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ARTICLE

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Nucleoside Transport Inhibitors: Structure−Activity Relationships for Pyrimido[5,4-*d*]pyrimidine Derivatives That Potentiate Pemetrexed Cytotoxicity in the Presence of α_1-Acid Glycoprotein

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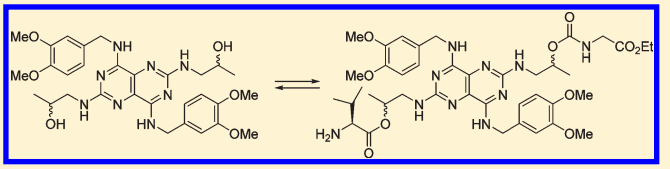
Supporting Information

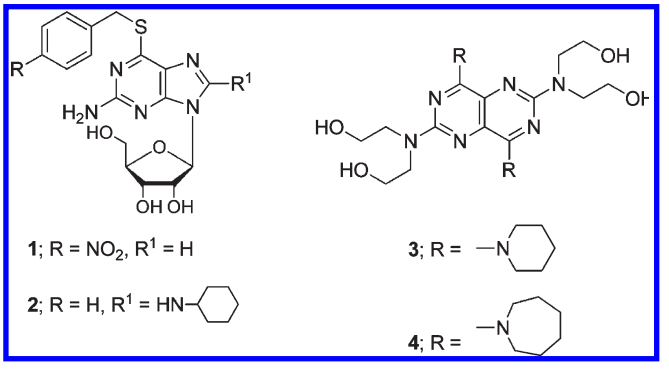
ABSTRACT: Membrane transport of nucleosides or nucleobases is mediated by transporters including the equilibrative nucleoside transporters (ENTs), and resistance to antitumor antimetabolite drugs may arise via salvage of exogenous purine or pyrimidine nucleosides or nucleobases by ENT transporters. The therapeutic utility of dipyridamole (**3**), a potent ENT inhibitor, is compromised by binding to the serum protein α_1-acid glycoprotein (AGP). Derivatives and prodrugs of the ENT inhibitor 4,8-bis[(3,4-dimethoxybenzyl)amino]-2,6-bis[(2-hydroxypropyl)amino]pyrimido[5,4-*d*]pyrimidine (**6**, NU3108) are described, with improved *in vivo* pharmacokinetic properties and reduced AGP binding relative to dipyridamole. The mono- and diglycine carbamate derivatives were at least as potent as **6** and showed no reduction in potency by AGP. In a [^{3}H]thymidine incorporation assay, employing COR-L23 cells, the diastereoisomers of **6** (IC_{50} = 26 nM) exhibited activity comparable with **3** (IC_{50} = 15 nM). The monophenyl carbamate and mono-4-methoxyphenyl carbamate exhibited the best ENT-inhibitory activity in the COR-L23 assay (IC_{50} = 8 and 4 nM, respectively). All of the new prodrugs were also highly effective at reversing thymidine/hypoxanthine rescue from pemetrexed cytotoxicity in the COR-L23 cell line.



INTRODUCTION

The bidirectional transport of hydrophilic nucleosides or nucleobases across cell membranes is mediated by a multitude of specialized nucleoside transporters that are divided into two general classes: equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs).[1,2] The ENTs are further classified as either equilibrative sensitive (*es* or ENT1) or equilibrative insensitive (*ei* or ENT2) based on their sensitivity to inhibition by 4-nitrobenzylmercaptopurine riboside (NBMPR, **1**), with two further isoforms, ENT3 and ENT4, having been identified more recently.[1,3] Modulation of nucleoside−nucleobase transport (NT) is recognized as of potential value for a number of diseases, including cardiovascular and inflammatory disorders, infectious disease, and cancer, and the development of small-molecule NT inhibitors as therapeutic agents has formed the subject of a comprehensive review.[4]

Some 60 years after their introduction, antimetabolites remain an important class of anticancer agents, with several promising new drugs having recently entered the clinic, including the multitargeted antifolate pemetrexed (PTX).[5] Resistance to these agents may arise via a variety of mechanisms, including the salvage of exogenous purine or pyrimidine nucleobases or nucleosides, which circumvent the blockade of *de novo* synthesis imposed by the antimetabolite drug. Importantly, there is evidence that the activities of salvage enzymes are higher than those of *de novo* synthesis[6] and that resistance attributable to salvage increases in parallel with malignancy.[7] The clinical relevance of salvage pathways in resistance to antifolates has also been demonstrated for several antitumor antifolate drugs, including PTX and raltitrexed.[8−10]



The initial step in nucleoside salvage requires membrane transport, which arises principally by carrier-mediated facilitated diffusion via the ENT1 and ENT2 nucleoside transporters. Although a potent ENT1 NT inhibitor, **1** is not a useful therapeutic agent owing to off-target activity and its polar nature. Efforts to overcome the unfavorable pharmaceutical properties of **1** through the synthesis of lipophilic derivatives (e.g., **2**) have

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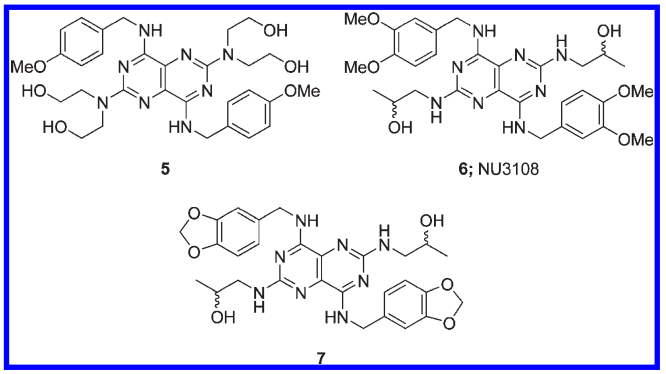
recently been reported.[11] The established antithrombotic and cardiovascular drug dipyridamole (DP, **3**) is a potent ENT inhibitor,[2,12] which has been demonstrated to potentiate the cytotoxicity and antitumor activity of a number of antimetabolite drugs by blocking nucleoside uptake for salvage.[13−18] Moreover, by abrogating cytotoxic nucleoside efflux, **3** can also act synergistically with base- or nucleoside-derived antimetabolite drugs, e.g., 5-fluorouracil, as well as exacerbating the nucleotide pool imbalances elicited by these agents.[19] Interestingly, **3** has very recently been reported to augment the replication of oncolytic *Herpes simplex* viral vectors utilized for tumor therapy, through a mechanism involving replicative amplification as a consequence of NT inhibition.[20] Unfortunately, the preclinical promise of **3** as a potentiator of antimetabolite cytotoxicity has not been realized in the clinic, and this is likely due, at least in part, to the avid binding of the drug to the serum protein α_1-acid glycoprotein (AGP). Levels of AGP are elevated in cancer patients,[21,22] and binding of **3** to AGP reduces free drug concentrations below those required to effectively inhibit NT, as has been demonstrated both in experimental systems employing physiological AGP concentrations[23] and in clinical trials.[24−28]

Numerous analogues of **3** have been synthesized to date, largely with the objective of improving antiplatelet and cardiovascular activity.[4,29,30] More recently, Lin and Buolamwini have reported structure−activity relationship studies (SARs) for a large series of derivatives of **3**, resulting in the identification of the analogue **4** as a potent NT inhibitor.[31] Our studies have also centered on the development of NT inhibitors based on the pyrimido[5,4-*d*]pyrimidine template of **3**, with a view to identifying agents with clinical utility as potentiators of antimetabolite drug activity. In particular, we have previously attempted to improve efficacy through structural modifications designed to abrogate AGP binding without detriment to potency. In addition to further delineating SARs for NT inhibition, these investigations have provided valuable information concerning structural determinants of AGP binding.[32,33] Thus, the 4,8-dibenzylaminopyrimidopyrimidines **5**−**7** retained NT-inhibitory activity comparable with **3** and potentiated the *in vitro* activity of PTX in human lung cancer cell lines in the presence of physiologically relevant concentrations of AGP that essentially abolished the activity of **3**.[34,35]



Unfortunately, subsequent comparative *in vivo* studies conducted with **5**, **6**, and **7** in human tumor-bearing mice at the maximum administrable dose were disappointing. Pharmacokinetic analysis revealed that plasma concentrations required for the prolonged inhibition of thymidine (TdR) incorporation into tumor xenografts, and required for effective chemosensitization, were not maintained for a sufficiently long time period, as previously indicated by our *in vitro* studies.[34] This observation suggested that improving the aqueous solubility of the 4,8-dibenzylaminopyrimidopyrimidine series (**5**−**7**) would enable repeat administration of the NT inhibitors at higher doses. In this paper, we describe the results of studies designed to improve the pharmaceutical properties of **6** (NU3108) employing a prodrug strategy and the identification of derivatives of **6** exhibiting improved NT-inhibitory activity compared with the parent pyrimidopyrimidine.

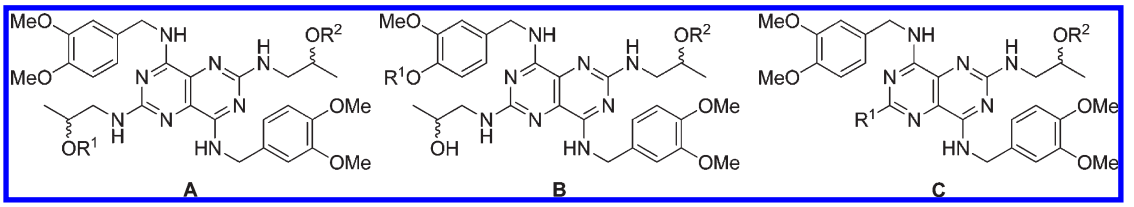
CHEMISTRY

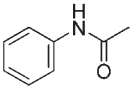
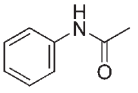
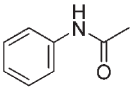
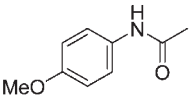
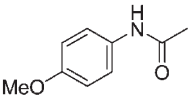
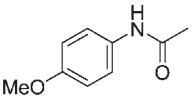
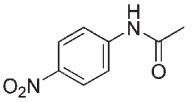
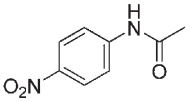
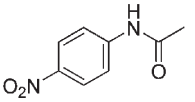
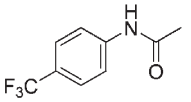
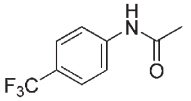
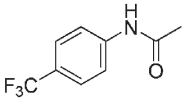
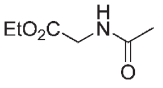
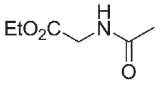
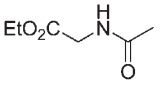
The structures and properties of all compounds synthesized and evaluated for biological activity are recorded in Table 1. Diastereoisomers (**10** and **11**) of **6** were prepared as described previously by reaction of 2,6-dichloro-*N*,*N*′-bis(3,4-dimethoxybenzyl)pyrimido[5,4-*d*]pyrimidine-4,8-diamine with an excess of the appropriate enantiomer of 1-amino-2-propanol.[35] The required aryl carbamate derivatives (**12**−**19**) were readily synthesized in satisfactory yield by treatment of **6** with the appropriate aryl isocyanate in THF, followed by separation of the mono- and symmetrical diaryl carbamates by chromatography on silica (Scheme 1). With the exception of the mono- and diglycine carbamates (**20** and **21**), a similar approach was also adopted for the preparation of the target amino acid carbamate derivatives (**22**−**26**). The monoglycine ethyl carbamate (**20**) was isolated in excellent yield on treatment of **6** with a stoichiometric quantity of ethyl isocyanatoacetate under mild reaction conditions, whereas reaction of **6** with the isocyanate under reflux conditions with catalytic DMAP gave the diglycine ethyl carbamate (**21**) in near quantitative yield.

Esterification of the side-chain hydroxyl groups of **6** was achieved under standard conditions, with the mono- (**27**) and disuccinate (**28**) derivatives being obtained in modest yield following treatment of **6** with succinic anhydride in the presence of catalytic DMAP. The corresponding glycine and valine esters were prepared by reaction of **6** with CDI or WSC, respectively, followed by treatment with the appropriate BOC-protected amino acid. Although only the diglycine BOC ester (**29**) was obtained, the mono- (**31**) and divaline (**32**) BOC esters were isolated, and subsequent deprotection under acidic conditions gave the required amino acid esters (**30**, **33**, **34**). Further derivatization of the monophenyl carbamate (**12**) used conditions analogous to those described above. Thus, acylation with succinic anhydride/DMAP gave the phenyl carbamate succinate ester (**35**), while coupling of **12** with BOC-valine to afford **36**, followed by deprotection with aqueous hydrochloric acid−THF, gave the target phenyl carbamate valine ester (**37**). Phosphate derivative **39** was synthesized by reaction of **12** with di-*tert*-butyldiethylphosphoramidite, oxidation of the phosphite intermediates with *m*-CPBA, and hydrolysis of the resulting phosphate diester (**38**) with TFA.

Functionalization of the 4-benzylamino ring on the pyrimidopyrimidine template required synthesis of the phenol (**47**), which was introduced as the *O*-allyl derivative (Scheme 2). Reaction of the tetrachloropyrimidopyrimidine **40** with 4-allyloxy-3-methoxybenzylamine (**9**), readily available from the corresponding benzonitrile (**8**), under conditions previously optimized,[36] gave the 4-monosubstituted derivative **41**. Subsequent stepwise substitution with 3,4-dimethoxybenzylamine

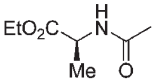
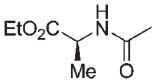
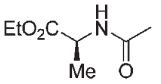
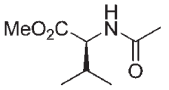
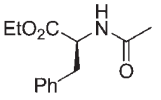
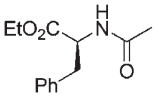
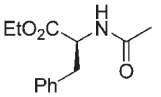
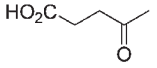
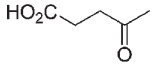
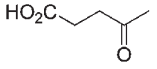
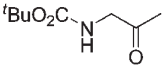
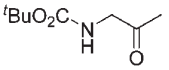
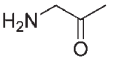
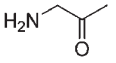
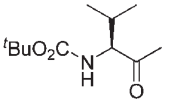
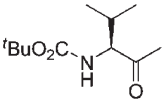
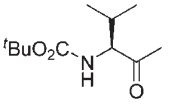
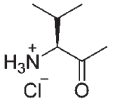
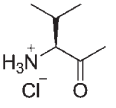
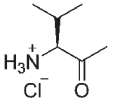
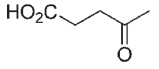
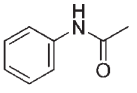
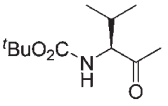
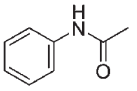
Table 1. Physical and Synthetic Data for Pyrimido[5,4-*d*]pyrimidines



No.	General Structure	R^1	R^2	Method	*Solvent system*	yield (%)	mp (°C)
6	**A**	H	H	—	—	—	—
10[a]	**A**	H	H	—	*C*	23	144-46
11[b]	**A**	H	H	—	*C*	57	144-45
12	**A**	H		I		38	80-81
13	**A**			I	*A*	44	103-104
14	**A**	H		I	*A*	28	86-87
15	**A**			I	*A*	32	94-95
16	**A**	H		I	*A*	38	110-112
17	**A**			I	*A*	17	186
18	**A**	H		I	*A*	23	93-94
19	**A**			I	*A*	63	179
20	**A**	H		—	*B*	80	70-71
21	**A**			—	*D*	93	92-93

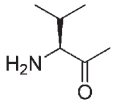
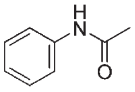
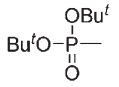
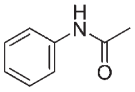
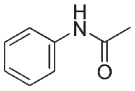
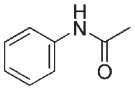
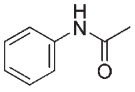
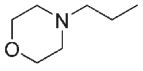
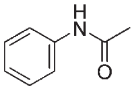
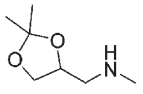
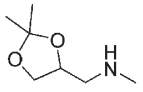
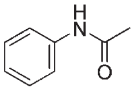
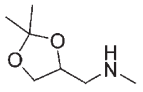
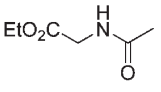
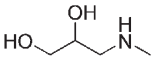
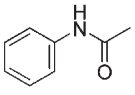
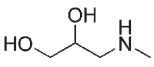
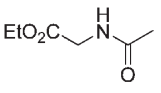
dx.doi.org/10.1021/jm101493z |*J. Med. Chem.* 2011, 54, 1847–1859

Table 1. Continued

No.	General Structure	R^1	R^2	Method	*Solvent system*	yield (%)	mp (°C)
22	A	H		I	*A*	30	66
23	A			I	*A*	40	74
24	A	H		I	*A*	16	78
25	A	H		I	*A*	28	69-70
26	A			I	*A*	35	63-64
27	A	H		—	*C,E*	17	85-87
28	A			—	*C,E*	32	148-150
29	A			—	*B*	56	89-91
30	A			—	*C*	36	111-112
31	A	H		—	*A*	40	78-79
32	A			—	*A*	41	138-39
33	A	H		II	—	100	151-52
34	A			II	—	100	162-63
35	A			—	*C*	84	114-115
36	A				*B*	42	81-82

dx.doi.org/10.1021/jm101493z |*J. Med. Chem.* 2011, 54, 1847–1859

Table 1. Continued

No.	General Structure	R^1	R^2	Method	*Solvent system*	yield (%)	mp (°C)
37	**A**			II	—	100	81-82
38	**A**			III	*A*	58	76-77
39	**A**			IV	—	98	90-91
43	**B**		H	—	*C*	40	138-39
44	**B**	H	H	—	*C*	20	141-42
45	**B**			I	*C*	39	80-81
47	**B**	H		—	*C*	90	93-94
48	**B**				*C*	40	115-117
51	**C**		H	—	*A*	75	80-81
52	**C**			I	*A*	79	71-72
53	**C**			—	*A*	58	79-80
54	**C**				*A*	67	95-96
55	**C**			—	*A*	85	67-68

[a] Prepared using (*R*)-(+)-1-amino-2-propanol. [b] Prepared using (*S*)-(+)-1-amino-2-propanol. Chromatography solvents: A, 60−100% EtOAc:petrol; B, EtOH:EtOAc (99:1); C, DCM:MeOH (20:1). Recrystallization solvents: D, DCM:petrol; E, EtOAc:petrol.

and 2-aminopropanol afforded **42** and **43**, respectively. Carbamoylation of **43** to give a separable mixture of mono- and diphenyl carbamates, **45** and **46**, preceded palladium-mediated removal of the allyl protecting group from **45** to furnish **47**. Interestingly, the 4-hydroxy-3-methoxybenzylaminopyrimidopyrimidine (**44**) was also isolated following treatment of **42** with 2-aminopropanol at 120 °C. Alkylation of the cesium salt of **47** with *N*-(2-chloroethyl)morpholine gave the 4-morpholinoethyl derivative **48** in

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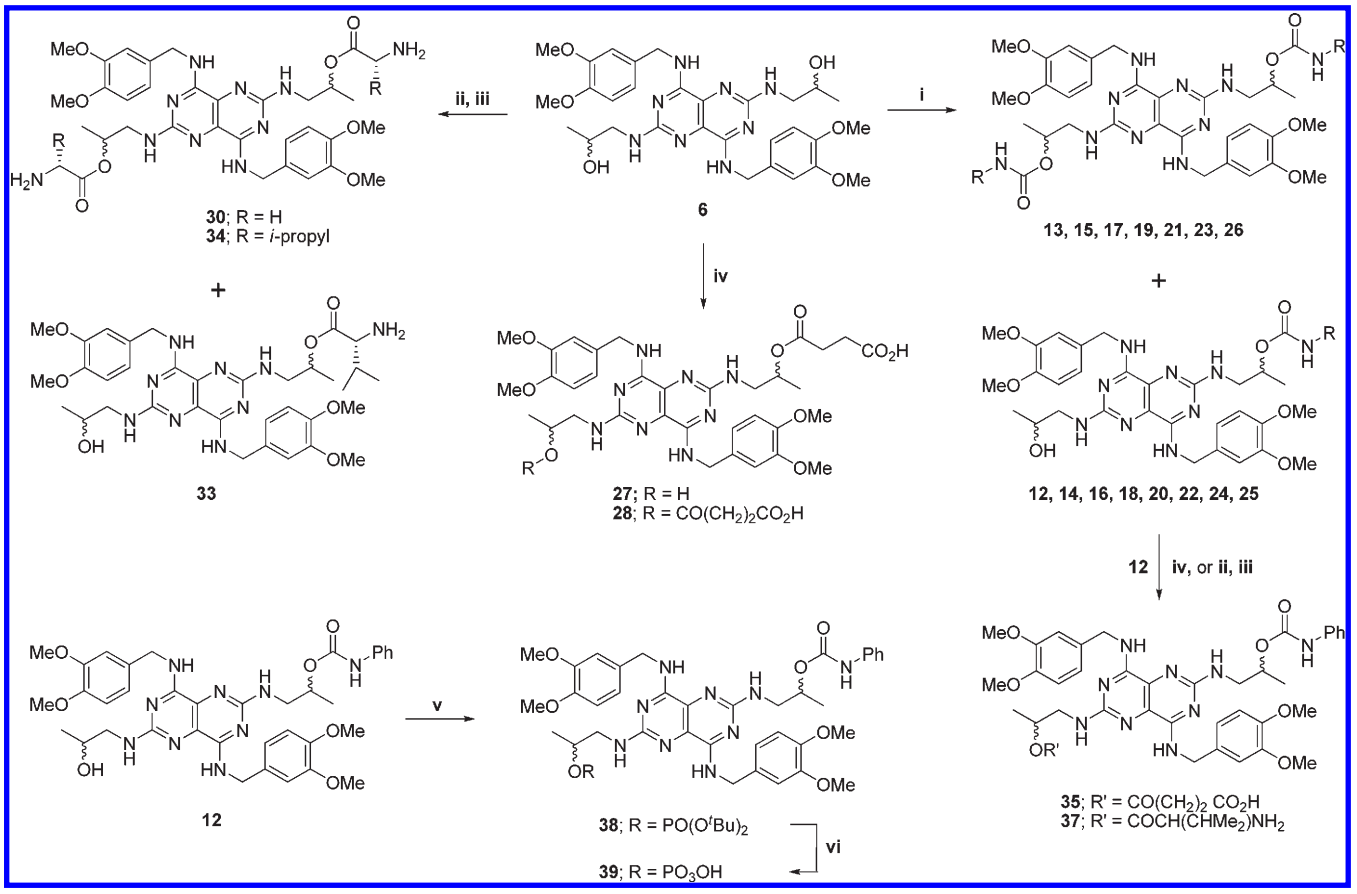
Scheme 1[a]



[a] Reagents and conditions: (i) RNCO, THF, reflux; (ii) CDI, *N*-BOC-glycine, DCM, 25 °C for **31**, WSC, *N*-Boc-valine, DMAP, NEt_3, DMF for **33**, **34**, and **38**; (iii) TFA, 25 °C for **32**, aqueous HCl, 25 °C for **35**, **36**, and **39**; (iv) $(COCH_2CH_2CO)O$, pyridine, DMAP, THF, reflux; (v) (a) 1*H*-tetrazole, $P(OEt)_2N(t\text{-}Bu)_2$, THF, 25 °C, (b) *m*-CPBA, −78 → 25 °C; (vi) TFA, 25 °C.

satisfactory yield. Introduction of a 2,3-dihydroxypropylamino group at the pyrimidopyrimidine 2-position entailed sequential substitution of 2,6-dichloro-*N*,*N*′-bis(3,4-dimethoxybenzyl)pyrimido[5,4-*d*]pyrimidine-4,8-diamine (**49**) with (2,2-dimethyl-1,3-dioxolan-4-yl)methanamine and 1-amino-2-propanol to give **50** and **51**, respectively (Scheme 2). Reaction of **51** with phenyl isocyanate or ethyl isocyanatoacetate gave the respective carbamate derivatives **52** and **53**, which were converted into the required 2,3-dihydroxypropylaminopyrimidopyrimidine salts (**54** and **55**) on treatment with TFA.

RESULTS AND DISCUSSION

The principal objective at the outset of this work was to identify more soluble derivatives of the lead NT inhibitor **6**, with a view to improving the *in vivo* pharmacokinetics of the agent. This objective was pursued by the preparation of potential prodrugs of **6** through reversible modification of the 2-hydroxypropylamino side chains at the pyrimidopyrimidine 2,6-positions, as well as via the synthesis of water-soluble derivatives. Although inherent NT-inhibitory activity was not a prerequisite for the candidate prodrugs, which by definition are subject to conversion into the parent **6** *in vivo*, we routinely screened these derivatives. By contrast, the introduction of functionality designed to improve intrinsic solubility will likely also influence biological activity, hence the necessity to evaluate these compounds as NT inhibitors.

Initial efforts to prepare water-soluble prodrugs of **6** entailed the synthesis of simple carbamate esters (**20**−**26**) derived from glycine, alanine, valine, and phenylalanine as a prelude to saponifying the carboxylate esters to provide water-soluble sodium salts. Poor aqueous solubility prevented the preliminary screening of dicarbamates **23** and **26** in a ^{3}H-TdR transport assay using L1210 murine leukemia cells. However, and to our surprise, the mono- and diglycine carbamate derivatives (**20**, **21**) were at least as potent as **6**, inhibiting ^{3}H-TdR transport by >90% at a concentration of 1 μM (Figure 1A). Unfortunately, more detailed studies demonstrated that the amino acid carbamate esters **20**−**26** lacked stability, decomposing rapidly in culture media,[37] and further investigations with this series were discontinued. The fortuitous observation that carbamoylated derivatives of **6** retained potent NT-inhibitory activity was investigated further through the synthesis of a small series of mono- and diaryl carbamates (**12**−**19**), in the expectation that these would also prove more stable.

Notwithstanding solubility problems with diaryl carbamates (**13**, **15**, **17**, **19**), it was evident that the monoaryl carbamates (**12**, **16**, **18**) retained NT-inhibitory activity at least comparable with **6**, **20**, and **21**, whereas the corresponding diaryl carbamates (**13**, **17**, **19**) were weakly active (**13**) or inactive (**17**, **19**) where evaluation was possible (Figure 1A). Crucially, the desired lack of significant AGP binding *in vitro* for NU3108 (**6**) was also observed for the monoglycine carbamate (**20**) and the monoaryl

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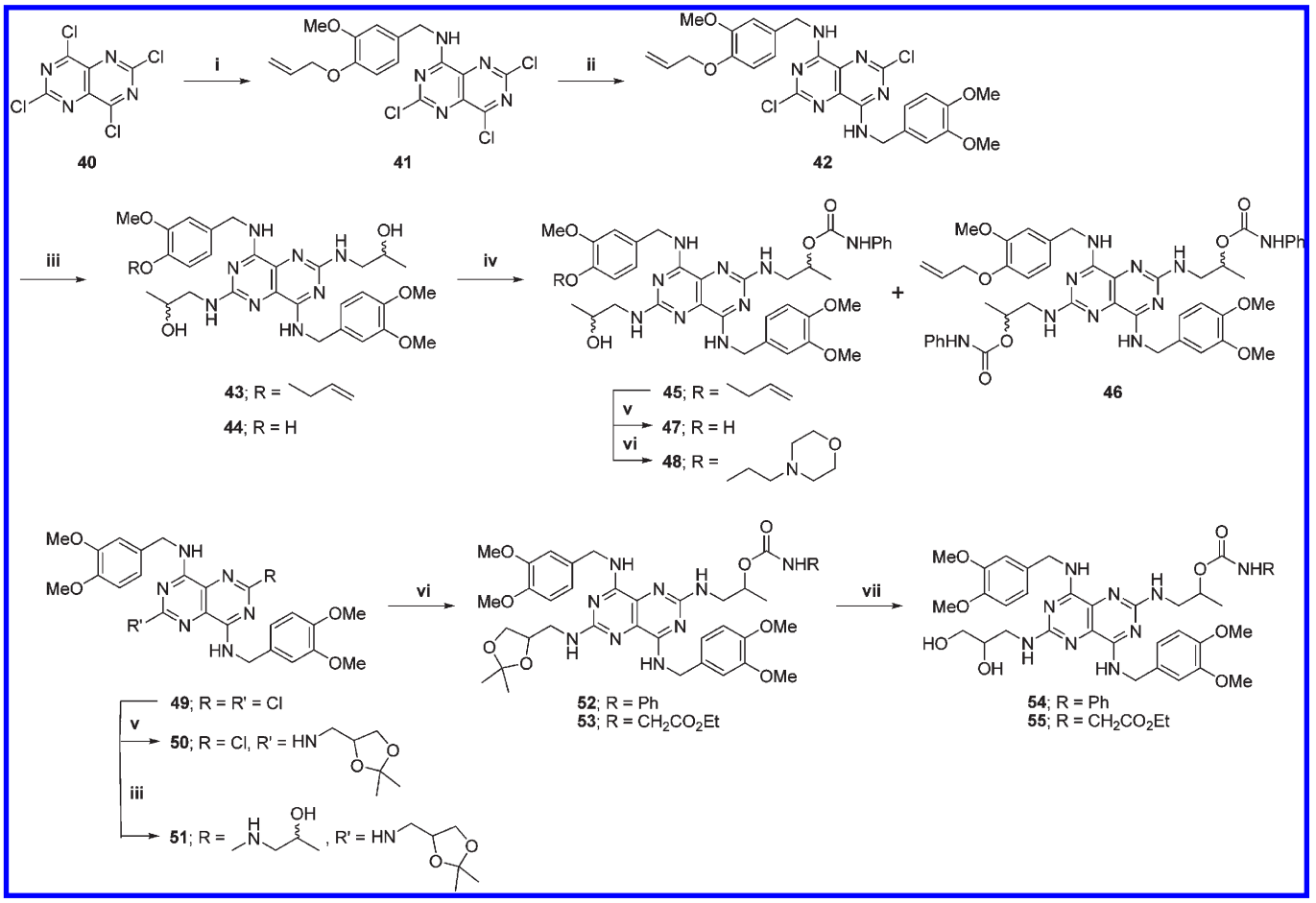
Scheme 2[a]



[a] Reagents and conditions: (i) **9**, K_2CO_3, THF, −78 → 25 °C; (ii) 3,4-dimethoxybenzylamine, THF, 25 °C; (iii) 1-amino-2-propanol, 120 °C; (iv) PhNCO, THF, 25 °C; (v) $Pd(PPh_3)_4$, MeOH; (vi) *N*-(2-chloroethyl)morpholine HCl, KI, $CsCO_3$, dioxane, reflux; (v) 4-aminomethyl-2,2-dimethyl-1,3-dioxolane, THF, 70 °C; (vi) for **55**, PhNCO, THF, reflux, for **56**, ethyl isocyanatoacetate, THF, 25 °C; (vii) TFA (aqueous), 25 °C.

carbamates **12**, **16**, and **18**, with no reduction in potency being evident in the presence of physiologically relevant concentrations (5 mg/mL) of AGP (Figure 1B).[37] By contrast, the NT-inhibitory activity of **3** was reduced by approximately 66% under these conditions. The monosuccinate ester (**27**) of **6** exhibited modest NT-inhibitory activity, whereas the disuccinate (**28**) was a very weak inhibitor, and a similar trend was observed for the *t*-BOC valine ester precursors (**31**, **32**). Interestingly, the potency of monovaline ester (**33**) was comparable to that of **3** in the ^{3}H-TdR transport assay, and reasonable NT-inhibitory activity was also evident for the valine diester (**34**).

While the ^{3}H-TdR transport assay served as a useful initial screen, a ^{3}H-TdR incorporation assay, employing the COR-L23 human lung carcinoma tumor cell line, was used for subsequent studies with selected compounds, as this assay provides a more representative value for NT inhibition.[37] As expected, the parent pyrimidopyrimidine **6** (IC_{50} = 26 ± 5 nM) exhibited activity comparable with **3** (IC_{50} = 15 ± 4 nM), with **6** and the homochiral derivative **11** proving equipotent. This result supports our previous observation that substituents at the pyrimidopyrimidine 2,6-positions do not appear to make important stereoselective interactions with the nucleoside transporter.[35] Encouragingly, the activity of **20** (IC_{50} = 59 nM) was confirmed in this assay, albeit that the compound was some 2-fold less potent than **6**, whereas the valine monocarbamate (**24**; IC_{50} = 7.0 nM) proved approximately 3-fold more potent than **6**. By contrast, the alanine monocarbamate (**22**; IC_{50} = 435 nM) was markedly less potent than **6** and **20**. The NT-inhibitory activity observed for the valine esters **33** and **34** was confirmed in the COR-L23 assay, with IC_{50} values of 39 ± 14 and 70 ± 9 nM, respectively, being obtained, and these derivatives were also noted to be more soluble than the parent **6**.

Consistent with the results from the initial screen, monoaryl carbamates (**12**, **14**, **16**, **18**) exhibited good NT-inhibitory activity in the COR-L23 cell-based assay (Table 2), with **12** (IC_{50} = 8 ± 1 nM) and **14** (IC_{50} = 4 ± 1 nM) proving more potent than **6** or **3**. A comparison of IC_{50} values for these compounds indicates that the phenyl (**12**) and 4-methoxyphenyl (**14**) carbamates are approximately 3-fold and 7-fold, respectively, more potent than **6**. By contrast, the 4-nitrophenyl (**16**) and 4-trifluoromethylphenyl (**18**) carbamates are 2−3-fold less potent than **6**, implying that electron-donating aryl groups are favored. The serendipitous observation that both simple monoaryl carbamate derivatives (e.g., **12**) and amino acid monoesters (e.g., **33**) of **6** exhibit potent inherent NT-inhibitory activity suggested that combining these structural modifications within a single pyrimidopyrimidine might be beneficial in terms of both potency and aqueous solubility. The monophenyl carbamate **12**

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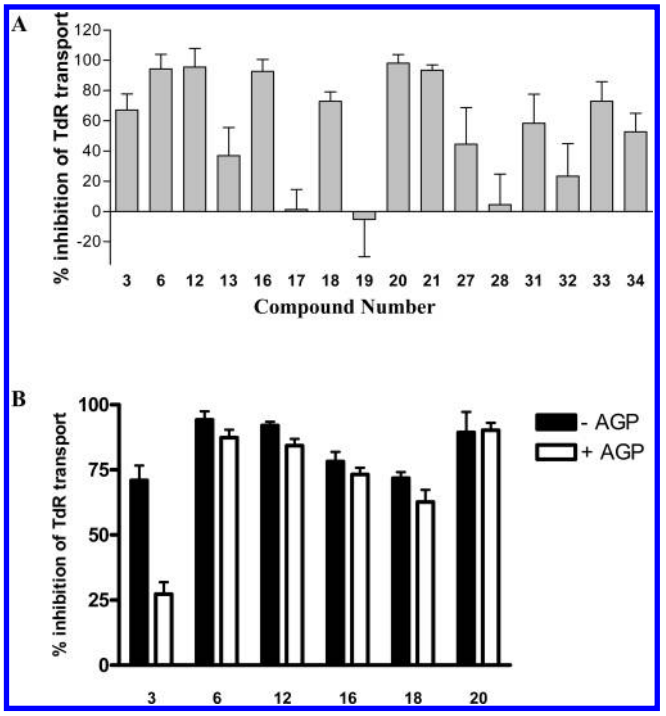

Figure 1. (A) Inhibition of ^{3}H-TdR transport into L1210 cells by selected pyrimido[5,4-*d*]pyrimidines (1.0 μM). (B) Inhibition of ^{3}H-TdR transport into L1210 cells by **3** (DP), **6**, **20**, **12**, **16**, and **18** (1.0 μM) in the absence (black columns) and presence (white columns) of AGP (5 mg/mL). Data are the mean values and SDs of three independent experiments. For assay details see ref 37.

Table 2. Inhibition of ^{3}H-TdR Incorporation into COR-L23 Cells by Selected Pyrimido[5,4-*d*]pyrimidine NT Inhibitors

	inhibition of TdR incorporation (%)		
compd no.	0.1 μM	1.0 μM	IC_{50} (nM)
3 (DP)	78 ± 3[a]	96 ± 1	15 ± 4
6	66 ± 1	90 ± 1	26 ± 5
11	68 ± 5	93 ± 2	31 ± 4
12	81 ± 2	97 ± 2	8 ± 1
14	84 ± 3	98 ± 1	4 ± 1
16	84[b]	96[b]	60[b]
18	75[b]	93[b]	67[b]
20	85[b]	94[b]	59[b]
22	39[b]	68[b]	435[b]
24	79 ± 1	95 ± 1	7 ± 1
33	64 ± 5	89 ± 7	39 ± 14
34	59 ± 1	87 ± 2	70 ± 9
35	75 ± 2	94 ± 2	7 ± 2
37	68 ± 4	92 ± 2	44 ± 13
39	53 ± 3	75 ± 3	218 ± 10
43	81 ± 3	96 ± 1	14 ± 4
48	80 ± 2	97 ± 1	22 ± 2
54	69 ± 3	91 ± 1	10 ± 2

[a] Mean ± SD of three independent experiments. [b] Single determination.

was selected for these studies, and whereas the valine ester (**37**) was approximately 5-fold less potent, the monosuccinate derivative (**35**) proved equipotent with the parent **12**. By contrast, the

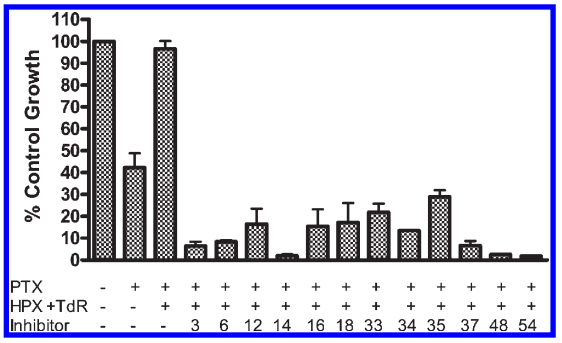

Figure 2. Growth of COR-L23 cells exposed to PTX (0.2 μM) + TdR (1.0 μM) + HPX (10 μM) + DP or selected inhibitors (1.0 μM). Data are the mean values and SDs of three independent experiments. For assay details see ref 37.

phosphate ester (**39**) was a weak NT inhibitor (IC_{50} = 218 ± 10 nM), and both **35** and **39** were chemically unstable, suffering rapid hydrolysis in solution.

Modification of **12** at other positions was also investigated. Thus, the introduction of a 4-(2-morpholino)ethoxy function on the pendant benzylamino group at the pyrimidopyrimidine 4-position was tolerated, with **48** (IC_{50} = 22 ± 2 nM) exhibiting only a 2-fold reduction in potency compared with **12**. Encouragingly, a similar tolerance was observed following introduction of an additional hydroxyl group on the side chains at the pyrimidopyrimidine 2,6-positions, with the bis(2,3-dihydroxypropyl)amino analogue **54** (IC_{50} = 10 ± 2 nM) proving equipotent with **12**.

The ability of selected pyrimidopyrimidines to modulate the growth-inhibitory activity of the antitumor antifolate drug PTX was assessed in COR-L23 tumor cells *in vitro* at a single concentration of 1.0 μM (Figure 2). Thus, growth inhibition following exposure of cells to PTX at a concentration (200 nM) estimated to reduce growth by approximately 50% (GI_{50}) was essentially completely reversed on addition of TdR and hypoxanthine (HPX). However, concomitant addition of **3** or **6** (1.0 μM) prevented TdR/HPX rescue from PTX-induced growth inhibition, and analogous results were obtained for all of the new pyrimidopyrimidine derivatives evaluated at the same concentration. Interestingly, all compounds elicited a > 100% reversal of TdR/HPX rescue; that is, growth in the presence of inhibitor plus TdR/HPX was less than that of PTX alone. This effect may arise as a consequence of the presence of TdR and HPX in the control medium, which contained serum with unknown quantities of nucleosides, or via additional direct growth-inhibitory activity produced by the NT inhibitors and possibly arising through DNA damage following dUTP misincorporation.[19]

All of the new pyrimidopyrimidines inhibited TdR/HPX rescue from PTX to a variable degree at 1.0 μM. Thus, the high potency of monoaryl carbamate **14** was not shared by the structurally related derivatives **12**, **16**, and **18**, which were approximately equipotent but less active than **3** and **6**. Similar potency was observed for valine esters **33** and **34**, whereas the monosuccinate ester **35** proved less active than expected given the potent NT-inhibitory activity seen in the TdR incorporation assay. By contrast, compounds **48** and **54** both demonstrated very high activity in reversing TdR/HPX rescue from PTX growth inhibition, despite the weaker activity of **48** as an NT inhibitor in the TdR incorporation assay compared with **12** or **54**. Clearly, for the ten pyrimidopyrimidines evaluated, there was no obvious direct correlation between potency in the PTX-mediated growth inhibition assay and inhibition of TdR incorporation.

This lack of correlation may arise as a consequence of the fact that all of the compounds selected were potent inhibitors of TdR incorporation and, hence, do not represent a sufficiently broad spectrum of activity. Alternatively, it is possible that modulation of PTX activity arises via additional mechanisms. For example **3** is known to enhance the activity of the antifolate methotrexate through inhibition of drug efflux processes.[38,39] It is thus conceivable that a similar effect contributes to the potentiation of PTX activity observed for the pyrimidopyrimidine derivatives, to a degree that reflects the individual potencies of the compounds as drug efflux inhibitors.

CONCLUSIONS

The initial aim of the work described in this paper was to circumvent problems with the aqueous solubility of the lead NT inhibitor **6** to facilitate *in vivo* studies. However, the serendipitous identification of pyrimidopyrimidines that were more potent than **6** and the parent **3** resulted in an expansion of the studies to include the evaluation of derivatives of **6** as novel NT inhibitors *per se*. Importantly, members of the newly identified aryl carbamate series exemplified by **12** combined potent NT-inhibitory activity in L1210 and COR-L23 tumor cells with very poor avidity for AGP, a prerequisite for *in vivo* activity. Further elaboration was achieved by combining beneficial substituents from different compound series, enabling the identification of very potent NT inhibitors as candidate prodrugs of **12** (e.g., **33**−**35**, **37**) or derivatives (**48**, **54**) with improved pharmaceutical properties. All of the new derivatives were highly effective at reversing TdR/HPX rescue from PTX cytotoxicity in the COR-L23 tumor cell line, with **14**, **48**, and **54** proving more potent than **3** or **6**, albeit that this activity did not necessarily correlate with NT-inhibitory activity. As a result of these initial studies, **12** and related compounds (**16**, **18**) were selected for more detailed biological evaluation together with **37**, the valine ester prodrug of **12**.[37] Pharmacokinetic studies demonstrated that **37** was efficiently converted into the parent **12** *in vivo*, with pharmacologically relevant plasma concentrations being maintained for at least 16 h following intraperitoneal administration of **37** (120 mg/kg). Unfortunately, potentiation of the antitumor activity of PTX by **12** was disappointing in this study, probably as a consequence of insufficient inhibition of TdR incorporation into tumors.

EXPERIMENTAL SECTION

Reagents were purchased from standard vendors and used as received unless otherwise stated. Solvents were purified and stored according to standard procedures. "Petrol" refers to that fraction of hexanes boiling in the range 40−60 °C. Melting points were obtained on a Stuart Scientific SMP3 apparatus and are uncorrected. Thin-layer chromatography was performed using Merck 1.05554 aluminum sheets precoated with Kieselgel 60F_{254} (0.2 mm) as the adsorbent, with visualization by potassium permanganate or UV light at 254 and 365 nm. Column chromatography was conducted under medium pressure on silica (Merck silica gel 40−63 μm). HPLC refers to purification on Gilson LC instruments, with a 15 min gradient of 0.1% aqueous TFA and 10−97% acetonitrile, at a flow rate of 6 mL/min using as the stationary phase a Jones Chromatography Genesis 4 μm C18 column, 10 mm × 250 mm, and peak acquisition based on UV detection at 254 nm.

Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Spectrospin AC 200E spectrometer (200 MHz for ^{1}H, 50 MHz for ^{13}C), a Bruker AMX (500 MHz for ^{1}H, 126 MHz for ^{13}C), or a Bruker Advance 300 MHz Ultrashield spectrometer (300 MHz for ^{1}H, 75 MHz for ^{13}C), using tetramethylsilane or the deuterated solvent as the internal standard for ^{1}H and ^{13}C spectra, respectively. Unless indicated otherwise, spectra were recorded in $CDCl_3$ as solvent. Chemical shift values are quoted in parts per million (ppm) and coupling constants (J) in hertz (Hz). Key: s = singlet, d = doublet, t = triplet, q = quartet, dd = double doublet, and m = multiplet. NH signals appeared as broad singlets (br s) exchangeable with D_2O. Mass spectra were determined on a Micromass Autospec M spectrometer in electron ionization (EI) mode. Liquid chromatography−mass spectrometry (LCMS) was carried out on either a Micromass Platform instrument operating in positive and negative ion electrospray mode, employing a 50 × 4.6 mm C18 column (Supelco Discovery or Waters Symmetry) and a 15 min gradient elution of 0.05% formic acid and methanol (10−90%), or a Finnegan LCQ instrument in positive ion mode with a Phenomenex 5 μm Luna C18 column, 4.6 mm × 50 mm, and an 8 min gradient of 0.1% aqueous formic acid and acetonitrile (5−98%) with a flow rate of 2 mL/min. IR spectra were recorded on a Bio-Rad FTS 3000MX diamond ATR. Compound purity was assessed by elemental analysis (Butterworth Laboratories, Middlesex, U.K.), and values are within ±0.4% of theory unless otherwise specified.

4-(Allyloxy)-3-methoxybenzonitrile (8). A mixture of 4-hydroxy-3-methoxybenzonitrile (6.0 g, 40.2 mmol), K_2CO_3 (8.34 g, 60.3 mmol), and allyl bromide (5.84 g, 48.3 mmol) in acetone (100 mL) was heated at reflux for 12 h. After cooling, the reaction mixture was poured into water (200 mL) and extracted with diethyl ether (3 × 100 mL), and the combined organic layers were washed with aqueous NaOH solution (2 M) and dried (Na_2SO_4). Evaporation of the solvent under reduced pressure afforded the title compound as a white solid (5.0 g, 66%): mp 65−66 °C; IR 3088, 3020, 2980, 2220, 1589, 1506 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.90 (3H, s, OMe), 4.67 (2H, m, OCH_2), 5.38 (2H, m, $=CH_2$), 6.07 (1H, m, =CH), 6.90 (1H, d, ArH), 7.09 (1H, d, ArH), 7.26 (1H, m, ArH); LCMS m/z 190.21 ($[M + 1]^+$). Anal. ($C_{11}H_{11}NO_2$) C, H, N.

4-(Allyloxy)-3-methoxybenzylamine (9). To a stirred suspension of $LiAlH_4$ (1.20 g, 31.7 mmol) in THF (70 mL) under N_2 was added a solution of **8** (3.0 g, 15.9 mmol) in THF (10 mL) dropwise at room temperature. The reaction mixture was stirred for 12 h and quenched by the sequential addition of water (1.2 mL), aqueous NaOH (15%, 1.2 mL), and water (3.6 mL). The white solid that deposited was collected, dissolved in water (50 mL), and extracted with DCM (3 × 20 mL). The combined organic fractions were dried (Na_2SO_4), and the solvent was removed *in vacuo* to afford the title compound as a red viscous oil (2.10 g; 70%): IR 3370, 2933, 2858, 1590, 1509 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.79 (2H, s, OCH_2), 3.86 (3H, s, OCH_3), 4.58 (2H, d, $NHCH_2Ar$), 5.29 (1H, d, =C*H*H), 5.39 (1H, d, =CH*H*), 6.08 (1H, m, =CH), 6.81 (2H, m, 2 × ArH), 6.87 (1H, m, ArH); LCMS m/z 194.13 ($[M + 1]^+$). Anal. ($C_{11}H_{15}NO_2$) C, H, N.

Mono- and Diaryl Carbamate Derivatives of 6: Method I. General Procedure. A solution of **6** (1.0 mol equiv) and the aryl isocyanate (1.0 mol equiv) in THF (60 mL) was heated at reflux for 48 h under N_2. The solvent was removed under reduced pressure, and the residue was dissolved in EtOAc (200 mL), washed sequentially with water (2 × 50 mL) and brine (50 mL), dried (Na_2SO_4), and concentrated *in vacuo*. The monoaryl and diaryl carbamates were isolated by chromatography on silica employing a gradient eluent of EtOAc−petrol (50 → 100% EtOAc).

Removal of the *N*-Boc Group: Method II. General Procedure. To a solution of the *N*-Boc-valine ester in dry THF (15 mL) was added aqueous HCl (3.0 M, 30 mL). The reaction mixture was stirred at ambient temperature for 24 h. The volatile substances were removed under reduced pressure to afford the required compound. Where necessary, purification was achieved by chromatography on silica employing a gradient eluent of EtOAc−petrol (50 → 100% EtOAc).

Phenyl Carbamate Derivatives of 6. Prepared from **6** (1.0 g, 1.64 mmol) and phenyl isocyanate (0.20 g, 1.64 mmol) in THF

(100 mL), using method I, except that the reaction mixture was stirred at room temperature for 5 h.

1-((4,8-Bis((3,4-dimethoxybenzyl)amino)-6-((2-hydroxypropyl)amino)pyrimido[5,4-*d*]pyrimidin-2-yl)amino)propan-2-yl Phenylcarbamate (12). Yellow powder; IR 3387, 3321, 2928, 2833, 1718, 1556, 1501 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.18 (3H, d, J = 6.3 Hz, CH_3), 1.27 (3H, d, J = 6.3 Hz, CH_3), 3.30 (1H, m, NHC*H*H), 3.52 (1H, m, NHCH*H*), 3.81 (3H, s, OCH_3), 3.84 (9H, s, 3 × OCH_3), 4.60 (2H, m, NHCH_2Ar), 5.11 (2H, m, NH, OH), 5.17 (1H, m, CHOCONH), 6.70 (1H, m, N*H*CH_2Ar), 6.78 (1H, d, ArH), 6.89 (4H, m, 4 × ArH), 7.03 (1H, t, ArH), 7.25 (3H, m, 3 × ArH), 7.33 (2H, d, 2 × ArH); LCMS 728.3 ($[M + 1]^+$). Anal. ($C_{37}H_{45}N_9O_7$) C, H, N.

((4,8-Bis((3,4-dimethoxybenzyl)amino)pyrimido[5,4-*d*]pyrimidine-2,6-diyl)bis(azanediyl))bis(propane-2,1-diyl) Bis(phenylcarbamate) (13). Yellow flakes; IR 3324, 2934, 1717, 1597, 1558, 1500 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.29 (6H, d, 2 × CH_3), 3.54 (4H, m, 2 × NHCH_2), 3.81 (6H, s, 2 × OCH_3), 3.84 (6H, s, 2 × OCH_3), 4.60 (2H, m, 2 × N*H*CH_2Ar), 5.07 (1H, br s, 2 × NH), 5.19 (1H, q, 2 × C*H*CH_3), 6.79 (2H, d, 2 × OCONH), 6.91, 6.91, 7.06, 7.27, 7.27, 7.33 (16H, all ArH); LCMS 847.4 ($[M + 1]^+$). Anal. ($C_{44}H_{50}N_{10}O_8$) C, H, N.

Succinate Esters of 6. Succinic anhydride (0.16 g, 1.64 mmol) was added to **6** (0.50 g, 0.41 mmol), pyridine (0.14 mL, 1.80 mmol), and DMAP (0.03 g) in THF (20 mL). The reaction mixture was heated under reflux for 48 h, and the volatiles were removed *in vacuo* to give a yellow oil. Water (15 mL) was added, and the mixture was extracted with EtOAc (3 × 30 mL). The combined organic layers were dried (Na_2SO_4), and the solvent was removed under reduced pressure to give a yellow solid. Purification by chromatography on silica gel, employing DCM−MeOH (20:1) as eluent, followed by recrystallization from EtOAc−petrol furnished the mono- and disuccinate esters as yellow solids.

4-((1-((4,8-Bis((3,4-dimethoxybenzyl)amino)-6-((2-hydroxypropyl)amino)pyrimido[5,4-*d*]pyrimidin-2-yl)amino)propan-2-yl)oxy)-4-oxobutanoic Acid (27). Yellow powder; IR 3394, 3065, 2930, 2835, 1696, 1516 cm^{-1}; ^1H NMR (d_6-DMSO) δ 1.16 (3H, d, CH_3, J = 6.2 Hz), 1.26 (3H, d, CH_3, J = 7.1 Hz), 2.57 (4H, m, CH_2CH_2), 3.22−3.50 (4H, m, 2 × CH_3CHOCH_2), 3.81 (6H, s, 2 × OCH_3), 3.82 (6H, s, 2 × OCH_3), 3.88 (1H, m, CH_3CHOCH_2), 4.66 (4H, s, 2 × $ArCH_2$), 5.15 (1H, m, CH_3CHOCO), 6.12 (1H, t, OH), 6.49 (2H, t, 2 × NH), 6.97−7.14 (6H, m, 6 × Ar−H), 7.61 (2H, br s, 2 × NH); LCMS 708 (M^+). Anal. ($C_{34}H_{44}N_8O_9$) C, H, N.

4,4′-((((4,8-Bis((3,4-dimethoxybenzyl)amino)pyrimido[5,4-*d*]pyrimidine-2,6-diyl)bis(azanediyl))bis(propane-2,1-diyl))bis(oxy))bis(4-oxobutanoic acid) (28). Bright yellow solid; IR 3373, 3348, 3066, 2934, 2835, 1696, 1594 cm^{-1}; ^1H NMR (d_6-DMSO) δ 1.26 (6H, d, 2 × CH_3), 2.52 (8H, s, 2 × CH_2CH_2), 3.48 (4H, m, 2 × CH_3CHOCH_2-NH), 3.81 (6H, s, 2 × OCH_3), 4.66 (4H, br s, 2 × $ArCH_2$), 5.15 (2H, m, 2 × CH_3CHOCH_2NH), 6.51 (2H, br s, 2 × NH), 6.98−7.15 (6H, m, 6 × Ar−H), 7.68 (2H, br s, 2 × NH); LCMS 809.1 ($[M + 1]^+$). Anal. ($C_{38}H_{48}N_8O_{12}$) C, H, N.

***N*-Boc-valine Esters of 6.** *N*-Boc-valine (0.89 g, 4.1 mmol) was added to **6** (1.00 g, 1.64 mmol), DMAP (0.12 g, 0.98 mmol), 1-[(3-dimethylaminopropyl)]-3-ethylcarbodiimide hydrochloride (1.20 g, 5.75 mmol) and NEt_3 (0.67 g, 6.57 mmol) in DMF (20 mL). The reaction mixture was stirred for 48 h at 25 °C, water (100 mL) was added, and the resulting solution was extracted with EtOAc (5 *x* 50 mL). The combined organic layers were washed sequentially with water (4 × 100 mL), saturated aqueous $NaHCO_3$ solution (100 mL), and brine (100 mL), dried (Na_2SO_4), and the solvent was removed *in* vacuo. Product isolation was achieved by chromatography on silica.

(2*S*)-1-((4,8-Bis((3,4-dimethoxybenzyl)amino)-6-((2-hydroxypropyl)amino)pyrimido[5,4-*d*]pyrimidin-2-yl)amino)propan-2-yl 2-((*tert*-Butoxycarbonyl)amino)-3-methylbutanoate (31). Yellow solid; IR 3374, 2966, 2931, 1707, 1556, 1504 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.82 (3H, dd, J = 6.8 Hz, Me), 0.92 (3H, t, J = 6.8 Hz, Me), 1.17 (3H, d, J = 6.2 Hz, Me), 1.25 (3H, t, J = 6.2 Hz, Me), 1.42 (9H, s, *t*-Bu), 2.09 (1H, m, *CH*Me_2), 3.29 (1H, m, N*H*CH_2), 3.47 (2H, m, −NHCH_2), 3.85 (12H, s, 4 × OCH_3), 3.99 (1H, t, J = 4.59 Hz, CHOCO), 4.17 (1H, q, J = 4.59 Hz, −NH−*CH*−CO), 4.62 (4H, m, 2 × $ArCH_2$), 5.01 (2H, m, −NH/OH), 5.24 (2H, m, −CH−OH, NH), 6.73 (1H, br s, NH), 6.78−6.93 (6H, m, Ar), 7.12 (1H, br s, −NH); LCMS 808.5 ($[M + 1]^+$). Anal. $C_{40}H_{57}N_9O_9$ C, H, N.

(2*S*,2′*S*)-((4,8-Bis((3,4-dimethoxybenzyl)amino)pyrimido[5,4-*d*]pyrimidine-2,6-diyl)bis(azanediyl))bis(propane-2,1-diyl) Bis(2-((*tert*-butoxycarbonyl)amino)-3-methylbutanoate) (32). Yellow solid; IR 3372, 2962, 2932, 1707, 1559, 1504 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.79 (3H, d, J = 6.7 Hz, Me), 0.85 (3H, d, J = 6.7 Hz, Me), 0.92 (6H, t, 2 × Me), 1.26 (6H, t, Me), 1.42 (9H, s, *t*-Bu), 1.43 (9H, s, *t*-Bu), 2.09 (2H, m, 2 × *CH*Me_2), 3.44 (1H, m, −N*H*−CH_2), 3.54 (3H, m, −NH−CH_2), 3.85 (6H, s, 2 × OMe), 3.87 (6H, 2 × OMe), 4.18 (2H, m, −NH−CH−CO), 4.64 (4H, m, −NH−CH_2−Ar), 5.02 (4H, m, NH), 5.24 (2H, m, CH−O−CO), 6.83 (2H, m, Ar), 6.91 (4H, m, Ar), 6.99 (2H, br s, −NH); LCMS 1007.5 (M^+). Anal. $C_{50}H_{74}N_{10}O_{12}$ C, H, N.

(2*S*)-1-((4,8-Bis((3,4-dimethoxybenzyl)amino)-6-((2-hydroxypropyl)amino)pyrimido[5,4-*d*]pyrimidin-2-yl)amino)propan-2-yl 2-Amino-3-methylbutanoate (33). Prepared from **31** (0.50 g, 0.62 mmol) in accordance with method II to furnish **33** as a bright yellow solid: IR 3331, 3207, 2933, 2834, 1741, 1633, 1537, 1512 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.86 (3H, q, J = 6.0 Hz, Me), 0.96 (3H, t, J = 6.0 Hz, Me), 1.20 (3H, d, J = 6.0 Hz, Me), 1.27 (3H, dd, J = 6.0 Hz, Me), 2.18 (2H, br s, $−NH_2$), 1.99 (1H, m, $−CH(Me)_2$), 3.25 (2H, m, −NH−CH_2−), 3.33 (1H, m, −*CH*−NH_2), 3.88 (12H, s, 4 × OMe), 3.46 (2H, m, −NH−CH_2−), 4.01 (1H, m, −CH(OH)), 4.65 (4H, m, −NH−CH_2−Ar), 4.96 (1H, m, OH), 5.24 (1H, m, −CH(O)−CO−), 6.68 (1H, br s, −NH), 6.83 (2H, m, Ar), 6.92 (4H, m, Ar), 7.10 (1H, br s, −NH); LCMS 708.4 ($[M + 1]^+$). Anal. ($C_{35}H_{49}N_9O_7.0.9H_2O$) C,H, N.

(2*S*)-1-((6-((2-(((*R*)-2-Amino-3-methylbutanoyl)oxy)propyl)amino)-4,8-bis((3,4-dimethoxybenzyl)amino)pyrimido[5,4-*d*]pyrimidin-2-yl)amino)propan-2-yl 2-Amino-3-methylbutanoate (34). Prepared from **32** (0.5 g, 0.50 mmol) by method II to furnish **34** as a yellow powder: IR 3318, 2933, 1718, 1559, 1510 cm^{-1}; ^1H NMR (CD_3OD) δ 1.02 (12H, m, 4 × Me), 1.33 (6H, t, 2 × Me), 2.31 (2H, m, $−CHMe_2$), 3.32 (2H, m, −NH−CH_2), 3.61 (1H, br s, −NH), 3.75 (2H, d, −NH−CH_2), 3.81 (6H, s, 2 × OMe), 3.85 (6H, s, 2 × OMe), 3.92 (1H, d, −NH−CH−CO), 4.02 (1H, d, −NH−CH−CO), 4.83 (4H, m, −NH−CH_2−Ar), 5.21 (2H, d, −CH−O−CO), 6.92 (2H, d, Ar), 7.00 (2H, d, Ar), 7.10 (2H, br s, Ar); LCMS 807.6 ($[M + 1]^+$). Anal. ($C_{40}H_{58}N_{10}O_8 \cdot 0.5H_2O$) C, H, N.

1-((6-((2-((Di-*tert*-butoxyphosphoryl)oxy)propyl)amino)-4,8-bis((3,4-dimethoxybenzyl)amino)pyrimido[5,4-*d*]pyrimidin-2-yl)amino)propan-2-yl Phenylcarbamate (38). To a stirred solution of **12** (1.00 g, 1.37 mmol) in THF (20 mL) were added 1*H*-tetrazole (0.45 M in MeCN, 9.0 mL, 4.12 mmol) and di-*tert*-butyl diethylphosphoramidite (0.411 g, 1.64 mmol). The solution was stirred for 3 h at room temperature and cooled to −78 °C, whereupon *m*-CPBA (0.31 g, 1.78 mmol) was added, and stirring was continued for a further 30 min at −78 °C, followed by 1 h at room temperature. After the addition of aqueous $NaHSO_3$ solution (10%, 25 mL), the reaction mixture was stirred for 15 min, and EtOAc (100 mL) was added. The solution was washed successively with aqueous $NaHSO_3$ solution (10%, 2 × 20 mL), aqueous $NaHCO_3$ solution (5%, 1 × 20 mL), and brine (20 mL), dried (Na_2SO_4), and concentrated *in vacuo*. Product purification was achieved by chromatography on silica, employing a gradient eluent of EtOAc−petrol (50%−100% EtOAc), to furnish **38** as a pale yellow powder: IR 3404, 3331, 2980, 2934, 1718, 1558 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.28 (3H, d, J = 6.3 Hz, Me), 1.33 (3H, d, J = 6.3 Hz, Me), 1.46 (9H, s, *t*-Bu), 1.47 (9H, s, *t*-Bu), 3.53 (4H, m, −NH−CH_2), 3.82 (3H, s, OMe), 3.85 (6H, s, 2 × OMe), 3.88 (3H, s, OMe), 4.62 (4H, m, −NH−CH_2−Ar),

4.71 (1H, m, −CH−O−P), 5.16 (1H, m, −CH−O−CO), 5.23 (1H, m, −NH), 6.79 (2H, dd, Ar), 6.90 (3H, s, Ar), 7.02 (2H, m, Ar), 7.10 (1H, br s, −NH), 7.25 (2H, t, Ar), 7.35 (2H, d, Ar); LCMS 920.8 ($[M + 1]^+$). ($C_{45}H_{62}N_9O_{10}P \cdot 0.5H_2O$) C, H, N.

1-((4,8-Bis((3,4-dimethoxybenzyl)amino)-6-((2-(phosphonooxy)propyl)amino)pyrimido[5,4-*d*]pyrimidin-2-yl)amino)propan-2-yl Phenylcarbamate (39). A solution of **38** (0.50 g 0.54 mmol) in a mixture of TFA (5 mL) and aqueous HCl (3.0 M, 10 mL) was stirred at room temperature for 3 h. Evaporation of volatiles *in vacuo* afforded the title compound as a pale yellow solid: IR 3300, 2971, 1721, 1608, 1537, 1514 cm^{-1}; ^1H NMR (CD_3OD) δ 1.32 (3H, s, Me), 1.35 (3H, s, Me), 3.52 (4H, m, $-NCH_2$), 3.74 (14H, m, $-OCH_3$, $-OCH-$), 4.60 (4H, m, Ph$-CH_2$), 6.76 (2H, m, Ar), 6.93 (4H, m, Ar), 7.02 (1H, s, Ar), 7.15 (2H, t, Ar), 7.36 (2H, d, Ar); LCMS 807.22 (M^+). Anal. ($C_{37}H_{46}N_9O_{10}P \cdot H_2O$) C, H, N.

1-((4-((3,4-Dimethoxybenzyl)amino)-8-((4-hydroxy-3-methoxybenzyl)amino)-6-((2-hydroxypropyl)amino)pyrimido[5,4-*d*]pyrimidin-2-yl)amino)propan-2-yl Phenylcarbamate (47). To a solution of **45** (0.25 g, 0.34 mmol) in MeOH (20 mL) was added $Pd(PPh_3)_4$ (20 mg, 0.017 mmol). The reaction mixture was stirred under N_2 for 5 min, K_2CO_3 (0.23 g, 1.70 mmol) was added, and stirring was continued for a further 4 h. After evaporation of the solvent *in vacuo*, the residue was dissolved in aqueous HCl (2.0 M, 100 mL), and the solution was stirred for 30 min. The aqueous layer was extracted with DCM (4 × 25 mL), the combined organic layers were washed with saturated aqueous NaCl solution (50 mL) and dried (Na_2SO_4), and the solvent was evaporated. The product was purified by chromatography on silica to furnish **47** as a yellow solid: IR 3416, 3369, 2969, 2931, 1716, 1664, 1558, 1502 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.21 (3H, d, J = 6.0 Hz, Me), 1.31 (3H, d, J = 6.0 Hz, Me), 3.34 (1H, q, J = 6.0 Hz, $-NHCH_2$), 3.48 (1H, dd, J = 3.0 Hz, $NHCH_2$), 3.55 (2H, m, $NHCH_2$), 3.84 (3H, s, OMe), 3.86 (3H, s, −OMe), 3.87 (3H, s, OMe), 4.01 (1H, m, CHOH), 4.61 (4H, m, $NHCH_2Ar$), 5.18 (1H, q, J = 6.0 Hz, −CHOCO), 5.59 (2H, br s, −NH/OH), 6.79−6.94 (5H, m, Ar), 7.04 (1H, t, J = 9.0 Hz, Ar), 7.24−7.36 (4H, m, Ar), 7.59 (1H, br s, −NH); LCMS 713.78 (M^+). Anal. ($C_{36}H_{43}N_9O_7$) C, H, N.

1-((4-((3,4-Dimethoxybenzyl)amino)-6-((2-hydroxypropyl)amino)-8-((3-methoxy-4-(3-morpholinoethoxy)benzyl)amino)-pyrimido[5,4-*d*]pyrimidin-2-yl)amino)propan-2-yl Phenylcarbamate (48). To **47** (0.05 g, 0.07 mmol) in dry dioxane (5 mL) were added cesium carbonate (0.07 g, 0.21 mmol), potassium iodide (~5 mg), and *N*-(2-chloroethyl)morpholine hydrochloride (0.02 g, 0.091 mmol), and the reaction mixture was heated under reflux for 15 h. After cooling, EtOAc (60 mL) was added, the organic phase was washed successively with water (3 × 25 mL) and saturated aqueous NaCl solution (25 mL) and dried (Na_2SO_4), and the volatiles were removed under reduced pressure. Purification by chromatography on silica afforded the title compound as a dark red oil: ^1H NMR ($CDCl_3$) δ 1.20 (3H, d, J = 6.0 Hz, Me), 1.31 (3H, d, J = 6.0 Hz, Me), 2.60 (4H, t, J = 6.0 Hz, NCH_2), 2.84 (2H, t, J = 6.0 Hz, NCH_2), 3.32 (1H, m, $NHCH_2$), 3.49 (1H, m, $NHCH_2$), 3.55 (2H, m, $NHCH_2$), 3.74 (4H, t, J = 6.0 Hz, OCH_2), 3.81 (3H, s, OMe), 3.84 (3H, s, OMe), 3.86 (3H, t, J = 3.0 Hz, OMe), 4.01 (1H, m, CHOH), 4.13 (2H, dt, J = 6.0 Hz, OCH_2), 4.63 (4H, m, $NHCH_2Ar$), 5.19 (1H, q, J = 6.0 Hz, CHOCO), 5.40 (1H, br s, NH), 6.93−6.79 (6H, m, ArH), 7.04 (1H, t, J = 6.0 Hz, ArH), 7.28 (2H, m, ArH), 7.35 (2H, d, J = 6.0 Hz, ArH), 7.45 (1H, br s, NH); LCMS 827.6 ($[M + 1]^+$). Anal. ($C_{42}H_{54}N_{10}O_8$) C, H, N.

1-((6-((2,3-Dihydroxypropyl)amino)-4,8-bis((3,4-dimethoxybenzyl)amino)pyrimido[5,4-*d*]pyrimidin-2-yl)amino)propan-2-yl Phenylcarbamate (54). A solution of **52** (0.42 g, 0.54 mmol) in TFA (12 mL) containing water (0.05 mL) was stirred at room temperature for 3 h. The solvent was removed under reduced pressure, and the product was purified by chromatography on silica. Yellow solid; IR 3327, 2935, 2837, 1686, 1568, 1510 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.21 (3H, d, J = 6.30 Hz, Me), 3.50 (4H, m, $NHCH_2$), 3.71 (2H, s, CH_2OH), 3.74 (12H, s, OMe), 3.79 (1H, m, −CHOCO), 4.50 (4H, m, $NHCH_2Ar$), 6.69 (2H, d, ArH), 6.80 (4H, t, ArH), 6.93 (1H, t, J = 7.20 Hz, ArH), 7.16 (2H, t, J = 7.20 Hz, ArH), 7.27 (2H, d, ArH), 7.80 (1H, brs, NH); LCMS 744.15 ($[M + 1]^+$). Anal. ($C_{37}H_{45}N_9O_8$) C, H, N.

[^{3}H]Thymidine (TdR) Incorporation Assays. Pemetrexed, a kind gift from Eli Lilly and Co., Indianapolis, IN, was prepared as a solution in water. Thymidine (TdR), hypoxanthine (HPX), and dipyridamole (DP, 3) were dissolved in water, 0.1 M NaOH, and 100% DMSO, respectively. Stock solutions of pyrimidopyrimidines for evaluation were prepared in DMSO. ^{3}H-TdR (final specific activity 6.2 GBq/mmol) and [^{14}C]sucrose (final specific activity 7.4 GBq/mmol) were obtained from Amersham International (Amersham International, Amersham, U.K.). ^{3}H-TdR incorporation into COR-L23 cells in logarithmic phase growth was determined at a concentration of 1 μM ^{3}H-TdR for 2 h, in the presence or absence of inhibitor as described previously.[37] Briefly, COR-L23 cells were harvested and seeded at 2 × 10^4 cells/well in 100 μL of medium in 96-well plates and left overnight. The medium was removed, the cells were washed with phosphate-buffered saline (PBS), and 80 μL of serum-free medium was supplemented with **3** or a novel TdR transport inhibitor at 2 μM was added (1 μM final concentration). Twenty microliters of 500 nM ^{3}H-TdR (final concentration 100 nM with specific activity 6.2 Gbq/mmol) was added into each well and incubated at 37 °C for 2 h. After 2 h exposure, the medium was aspirated, and the cells were washed twice with ice-cold PBS and solubilized with 50 μL of 0.5 M NaOH for 30 min. The content of each well was transferred to glass fiber filters (Flow Laboratories Ltd., Scotland) using the Titertek cell harvester apparatus (Flow Laboratories) by sequentially rinsing with 10% (w/v) trichloroacetic acid for 5 s, H_2O for 20 s, and MeOH for 10 s before air-drying for 10 s. The filters air-dried were placed in scintillation vials containing Optiphase Hisafe 2 scintillation fluid (Pharmacia Wallac, 4.0 mL). The samples were counted on a Wallac 1410 liquid scintillation counter (Pharmacia Wallac), with 20 μL of 500 nM ^{3}H-TdR (final concentration = 100 nM, specific activity 6.2 Gbq/mmol) employed as a standard for concentration calculations.

Growth Inhibition Assays. COR-L23 lung cancer cells (a gift from the late Dr. P. Twentyman, MRC Clinical Oncology and Radiotherapeutics Unit, Cambridge, U.K.) were grown in RPMI 1640 medium supplemented with 1000 units/mL penicillin, 100 μg/mL streptomycin (Gibco, Paisley, U.K.), and 10% (v/v) fetal calf serum. Cells were maintained as exponentially growing cultures. Logarithmically growing COR-L23 cells were seeded at 1 × 10^3 cells in 100 μL of medium/well in 96-well plates, and after being allowed to attach overnight the medium was replaced with that containing 200 nM pemetrexed (approximate GI_{50}) in the presence or absence of 1 μM TdR, 10 μM HPX, and 1% (v/v) DMSO, with or without 1 μM inhibitor, for three cell doublings. Cells were then fixed, and cell growth was measured by the SRB assay. Ability to reverse TdR and hypoxanthine rescue was expressed as a percentage of reversal using the formula:

$$\%\ \text{reversal} = [(\text{growth arrest caused by pemetrexed in the presence of TdR, HPX, and inhibitor})/(\text{growth arrest caused by pemetrexed alone})] \times 100$$

■ ASSOCIATED CONTENT

Supporting Information. Synthetic and analytical data for compounds **10**, **11**, **14**−**26**, **29**, **30**, **35**−**37**, **41**−**46**, **50**−**53**, and **55**; elemental analyses for all target compounds; nucleoside-inhibition data for Table 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

ENTs, equilibrative nucleoside transporters; CNTs, concentrative nucleoside transporters; NBMPR, 4-nitrobenzylmercaptopurine riboside; NT, nucleoside−nucleobase transport; PTX, pemetrexed; DP, dipyridamole; AGP, α_1-acid glycoprotein; TdR, thymidine; HPX, hypoxanthine.

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