2-(trifluoromethyl)sulfanilyl)glycine **(7u).** The methyl glycinate **7t** (0.138 g, 0.264 mmol) was suspended in a 1:1 mixture of THF:H_2O (8 mL) and treated with 1 N LiOH (1.32 mL, 1.32 mmol) to give a solution which was kept at 20 °C for 3 h. The solution was filtered and brought to pH 2.0 with 1 N HCl to produce a gelatinous white precipitate. This was centrifuged and washed by three cycles of resuspension (H_2O, 15 mL)–centrifugation–decantation. The product was dried over P_2O_5 in vacuo at 20 °C and obtained as an amorphous white solid (0.087 g, 65%): mp 237 °C dec. NMR (Me_2SO-d_6) satisfactory. Anal. ($C_{22}H_{19}F_3N_4O_5S$) C,H,N.

to prevent anion exchange. Removal of the protecting group with LiOH during workup gave the desired antifolates **17** and **18**.

Displacement of fluoride from 4-fluorophenyl phenyl sulfoxide **19** gave 4-(propargylamino)phenyl phenyl sulfoxide **20** that was coupled with the quinazoline **6** to provide the target sulfoxide **21**. Morpholine and piperazine (the latter protected by *tert*-butyloxycarbonyl) were treated with 4-nitrobenzenesulfonyl chloride, and the resulting sulfonamides were taken on in familiar reduction, propargylation, and coupling steps to give the quinazolines **25** and **30**, the latter following removal of the *t*-BOC with trifluoroacetic acid. The piperazine **30** was *N*-methylated to give the additional target compound **31**. The N^{10}-unsubstituted diphenyl sulfone **32** was prepared by N-alkylation. Alkylation of the aniline **4n** gave 4-(ethylamino)phenyl phenyl sulfone **33**, a compound that was alternatively prepared from 4-fluorophenyl phenyl sulfone. A similar fluoride displacement reaction gave 4-(ethylamino)trifluoroacetophenone **34**. Coupling of these amines with **6** gave the targeted N^{10}-ethylated compounds **35** and **36**. Coupling of 4-(phenylmethyl)aniline with **6** gave the amine **37** which on reductive methylation gave the target compound **38** containing the 4-benzyl substituent. Finally, the 4-thioquinazoline derivative **39** was prepared by P_2S_5 treatment of the antifolate **7f**.

Inhibition of Cellular Growth. The target compounds were assessed for cytotoxicity by measuring their effect on the growth in tissue culture of three cell lines: murine (L1210) and human (CCRF-CEM) leukemias and a thymidine kinase-deficient human adenocarcinoma cell line (GC_3/M TK^-). The results are shown in Table 2. The insolubility of certain compounds limited or prevented their proper assay, which was an unwelcome first sign. Potent growth inhibition was not expected of the weaker TS inhibitors, nor was it found; but it was disappointing that it was also not found for most of the stronger TS inhibitors, for example, **7k**,**l**,**o**–**s** and **17**. However, a group of five inhibitors (**7n**, **18**, **21**,

Table 2. Inhibition of Thymidylate Synthase and of Growth of Cells in Tissue Culture

	K_i[a] (μM)		IC_{50}[b] (μM)		
compd	*E. coli* TS	human TS	L1210	CCRF-CEM	GC_3/M TK^-
1[c]	0.0030 ± 0.0003	0.012 ± 0.003			
	0.0051 ± 0.0017	0.011 ± 0.003	1.57	0.75	2.13
3[d]	0.0048 ± 0.0011	0.0085 ± 0.0044			
	0.013 ± 0.003	0.0071 ± 0.0035	0.045	0.029	0.048
7a	4.0 ± 1.2	2.2 ± 1.6			
	15 ± 7	5.7 ± 1.9	>6.5 (35%)	>10.7 (29%)	>10.7 (24%)
7b	3.4 ± 1.1	1.4 ± 0.4			
	16 ± 7	3.4 ± 0.3	>5 (30%)	>5 (20%)	>5 (34%)
7c	5.0 ± 2.4	2.7 ± 0.7			
	24 ± 8	3.8 ± 0.2	>5 (25%)	>5 (26%)	>5 (20%)
7d	1.7 ± 0.7	0.62 ± 0.25			
	5.1 ± 1.3	1.2 ± 0.6	48	>54 (30%)	>54 (22%)
7e	2.4 ± 1.1	1.7 ± 0.3			
	8.3 ± 4.3	3.6 ± 0.7	>49 (35%)	>49 (33%)	49
7f	0.48 ± 0.22	0.39 ± 0.4			
	2.2 ± 0.8	1.1 ± 0.4	>2.2 (35%)	>4.3 (41%)	>4.3 (25%)
7g	0.92 ± 0.35	0.65 ± 0.16			
	3.3 ± 1.9	0.84 ± 0.07	1.8	>5.7 (20%)	5.3
7h	0.33 ± 0.08	0.16 ± 0.02			
	1.4 ± 0.5	0.44 ± 0.03	>5.3 (none)	>5.3 (10%)	>5.3 (35%)
7i	0.86 ± 0.46	0.41 ± 0.08			
	2.7 ± 1	0.68 ± 0.19	>2.4 (5%)	>2.4 (15%)	NT
7j	0.85 ± 0.12	0.36 ± 0.02			
	3.7 ± 0.8	0.77 ± 0.19	>4 (25%)	>4 (20%)	NT
7k	0.15 ± 0.05	0.076 ± 0.063			
	0.33 ± 0.1	0.17 ± 0.08	>5 (35%)	>5 (10%)	>5 (none)
7l	0.068 ± 0.02	0.056 ± 0.013			
	0.17 ± 0.04	0.098 ± 0.018	4.7	>4.8 (8%)	>4.8 (none)
7m	0.094 ± 0.049	0.11 ± 0.04			
	0.47 ± 0.22	0.16 ± 0.07	7.7	>7.7 (40%)	6.2
7n	0.025 ± 0.006	0.012 ± 0.005			
	0.068 ± 0.05	0.021 ± 0.009	0.86	1.44	1.76
7o	0.10 ± 0.04	0.064 ± 0.015			
	0.41 ± 0.17	0.084 ± 0.027	20	>26 (none)	>26 (none)
7p	0.035 ± 0.01	0.037 ± 0.016			
	0.10 ± 0.03	0.079 ± 0.028	4.5	>6.7 (10%)	>6.7 (25%)
7q	0.015 ± 0.007	0.081 ± 0.023			
	0.047 ± 0.006	0.14 ± 0.06	>2.8 (15%)	>2.8 (none)	NT
7r	0.038 ± 0.019	0.074 ± 0.035			
	0.073 ± 0.057	0.15 ± 0.02	>5 (none)	10.5	NT
7s	0.024 ± 0.007	0.036 ± 0.011			
	0.061 ± 0.024	0.070 ± 0.021	insoluble	insoluble	insoluble
7u	0.0059 ± 0.0021	0.0096 ± 0.0045			
	0.015 ± 0.002	0.019 ± 0.007	>75 (none)	>77 (none)	>77 (28%)
17	0.042 ± 0.015	0.021 ± 0.003			
	0.064 ± 0.008	0.028 ± 0.006	>2.75 (45%)	>2.75 (none)	>2.75 (none)
18	0.037 ± 0.019	0.050 ± 0.031			
	0.040 ± 0.008	0.084 ± 0.001	2.0	3.5	7.5
21	0.16 ± 0.08	0.061 ± 0.023			
	0.55 ± 0.04	0.11 ± 0.02	1.7	4.5	3.9
25	0.039 ± 0.001	0.045 ± 0.02			
	0.15 ± 0.05	0.091 ± 0.033	3.1	4.5	5.2
30	0.10 ± 0.02	0.063 ± 0.013			
	0.28 ± 0.11	0.071 ± 0.02	>50 (47%)	48	>50 (47%)
31	0.13 ± 0.08	0.083 ± 0.012			
	0.28 ± 0.08	0.19 ± 0.03	>10 (27%)	>10 (24%)	>10 (17%)
32	2.0 ± 0.6	1.0 ± 0.2			
	17 ± 4	2.2 ± 0.4	>14.7 (40%)	>14.7 (33%)	>14.7 (10%)
35	0.077± 0.044	0.034 ± 0.018			
	0.24 ± 0.09	0.13 ± 0.08	0.66	2.7	2.6
36	0.18 ± 0.03	0.13 ± 0.01			
	1.0 ± 0.1	0.49 ± 0.08	3.4	>4 (10%)	>4 (10%)
38	>1	>1			
	>1	>1	2.8	3.4	>5 (25%)
39	0.18 ± 0.07	1.3 ± 0.6			
	0.36 ± 0.2	2.3 ± 1.4	>5.6 (none)	>5.6 (30%)	>5.6 (none)

[a] For each compound the uppermost value is K_{is} and the lower K_{ii}. The insolubility of compound **38** prevented any value from being determined. [b] For entries of the form > *x* (*y*%), *x* is the limiting solubility of the compound and *y* the percent inhibition at that concentration. NT means not tested. [c] Reference 2. [d] Reference 8.

25, and **35**) containing large cyclic groups linked to position 4 by SO or SO_2 groups did show reasonable cytotoxicity with most IC_{50} values falling in the range 1–5 μM. The cytoxicity of all of these compounds was reversible by thymidine. The IC_{50} ratios (see the Experimental Section) were >5.8, 2.25, >5.3, 10, and 10, respectively, showing that their locus of action was indeed TS. The most potent of these compounds was the diphenyl sulfone **7n**, which had IC_{50} values of about 1 μM.

Conclusions

This manuscript describes our first study in which we have exploited the three-dimensional architecture of thymidylate synthase in the design of novel inhibitors. There are five main findings to report. (1) A substituent put *meta* to the N^{10} nitrogen in the glutamate-lacking antifolate structure **7a** exploits a pocket in TS and enhances binding to by up to 1 order of magnitude. The best substituents are iodo, trifluoromethyl, and ethynyl. (2) A simple, strongly electron-withdrawing substituent, such as nitro or (trifluoromethyl)sulfonyl, put *para* to N^{10} in **7a** enhances binding by 2 orders of magnitude. This effect was independently discovered by another research group[39] while this work was in progress. The enhanced binding is attributed to a favorable interaction between the protein and the transannular dipole that the substituent generates. (3) An attempt at additively combining the results of 1 and 2 by incorporating two substituents in one molecule gave mixed results for *E. coli* TS—unsuccessful in the case of the (trifluoromethyl)phenyl sulfone **18** but successful for the trifluoromethyl nitrile **7q**. Disubstituted compounds all failed to show the expected binding for the human enzyme, perhaps revealing a failing of using the bacterial structure as a surrogate. (4) Strong electron withdrawal allied with a well-positioned bulky hydrophobic group as embodied in a 4-phenylsulfonyl substituent brought enhancement of binding by over 2 orders of magnitude. Thus a comparison of the human TS K_{is} values for compounds **3** and **7n** (0.0085 and 0.012, respectively) shows that the doubly ionic glutamate residue can be replaced essentially by a hydrophobic phenyl ring with *no change* in inhibition. Unsuccessful variants of the glutamate residue had been previously reported[40] before the TS structure became known. Once the ligand-bound enzyme structure was on the graphics, it became possible to devise quickly a totally different inhibitor structure—an illustration of the remarkable power of structure-based design. This result achieved the stated goal of winning back the inhibition lost by removal of the glutamate. (5) The inhibition of the growth of cells in tissue culture by these quinazoline compounds was not impressive. As a class, they had poor water solubility, and moreover, the reasonably potent enzyme inhibition shown by several of them did not translate into good cytotoxicity. Possible reasons for this outcome are that the compounds did not penetrate cells sufficiently well and that if they did, did not remain within. The former explanation is the more probable. Many additional properties are required of an enzyme inhibitor before it can begin to qualify as a drug candidate, and unfortunately this series of compounds seemed generally to lack them. However, the results for the diphenyl sulfone **7n** showed that it was an exception since it was both the most potent enzyme inhibitor and also the most cytotoxic to cells. Attempts to develop it into an even more potent TS inhibitor having better cytotoxicity will be described in forthcoming manuscripts.

Experimental Section

Proton magnetic resonance spectra were determined using a General Electric QE-300 spectrometer operating at a field strength of 300 MHz. Chemical shifts are reported in parts per million (δ) downfield from tetramethysilane as an internal standard, and peak multiplicities are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; br s, broad singlet; br d, broad doublet; br, broad signal; m, multiplet. Mass spectra were determined at the University of California, Riverside, using a VG 7070E-HF high-resolution mass spectrometer using the direct insertion method, an ionizing voltage of 70 eV, and an ion source temperature of 200 °C. For HRMS the measured mass was within ±13 millimass units of the theoretical value. Infrared absorption spectra were taken on either a Perkin-Elmer 457 spectrometer or a MIDAC M2000 FTIR instrument. Elemental microanalyses, performed by MHW Laboratories (Phoenix, AZ) or Atlantic Microlab Inc. (Norcross, GA), gave results for the elements stated within ±0.4% of the theoretical values. Propargyl bromide was used as an 80% (w/w) solution in toluene. *N,N*-Dimethylformamide (DMF) was dried over activated (250 °C) 3 Å molecular sieves; *N,N*-dimethylacetamide (DMA) (Aldrich Gold Label grade) was similarly dried. 6-(Bromomethyl)-3,4-dihydro-2-methyl-4-oxoquinazoline, **6**, was prepared as published.[8] Hünig's base refers to *N,N*-diisopropylethylamine. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl under nitrogen. Ether refers to diethyl ether. Petrol refers to petroleum ether of bp 36−53 °C. Flash chromatography was performed using silica gel 60 (Merck Art 9385). Where the crude solid (*x* g) was insoluble in the chosen eluant, it was dissolved in a more polar solvent and Merck Art 7734 silica gel (4*x* g) added. The slurry was evaporated to dryness on a rotary evaporator fitted with a coarse glass frit to prevent spraying of the silica gel. The coated silica gel was then applied to the column. Thin layer chromatographs (TLC) were performed on precoated sheets of silica gel $60F_{254}$ (Merck Art 5719). Extracts were dried over Na_2SO_4 or $MgSO_4$. Melting points were determined on a Mel-Temp apparatus and are corrected.

4-Cyano-3-(trifluoromethyl)aniline (4q). This compound was prepared by a published[31] method: A mixture of 5-amino-2-bromobenzotrifluoride (12.00 g, 50 mmol), CuCN (5.37 g, 60 mmol), and DMF (7.5 mL) was heated under reflux for 2 h. It was poured into 30% (w/v) $NaCN_{aq}$ (150 mL) and extracted with ether (75 mL). The ether layer was washed sequentially with 10% $NaCN_{aq}$ (100 mL), H_2O (100 mL), and brine (100 mL), dried (Na_2SO_4), and evaporated to dryness to give the crude product (8.00 g). This was flash chromatographed on silica gel (700 g) using 20% ether in CH_2Cl_2 as the eluant to give the pure product as a light tan solid (7.03 g, 76%) suitable for further use. An analytical sample recrystallized from EtOH/H_2O as off-white needles: mp 142−143 °C; NMR satisfactory. Anal. ($C_8H_5F_3N_2$) C,H,N,F.

4-Nitro-2-(trifluoromethyl)benzenesulfonyl Chloride (8). 2-Amino-5-nitrobenzotrifluoride (82.45 g, 0.4 mol) was added to a mechanically stirred, almost boiling mixture of concentrated HCl (120 mL) and H_2O (120 mL) in a 2 L beaker. Glacial HOAc (200 mL) was added to give a clear yellow solution which was then cooled to −10 °C; the yellow hydrochloride salt precipitated. A solution of $NaNO_2$ (29.67 g, 0.43 mol) in H_2O (200 mL) was added during 15 min keeping the temperature below −5 °C, and the resulting mixture was stirred at −5 to −10 °C for 15 min. Meanwhile, glacial HOAc (400 mL) contained in a 4 L beaker was saturated (45 min) with SO_2 gas and CuCl (9.90 g, 0.1 mol) then added. Passage of SO_2 was continued for 30 min until most of the solid had dissolved to give a thin blue-green suspension which was then cooled to 5 °C. The diazonium preparation was added to the cuprous preparation during 11 min, causing vigorous evolution of N_2; the resulting green mixture was stirred for 1.5 h while it attained ambient temperature. Crushed ice (1 kg) was added to give the product as a flocculent yellow solid which was filtered off, washed well with water, and dried in vacuo (51.36 g). Careful recrystallization from cyclohexane (400 mL) with seeding gave the pure product as cubic crystals (38.26 g, 33%): mp 81−83 °C; NMR ($CDCl_3$) satisfactory. Anal. ($C_7H_3ClF_3NO_4S$) C,H,N,Cl.

4-Nitro-2-(trifluoromethyl)benzenesulfonamide (9a). 4-Nitro-2-(trifluoromethyl)benzenesulfonyl chloride (11.58 g, 40 mmol) dissolved in EtOH (150 mL) was treated with concentrated NH_4OH_{aq} (13.5 mL, 200 mmol). The mixture was kept for 1 h at ambient temperature, the reaction quenched with H_2O (600 mL), and the mixture acidified to pH 4 with 1

N HCl to give a precipitate which was kept thus overnight at 4 °C. The product was filtered off, washed, and dried to give pale yellow needles (7.54 g, 69.8%): mp 163−164 °C; NMR (Me_2SO-d_6) satisfactory. Anal. ($C_7H_5N_2F_3O_4S$) C,H,N,F,S.

Methyl *N*-((4-Nitro-2-(trifluoromethyl)phenyl)sulfonyl)-glycinate (9b). 4-Nitro-2-(trifluoromethyl)benzenesulfonyl chloride (13.03 g, 45 mmol) dissolved in EtOH (175 mL) was added during 3 min to a slurry formed from methyl glycinate hydrochloride (11.30 g, 90 mmol) in a mixture of EtOH (30 mL) and Et_3N (25.1 mL, 180 mmol) keeping the temperature between 0 and 5 °C. The mixture was stirred for 50 min at 20 °C and then poured into H_2O (1600 mL) to precipitate the product. The pH was adjusted to 1.5 with 1 N HCl, and the resulting white needles were filtered off, washed with water, and dried (9.28 g, 60%): mp 139.5−140 °C; NMR (Me_2SO-d_6) satisfactory. Anal. ($C_{10}H_9F_3N_2O_6S$) C,H,N,F,S.

2-(Trifluoromethyl)sulfanilimide (4s). 4-Nitro-2-(trifluoromethyl)benzenesulfonamide (**9a**; 6.89 g, 25.5 mmol) was added during 4 min to a warm solution of $SnCl_2 \cdot 2H_2O$ (28.77 g, 127.5 mmol) in EtOH (35 mL). The mixture was heated to 55 °C for 10 min to give a solution to which concentrated HCl (35 mL) was added. The EtOH was boiled away during 20 min, and the mixture was cooled and treated with 2.5 N NaOH to pH 6.5 and then extracted with EtOAc (3 × 250 mL). The combined extracts were washed with brine, dried, and evaporated to dryness to give the product (5.44 g, 89%). An analytical sample was recrystallized from H_2O to give beige plates: mp 186−187 °C; NMR (Me_2SO-d_6) satisfactory. Anal. ($C_7H_7F_3N_2O_2S$) C,H,N,S.

Methyl *N*-((4-Amino-2-(trifluoromethyl)phenyl)sulfonyl)glycinate (4t). The above procedure was repeated starting from methyl *N*-((4-nitro-2-(trifluoromethyl)phenyl)sulfonyl)-glycinate (**9b**; 7.87 g, 23 mmol), $SnCl_2 \cdot 2H_2O$ (25.95 g, 115 mmol), MeOH (35 mL), and concentrated HCl (35 mL). Basification to pH 6.0 was carefully done. The product was obtained as a white solid (3.46 g, 48%). An analytical sample was recrystallized from H_2O to give beige crystals: mp 133−134 °C; NMR (Me_2SO-d_6) satisfactory. Anal. ($C_{10}H_{11}F_3N_2O_4S$) C,H,N,F,S.

General Method for Preparing the Propargylanilines. The starting aniline and propargyl bromide were allowed to react in the solvent stated in the presence of K_2CO_3. Conditions re given in the Supporting Information. The corresponding dipropargylaniline was present as a byproduct along with some unreacted starting aniline. 4-(Dimethylamino)cinnamaldehyde spray reagent was useful in differentiating these amines to allow an optimal yield. The reaction mixture was poured into water and the organic material extracted with ether. The washed and dried extract was evaporated in vacuo to provide the crude product that was flash chromatographed. In each case, the pure propargylamine thus obtained had a satisfactory NMR spectrum and a satisfactory elemental microanalysis for the elements stated.

***N*-Prop-2-ynyl-3-(hydroxymethyl)aniline (5d).** A mixture of 3-aminobenzyl alcohol (3.42 g, 24.76 mmol), propargyl tosylate[32] (5.21 g, 24.76 mmol), K_2CO_3 (3.42 g, 24.76 mmol), and DMF (75 mL) was stirred at ambient temperature for 24 h. The reaction mixture was partitioned between ether (250 mL) and H_2O (250 mL). The organic layer was washed with H_2O (2 × 250 mL) and then extracted with 0.5 N HCl (200 mL). The acidic extract was basified with 5 N NaOH and extracted with ether (2 × 200 mL). The combined ether layers were dried (Na_2SO_4) and evaporated to yield the crude solid product (1.11 g, 28%). Flash chromatography (5% MeOH in $CHCl_3$) gave the pure product as a solid (0.83 g): mp 77−79 °C; NMR satisfactory. Anal. ($C_{10}H_{11}NO$) C,H,N.

***N*-Prop-2-ynyl-3-(1-hydroxyethyl)aniline (5e).** The above reaction was repeated with 3-(1-hydroxyethyl)aniline (4.00 g, 29.15 mmol), propargyl tosylate[32] (6.13 g, 29.15 mmol), K_2CO_3 (4.03 g, 29.15 mmol), and DMF (75 mL): thick oil which crystallized when stored at 0 °C (1.83 g, 36%); NMR satisfactory. Anal. ($C_{11}H_{13}NO$) C,H,N.

4-Chloro-*N*-prop-2-ynylaniline (5j). 4-Chlorotrifluoroacetanilide[33] (111.79 g, 0.50 mol) dissolved in DMF (100 mL) was added to sodium hydride (15.00 g, 0.625 mol) slurried in DMF (200 mL) during 30 min keeping the temperature at 10−15 °C. The mixture was stirred at 10 °C for 45 min. Propargyl bromide (74.36 g, 0.50 mol) was then added during 10 min at 8 °C. The mixture was stirred for 2 h as it attained ambient temperature and then left to stand thus overnight for 19.5 h. The mixture was partitioned between ether (2 L) and H_2O (2 L). The ether layer was washed with H_2O (3 × 2 L) and evaporated to give a brown oil (139.7 g). This oil was treated with a solution of KOH (84.16 g, 1.50 mol) in CH_3OH (750 mL) and the whole then heated under reflux for 3 h. Most (500 mL) of the CH_3OH was removed in vacuo leaving a residue which was partitioned between ether (1.5 L) and H_2O (1.5 L). The ether extract was washed with saturated brine (2 × 1 L), dried ($MgSO_4$), and evaporated to dryness in vacuo. The resulting oil (79.3 g) was vacuum distilled to give the product (bp 126 °C/5 mmHg) as a colorless oil (46.9 g, 56.6%). Anal. (C_9H_8ClN) C,H,N,Cl.

4-(Phenylsulfonyl)-*N*-prop-2-ynylaniline (5n). A solution of 4-(phenylsulfonyl)fluorobenzene (40.00 g, 0.169 mol) and propargylamine (79.6 g, 1.445 mol, 8.55 equiv) in Me_2SO (900 mL) containing $CaCO_3$ (18.6 g, 0.186 mol) in suspension was stirred at 125 °C for 32 h under argon. The mixture was cooled and filtered through Celite. The filtrate was evaporated to dryness in vacuo to leave a residue which was partitioned between H_2O (2 L) and CH_2Cl_2 (2 L). The organic layer was dried and evaporated to dryness to give the crude product (89.6 g). This was thrice flash chromatographed with CH_3CN on silica gel (3 × 1 kg) taking pure product out in the first two stages and recycling fractions containing less pure material. The total yield was 34.53 g (75.3%) of a pale yellow solid identical by NMR with the product (Supporting Information) prepared as in Scheme 1.

***N*^{1},*N*^{1}-Dimethyl-*N*^{3}-(4-nitro-3-(trifluoromethyl)-phenyl)formamidine (10).** During the purification of the amine **5r** (Supporting Information) this byproduct eluted last and was characterized as a yellow oil (2.56 g, 33%): NMR ($CDCl_3$) δ 3.08 (s, 3H, CH_3), 3.11 (s, 3H, CH_3), 7.12 (dd, 1H, J = 8.7, 2.4 Hz, H^6), 7.32 (d, 1H, J = 2.4 Hz, H^2), 7.63 (s, 1H, CH=N), 7.93 (d, 1H, J = 8.7 Hz, H^5). Anal. ($C_{10}H_{10}F_3N_3O_2$) C,H,N.

General Method for Preparing the Antifolates 7. A solution of the propargylaniline **5** and the (bromomethyl)-quinazoline **6**[8] in the solvent stated was stirred over $CaCO_3$. Conditions are collected in Table 1. SiO_2GF/EtOAc was in most cases a useful TLC system, and the Epstein spray reagent[41] was used to monitor consumption of the (bromomethyl)quinazoline. The mixture was filtered through Celite, and the filtrate was poured into iced water (1 L). The product precipitated and, in most cases, was filtered off on a Whatmans no. 5 filter, washed with H_2O, and then dried over P_2O_5 in vacuo. In three cases filtration was unsuccessful, and the product was therefore obtained by extractive workup. Eight of the compounds were purified by recrystallization, 10 by flash chromatography, and two by reprecipitation. In some of the recrystallizations, $MgSO_4$ in equal weight to the crude product was used to dry the hot solution (and removed at the filtration stage) to improve crystal formation. All of the compounds in the table gave satisfactory ^{1}H NMR spectra, and these are not recorded, with the exception of the spectrum of the 4-chloro compound **7j** to serve as an example: (Me_2SO-d_6) δ 2.33 (s, 3H, CH_3), 3.22 (t, 1H, J = 2.0 Hz, ≡CH), 4.24 (d, 2H, J = 2.0 Hz, CH_2-C≡), 4.66 (s, 2H, CH_2), 6.81 (d, 2H, J = 9.1 Hz, aromatic), 7.21 (d, 2H, J = 9.1 Hz, aromatic), 7.54 (d, 1H, J = 8.3 Hz, H^8), 7.69 (dd, 1H, J = 8.3, 1.7 Hz, H^7), 7.95 (d, 1H, J = 1.7 Hz, H^5), 12.20 (br s, 1H, NH). In like vein, the mass spectrum of this compound typifies the series: m/z 339 and 337 (M^+), 173 (M − C_9H_7ClN), 132 (173 − CH_3CN), 104 (132 − CO).

4-Nitro-2-(trifluoromethyl)diphenyl Phenyl Sulfone (11). A mixture of sodium benzenesulfinate (18.06 g, 0.11 mol), ethylene glycol (75 mL), and 2-(2-ethoxyethoxy)ethanol (120 mL) was heated to 125 °C in a flask equipped with an overhead stirrer. 2-Fluoro-5-nitrobenzotrifluoride (20.91 g, 0.1 mol) was added, and the mixture was heated at 135 °C for 3.5 h. The resulting solution was cooled, and H_2O (20 mL) was added to throw down an oil which eventually solidified. The pasty solid was filtered off, washed with hot H_2O (75 mL),

partially dried, and recrystallized from EtOH–H_2O with carbon treatment to provide a tan-colored solid (7.85 g, 23.7%). Flash chromatography (70% CH_2Cl_2 in petrol) of a small portion gave the analytical sample as a cream-colored solid: mp 167–168 °C; NMR satisfactory. Anal. ($C_{13}H_8F_3NO_4S$) C,H,N,F,S.

4-Amino-2-(trifluoromethyl)phenyl Phenyl Sulfone (12). The nitro compound **11** (7.00 g, 21 mmol) was added during 4 min to a warm solution of $SnCl_2 \cdot 2H_2O$ (28.43 g, 126 mmol) in EtOH (33 mL). Heating for 8 min at 54 °C gave a solution to which concentrated HCl (33 mL) was added. The ethanol was boiled away during 20 min; the residue was cooled in ice and treated with 2.5 N NaOH to pH 14 to precipitate the product which was filtered off, washed with water, and dried (5.62 g, 89%)-suitable for further use. The analytical sample was obtained by flash chromatography (3% CH_3CN in CH_2Cl_2) as a white solid: mp 186–188 °C; NMR satisfactory. Anal. ($C_{13}H_{10}F_3NO_2S$) C,H,N,F,S.

***N*-Prop-2-ynyl-4-(phenylsulfonyl)-3-(trifluoromethyl)-aniline (15):** prepared from **12** (Supporting Information).

4-((Trifluoromethyl)sulfonyl)fluorobenzene (13). Trifluoromethanesulfonyl anhydride (50 g, 177 mmol) was syringed during 10 min into a mixture of fluorobenzene (49.84 mL, 531 mmol) and $AlCl_3$ (29.37 g, 220 mmol) stirred at 0 °C under argon. The resulting orange-colored mixture was stirred for 10 min at 0 °C and then for 23 h at 25 °C. The mixture was partitioned between H_2O (1000 mL) and ether (2 × 400 mL). The combined ether layers were washed with H_2O (2 × 500 mL) and brine (800 mL) and dried (Na_2SO_4) to give the crude product as an orange-colored oil (17.5 g). This was chromatographed on SiO_2 (1 kg) using ether/CCl_4 (1.5/98.5) as the eluant. Fractions containing the second component to elute were pooled and evaporated to give the technical quality product (3.69 g, 9.1%). This was 88% pure: NMR ($CDCl_3$) δ 7.37 (dd, 2H, J_H = 8.9 Hz, J_F = 8.1 Hz, H^2, H^6), 8.09 (dd, 2H, J_H = 8.9 Hz, J_F = 4.9 Hz, H^3, H^5). The single impurity was 12% of 3-((trifluoromethyl)sulfonyl)fluorobenzene: NMR δ 7.55 (dddd, 1H, $J_{6,F}$ = 10.7 Hz, $J_{6,5}$ = 7.7 Hz, $J_{6,4}$ = 2.5 Hz, $J_{6,2}$ = 1.0 Hz, H^6), 7.70 (ddd, 1H, $J_{5,4}$ = $J_{5,6}$ = 7.7 Hz, $J_{5,F}$ = 5.0 Hz, H^5), 7.76 (ddd, 1H, $J_{4,5}$ = 7.7 Hz, $J_{4,6}$ = 2.5 Hz, $J_{4,2}$ = 2.2 Hz, H^4), 7.87 (br d, 1H, $J_{2,F}$ = 7.8 Hz, H^2).

***N*-Prop-2-ynyl-4-((trifluoromethyl)sulfonyl)aniline (14).** A solution of **13** (technical quality, 88%, 1.16 g, 4.47 mmol) and propargylamine (0.51 g, 9.26 mmol) in DMSO (15 mL) was stirred over K_2CO_3 (0.80 g, 5.79 mmol) at 25 °C for 24 h. The mixture was poured into H_2O (400 mL) and basified with 0.1 N NaOH to pH 11; extractive workup (Et_2O) gave an essentially pure amber oil which slowly crystallized (0.79 g, 67%). A small sample was flash chromatographed using CH_2Cl_2 to give the analytical sample as a white solid: mp 86–87 °C; NMR ($CDCl_3$) δ 2.31 (t, 1H, J = 2.4 Hz, ≡CH), 4.04 (d, 2H, J = 2.4 Hz, CH_2-C≡), 4.4–5.2 (br, 1H, NH), 6.77 (d, 2H, J = 8.9 Hz, H^2, H^6), 7.82 (d, 2H, J = 8.9 Hz, H^3, H^5). Anal. ($C_{10}H_8F_3NO_2S$) C,H,N,F,S.

***N*-((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)-*N*-prop-2-ynyl-4-((trifluoromethyl)sulfonyl)aniline (17).** Sodium hydride (60% oil dispersion, 0.71 g, 1.77 mmol) was placed in a flame dried flask and covered with argon. The propargylaniline **14** (0.453 g, 1.72 mmol) in DMF (9 mL) was cannulated in during 1 min with stirring. An orange-red solution formed which was stirred for 30 min. To it was added 6-(bromomethyl)-3, 4-dihydro-2-methyl-4-oxo-3-((pivaloyloxy)-methyl)quinazoline (**16**;[38] 0.316 g, 0.86 mmol) in DMF (10 mL), and the solution was stirred at 25 °C for 22.5 h. The mixture was poured into saturated $NaHCO_{3aq}$ (100 mL) and extracted with CH_2Cl_2 (4 × 100 mL). The combined organic layers were dried (Na_2SO_4) and evaporated to dryness under oil pump vacuum. The brown residue was treated with MeOH (20 mL) followed by 1 N LiOH (2 mL, 2 mmol), and the resulting mixture was stirred for 37 min, acidified to pH 3 with 1 N HCl, poured into H_2O (100 mL), and extracted with CH_2Cl_2 (4 × 50 mL). The combined, dried extract was taken to dryness to give the crude product (0.350 g) which was loaded onto a silica gel column (60 g) in 1,2-dichloroethane and eluted off with 40% CH_3CN in CCl_4 to give the pure product as a white solid (0.127 g, 34%): mp 250–251.5 °C; NMR (Me_2SO-d_6) δ 2.33 (s, 3H, CH_3), 3.34 (t, 1H, J = 2.2 Hz, ≡CH), 4.50 (d, 2H, J = 2.2 Hz, CH_2-C≡), 4.93 (s, 2H, CH_2), 7.07 (d, 2H, J = 9.2 Hz, aromatic), 7.56 (d, 1H, J = 8.4 Hz, H^8), 7.68 (dd, 1H, J = 8.4, 2.0 Hz, H^7), 7.83 (d, 2H, J = 9.2 Hz, aromatic), 7.96 (d, 1H, J = 2.0 Hz, H^5), 12.22 (br s, 1H, NH). Anal. ($C_{20}H_{16}F_3N_3O_3S$) C,H,N,F.

***N*-((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)-4-(phenylsulfonyl)-*N*-prop-2-ynyl-3-(trifluoromethyl)aniline (18).** The above procedure was repeated starting from the aniline **15** (1.27 g, 3.74 mmol) in DMF (20 mL) with reagents in the following quantities: 60% sodium hydride (0.15 g, 3.70 mmol), **16**[38] (1.36 g, 3.70 mmol) in DMF (20 mL), $NaHCO_{3aq}$ (400 mL), and MeOH (90 mL)/1 N LiOH (9 mL, 9 mmol). Exhaustive extraction with CH_2Cl_2 (11 × 100 mL) was needed to obtain the crude product as an amber oil (1.37 g). Flash chromatography (50% CH_3CN in CCl_4) gave the pure product as an off-white solid (0.207 g, 10.9%): mp 235.5–237 °C; NMR satisfactory. Anal. ($C_{26}H_{20}F_3N_3O_3S$) C,H,N,F,S.

4-Fluorophenyl Phenyl Sulfoxide (19). To a rapidly stirred solution of *p*-fluorodiphenyl sulfide[42] (11.0 g, 53.9 mmol) in HOAc (125 mL) at 25 °C was added 30% H_2O_2 (6.10 g, 53.9 mmol). The mixture was stirred for 28 h and then poured into H_2O (500 mL); extractive workup (Et_2O) followed by flash chromatography with 50% Et_2O in petrol gave the desired sulfoxide (11.8 g, 100%) identical with that previously described.[43]

4-(*N*-Prop-2-ynylamino)phenyl Phenyl Sulfoxide (20). To a rapidly stirred solution of **19** (2.0 g, 9.1 mmol) in dry DMSO (15 mL) were added propargylamine (6.2 mL, 91 mmol, 10 equiv) and $CaCO_3$ (1.0 g, 9.99 mmol). The mixture was heated at 95 °C in a sealed tube for 18 h, at which time further propargylamine (6.2 mL, 91 mmol) was added. The mixture was heated at 125 °C for an additional 24 h and then poured into H_2O (500 mL); extractive workup (EtOAc) followed by flash chromatography with 20% EtOAc in CH_2Cl_2 gave an off-white solid (444 mg, 19%) which was then recrystallized from CH_2Cl_2: mp 106–107 °C; NMR ($CDCl_3$) δ 2.23 (t, 1H, J = 2.4 Hz, CH), 3.93 (dd, 2H, J = 6.0, 2.4 Hz, CH_2), 4.29 (br s, 1H, NH), 6.68 (d, 2H, J = 8.8 Hz), 7.45 (m, 5H), 7.60 (d, 2H, J = 8.8 Hz); IR (KBr, cm^{-1}) 3395 (s), 3250 (m), 1585 (m), 1320 (m), 1085 (m), 1025 (m), 823 (m). Anal. ($C_{15}H_{13}NOS$) C,H,N,S.

4-(*N*-((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)-*N*-prop-2-ynylamino)phenyl Phenyl Sulfoxide (21). To a rapidly stirred solution of **20** (4.0 g, 15.7 mmol) in DMF (40 mL) were added **6** (4.76 g, 18.8 mmol) and diisopropylethylamine (3.4 mL, 23.6 mmol). The resulting mixture was heated at 90 °C for 4 h. It was cooled and poured into H_2O (700 mL), and the resulting solid was filtered off. It was coated onto SiO_2 (20 g), from DMF (20 mL), and chromatographed on SiO_2 (500 g) with a gradient of 0–3.5% MeOH in CH_2Cl_2 to give the product (4.4 g, 66%) as a white solid. Recrystallization from 20% CH_2Cl_2 in Et_2O gave an analytically pure sample: mp 219–221 °C; NMR (Me_2SO-d_6) δ 2.30 (s, 3H, CH_3), 3.21 (br s, 1H, CH), 4.29 (s, 2H, CH_2), 4.72 (s, 2H, CH_2), 6.87 (d, 2H, J = 9.0 Hz), 7.40–7.75 (m, 9H), 7.91 (s, 1H), 12.17 (s, 1H, NH); IR (KBr, cm^{-1}) 3160 (m), 1670 (s), 1620 (m), 1590 (m), 1090 (w), 1018 (w). Anal. ($C_{25}H_{21}N_3O_2S$) C,H,N,S.

***N*-((4-Nitrophenyl)sulfonyl)morpholine (22).** Morpholine (8.6 mL, 98.73 mmol) was added dropwise to a stirred solution of 4-nitrobenzenesulfonyl chloride (10.4 g, 46.93 mmol) in CH_2Cl_2 (150 mL) at 0 °C. After stirring for 10 min, 0.5 N HCl (150 mL) was added; extractive workup with CH_2Cl_2 gave the product as a light brown solid (12.13 g, 95%): mp 172–174 °C (lit.[44] mp 172.5–173 °C); NMR ($CDCl_3$) δ 3.05 (m, 4H), 3.76 (m, 4H), 7.94 (d, 2H, J = 9.0 Hz), 8.41 (d, 2H, J = 9.0 Hz). Anal. ($C_{10}H_{12}N_2O_5S$) C,H,N,S.

***N*-((4-Aminophenyl)sulfonyl)morpholine (23).** A solution of **22** (9.2 g, 33.79 mmol) in CH_3OH (250 mL) containing concentrated HCl (1.4 mL, 16.90 mmol) and with 5% Pd on activated carbon (1.0 g) in suspension, in a Parr apparatus, was shaken under 40 psi of hydrogen gas for 18 h. The reaction mixture was filtered through Celite, the catalyst washed with CH_3OH (500 mL), and the solvent removed under reduced pressure. Extractive workup (EtOAc) gave the product as a pale yellow solid (7.5 g, 92%): mp 210–213 °C (lit.[45]

mp 217 °C); NMR (Me_2SO-d_6) δ 2.77 (t, 4H, J = 4.6 Hz), 3.62 (t, 4H, J = 4.6 Hz), 6.15 (s, 2H), 6.67 (d, 2H, J = 8.7 Hz), 7.35 (d, 2H, J = 8.7 Hz). Anal. ($C_{10}H_{14}N_2O_3S$) C,H,N,S.

***N*-((4-(*N*-Prop-2-ynylamino)phenyl)sulfonyl)morpholine (24).** A solution of **23** (1.001 g, 4.14 mmol), *N,N*-diisopropylethylamine (0.86 mL, 4.93 mmol), and propargyl bromide (80% (w/w) in toluene, 0.46 mL, 4.14 mmol) in DMF (10 mL) was heated at 75 °C. At 5 h, additional diisopropylethylamine (0.36 mL, 2.5 mmol) and propargyl bromide (0.23 mL, 2.07 mmol) were added, and the mixture was heated for 3 h more. It was poured into 0.5 N HCl (200 mL), and extractive workup (EtOAc) followed by flash chromatography with CH_2Cl_2−EtOAc (20:1) gave the desired product as a pale yellow solid (0.47 g, 41%): mp 117−120 °C; NMR ($CDCl_3$) δ 2.27 (t, 1H, J = 2.5 Hz), 2.97 (m, 4H), 3.73 (m, 4H), 4.00 (dd, 2H, J = 2.5, 6.0 Hz), 4.47 (m, 1H), 6.71 (d, 2H, J = 8.8 Hz), 7.58 (d, 2H, J = 8.8 Hz). Anal. ($C_{13}H_{16}N_2O_3S$) C,H,N,S.

***N*-((4-(*N*-((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)-*N*-prop-2-ynylamino)phenyl)sulfonyl)morpholine (25).** A solution of **24** (1.314 g, 4.69 mmol), **6** (1.185 g, 4.69 mmol), and 2,6-lutidine (0.85 mL, 7.3 mmol) in DMF (15 mL) was heated at 100 °C. At 1.5 h, further **6** (0.885 g, 3.52 mmol) and 2,6-lutidine (0.41 mL, 3.52 mmol) were added. At 4.5 h, the reaction mixture was poured into 0.5 N HCl (150 mL), and extractive workup (EtOAc) followed by flash chromatography with 50% EtOAc in CH_2Cl_2 gave the desired product as an off-white solid (0.630 g, 30%): mp 222−225 °C slight dec; NMR (Me_2SO-d_6) δ 2.31 (s, 3H), 2.77 (m, 4H), 3.26 (t, 1H, J = 1.9 Hz), 3.59 (m, 4H), 4.37 (d, 2H, J = 1.7 Hz), 4.81 (s, 2H), 6.95 (d, 2H, J = 9.0 Hz), 7.52 (m, 3H), 7.68 (dd, 1H, J = 1.9, 8.3 Hz), 7.96 (d, 1H, J = 1.5 Hz), 12.18 (s, 1H); HRMS $(C_{23}H_{24}N_4O_4S)^+$ calcd 452.1518, found 452.1497. Anal. ($C_{23}H_{24}N_4O_4S$).

4-((4-Nitrophenyl)sulfonyl)-1-(*tert*-butyloxycarbonyl)piperazine (26). To a stirred solution of *tert*-butyl 1-piperazinecarboxylate (3.069 g, 16.48 mmol) and diisopropylethylamine (3.16 mL, 18.13 mmol) in CH_2Cl_2 (80 mL) at 0 °C was slowly added a solution of 4-nitrobenzenesulfonyl chloride (3.652 g, 16.48 mmol) in CH_2Cl_2 (50 mL). When the addition was complete, the reaction mixture was allowed to warm to room temperature, and then it was poured into 0.5 N HCl (75 mL); extractive workup (CH_2Cl_2) gave the desired sulfonamide as a tan solid (5.78 g, 94%): mp 169−171 °C; NMR ($CDCl_3$) δ 1.40 (s, 9H), 3.03 (t, 4H, J = 5.0 Hz), 3.53 (t, 4H, J = 5.1 Hz), 7.94 (d, 2H, J = 8.8 Hz), 8.39 (d, 2H, J = 8.8 Hz). Anal. ($C_{15}H_{21}N_3O_6S$) C,H,N,S.

4-((4-Aminophenyl)sulfonyl)-1-(*tert*-butyloxycarbonyl)piperazine (27). A solution of **26** (5.00 g, 13.46 mmol) in EtOAc (50 mL) with 5% Pd on activated carbon (1.0 g) in suspension, in a Parr apparatus, was shaken under 44 psi of hydrogen gas for 18 h. At this time further catalyst (0.60 g) was added, and the mixture was hydrogenated at 44 psi for a further 4 h. The reaction mixture was filtered through Celite, the catalyst washed with EtOAc (1 L), and the solvent removed under reduced pressure to give the desired aniline as a white solid (4.58 g, ~100%): mp 194−197 °C; NMR ($CDCl_3$) δ 1.40 (s, 9H), 2.92 (t, 4H, J = 5.0 Hz), 3.49 (t, 4H, J = 5.1 Hz), 4.15 (s, 2H), 6.69 (d, 2H, J = 8.7 Hz), 7.50 (d, 2H, J = 8.7 Hz); HRMS $(C_{15}H_{23}N_3O_4S)^+$ calcd 341.1409, found 341.1419. This material was used without further purification.

4-((4-(Prop-2-ynylamino)phenyl)sulfonyl)-1-(*tert*-butyloxycarbonyl)piperazine (28). A solution of the aniline **27** (1.50 g, 4.39 mmol), propargyl bromide (80% (w/w) in toluene, 0.49 mL, 4.39 mmol), and *N,N*-diisopropylethylamine (0.80 mL, 4.61 mmol) in DMF (20 mL) was heated at 75 °C. At 4 h, additional propargyl bromide (0.49 mL, 4.39 mmol) and diisopropylethylamine (0.80 mL, 4.61 mmol) were added, and the mixture was heated for 4 h more. It was poured into 0.5 N HCl (100 mL); extractive workup (EtOAc) followed by flash chromatography with CH_2Cl_2−EtOAc (20:1) gave the desired propargylamine as a pale yellow solid (0.923 g, 55%): mp 142−143 °C; IR (KBr, cm^{-1}) 3390, 1680, 1595, 1325, 1160; NMR ($CDCl_3$) δ 1.41 (s, 9H), 2.27 (t, 1H, J = 2.5 Hz), 2.94 (t, 4H, J = 5.0 Hz), 3.49 (t, 4H, J = 5.0 Hz), 4.00 (dd, 2H, J = 2.3, 5.6 Hz), 4.45 (t, 1H, J = 5.7 Hz), 6.71 (d, 2H, J = 8.8 Hz), 7.57 (d, 2H, J = 8.8 Hz). Anal. ($C_{18}H_{25}N_3O_4S$) C,H,N,S.

4-((4-(*N*-((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)-*N*-prop-2-ynylamino)phenyl)sulfonyl)-1-(*tert*-butyloxycarbonyl)piperazine (29). A solution of **28** (0.678 g, 1.79 mmol), **6** (0.452 g, 1.79 mmol), and 2,6-lutidine (0.31 mL, 2.66 mmol) in DMF (10 mL) was heated at 100 °C. At 1 h, further **6** (0.452 g, 1.79 mmol) and lutidine (0.21 mL, 1.80 mmol) were added. At 2 h, further **6** (0.226 g, 0.89 mmol) and lutidine (0.1 mL, 0.90 mmol) were added. At 4 h, the reaction mixture was poured into 0.5 N HCl (75 mL); extractive workup (EtOAc) followed by flash chromatography with benzene−EtOAc (1:2) gave the desired product as an off-white solid (330 mg, 33%): mp 193−195 °C; IR (KBr, cm^{-1}) 1680, 1590, 1160; NMR ($CDCl_3$) δ 1.4 (s, 9H), 2.31 (t, 1H, J = 2.3 Hz), 2.55 (s, 3H), 2.95 (m, 4H), 3.49 (t, 4H, J = 4.8 Hz), 4.18 (d, 2H, J = 2.3 Hz), 4.77 (s, 2H), 6.88 (d, 2H, J = 9.0 Hz), 7.58 (d, 2H, J = 9.0 Hz), 7.68 (s, 2H), 8.15 (s, 1H), 10.72 (s, 1H). Anal. ($C_{28}H_{33}N_5O_5S$) C,H,N,S.

***N*-((4-(*N*-((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)-*N*-prop-2-ynylamino)phenyl)sulfonyl)piperazine (30).** CF_3COOH (0.50 mL, 6.49 mmol) was added to a stirred solution of **29** (0.200 g, 0.363 mmol) in CH_2Cl_2 (4 mL). After 1 h, volatile components were removed under reduced pressure and the resulting residue was dissolved in EtOAc (25 mL) and washed with 2 N NaOH (10 mL). Exhaustive extractive workup (EtOAc) followed by flash chromatography with 10% CH_3OH in CH_2Cl_2 gave the product as a white, hygroscopic solid (150 mg, 92%): mp indeterminate; NMR ($CDCl_3$) δ 2.30 (t, 1H, J = 2.3 Hz), 2.52 (s, 3H), 3.02 (br s, 8H), 4.13 (d, 2H, J = 2.3 Hz), 4.82 (s, 2H), 6.93 (d, 2H, J = 9.0 Hz), 7.62 (d, 2H, J = 9.0 Hz), 7.66 (m, 2H), 7.88 (m, 1H), NH's not discernable; HRMS $(C_{23}H_{25}N_5O_3S)^+$ calcd 451.1688, found 451.1678. HCl salt: mp 225−227 °C.

4-((4-(*N*-((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)-*N*-prop-2-ynylamino)phenyl)sulfonyl)-1-methylpiperazine (31). Iodomethane (0.017 mL, 0.273 mmol) was added to a stirred solution of **30** (123 mg, 0.272 mmol) and *N,N*-diisopropylethylamine (0.048 mL, 0.276 mmol) in DMF (5 mL). After 18 h at 25 °C, the mixture was poured into H_2O (20 mL); extractive workup (CH_2Cl_2) followed by chromatography with CH_2Cl_2−CH_3OH (10:1) gave the product as a white solid (75 mg, 59%): mp 139−141 °C; NMR ($CDCl_3$) δ 2.26 (s, 3H), 2.31 (t, 1H, J = 2.3 Hz), 2.47 (br t, 4H), 2.99 (br t, 4H), 4.18 (d, 2H, J = 2.3 Hz), 4.77 (s, 2H), 6.88 (d, 2H, J = 7.2 Hz), 7.61 (d, 2H, J = 7.2 Hz), 7.68 (d, 2H, J = 1.2 Hz), 8.19 (s, 1H), 11.09 (br s, 1H); HRMS $(C_{24}H_{27}N_5O_3S)^+$ calcd 465.1835, found 465.1830. Anal. ($C_{24}H_{27}N_5O_3S$) C,H,N,S.

***N*-((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)-4-(phenylsulfonyl)aniline (32).** A mixture of 4-aminophenyl phenyl sulfone (**4n**; 7.0 g, 30 mmol), **6** (technical grade, 7.91 g, 25 mmol), 2,6-lutidine (2.95 g, 28 mmol), and DMA (50 mL) was stirred under argon at 60 °C for 17 h. The solvent was evaporated (90 °C/2 mmHg) to leave a yellow solid which was flash chromatographed on SiO_2 (1 kg) with 6% MeOH in EtOAc to give the pure product as a tan powder (4.33 g, 43%): mp 300−301 °C; NMR (Me_2SO-d_6) satisfactory. Anal. ($C_{22}H_{19}N_3O_3S$) C,H,N,S.

4-(Phenylsulfonyl)-*N*-ethylaniline (33). Method A. A mixture of 4-aminophenyl phenyl sulfone (**4n**; 5.83 g, 25 mmol), iodoethane (7.80 g, 50 mmol), K_2CO_3 (3.45 g, 25 mmol), and DMA (125 mL) was stirred at 110 °C for 3.5 h. The DMA was removed in vacuo at 80 °C leaving a white residue; extractive workup (EtOAc/H_2O) (500 mL) gave an oil that was flash chromatographed using 10% MeOH in toluene to give the pure product as a white solid (3.17 g, 48.5%): mp 134.5−136 °C; NMR satisfactory. Anal. ($C_{14}H_{15}NO_2S$) C,H,N,S.

Method B. A stirred solution of 4-(phenylsulfonyl)fluorobenzene (25.00 g, 0.106 mol) and $C_2H_5NH_2$ (20.8 mL, 0.318 mol, 3 equiv) in DMSO (125 mL) in a flask fitted with a dry ice condenser was heated at 130 °C for 3 h under argon. The resulting amber solution was allowed to cool, poured into H_2O (1.3 L), and extracted with CH_2Cl_2 (2 × 200 mL) and finally with EtOAc (2 × 200 mL). The organic layers were combined, dried, and evaporated in vacuo to give the crude beige-colored product. Recrystallization from ethanol gave off-white crystals (23.03 g, 83.1%) identical by TLC with the product prepared as above.

4-(Ethylamino)trifluoroacetophenone (34). A clear solution of 4-fluorotrifluoroacetophenone[46] (10.0 g, 52 mmol) and $C_2H_5NH_2$ (7.04 g, 156 mmol, 3 equiv), in DMSO (50 mL) in a flask fitted with a dry ice condenser was stirred at 140 °C for 1 h 40 min under an inert atmosphere. The mixture was poured into H_2O (500 mL); extractive workup (CH_2Cl_2) gave a yellow oil which was flash chromatographed with CH_2Cl_2 as eluant to give the pure product as a yellow solid (6.87 g, 61%): mp 80−81 °C; NMR satisfactory. Anal. ($C_{10}H_{10}F_3NO$) C,H,N,F.

4-(*N*-((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)-*N*-ethylamino)phenyl Phenyl Sulfone (35). A mixture of **33** (1.41 g, 5.4 mmol), **6** (1.51 g, 6 mmol), $CaCO_3$ (0.060 g, 6 mmol), and DMA (25 mL) was stirred at 100 °C. Further **6** was added at 2 h (0.1 g) and at 3 h (0.15 g). At 6 h the mixture was cooled and then filtered through Celite. The solution was slowly pipetted into H_2O (700 mL) to produce a cream-colored flocculent solid. This was filtered off, washed carefully with water, dried over P_2O_5 in vacuo, and purified by flash column chromatography twice (300 g of SiO_2, 15% MeOH in benzene; 200 g of SiO_2, 6% MeOH in CH_2Cl_2) to give the product as a cream-colored solid (0.87 g, 37%): mp 193−194 °C; NMR (Me$_2$SO-d_6) satisfactory. Anal. ($C_{24}H_{23}N_3O_3S{\cdot}0.5H_2O$) C,H,N,S.

4-(*N*-((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)-*N*-ethylamino)trifluoroacetophenone (36). A mixture of **34** (2.17 g, 10 mmol), **6** (3.23 g, 12.8 mmol), $CaCO_3$ (1.28 g, 12.8 mmol), and DMA (30 mL) was stirred under argon at 100−110 °C for 3.5 h. The orange-colored mixture was filtered through Celite and the solvent then evaporated at 90 °C (2 mmHg), to leave an amber resin which was flash chromatographed with 40% CH_3CN in CCl_4 to give the pure product as an off-white solid (0.870 g, 22%): mp 273.5−275.5 °C; NMR (Me$_2$SO-d_6) satisfactory. Anal. ($C_{20}H_{18}F_3N_3O_2$) C,H,N,F.

(4-(*N*-((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)amino)phenyl)phenylmethane (37). A solution of 4-(phenylmethyl)aniline (0.46 g, 2.53 mmol), **6** (0.64 g, 2.53 mmol), and *N,N*-diisopropylethylamine (0.36 g, 2.78 mmol, 1.1 equiv) in DMA (15 mL) was stirred at 85−90 °C for 1.5 h and then pipetted into rapidly stirring H_2O (150 mL). The resulting beige-colored solid was filtered off, washed with H_2O (25 mL), and then dissolved in boiling EtOH (50 mL). This solution was charcoal treated, filtered, brought back to the boil, diluted with H_2O (25 mL), and cooled in ice for 15 min. The resulting pale yellow, flocculent solid was filtered off and washed with EtOH (25 mL) followed by Et_2O (25 mL). This slightly impure product (0.30 g) was coated onto SiO_2 (1.50 g) from 50% MeOH in CH_2Cl_2 (25 mL) and flash chromatographed with 5% MeOH in EtOAc to give the pure product as a cream-colored solid (0.118 g, 13%): mp 223−226 °C dec; NMR (Me$_2$SO-d_6) δ 2.32 (s, 3H, CH_3), 3.72 (s, 2H, CH_2), 4.34 (d, 2H, J = 5.2 Hz, CH_2), 6.26 (t, 1H, J = 5.2 Hz, NH), 6.48 (d, 2H, J = 8.4 Hz), 6.88 (d, 2H, J = 8.4 Hz), 7.10−7.27 (m, 5H, phenyl), 7.51 (d, 1H, J = 8.4 Hz, H^8), 7.72 (dd, 1H, J = 8.4, 1.9 Hz, H^7), 8.02 (d, 1H, J = 1.9 Hz, H^5), 12.14 (s, 1H, lactam NH). Anal. ($C_{23}H_{21}N_3O{\cdot}0.3H_2O$) C,H,N.

(4-(*N*-((3,4-Dihydro-2-methyl-4-oxo-6-quinazolinyl)methyl)methylamino)phenyl)phenylmethane (38). A suspension of **37** (108 mg, 0.3 mmol) in EtOH (5 mL) was treated with formaldehyde (37%, 142 mg, 1.7 mmol) and then $NaCNBH_3$ (27 mg, 0.4 mmol). $HC1_{aq}$ (0.1 N) was added dropwise during 45 min to maintain the pH at just below 7. The suspension was stirred at 25 °C for 15 h; 1 N HC1 (2 mL) was added to quench excessive hydride. The resulting precipitate was filtered off, washed, and dried to give the product as an off-white solid (87 mg, 79%): mp 225 °C dec; NMR (Me$_2$SO-d_6) δ 2.27 (s, 3H, CH_3), 2.94 (s, 3H, N-CH_3), 3.74 (s, 2H, CH_2), 4.57 (s, 2H, N-CH_2), 6.62 (d, 2H, J = 8.5 Hz), 6.96 (d, 2H, J = 8.5 Hz), 7.09−7.23 (m, 5H, phenyl), 7.48 (d, 1 H, J = 8.3 Hz, H^8), 7.55 (dd, 1H, J = 8.3, 1.5 Hz, H^7), 7.82 (d, 1H, J = 1.5 Hz, H^5), 12.17 (br s, 1H, NH); HRMS ($C_{24}H_{23}N_3O$)$^+$ calcd 369.1841, found 369.1843. Anal. ($C_{24}H_{23}N_3O{\cdot}0.6H_2O$) C,H,N.

***N*-((3,4-Dihydro-2-methyl-4-thio-6-quinazolinyl)methyl)-*N*-prop-2-ynyl-3-(trifluoromethyl)aniline (39).** In a flame-dried two-necked flask were placed **7f** (0.371 g, 1.0 mmol), CH_3CN (4 mL), Et_3N (0.56 mL, 4.0 mmol), and P_2S_5 (0.445 g, 2.0 mmol) while under an argon atmosphere. The stirred mixture was heated to 50 °C under reflux whereupon most of the P_2S_5 dissolved. Heating was continued overnight. The mixture was cooled and poured into H_2O (100 mL). A solid precipitated which was filtered off, washed with cold H_2O, dried over P_2O_5 in vacuo, and flash chromatographed (2% CH_3CN in CH_2Cl_2) to give the thiolactam as a pale yellow solid (0.368 g, 95%): mp 232−234 °C; R_f = 0.34 (silica gel, 5% CH_3CN in CH_2Cl_2); NMR (Me$_2$SO-d_6) δ 2.44 (s, 3H, CH_3), 3.24 (t, 1H, J = 2.1 Hz, ≡CH), 4.33 (d, 2H, J = 2.1 Hz, CH_2-C≡), 4.79 (s, 2H, N-CH_2-Ph), 7.00−7.1 (m, 3H, aromatic), 7.39 (t, 1H, J = 8.25 Hz, aromatic), 7.61 (d, 1H, J = 8.4 Hz, H^8), 7.76 (dd, 1H, J = 8.4, 2.0 Hz, H^7), 8.48 (d, 1H, J = 2.0 Hz, H^5), 13.68 (br s, 1H, NH). Anal. ($C_{20}H_{16}F_3N_3S$) C,H,N,S.

Protein Crystallization. Small crystals (~0.1 mm) were grown by vapor diffusion using 1.5 M sodium/potassium phosphate as the precipitant according to the protocol described.[24] Fresh protein was then concentrated to 27 mg/mL in 0.002 M Tris buffer, 0.66 M phosphate, and 1% poly(ethylene glycol) (PEG400) at pH 8.0; 10 μL drops were placed on plastic cover slips and inverted over wells containing 0.005 M Tris, 2.0 M phosphate, and 1% PEG400 at pH 8.0. After 24 h of equilibration at 4 °C, a single 0.1 mm seed crystal was introduced into each drop. Over the course of 1−2 weeks the seed crystals grew to final dimensions of around 0.8 mm on an edge.

Biochemical Assays. TS activity was assayed by a modified procedure of the tritium release method of Lomax and Greenberg.[47] Inhibition constants K_{is} and K_{ii} were as described by Cleland[48] and determined by steady state analysis against 5,10-methylenetetrahydrofolate as the variable cosubstrate under conditions of saturating dUMP. Values listed are averaged over multiple data sets. Reaction conditions in 0.1 mL were 50 mM Tris (pH 7.6), 10 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, 25 mM $MgCl_2$, 15 mM formaldehyde, 25 μM dUMP ([5-^{3}H]; specific activity $\cong 2 \times 10^8$ cpm/μmol), and tetrahydrofolate (eight concentrations ranging from 5 to 150 μM). Bovine serum albumin at up to 100 μg/mL was present when human TS was assayed. These reactions were conducted either in the absence of inhibitor or in the presence of inhibitor at concentrations ranging, at a minimum, between 0.5 K_i and 2.0 K_i except when the solubility of the inhibitor was limiting. Reactions were run at room temperature by initiating with the addition of enzyme. After 5 min, the reactions were quenched by the addition of charcoal, the mixtures were centrifuged to remove unreacted dUMP, and the supernatant was counted to determine the release of tritium from the 5-position of dUMP. Experimental results were analyzed by a nonlinear regression analysis program[49] which fit the data to a mixed noncompetitive inhibition scheme.

Measurement of Tissue Culture IC_{50}'s. IC_{50} values for the inhibition of cellular growth were measured using a modification[50] of the MTT[51] colorimetric assay of Mosmann[52] using mouse (L1210) and human (CCRF-CEM) leukemia lines (ATCC) and a human adenocarcinoma (GC_3/M TK^-) deficient in thymidine kinase.[53] Cells were seeded at 1000 (L1210) or 10 000 (CCRF-CEM, GC_3/M TK^-) cells/well in 96-well plates, and growth was measured over a range of nine 2-fold serial dilutions of each compound. Culture medium (RPMI-1640) contained 5% (L1210, CCRF-CEM) or 10% (GC_3/M TK^-) fetal calf serum and 0.5% DMSO. Following a 3 day (L1210) or 5 day (CCRF-CEM, GC_3/M TK^-) incubation and a 4 h treatment with MTT, cells were harvested and growth was measured spectrophotometrically after dissolution of the deposited formazan in DMSO. IC_{50} values were determined from semilogarithmic plots of compound concentration vs the mean of the four growth assessments made at each serial dilution of the agent relative to the growth of control cultures.

Measurement of IC_{50} Shift Due to Thymidine. The ability of thymidine to reverse cell growth inhibition induced by inhibitors was assessed in L1210 cells by comparing the IC_{50} measured under standard conditions (RPMI-1640 medium containing 5% fetal calf serum) with that obtained in the presence of 10 μM thymidine which was replenished daily

during the 3 days of growth. The magnitude of the ratio of the IC_{50} measured in the presence of thymidine to that measured without added nucleoside was used as a measure of the extent to which the inhibition of growth could be attributed to intracellular inhibition of thymidylate synthase. A value of ≤ 1.0 under these conditions would suggest a locus of action other than TS, while larger values probably indicate a direct relationship between growth inhibition and TS targeting.

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Supporting Information Available: One table giving the preparation and physical properties of the propargylanilines **5a**−**c**,**f**−**i**,**k**,**m**−**t**, and **15** (3 pages). Ordering information is given on any current masthead page.

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