Potent Inhibition of Thymidylate Synthase by Two Series of Nonclassical Quinazolines

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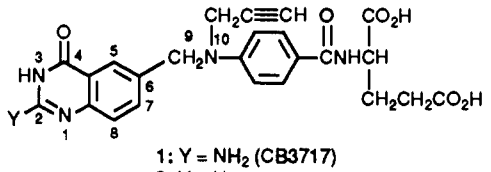
The synthesis and biological activity of two series of nonclassical thymidylate synthase (TS) inhibitors are described. The first is a series of 10-propargyl-5,8-dideazafolic acid derivatives (**10a–j**) and the second is a series of the analogous 2-desamino derivatives (**13a–c,k**), both bearing a more lipophilic substituent on the phenyl ring than the CO-glutamate of classical antifolates. Compounds **10a–j** were prepared in a straightforward manner, generally by treatment of *N*-[6-(bromomethyl)-3,4-dihydro-4-oxo-2-quinazolinyl]-2,2-dimethylpropanamide (**6**) with various phenyl-substituted *N*-propargylanilines (**8**), followed by deprotection. Compounds **13a–c,k** were prepared similarly from [6-(bromomethyl)-4-oxo-3(4*H*)-quinazolinyl]methyl 2,2-dimethylpropanoate (**11**). The compounds were tested for inhibition of purified L1210 TS and for inhibition of L1210 cell growth in vitro. Several of these nonclassical analogues approached the TS inhibitory potency of 10-propargyl-5,8-dideazafolic acid (**1**, CB3717), a glutamate-containing TS inhibitor. 2-Amino target compounds **10a–j** were generally potent inhibitors of L1210 TS, with IC_{50}s within the range of 0.51–11.5 μM, compared to 0.05 μM for **1**. The order of potency for phenyl substitution at the 4-position in this series was the following: $COCF_3 \geq NO_2 \geq CONH_2 \geq COCH_3 > SO_2NMe_2 > CN \gg OCF_3 \geq F$. The 2-desamino target compounds **13a–c,k** also exhibited significant, although diminished, TS inhibition. Both series were growth inhibitory to cells in tissue culture and this inhibition could be reversed by thymidine alone, indicating that the primary target was TS. None of the compounds was a potent inhibitor of dihydrofolate reductase. These studies indicate that the presence of the glutamate moiety in folate analogues is not an absolute requirement for potent inhibition of TS.

The recent discovery of potent and selective inhibitors of thymidylate synthase (TS) and glycinamide ribonucleotide (GAR) transformylase[1] provides the impetus for renewed interest in the unexplored potential of antifolates in cancer chemotherapy.[2a] New opportunities for direct and selective intervention in processes in the folate pathway, other than that mediated by dihydrofolate reductase (DHFR), now exist.

In 1981, Jones and co-workers reported[3] that 10-propargyl-5,8-dideazafolic acid (**1**, CB3717) was a selective,



1: Y = NH_2 (CB3717)
2: Y = H

tight-binding inhibitor of TS, the enzyme mediating the de novo biosynthesis of thymidylate for DNA synthesis. Long considered an appropriate mechanistic approach for cancer chemotherapy, the induction of a "thymineless death" with a cofactor analogue had yet to be tested for lack of a selective, potent inhibitor. Although circumvention of the effects of a TS inhibitor through salvage of exogenous thymidine makes preclinical evaluation in murine models (where circulating thymidine levels are high) less than straightforward, antitumor activity seen in one vivo model[3,4] was indeed confirmed in phase 1 and 2 clinical trials with **1**. Antitumor activity was observed in patients with ovarian cancer and breast cancer.[5,6a]

Unfortunately, dose-limiting renal toxicity and dose-unrelated hepatotoxicity and malaise may limit the clinical utility of **1**.[6a] It has been speculated that the kidney toxicity may be attributable to accumulation of precipitated parent drug or polyglutamates thereof.

An analogue program designed to increase aqueous solubility may have found early success. Although extensive modifications of the N^{10}-position,[7,8] the benzoyl ring,[9,10,13] and the amino acid terminus[11] provided no advantage, modification of the substituent on the 2-carbon has shown promise. In particular 2-desamino analogue **2** exhibited excellent TS inhibitory activity with significantly enhanced aqueous solubility.[12]

In further considering the structure–activity relationships for this series of TS inhibitors, our experience with the nonclassical DHFR inhibitor trimetrexate[2a] prompted

† Department of Chemistry.
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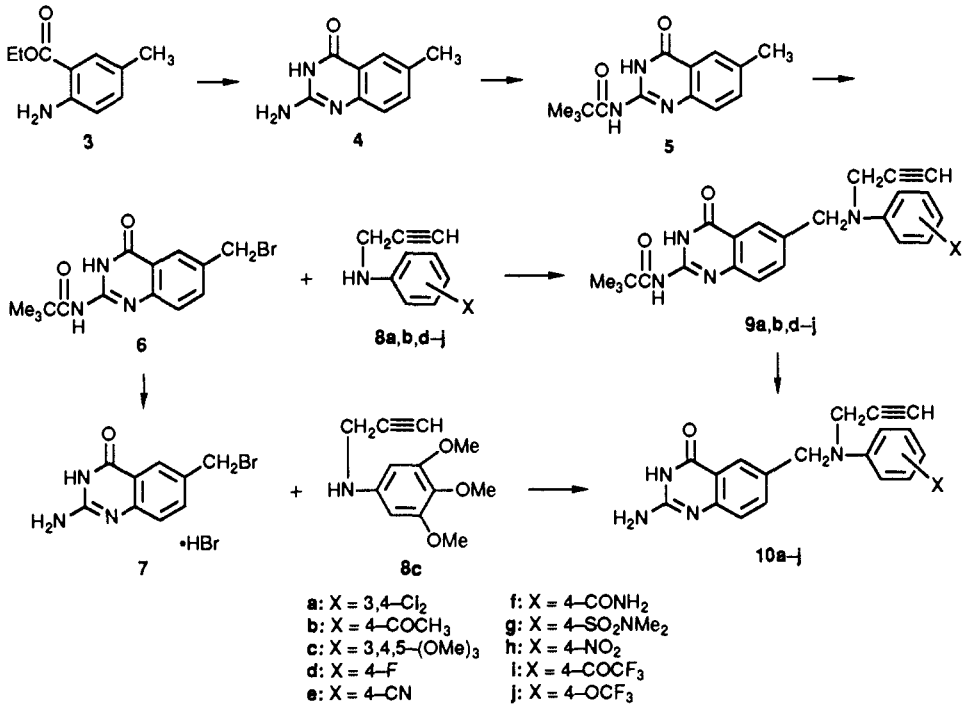
Scheme I



our laboratory to investigate further the role of the glutamate moiety in TS inhibition and cytotoxicity. Our curiosity was, in part, prompted by two observations. Firstly, the role of the glutamate in the transport of **1** is unclear. Compound **1** does not use the reduced-folate carrier system required for methotrexate transport.[2a-c] Transport of trimetrexate is in part attributable to passive diffusion, although some energy-dependent component also has been suggested.[2a] We extrapolated from this to investigate quinazoline-based TS inhibitors which would not necessarily require active transport. This seemed appealing since active transport mechanisms can open the door to the development of resistance. Interestingly, this does not seem to be the case for **1**. Secondly, and more importantly, it has been demonstrated that the polyglutamates of **1** are more potent inhibitors of TS than the parent drug, in particular the compound with three additional glutamates attached to **1**.[6a-c] For this reason, it has always been assumed that the glutamic acid moiety was required for potent activity, if not for transport.

With this in mind, we wondered whether potent TS inhibition could be achieved with a nonclassical quinazoline analogue which did not bear the appended amino acid. Toward that end, we synthesized two series of nonclassical quinazoline analogues, one (**10a–j**) analogous to **1** and bearing a 2-amino substituent, the other (**13a–c,k**) analogous to **2**, lacking the 2-amino substituent. Although we will develop expanded structure–activity relationships in these two series, our early emphasis was to maintain the electron-withdrawing property of the 4-carboxamide substituent of the natural cofactor, N^5,N^{10}-methylenetetrahydrofolate, while increasing its lipophilicity and decreasing its steric bulk. Herein we report on the first nonclassical quinazolines to achieve potent TS inhibition.[14]

Chemistry

The 2-amino-6-[(phenyl-2-propynylamino)methyl]-4-(3*H*)-quinazolinone target compounds **10a–j** were synthesized by one of the two published variations illustrated in Scheme I.[7] In Scheme I, the 2-amino-6-methyl-4-(3*H*)-quinazolinone (**4**) had previously been made in yields of 79–82% by treating ethyl 2-amino-5-methylbenzoate (**3**) with guanidine.[15] By substituting chloroformamidine hydrochloride[16] for guanidine in this reaction we increased the yield of 4 to 99%.[17] In the first variation, treatment of 3,4,5-trimethoxy-*N*-2-propynylbenzenamine (**8c**) with 2-amino-6-(bromomethyl)-4(3*H*)-quinazolinone hydrobromide (**7**)[18] gave the target compound **10c**. Purification of **10c** made by this route required both chromatography and recrystallization. When other *N*-2-propynylbenzenamines (**8**) were similarly treated with **7**, purification of the target compounds was more difficult due to their decreased solubility. Therefore the variation in Scheme I where **8** was treated with the protected bromomethyl derivative **6**[15] rather than the deprotected bromomethyl derivative **7** was employed. This reaction gave the pen-

Table I. Preparation of Intermediates **9a,b,d–j** and **12a–c,k**

no.	% yield	mp, °C	no.	% yield	mp, °C
9a	66	*a*	**9i**	41.3	*b*
9b	63	190–192	**9j**	65	174–176
9d	80.5	187–190	**12a**	88	*c*
9e	66.6	183–186	**12b**	42	*b*
9f	44	147–153	**12c**	100	*d*
9g	58.6	217–220	**12k**	60	*c*
9h	32.6	210–215			

[a] Not recorded. [b] Off-white foam. [c] Colorless oil. [d] Light brown oil.

(14) Preliminary communication of this work appeared previously: Fry, D. W.; McNamara, D. J.; Werbel, L. M.; Berman, E. M. *Proc. Am. Assoc. Cancer Res.* **1989**, *30*, Abstract 1905, 479.

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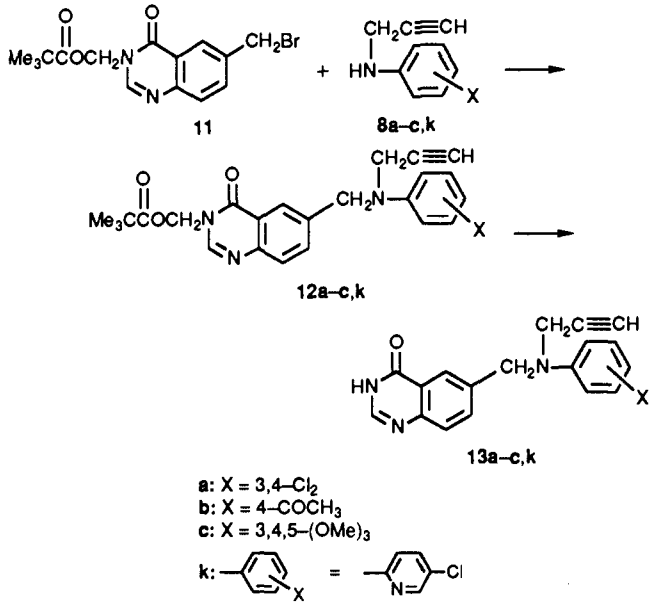
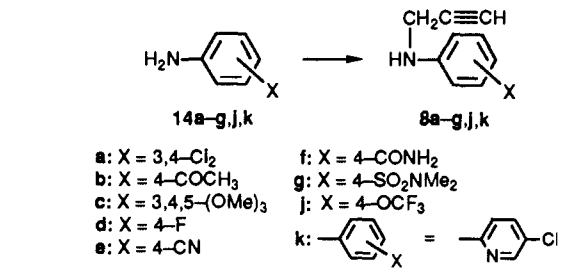
Scheme II



Scheme III

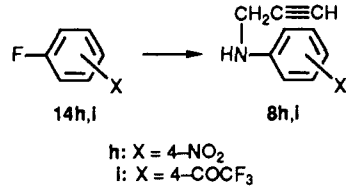


ultimate intermediates **9**, which were very soluble and easily purified by chromatography. The yields and melting points of **9a,b,d–j** are listed in Table I. These intermediates **9** were then treated with NH_3/MeOH to provide the target compounds **10**, which generally required no purification. Target compounds **10a,b,d–j** were prepared by this variation.

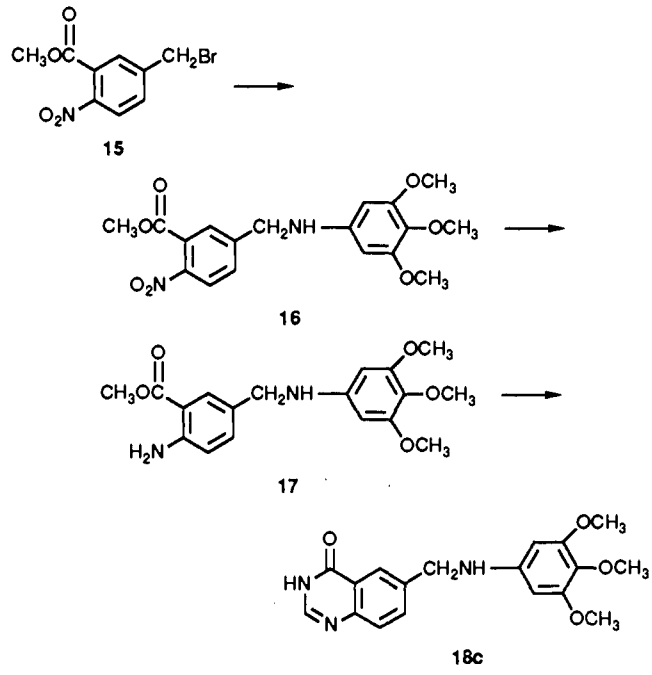
The 6-[(phenyl-2-propynylamino)methyl]-4(3*H*)-quinazolinone target compounds **13a–c,k**, lacking the 2-amino group, were synthesized by the published route illustrated in Scheme II.[12] The yields and appearances of the intermediates **12a–c,k** are listed in Table I. Our only departure from the literature procedures was the use of NH_3/MeOH rather than aqueous NaOH in the conversion of the penultimate intermediates **12** to the target compounds **13**.

The required *N*-2-propynylbenzenamines (**8**) were made by one of two different mehods (Scheme III or IV). Compounds **8a–g,j,k** were synthesized as illustrated in Scheme III by alkylating the appropriately substituted benzenamine (**14a–g,j,k**) with 3-bromo-1-propyne. The desired monoalkylated products were usually contaminated by dialkylated byproducts, but these were easily removed by chromatography. Compounds **8h,i** could not be synthesized by this method due to the low nucleophilicity of the starting benzenamines. They were therefore synthesized as illustrated in Scheme IV by displacing the aromatic fluorine atom from 4-fluoronitrobenzene (**14h**)[19] and 2,2,2-trifluoro-1-(4-fluorophenyl)ethanone (**14i**), respectively, with 2-propynylamine.

Scheme IV



Scheme V



The despropargylated 2-desamino compound **18c** was prepared by the novel route illustrated in Scheme V. Thus the known 5-(bromomethyl)-2-nitrobenzoic acid methyl ester (**15**)[20] was treated with 3,4,5-trimethoxybenzenamine (**14c**) to afford 2-nitro intermediate **16**. This was catalytically reduced to 2-amino intermediate **17**, which was then cyclized with formamidine[21] to yield the target **18c**. As discussed above, the other 2-desamino compounds **13a–c,k** were not obtained by this method but rather by that in Scheme II, which was more amenable to the synthesis of analogues.

Results and Discussion

The nonclassical target compounds **10a–j**, **13a–c,k**, and **18c** synthesized here, as well as the classical reference compound **1**, were tested for inhibition of L1210 TS, inhibition of L1210 growth in culture, and inhibition of WI-L2 DHFR. These results are summarized in Table II. Also included are the literature values for the 2-desamino reference compound **2**.[12] The 2-amino target compounds **10a–j** were generally potent inhibitors of L1210 TS, with IC_{50}s within the narrow range of 0.51–11.5 μM. Compound **10i** (X = 4-$COCF_3$) exhibited the most potent inhibition with an IC_{50} of 0.51 μM, which shows it to be 10-fold less potent than **1**. These compounds also inhibited the growth of L1210 cells in culture, with IC_{50}s of 2.4–$\gg$20 μM. Compound **10h** (X = 4-NO_2) exhibited the most potent

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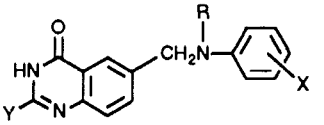
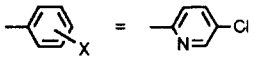
(21) The use of formamidine acetate to effect the ring closure of anthranilic acid esters to 4(3*H*)-quinazolinones has been reported: Stevenson, T. M.; Kazmierczak, F.; Leonard, N. J. *J. Org. Chem.* **1986**, *51*, 616.

Table II. Biological Properties of 5,8-Dideazafolates



compd	Y	R	X	IC$_{50}$, μM: TS (L1210) inhibn	IC$_{50}$, μM: L1210 growth inhibn	IC$_{50}$, μM: DHFR (WI-L2) inhibn
10a	NH_2	$CH_2C{\equiv}CH$	3,4-Cl_2	insol[a]	insol	insol
10b	NH_2	$CH_2C{\equiv}CH$	4-$COCH_3$	0.77	4.3	37.3
10c	NH_2	$CH_2C{\equiv}CH$	3,4,5-$(OMe)_3$	insol	insol	insol
10d	NH_2	$CH_2C{\equiv}CH$	4-F	11.5	26.1	insol
10e	NH_2	$CH_2C{\equiv}CH$	4-CN	1.3	5.9	66.6
10f	NH_2	$CH_2C{\equiv}CH$	4-$CONH_2$	0.64	≫20	72.6
10g	NH_2	$CH_2C{\equiv}CH$	4-SO_2NMe_2	1.0	4.1	20.3
10h	NH_2	$CH_2C{\equiv}CH$	4-NO_2	0.62	2.4	22.6
10i	NH_2	$CH_2C{\equiv}CH$	4-$COCF_3$	0.51	11.8	10.2
10j	NH_2	$CH_2C{\equiv}CH$	4-OCF_3	11.3	25.1	ND[c]
13a	H	$CH_2C{\equiv}CH$	3,4-Cl_2	11	4.0	ND
13b	H	$CH_2C{\equiv}CH$	4-$COCH_3$	2.4	7.0	100
13c	H	$CH_2C{\equiv}CH$	3,4,5-$(OMe)_3$	96	>10	66.6
13k	H	$CH_2C{\equiv}CH$		≫100	>10	ND
18c	H	H	3,4,5-$(OMe)_3$	≫500	>10	63.3
1	NH_2	$CH_2C{\equiv}CH$	4-COGlu	0.05	3.0	7.2
2	H	$CH_2C{\equiv}CH$	4-COGlu	0.16[b]	0.4[b]	ND

[a] Insol = not tested due to insolubility. [b] Literature[12] value. [c] ND = not determined.

inhibition with an IC$_{50}$ of 2.4 μM, comparable to that of **1** with an IC$_{50}$ of 3.0 μM.

The less well represented 2-desamino target compounds **13a–c,k** also exhibited significant inhibition of L1210 TS and L1210 growth in culture. Compound **13b** (X = 4-$COCH_3$) exhibited the most potent TS inhibition with an IC$_{50}$ of 2.4 μM, which was 15-fold less potent than the literature value for **2**. Within this 2-desamino series, a comparison of the modest TS inhibition of the propargylated **13c** (X = 3,4,5-$(OCH_3)_3$) with the complete lack of inhibition of despropargylated **18c** (X = 3,4,5-$(OCH_3)_3$) emphasizes the importance of the N-10 propargyl group. This is similar to that reported in the 2-amino- and 2-desaminoglutamate-containing series.[7,12] Compound **13a** (X = 3,4-Cl_2) exhibited the most potent L1210 growth inhibition in this series with an IC$_{50}$ of 4.0 μM, 10-fold less potent than the literature value for the reference compound **2**.

2-Amino compounds **10a–j** and 2-desamino compounds **13a–c,k** did inhibit WI-L2 DHFR as shown in Table II, but with much less potency than TS. Furthermore, the cytotoxicity of both classes of these compounds against L1210 cells in culture was completely reversed by the addition of thymidine to the culture medium as illustrated in Table III. These studies indicate that **10** and **13** are cytotoxic by virtue of their inhibition of TS and not some other enzyme, for example DHFR.

These results indicate that the glutamate moieties of **1** and **2** are not absolutely necessary for potent inhibition of L1210 TS and L1210 growth in culture. We are presently expanding the scope of this finding in an effort to discover more potent nonclassical inhibitors of TS.

Experimental Section

Melting points were determined on a Thomas-Hoover Unimelt apparatus and are uncorrected. Mass spectra were determined on a Finnigan 4000 mass spectrometer with an INCOS 2300 data system using direct introduction and electron impact at 70 eV and 150 °C. ^{1}H NMR spectra were determined at 90 MHz on a Varian EM-390, at 100 MHz on an IBM WP100SY, or at 200 MHz on a Varian XL-200 spectrometer in Me_2SO-d_6 or $CDCl_3$ with tetramethylsilane as an internal standard. All compounds with reported NMR spectra also had IR spectra consistent with their structure as determined on a Nicolet 205X FT/IR. Elemental analyses were determined by the microanalytical laboratory at Parke-Davis. TLC was performed with E. Merck silica gel 60 F-254 precoated glass plates (0.25 mm). Flash column chromatography was performed with E. Merck silica gel (230–400 mesh). Concentrations were performed on a rotary evaporator at ≤45 °C (20 Torr) unless otherwise noted.

Table III. Effects of Thymidine on L1210 Growth Inhibition of 5,8-Dideazafolates

compd[a]	without thymidine, % of control	with thymidine,[b] % of control
10b	34	102
10d	69	101
10g	20	96
10h	7	95
13b	35	90
1	11	92

[a] Drug concentration was 10 μM. [b] Thymidine concentration was 10 μM.

Scheme I. 2-Amino-6-methyl-4(3*H*)-quinazolinone (4). A suspension of ethyl 2-amino-5-methylbenzoate (17.9 g, 0.1 mol, **3**), chloroformamidine hydrochloride[16] (17.2 g, 0.15 mol), and dimethyl sulfone (50 g) was stirred and heated at 150 °C for 1 h. Water was added to the thick mixture, followed by concentrated NH_4OH to make the suspension basic. The solid was filtered off, washed with H_2O, and dried to give 17.32 g (99%) of the product as a white solid, mp >250 °C. This material was identical with that prepared with guanidine.[15]

2-Amino-6-[[2-propynyl(3,4,5-trimethoxyphenyl)amino]methyl]-4(3*H*)-quinazolinone 0.3-Hydrate (10c). A suspension of 2-amino-6-(bromomethyl)-4(3*H*)-quinazolinone hydrobromide (1.00 g, 2.99 mmol, **7**),[18] 3,4,5-trimethoxy-*N*-2-propynylbenzenamine (0.66 g, 2.99 mmol, **8c**), and dry $CaCO_3$ (0.30 g, 2.99 mmol) in *N*,*N*-dimethylacetamide (25 mL, DMA) was stirred at room temperature for 18 h. The suspension was filtered and the solid was washed with DMA. The filtrate and washings were concentrated (50 °C, 1 mm) to give a brown syrup. This was dissolved in EtOH and treated with silica gel (15 g, 230–400 mesh). The EtOH was evaporated and the powder was applied to a column of silica gel (225 g, 230–400 mesh) packed in CH_2Cl_2/MeOH (10:1). Elution with the same solvent gave 0.75 g of

material. Recrystallization from EtOH gave 0.61 g (52%) of the product as a grey solid: mp 236–238 °C; NMR (100 MHz, Me_2SO-d_6) δ = 3.20 (distorted t, 1, C≡CH), 3.57 (s, 3, 4-OCH_3), 3.70 (s, 6, 3- and 5-$(OCH_3)_2$), 4.13 (distorted d, 2, $CH_2C{\equiv}CH$), 4.50 (s, 2, NCH_2), 6.17 (s, 2, H2 and -6 of Ph), 6.40 (br s, 2, NH_2, exchangeable), 7.20 (d, J = 9.2 Hz, 1, H8), 7.57 (dd, J = 9.2 Hz, 2.5 Hz, 1, H7), 7.87 (distorted d, 1, H5), 10.97 (br s, 1, NH, exchangeable); MS m/e = 394 (M^+). Anal. ($C_{21}H_{22}N_4O_4 \cdot 0.3H_2O$) C, H, N, H_2O.

***N*-[6-[[(3,4-Dichlorophenyl)-2-propynylamino]methyl]-3,4-dihydro-4-oxo-2-quinazolinyl]-2,2-dimethylpropanamide (9a).** A suspension of *N*-[6-(bromomethyl)-3,4-dihydro-4-oxo-2-quinazolinyl]-2,2-dimethylpropanamide (1.00 g, 2.96 mmol, **6**),[15] 3,4-dichloro-*N*-2-propynylbenzeneamine (0.65 g, 3.2 mmol, **8a**), and dry $CaCO_3$ (0.59 g, 5.9 mmol) in DMA (20 mL) was stirred at 60 °C for 24 h. The suspension was poured into an H_2O/ice mixture and stirred. The suspension was extracted with EtOAc. The EtOAc solution was dried ($MgSO_4$) and concentrated to give 1.79 g of an oil. Crystallization from EtOH (50 mL) gave 0.89 g (66%) of the product as an off-white solid. This was not characterized but used directly in the next reaction.

Compounds **9b,d–j** were prepared similarly to **9a**. Their melting points and yields are listed in Table I.

2-Amino-6-[[(3,4-dichlorophenyl)-2-propynylamino]methyl]-4(3*H*)-quinazolinone (10a). To MeOH (150 mL) saturated with NH_3 at 0 °C was added **9a** (0.89 g, 1.9 mmol). The solution was stirred at room temperature for 18 h, then cooled, resaturated with NH_3, and stirred at room temperature another 18 h. The solution was filtered and concentrated to give a solid. This was dissolved in boiling EtOH (500 mL). The solution was concentrated to 100 mL, cooled, and filtered to give 0.61 g (84%) of the product as an off-white solid: mp 251–253 °C; NMR (200 MHz, Me_2SO-d_6) δ = 3.24 (t, J = 2.1 Hz, 1, C≡CH), 4.25 (d, J = 2.0 Hz, 2, $CH_2C{\equiv}CH$), 4.60 (s, 2, NCH_2), 6.40 (br s, 2, NH_2, exchangeable), 6.80 (dd, J = 9.1 Hz, 2.9 Hz, 1, H6 of Ph), 7.01 (d, J = 2.9 Hz, 1, H2 of Ph), 7.18 (d, J = 8.5 Hz, 1, H8), 7.38 (d, J = 9.0 Hz, 1, H5 of Ph), 7.51 (dd, J = 8.5 Hz, 2.2 Hz, 1, H7), 7.79 (d, J = 2.0 Hz, 1, H5), 11.07 (br s, 1, NH, exchangeable); MS m/e = 372 (M^+). Anal. ($C_{18}H_{14}Cl_2N_4O$) C, H, N.

The following compounds (**10b,d–j**) were prepared similarly to **10a**.

6-[[(4-Acetylphenyl)-2-propynylamino]methyl]-2-amino-4(3*H*)-quinazolinone (10b): 84.8% yield; off-white solid; mp 255–258 °C; NMR (100 MHz, Me_2SO-d_6) δ = 3.45 (s, 3, CH_3), 3.27 (distorted t, 1, C≡CH), 4.37 (d, J = 2 Hz, 2, $CH_2C{\equiv}CH$), 4.70 (s, 2, NCH_2), 6.40 (br s, 2, NH_2, exchangeable), 6.87 (d, J = 10 Hz, 2, H2 and -6 of Ph), 7.18 (d, J = 9 Hz, 1, H8), 7.50 (dd, J = 2 Hz, 9 Hz, 1, H7), 7.67 (distorted s, 1, H5), 7.82 (d, J = 9 Hz, 2, H3 and -5 of Ph), 10.89 (br s, 1, NH, exchangeable); MS m/e = 346 (M^+). Anal. ($C_{20}H_{18}N_4O_2$) C, H, N.

2-Amino-6-[[(4-fluorophenyl)-2-propynylamino]methyl]-4(3*H*)-quinazolinone (10d): 56.8% yield; white solid; mp 251–252 °C; NMR (200 MHz, Me_2SO-d_6) δ = 3.17 (t, J = 2.1 Hz, 1, C≡CH), 4.11 (d, J = 2.1 Hz, 2, $CH_2C{\equiv}CH$), 4.49 (s, 2, NCH_2), 6.35 (br s, 2, NH_2, exchangeable), 6.88 (m, 2, H2 and -6 of Ph), 7.04 (m, 2, H3 and -5 of Ph), 7.18 (d, J = 8.5 Hz, 1, H8), 7.50 (dd, J = 2.1 Hz, 8.4 Hz, 1, H7), 7.81 (d, J = 1.6 Hz, 1, H5), 11.0 (br s, 1 NH, exchangeable); MS m/e = 322 (M^+). Anal. ($C_{18}H_{15}FN_4O$) C, H, N.

4-[[(2-Amino-3,4-dihydro-4-oxo-6-quinazolinyl)methyl]-2-propynylamino]benzonitrile hemihydrate (10e): 82.2% yield; cream solid; mp 251–253 °C; NMR (100 MHz, Me_2SO-d_6) δ = 3.27 (distorted t, 1, C≡CH), 4.35 (d, J = 2 Hz, 2, $CH_2C{\equiv}CH$), 4.70 (s, 2, NCH_2), 6.37 (br s, 2, NH_2, exchangeable), 6.87 (d, J = 10 Hz, 2, H3 and -5 of Ph), 7.17 (d, J = 9 Hz, 1, H8), 7.48 (dd, J = 2 Hz, 10 Hz, 1, H7), 7.59 (d, J = 10 Hz, 2, H2 and -6 of Ph), 7.77 (d, J = 2 Hz, 1, H5), 11.03 (br s, 1, NH, exchangeable); MS m/e = 329 (M^+). Anal. ($C_{19}H_{15}N_5O \cdot 0.5H_2O$) C, H, N (insoluble for Karl Fischer H_2O analysis).

4-[[(2-Amino-3,4-dihydro-4-oxo-6-quinazolinyl)methyl]-2-propynylamino]benzamide (10f): 76% yield; off-white solid; mp >275 °C; NMR (200 MHz, Me_2SO-d_6) δ = 3.21 (distorted t, 1, C≡CH), 4.28 (d, J = 1.7 Hz, 2, $CH_2C{\equiv}CH$), 4.66 (s, 2, NCH_2), 6.34 (br s, 2, NH_2, exchangeable), 6.82 (d, J = 8.7 Hz, 2, H3 and -5 of Ph), 7.00 (br s, 1, CONH, exchangeable), 7.18 (d, J = 8.5 Hz, 1, H8), 7.51 (dd, J = 2.1 Hz, 8.5 Hz, 1, H7), 7.67 (br s, 1, CONH, exchangeable), 7.73 (d, 2, J = 8.9 Hz, 2, H2 and -6 of Ph), 7.80 (d, J = 2.7 Hz, 1, H5), 11.0 (br s, 1, NH, exchangeable); MS m/e = 347 (M^+). Anal. ($C_{19}H_{17}N_5O_2$) H, N; C: calcd, 65.69; found, 65.14.

4-[[(2-Amino-3,4-dihydro-4-oxo-6-quinazolinyl)methyl]-2-propynylamino]-*N*,*N*-dimethylbenzenesulfonamide 1.7-hydrate (10g): 72.9% yield; white solid; mp 247–250 °C dec; NMR (200 MHz, Me_2SO-d_6) δ = 3.28 (distorted t, 1, C≡CH), 3.36 (s, 6, $N(CH_3)_2$), 4.35 (distorted d, 2, $CH_2C{\equiv}CH$), 4.72 (s, 2, NCH_2), 6.36 (br s, 2, NH_2, exchangeable), 6.97 (d, J = 9.0 Hz, 2, H3 and -5 of Ph), 7.21 (d, J = 8.4 Hz, 1, H8), 7.55 (m, 3, H2 and -6 of Ph and H7), 7.81 (distorted d, 1, H5), 11.01 (br s, 1, NH, exchangeable). Anal. ($C_{20}H_{21}N_5O_3S \cdot 1.7H_2O$) C, H, N, S, H_2O.

2-Amino-6-[[(4-nitrophenyl)-2-propynylamino]methyl]-4(3*H*)-quinazolinone 0.25-hydrate (10h): 47% yield; yellow solid; mp 254–259 °C; NMR (100 MHz, Me_2SO-d_6) δ = 3.30 (distorted t, 1, C≡CH), 4.43 (d, J = 2 Hz, 2, $CH_2C{\equiv}CH$), 4.78 (s, 2, NCH_2), 6.35 (br s, 2, NH_2, exchangeable), 6.88 (d, J = 9 Hz, 2, H2 and -6 of Ph), 7.17 (d, J = 9 Hz, 1, H8), 7.49 (dd, J = 9 Hz, 2 Hz, 1, H7), 7.77 (d, J = 2 Hz, 1, H5), 8.10 (d, J = 9 Hz, 2, H3 and -5 of Ph); MS m/e = 349 (M^+). Anal. ($C_{18}H_{15}N_5O_3 \cdot 0.25H_2O$) C, H, N (insoluble for Karl Fischer H_2O analysis).

2-Amino-6-[[2-propynyl[4-(trifluoroacetyl)phenyl]-amino]methyl]-4(3*H*)-quinazolinone 0.3-hydrate (10i): 94.5% yield; off-white solid; mp 231–234 °C; NMR (100 MHz, Me_2SO-d_6) δ = 3.33 (distorted t, 1, C≡CH), 4.47 (d, J = 2 Hz, 2, $CH_2C{\equiv}CH$), 4.82 (s, 2, NCH_2), 6.40 (br s, 2, NH_2, exchangeable), 7.00 (d, J = 9 Hz, 2, H2 and -6 of Ph), 7.18 (d, J = 9 Hz, 1, H8), 7.50 (dd, J = 2 Hz, 9 Hz, 1, H7), 7.78 (d, J = 2 Hz, 1, H5), 7.88 (d, J = 9 Hz, 2, H3 and -5 of Ph), 10.89 (br s, 1, NH, exchangeable); MS m/e = 400 (M^+). Anal. ($C_{20}H_{15}F_3N_4O_2 \cdot 0.3H_2O$) C, H, N (insoluble for Karl Fischer H_2O analysis).

2-Amino-6-[[2-propynyl[4-(trifluoromethoxy)phenyl]-amino]methyl]-4(3*H*)-quinazolinone (10j): 95% yield; white solid; mp 247–249 °C; NMR (100 MHz, Me_2SO-d_6) δ = 3.23 (distorted t, 1, C≡CH), 4.23 (d, J = 2 Hz, 2, $CH_2C{\equiv}CH$), 4.58 (s, 2, NCH_2), 6.68 (br s, 2, NH_2, exchangeable), 6.87 (d, J = 10 Hz, 2, H2 and -6 of Ph), 7.20 (d, J = 10 Hz, 3, H3 and -5 of Ph and H8), 7.53 (dd, J = 2 Hz, 8 Hz, 1, H7), 7.83 (d, J = 2 Hz, 1, H5), 11.12 (br s, 1, NH, exchangeable); MS m/e = 388 (M^+). Anal. ($C_{19}H_{15}F_3N_4O_2$) C, H, N.

Scheme II. [4-Oxo-6-[[2-propynyl(3,4,5-trimethoxyphenyl)amino]methyl]-3(4*H*)-quinazolinyl]methyl 2,2-Dimethylpropanoate (12c). A suspension of [6-(bromomethyl)-4-oxo-3(4*H*)-quinazolinyl]methyl 2,2-dimethylpropanoate (1.00 g, 2.16 mmol, **11**),[12] **8c** (0.64 g, 2.88 mmol), and dry $CaCO_3$ (0.58 g, 5.8 mmol) in DMA (20 mL) was stirred at room temperature for 18 h. The suspension was poured into an H_2O/ice mixture and extracted with EtOAc. The EtOAc solution was dried ($MgSO_4$) and concentrated to give 1.84 g of a brown oil. This was dissolved in a minimum amount of $PhCH_3$ and applied to a column of silica gel (100 g, 230–400 mesh) packed in $PhCH_3$/EtOAc (5:1). Elution with the same solvent gave 1.08 g (100% assuming 75% pure **11**) of the product as a light brown oil. This was not characterized but used directly in the next reaction.

Compounds **12a,b,k** were prepared similarly to **12c**. Their appearance and yields are listed in Table I.

The following compounds (**13a–c,k**) were prepared similarly to **10a**.

6-[[(3,4-Dichlorophenyl)-2-propynylamino]methyl]-4-(3*H*)-quinazolinone (13a): 79% yield; white solid; mp 211–213 °C; NMR (200 MHz, Me_2SO-d_6) δ = 3.26 (t, J = 2.3 Hz, 1, C≡CH), 4.30 (d, J = 2.4 Hz, 2, $CH_2C{\equiv}CH$), 4.74 (s, 2, NCH_2), 6.78 (dd, J = 9.0 Hz, 3.0 Hz, 1, H6 of Ph), 7.01 (d, J = 3.0 Hz, 1, H2 of Ph), 7.38 (d, J = 9.0 Hz, 1, H5 of Ph), 7.65 (d, J = 8.5 Hz, 1, H8), 7.73 (dd, J = 8.5 Hz, 2.1 Hz, 1, H7), 8.01 (d, J = 1.5 Hz, 1, H5), 8.07 (d, J = 2.8 Hz, 1, H2, coalesces to s upon D_2O addition), 12.2 (br s, 1, NH, exchangeable); MS m/e = 357 (M^+). Anal. ($C_{18}H_{13}Cl_2N_3O$) C, H, N.

6-[[(4-Acetylphenyl)-2-propynylamino]methyl]-4(3*H*)-quinazolinone (13b): 73.0% yield; pink solid; mp 197–199 °C; NMR (100 MHz, Me_2SO-d_6) δ = 2.42 (s, 3, CH_3), 3.27 (t, J = 2 Hz, 1, C≡CH), 4.42 (d, J = 2 Hz, 2, $CH_2C{\equiv}CH$), 4.85 (s, 2, NCH_2), 6.85 (d, J = 9.3 Hz, 2, H2 and -6 of Ph), 7.69 (m, 2, H7 and -8), 7.81 (d, J = 9.3 Hz, 2, H3 and -5 of Ph), 8.00 (distorted s, 1, H5), 8.07 (s, 1, H2), 12.2 (br s, 1, NH, exchangeable); MS m/e = 331

(M^+). Anal. ($C_{20}H_{17}N_3O_2$) C, H, N.

6-[[2-Propynyl(3,4,5-trimethoxyphenyl)amino]methyl]-4-(3*H*)-quinazolinone (13c): 73% yield; white solid; mp 167–169 °C; NMR (200 MHz, Me_2SO-d_6) δ = 3.20 (t, J = 2.1 Hz, 1, C≡CH), 3.56 (s, 3, 4-OCH_3), 3.69 (s, 6, 3- and 5-$(OCH_3)_2$), 4.19 (d, J = 2.1 Hz, 2, CH_2C≡CH), 4.64 (s, 2, NCH_2), 6.15 (s, 2, H2 and -6 of Ph), 7.66 (d, J = 8.4 Hz, 1, H8), 7.80 (dd, J = 8.4 Hz, 2.0 Hz, 1, H7), 8.07 (s, 1, H2), 8.10 (d, J = 1.9 Hz, 1, H5), 12.2 (br s, 1, NH, exchangeable); MS m/e = 379 (M^+). Anal. ($C_{21}H_{21}N_3O_4$) C, H, N.

6-[[(5-Chloro-2-pyridinyl)-2-propynylamino]methyl]-4-(3*H*)-quinazolinone (13k): 64.5% yield; white solid; mp 178–181 °C; NMR (200 MHz, Me_2SO-d_6) δ = 3.15 (t, J = 2.3 Hz, 1, C≡CH), 4.39 (d, J = 2.2 Hz, 2, CH_2C≡CH), 4.91 (s, 2, NCH_2), 6.77 (d, J = 9.1 Hz, 1, H3), of pyridine), 7.66 (m, 2, H8 and H4 of pyridine), 7.74 (dd, J = 8.4 Hz, 1.9 Hz, 1, H7), 8.01 (d, J = 1.6 Hz, 1, H5), 8.07 (s, 1, H2), 8.17 (d, J = 2.6 Hz, 1, H6 of pyridine); 12.24 (br s, 1, NH, exchangeable; MS m/e = 324 (M^+). Anal. ($C_{17}H_{13}ClN_4O$) C, H, N.

Scheme III. 4-(2-Propynylamino)benzonitrile (8e). A suspension of 4-aminobenzonitrile (23.63 g, 0.20 mol), 3-bromo-1-propyne (80% solution in $PhCH_3$, 32.72 g, 0.22 mol), and *N*-ethyl-*N*-(1-methylethyl)-2-propanamine (38.3 mL, 0.22 mol) in $PhCH_3$ (500 mL) was heated at 90 °C for 18 h. EtOAc was added to the cooled reaction suspension, and it was washed three times with H_2O. The organic layer was dried ($MgSO_4$) and concentrated to give 32.5 g of a dark brown oil which crystallized on standing. This was dissolved in a minimum amount of $PhCH_3$ and applied to a column of silica gel (1 kg, 230–400 mesh) packed in $PhCH_3$/EtOAc (20:1). Elution with the same solvent gave 24.66 g (78.9%) of the product as an off-white solid: mp 68–70 °C; NMR (100 MHz, Me_2SO-d_6) δ = 3.15 (t, J = 2 Hz, 1, C≡CH), 3.95 (dd, J = 2 Hz, 7 Hz, 2, CH_2), 6.70 (d, J = 9 Hz, 2, H3 and -5), 7.03 (t, J = 7 Hz, 1, NH), 7.50 (d, J = 9 Hz, 2, H2 and -6); MS m/e = 156 (M^+). Anal. ($C_{10}H_8N_2$) C, H, N.

The following compounds (**8a–d,f,g,j,k**) were prepared similarly to **8e**.

3,4-Dichloro-*N*-2-propynylbenzenamine (8a): 79% yield; yellow liquid; NMR (200 MHz, Me_2SO-d_6) δ = 3.13 (t, J < 3 Hz, 1, C≡CH), 3.92 (d, J < 3 Hz, 2, CH_2), 6.48 (br s, 1, NH, exchangeable), 6.64 (dd, J = 8.9 Hz, 2.8 Hz, 1, H6), 6.84 (d, J < 3 Hz, 1, H2), 7.32 (d, J = 8.9 Hz, 1, H5); MS m/e = 199 (M^+). Anal. ($C_9H_7Cl_2N$) C, H, N.

1-[4-(2-Propynylamino)phenyl]ethanone (8b): 47% yield; yellow solid; mp 97–100 °C; NMR (90 MHz, $CDCl_3$) δ = 2.33 (t, J = 3 Hz, 1, C≡CH), 2.56 (s, 3, CH_3), 4.07 (d, J = 3 Hz, 2, CH_2), 4.25 (br s, 1, NH), 6.73 (m, 2, H3 and -5); 7.92 (m, 2, H2 and -6); MS m/e = 173 (M^+). Anal. ($C_{11}H_{11}NO$) C, H, N.

3,4,5-Trimethoxy-*N*-2-propynylbenzenamine (8c): 82.5% yield; yellow solid; mp 62–63 °C; NMR (100 MHz, Me_2SO-d_6) δ = 3.07 (t, J = 2.5 Hz, 1, C≡CH), 3.54 (s, 3, 4-OCH_3), 3.70 (s, 6, 3- and 5-$(OCH_3)_2$), 3.85 (dd, J = 2.5 Hz, 7.0 Hz, 2, CH_2, coalesces to d, J = 2.5 Hz, on D_2O addition), 5.76 (t, J = 7.0 Hz, 1, NH, exchangeable), 5.95 (s, 2, H2 and -6); MS m/e = 221 (M^+). Anal. ($C_{12}H_{15}NO_3$) C, H, N.

4-Fluoro-*N*-2-propynylbenzenamine (8d): 61% yield; yellow oil; NMR (200 MHz, Me_2SO-d_6) δ = 3.04 (t, J = 2.3 Hz, 1, C≡CH), 3.86 (dd, J = 2.4 Hz, 6.3 Hz, 2, CH_2, coalesces to d, J = 2.3 Hz, on D_2O addition), 5.90 (t, J = 6.2 Hz, 1, NH, exchangeable), 6.61 (m, 2, H2 and -6), 7.0 (m, 2, H3 and -5); MS m/e = 149 (M^+). Anal. (C_9H_8FN·0.15EtOAc) C, H, N.

4-(2-Propynylamino)benzamide (8f): 38.7% yield; yellow solid; mp 139–141 °C; NMR (100 MHz, Me_2SO-d_6) δ = 3.10 (t, J = 2 Hz, 1, C≡CH), 3.90 (dd, J = 2 Hz, 7 Hz, 2, CH_2, coalesces to d, J = 2 Hz, upon D_2O addition), 6.50 (t, J = 6 Hz, 1, NH, exchangeable), 6.60 (d, J = 9 Hz, 2, H3 and -5), 6.92 (br s, 1, CONH, exchangeable), 7.60 (br s, 1, CONH, exchangeable), 7.67 (d, J = 9 Hz, 2, H2 and -6); MS m/e 174 (M^+). Anal. ($C_{10}H_{10}N_2O$) C, H, N.

***N*,*N*-Dimethyl-4-(2-propynylamino)benzenesulfonamide (8g)**: 50% yield from *N*,*N*-dimethyl-4-aminobenzenesulfonamide;[22] off-white solid; mp 107–111 °C; NMR (100 MHz, Me_2SO-d_6) δ = 2.5 (s, 6, $N(CH_3)_2$), 3.1 (t, J = 2.7 Hz, 1, C≡CH), 3.9 (dd, J = 2.7 Hz, 6.3 Hz, 2, CH_2, coalesces to d, J = 2.7 Hz, upon D_2O addition), 6.76 (m, 2, H3 and -5), 6.98 (t, J = 6.3 Hz, 1, exchangeable), 7.5 (m, 2, H2 and -6); MS m/e = 238 (M^+). Anal. ($C_{11}H_{14}N_2O_2S$) C, H, N.

***N*-2-Propynyl-4-(trifluoromethoxy)benzenamine (8j)**: 66.2% yield; brown liquid; NMR (200 MHz, Me_2SO-d_6) δ = 3.07 (t, J = 2.3 Hz, 1, C≡CH), 3.89 (dd, J = 2.4 Hz, 6.2 Hz, 2, CH_2, coalesces to d, J = 2.4 Hz, upon D_2O addition), 6.28 (t, J = 6.2 Hz, 1, NH, exchangeable), 6.70 (d, J = 9.0 Hz, 2, H2 and -6), 7.11 (d, J = 8.2 Hz, 2, H3 and -5); MS m/e = 215 (M^+). Anal. ($C_{10}H_8F_3NO$) C, H, N.

5-Chloro-*N*-2-propynyl-2-pyridinamine (8k): 4% yield; white solid; mp 68–70 °C; NMR (90 MHz, $CDCl_3$) δ = 2.15 (t, J = 2 Hz, 1, C≡CH), 4.0 (dd, J = 2 Hz, 7 Hz, 2, CH_2), 4.6 (br s, 1, NH), 6.34 (d, J = 9 Hz, 1, H3), 7.3 (dd, J = 3 Hz, 9 Hz, 1, H4), 8.02 (d, J = 3 Hz, 1, H6).

Scheme IV. 4-Nitro-*N*-2-propynylbenzenamine (8h). A suspension of 4-fluoronitrobenzene (26.6 g, 0.189 mol, 14**h**), 2-propynylamine (20.8 g, 0.377 mol), potassium fluoride (11 g, 0.189 mol), and potassium carbonate (26 g, 0.188 mol) in DMSO (600 mL) was stirred at room temperature under N_2 for 20 h. The suspension was poured into H_2O (1.5 L) and stirred for 2 h. The yellow solid was collected and washed with H_2O and then 2-propanol to give 16.27 g (49%) of the product as a yellow solid: mp 150–153 °C (lit.[19] mp 149–151 °C); NMR (90 MHz, $CDCl_3$) δ = 2.30 (t, J = 2.4 Hz, 1, C≡CH), 4.04 (dd, J = 2.3 Hz, 5.7 Hz, 2, CH_2), 4.74 (br s, 1, NH), 6.65 (m, 2, H2 and -6), 8.14 (m, 2, H3 and -5). MS m/e = 176 (M^+). Anal. ($C_9H_8N_2O_2$) C, H, N.

2,2,2-Trifluoro-1-[4-(2-propynylamino)phenyl]ethanone (8i). To a solution of 2,2,2-trifluoro-1-(4-fluorophenyl)ethanone[23] (8.64 g, 0.045 mol, 14**i**) and Et_3N (7.53 mL, 0.054 mol) in CH_3CN (150 mL), cooled by an ice bath, was added 2-propynylamine (31.13 mL, 0.45 mol). The ice bath was removed and the solution was heated under reflux for 18 h. The reaction solution was cooled and concentrated to give a solid, which was partitioned between EtOAc and H_2O. The EtOAc layer was dried ($MgSO_4$) and concentrated to give 10.23 g of a red-brown oil. This was applied to a column of silica gel (250 g, 230–400 mesh) packed in *n*-heptane/CH_2Cl_2 (2:1). Elution with the same solvent gave 4.05 g (39.6%) of the product as a fluffy white solid: mp 96–98 °C; NMR (200 MHz, Me_2SO-d_6) δ = 3.22 (t, J = 2.4 Hz, 1, C≡CH), 4.06 (dd, J = 2.5 Hz, 5.9 Hz, 2, CH_2, coalesces to s, J = 2.5 Hz, upon D_2O addition), 6.80 (d, J = 9.1, 2, H3 and -5), 7.71 (t, J = 5.7 Hz, 1, NH, exchangeable), 7.86 (d, J = 9.0 Hz, 2, H2 and -6); MS m/e = 227 (M^+).

Scheme V. 2-Nitro-5-[[(3,4,5-trimethoxyphenyl)amino]methyl]benzoic Acid Methyl Ester (16). To a solution of 5-(bromomethyl)-2-nitrobenzoic acid methyl ester[20] (2.74 g, 0.01 mol, 15) in MeOH (50 mL) was added Et_3N (2.79 mL, 0.02 mol) and 3,4,5-trimethoxybenzenamine (1.83 g, 0.01 mol, 14**c**). The resulting solution was stirred at 25 °C for 18 h and then concentrated. The residue was dissolved in EtOAc, and the solution was washed with water, dried ($MgSO_4$), and concentrated. The residue was dissolved in a minimum amount of $PhCH_3$ and applied to a column of silica gel (100 g, 230–400 mesh) packed in $PhCH_3$/EtOAc (5:1). Elution with the same solvent gave 1.76 g (46.9%) of the product as a red gum which solidified on standing: mp 109–113 °C; NMR (200 MHz, Me_2SO-d_6) δ = 3.51 (s, 3, 4-OCH_3), 3.66 (s, 6, 3- and 5-$(OCH_3)_2$), 3.85 (s, 3, OCH_3), 4.42 (s, 2, CH_2), 5.89 (s, 2, H2 and -6 of Ph), 6.3 (br s, 1, NH, exchangeable), 7.76 (d, J = 1.3 Hz, 1, H6), 7.82 (d, J = 6.9 Hz, 1, H4), 8.07 (d, J = 8.2 Hz, 1, H3); MS m/e = 376 (M^+).

2-Amino-5-[[(3,4,5-trimethoxyphenyl)amino]methyl]benzoic Acid Methyl Ester (17). A suspension of **16** (1.64 g, 4.36 mmol) in THF (50 mL) and MeOH (50 mL) was shaken with Raney Ni (0.5 g) in a stainless steel Parr apparatus at an initial pressure of 51 psi for 21 h. The mixture was filtered to remove the catalyst and then concentrated to a gum. This was dissolved in a minimum amount of CH_2Cl_2 and applied to a column of silica gel (125 g, 230–400 mesh) packed in CH_2Cl_2/MeOH (50:1). Elution with the same solvent gave 1.0 g (67%) of the product as a light yellow oil which solidified on standing: mp 131–134 °C; NMR (100 MHz, Me_2SO-d_6) δ = 3.53 (s, 3, 4-OCH_3), 3.68 (s,

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6, 3- and 5-$(OCH_3)_2$), 3.80 (s, 3, OCH_3), 4.07 (d, J = 6 Hz, 2, CH_2, coalesces to s on D_2O addition), 5.90 (s, 2, H2 and -6 of Ph), 5.9 (m, 1, NH, exchangeable), 6.56 (s, 2, NH_2, exchangeable), 6.73 (d, J = 8 Hz, 1, H3), 7.30 (d, J = 8 Hz, 1 H4), 7.73 (s, 1, H6); MS m/e = 346 (M^+).

6-[[(3,4,5-Trimethoxyphenyl)amino]methyl]-4(3*H*)-quinazolinone (18c). A suspension of 17 (0.73 g, 2.1 mmol) and formamidine acetate (2.0 g, 19.2 mmol) in EtOH (75 mL) was heated under reflux for 5 h. More formamidine acetate (2.2 g, 2.1 mmol) was added to the reaction and it was heated under reflux for another 18 h. TLC (SiO_2: CH_2Cl_2/MeOH (10:1)) indicated the presence of much starting material. More formamidine acetate (10 g, 96.1 mmol) was added to the reaction, and it was again heated under reflux for 18 h. Formamidine acetate (10 g, 96.1 mmol) was again added to the reaction, and it was heated under reflux for another 18 h. The suspension was concentrated to give a semisolid which was partitioned between H_2O and EtOAc. Some material did not dissolve and this was collected to give 0.11 g of a white solid. The resulting layers were separated, and the H_2O layer was extracted with EtOAc. The organic layers were combined, dried ($MgSO_4$), and concentrated to give 1.46 g of a red oil which solidified on standing. This material was combined with the 0.11 g of white solid above and recrystallized first from 95% EtOH and then from CH_3CN to give 0.27 g (38%) of the product as a white solid: mp 205–208 °C; NMR (100 MHz, Me_2SO-d_6) δ = 3.49 (s, 3, 4-OCH_3), 3.61 (s, 6, 3- and 5-$(OCH_3)_2$) 4.39 (d, J = 6 Hz, 2, CH_2, coalesces to s on D_2O addition), 5.88 (s, 2, H2 and -6 of Ph), 6.24 (t, J = 6 Hz, 1, N*H*CH_2, exchangeable), 7.63 (d, J = 8 Hz, 1, H8), 7.84 (dd, J = 8 Hz, 2 Hz, 1, H7), 8.04 (s, 1, H2), 8.14 (d, J = 2 Hz, 1, H5), 12.20 (br s, 1, NH, exchangeable); MS m/e = 341 (M^+). Anal. ($C_{18}H_{19}N_3O_4$) C, H, N.

Purification and Assay of Thymidylate Synthase. Thymidylate synthase was isolated from a strain of L1210 cells (L1210:C15, kindly supplied by Dr. Hilary Calvert) which overproduce the enzyme by 45-fold.[24] Purification was achieved by affinity chromatography by the method described by Jackman et al.[25] Enzyme activity was determined by a modification of the tritium release assay as described by Roberts.[26] In brief, the reaction was performed at 37 °C over 30 min in 13 × 75 mm glass tubes containing reaction buffer (0.275 mL; 10 mM Na_2HPO_4, 10 mM NaF, 10 mM β-mercaptoethanol, pH 7.4), 5,10-methylenetetrahydrofolate (50 μL, final concentration 100 μmol), [^{3}H]deoxyuridine monophosphate (50 μL, final concentration 50 μmol, specific activity 40 μCi/μmol), appropriately diluted enzyme (100 μL), and inhibitor or solvent (25 μL). The reaction was terminated by the addition of acid-washed charcoal suspended in 1 N HCl (200 μL). The tubes were thoroughly mixed and spun for 5 min at 2000g, the supernatant (0.5 mL) was removed and added to Ready-gel (Beckman, 10 mL), and the radioactivity was determined in a Beckman LS 6800 scintillation counter.

Purification and Assay of Dihydrofolate Reductase. Dihydrofolate reductase was isolated from a strain of human lymphoblastoid cells (WI-L2/MTX, kindly supplied by Dr. James Freisheim) which overproduces the enzyme by 50-fold. The enzyme was purified by affinity chromatography by the method of Whiteley et al.[27] Enzyme assays were performed by the following procedure: in a 3-mL quartz cuvette, 0.05 M Tris Cl buffer (2.73 mL), 5 mM NADPH (0.02 mL), inhibitor or solvent (0.1 mL), and appropriately diluted enzyme (0.1 mL) were mixed and warmed to 37 °C. The reaction was started by addition of 2 mM dihydrofolate (0.05 mL) and the decrease in absorbance was monitored at 340 nm with a Cary Model 219 spectrophotometer.

Cell Growth Inhibition and Protection Assays. All experiments employed L1210 mouse leukemia cells grown as a suspension culture in RPMI 1640 medium (Gibco Laboratories) supplemented with 10% fetal bovine serum and 50 μg/mL gentamycin. The doubling time was 12–13 h and viability was greater than 95% by trypan blue exclusion. Dilutions of test compounds in 20 μL were placed into wells of 24-well Linbro plates (1.7 × 1.6 cm, flat bottom) followed by the addition of L1210 cells (3 × 10^4/mL) in 2 mL of media. Plates were incubated for 3 days at 37 °C in a humidified atmosphere containing 5% CO_2 in air. Cell growth was determined by cell count with a Coulter Model ZM electronic cell counter (Coulter Electronics, Inc., Hialeah, FL). Protection experiments were performed in an identical manner except protecting agents were included along with the drug.

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