Brief Articles

Structural Studies on Bioactive Compounds. 39.[1] Biological Consequences of the Structural Modification of DHFR-Inhibitory 2,4-Diamino-6-(4-substituted benzylamino-3-nitrophenyl)-6-ethylpyrimidines ('benzoprims')

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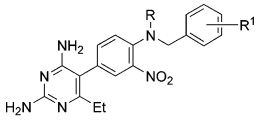
Benzimidazole-*N*-oxide modifications of potent lipophilic dihydrofolate reductase (DHFR) inhibitors (e.g., methylbenzoprim **1** and dichlorobenzoprim **2**) have been prepared by base-promoted cyclization of the nitrophenylbenzylamino groups to explore the possibility that abrogation of DHFR-inhibitory activity might reveal clues to an alternative anti-ras mechanism. Examples of the new series had only low growth inhibitory activities (GI_{50} generally >50 μM) against colon HCT116 and lung HT29 cell lines but, unlike methylbenzoprim, this activity was unaffected by hypoxanthine/thymidine rescue.

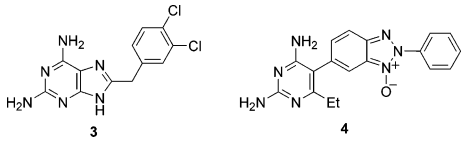
Introduction

The nonclassical antifolate 2,4-diamino-5-(4-(*N*-methylbenzylamino)-3-nitrophenyl)-6-ethylpyrimidine (**1**, methylbenzoprim) is representative of a family of potent lipophilic dihydrofolate reductase (DHFR) inhibitors ('benzoprims') with spectacular antitumor activity against the methotrexate (MTX)-resistant mouse M5076 reticulum cell sarcoma.[2] Recently we have reported that **1** and related structures such as the dichlorobenzylamino analogue (**2**: dichlorobenzoprim) (I_{50} for rat liver DHFR = 0.008 μM) demonstrate selective in vitro growth-inhibitory activities in cell lines of the non small cell lung (NSCL) and colon cancer subpanels of the National Cancer Institute (NCI) 60-cell panel which harbor mutations in codon 12 of the Ki-*ras* gene.[3] Significant Pearson correlation coefficients (PCCs) for methylbenzoprim (0.77) and dichlorobenzoprim (0.79) in the NCI COMPARE algorithm[4,5] link the GI_{50} values of these compounds with the *ras* status (wild or mutated) of NSCL and colon cancer cell lines. Moreover, compound **2** has potent inhibitory activity against the CCRF−CEM human T-cell leukemia growing in athymic mice: this tumor bears a mutation in codon 12 of the Ki-*ras* 2 gene (Gly to Asp) and is intrinsically resistant to MTX.[3]



1: R = Me, R^1 = H (methylbenzoprim)
2: R = H, R^1 = 3,4-di-Cl (dichlorobenzoprim)

Other observers [6] have concluded that lipophilic 2,4-diaminopyrimidine DHFR inhibitors might have an alternative nonfolate mechanism. Gangjee and colleagues[7] reported that 2,6-diamino-8-(3,4-dichlorobenzyl)purine **3**, which shares the 2,4-diamino-1,3-diazine motif with dichlorobenzoprim, and is only a weak inhibitor of rat liver DHFR (I_{50} 252 μM), showed GI_{50} values of <1 μM for growth inhibition of an unspecified "17 tumor cell lines." Furthermore, the benzotriazolium oxide **4**, which might be considered as a cyclic variant



3 **4**

of the benzoprim family,[8] is also a relatively weak inhibitor of rat liver DHFR (I_{50} = 2.7 μM) but gives a highly significant PCC of 0.72 relating its in vitro activity fingerprint to the Ki-*ras* status of NSCL and colon tumor cell lines.[3]

Because inhibition of DHFR has such a profound effect on cellular functions in vitro and in vivo,[9] design of experiments to explore if new 2,4-diaminopyrimidines might perturb other cancer-relevant molecular targets relies on reversal experiments applying exogenously the products of the inhibited folate mechanism. In a medicinal chemistry approach to address this question we have redesigned both substructures of the benzoprim pharmacophore to ablate DHFR-inhibitory potency: (a) by effecting a base-promoted intramolecular cyclization between the *o*-nitrophenyl substituent and the benzylamino residues to generate benzimidazole-*N*-oxides,[10] analogous in structure to the lead benzotriazole-*N*-oxide **4**; and (b) by removing the 4-amino and 6-ethyl groups to furnish 4-desaminobenzoprims of reduced basicity and lipophilicity compared to the lead benzoprims. Both these features are required for tight binding inhibition of DHFR.[11]

Chemistry

Several of the benzoprims required for this work have been reported in our earlier work from the interaction

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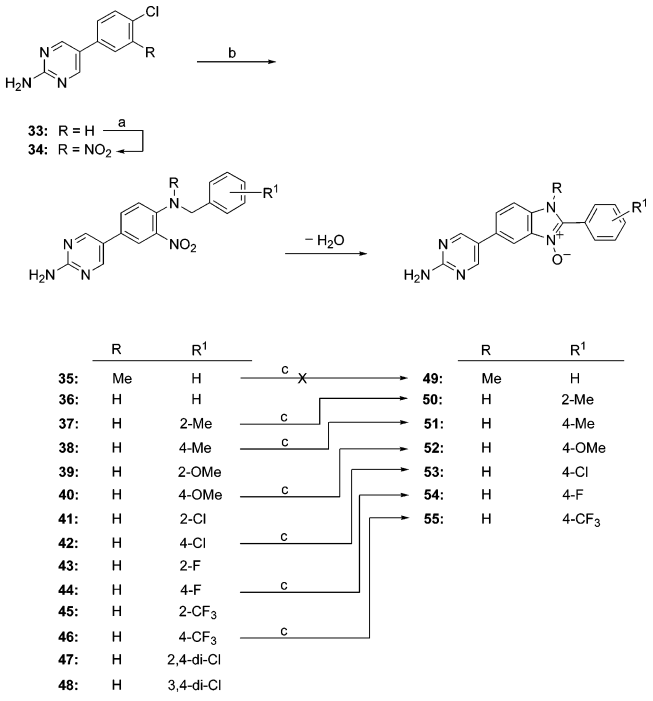
Table 1. Yields[a] and Melting Points[b] of 2,4-Diamino-5-(4-substituted benzylamino-3-nitrophenyl)-6-ethylpyrimidines, 2-Amino-5-(4-substituted benzylamino-3-nitrophenyl)pyrimidines, 5-(2,4-Diamino-6-ethylpyrimidin-5-yl)-2-(substituted phenyl)-1*H*-benzimidazole-3-oxides, and 5-(2-Aminopyrimidin-5-yl)-2-(substituted phenyl)-1*H*-benzimidazole-3-oxides

compound	yield (%)	mp (°C)	compound	yield (%)	mp (°C)
7	45	260	**36**	77	233
9	44	226	**37**	76	247
13	38	256	**38**	91	254
15	40	234	**39**	63	192
17	69	219	**40**	82	229
19	75	191[d]	**41**	60	229
20	92	273[d]	**42**	84	285
21	75	195[d]	**43**	67	211
22	95	212[d]	**44**	83	241
23	60	174[d]	**45**	71	225
24	98	174[d]	**46**	55	221
25	61	246[d]	**47**	66	249
26	65	262[d]	**48**	77	240
27	54	256[d]	**50**	81	290[d]
28	99[c]	232[d]	**51**	96	302[d]
29	72	184[d]	**52**	89	271[d]
30	61[c]	190[d]	**53**	95	302[d]
31	61	187	**54**	89	301[d]
35	84	216	**55**	77	220[d]

[a] For details of synthetic method, see Experimental Section. [b] For details of other physical data (IR, ^{1}H NMR, ^{13}C NMR, and mass spectra), see Supporting Information. [c] DMSO solvent used in synthesis. [d] Melts with decomposition.

of 2,4-diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine (**5**; 'nitropyrimethamine') and substituted benzylamines.[2] Yields and melting points of new benzoprims, 4-desaminobenzoprims, and their benzimidazole-*N*-oxide cyclization products are listed in Table 1. The model compound *N*-benzyl-*N*-methyl-2-*o*-nitroaniline does not cyclize in base[12] and attempts to cyclize methylbenzoprim **1** to the corresponding benzimidazole oxide **18** using inorganic bases NaOH or K_2CO_3 in hot 2-ethoxyethanol or DMSO were similarly unsuccessful. In contrast the benzylamines **2**, **6**−**17** all cyclized to benzimidazole oxides **19**−**31** (Scheme 1) in yields > 60% (Table 1). Generally, cyclization was complete within 2 h with NaOH but required 5 h using K_2CO_3. Attempts to prepare the benzimidazole oxides **28** and **30** in 2-ethoxyethanol containing K_2CO_3 directly from **5**, and the more weakly basic benzylamines bearing powerful electron-withdrawing F or CF_3 groups led to the isolation of the ether **32** (~50%), also obtained (65%) when nitropyrimethamine was boiled in 2-ethoxyethanol containing base. To avoid the possibility that the substituted benzylamino groups of **14** and **16** might suffer nucleophilic displacement under cyclization conditions in 2-ethoxyethanol, compounds **28** and **30** were most efficiently synthesized using NaOH in DMSO as solvent.

Scheme 2[a]

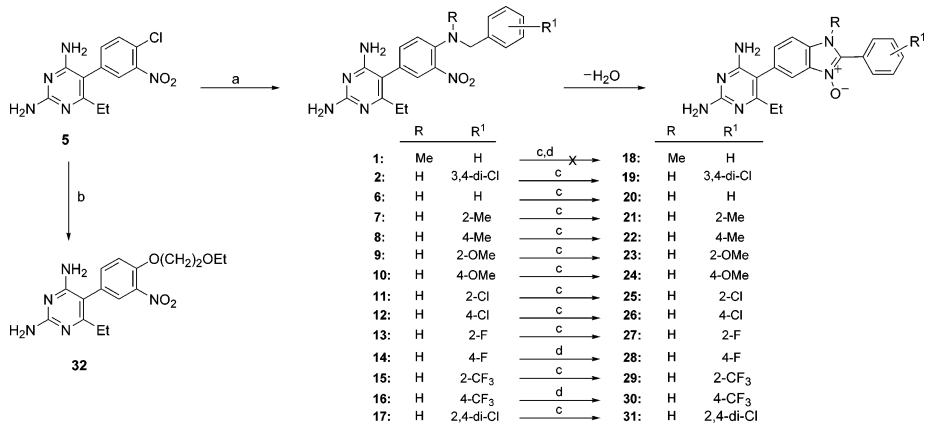


[a] Reagents and conditions: (a) $HNO_3-H_2SO_4$; (b) 50 °C, 2 h; **34**, substituted benzylamine, reflux, 5 h; (c) NaOH in 2-ethoxyethanol, reflux 5 h, then neutralize with HCl.

The starting point for the synthesis of 4-desaminobenzoprims was 2-amino-5-(4-chlorophenyl)pyrimidine **33** which we have prepared recently by a Suzuki reaction between 2-amino-5-bromopyrimidine and 4-chlorobenzeneboronic acid.[13] Nitration of **33** in a $HNO_3-H_2SO_4$ mixture at 45−50 °C gave 2-amino-5-(4-chloro-3-nitrophenyl)pyrimidine **34** which was reacted with neat benzylamines to furnish the new series **35**−**48**. Representative samples were then cyclized by base to afford the oxides **50**−**55** (Scheme 2 and Table 1). Again, the *N*-methylbenzylamine **35** could not be cyclized to a benzimidazole-*N*-oxide, **49**.

Unlike the highly crystalline orange-red benzoprims,

Scheme 1[a]



[a] Reagents and conditions: (a) substituted benzylamine, reflux, 5 h; (b) K_2CO_3 in 2-ethoxyethanol, reflux, 42 h; (c) NaOH in 2-ethoxyethanol, reflux 5 h, then neutralize with HCl; (d) NaOH in DMSO, 80 °C, 2 h, then neutralize with HCl.

Table 2. GI_{50} Values (μM)[a] of Methotrexate (Mtx), Trimetrexate (TMX), Methylbenzoprim (**1**), Nitropyrimethamine (**5**), and Novel Compounds against Colon HCT-116 and HT29 Cells ± Hypoxanthine-Thymidine (HT) Rescue[b]

compound	HCT116	HCT116 + HT	HT29	HT29 + HT
MTX	0.01	>50	0.07	>50
TMX	0.01	19	0.01	21
1	0.08	7.4	0.48	9.2
5	0.25	38	3.8	40
9	0.08	7.5	1.5	11.6
26	9.0	9.0	>50	>50
28	17	22	46	39
34	>50	>50	>50	>50
35	>50	>50	14	17
42	3	5	17	25
44	48	47	41	38
50	>50	>50	>50	>50
51	>50	>50	>50	>50
53	7	9	17	15

[a] MTT assays were carried out following 4 days exposure to increasing concentrations of drug. Results are the mean of three experiments. [b] H = hypoxanthine (100 μM); T = thymidine (20 μM).

the benzimidazole-*N*-oxides were off-white solids with a chalky consistency which decomposed on attempted crystallization. The compounds could be purified by precipitation from DMSO with water and, like many 2,4-diaminopyrimidines, did not give reliable elemental analyses because of partial hydration. Compounds were characterized by NMR and HRMS analysis. For example, in the ^{1}H NMR spectra the benzylic protons of the benzoprims (at ~δ 4.5−4.6) are generally coupled to the benzylamine NH protons: these features are absent in the spectra of the oxides (see Supporting Information).

Biological Results and Discussion

Growth-Inhibitory Activity of Compounds against Human Colon Cell Lines in Vitro. The inhibitory effects (GI_{50} values) of several new compounds were measured against the colon cell lines HCT-116, which bears a GGC (gly) to GAC (asp) mutation in Ki-*ras* codon 13, and HT29 with wild type *ras*, in a 4-day MTT assay. To ascertain if inhibitory mechanisms differed from those of the classical DHFR inhibitor methotrexate (MTX) and the lipophilic antifolate trimetrexate (TMX), methylbenzoprim **1**, nitropyrimethamine **5**, and several new compounds were also evaluated in the presence/absence of hypoxanthine-thymidine (HT) rescue (Table 2). Overall there was a 5000-fold range in potencies, and compounds were more growth-inhibitory toward the mutated HCT-116 line. The most active agents were MTX and TMX. Of the compounds with an *o*-nitro substituent, the most growth-inhibitory against HCT-116 were the lipophilic antifolates methylbenzoprim **1**, nitropyrimethamine **5**, and the novel 2-methoxy-substituted benzoprim **9**, a close analogue of **1**. Removal of the 4-amino and 6-ethyl groups in compounds **34**, **35**, **44** had a significant dyschemotherapeutic effect in comparison to the foregoing agents with only **42** showing activity in the low micromolar range. Similarly, compounds bearing the benzimidazole-*N*-oxide variation **26**, **28**, **50**−**51** displayed only weak growth-inhibitory properties against both cell lines with **53** being the most active but still approximately 100-fold less potent than methylbenzoprim **1**.

The fact that the antitumor activity of methylbenzoprim **1**, unlike that of MTX, cannot be reversed completely by HT rescue has suggested to us that methylbenzoprim may have an alternative non-folate mechanism.[3] Data for the 2-methoxy-derivative **9** showed a similar partial rescue by HT (Table 2). In contrast the GI_{50} values against HCT-116 and HT29 for all the new compounds, in their acyclic (**34**, **35**, **42**, and **44**) or cyclic modifications (**26**, **28**, **50**, **51,** and **53**), were uninfluenced by HT. Specimen dose−response curves for methylbenzoprim **1** and three compounds (**26**, **42**, and **53**) representative of the new structural types against HCT-116 cells ± HT rescue are included in Supporting Information. These data confirm that the chosen structural modifications abrogate DHFR-inhibitory properties leading to loss of growth-inhibitory activity but do not reveal clues to a novel cancer-relevant mechanism. In corroboration, flow cytometric analysis of HCT-116 and HT29 cells treated with compounds **26**, **42**, and **53** at their respective GI_{50} values (from Table 2) revealed no significant cell cycle perturbations (data not shown).

Experimental Section

Chemical Synthesis. Melting points (uncorrected) were measured using a Gallenkamp melting point apparatus.

2,4-Diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine (5; nitropyrimethamine). This compound was prepared by a published method.[14]

2-Amino-5-(4-chloro-3-nitrophenyl)pyrimidine (34). 2-Amino-5-(4-chlorophenyl)pyrimidine (2.402 g, 11.7 mmol)[13] was added to a stirred mixture of nitric (*d*, 1.42, 10 mL) and sulfuric acid (10 mL) over 20 min with the temperature maintained below 50 °C. The yellow syruplike solution was heated at 45−50 °C for 2 h and then stirred at room temperature for 1.5 h. The mixture was poured slowly into an ice-concentrated ammonia mixture (to >pH 10). The pale yellow product (84%) was collected, washed with water, and dried at 40 °C. The pyrimidine recrystallized from 50% ethanol as yellow crystals, mp 260 °C. Anal. Calcd for $C_{10}H_7ClN_4O_2$: C, H, N.

General Method for the Preparation of 2,4-Diamino-5-(4-substituted benzylamino-3-nitrophenyl)-6-ethylpyrimidines and 2-Amino-5-(4-substituted benzylamino-3-nitrophenyl)pyrimidines. A suspension of 2,4-diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine (10.0 g, 34 mmol)[14] or 2-amino-5-(4-chloro-3-nitro-phenyl)pyrimidine (8.52 g, 34 mmol) in the appropriate pure benzylamine (30 mL) was heated under reflux for 5 h. The red colored solutions were cooled and diluted with water (for water-miscible amines) or ether (for water immiscible amines) to liberate the required compounds. All products were recrystallized from aqueous 2-ethoxyethanol. Compounds **1**, **2**, **6**, **8**, **10**−**12**, **14**, and **16** have been prepared previously.[2] Yields and melting-points of new compounds are assembled in Table 1. IR, ^{1}H NMR, ^{13}C NMR, and mass spectroscopic data are included in Supporting Information.

General Method for the Synthesis of 5-(2,4-Diamino-6-ethylpyrimidin-5-yl)-2-(substituted phenyl)-1*H*-benzimidazole-3-oxides and 5-(2-Aminopyrimidin-5-yl)-2-(substituted phenyl)-1*H*-benzimidazole-3-oxides. 2,4-Diamino-5-(4-substituted benzylamino-3-nitrophenyl)-6-ethylpyrimidine (0.8 mmol) or 2-amino-5-(4-substituted benzylamino-3-nitrophenyl)pyrimidine (0.8 mmol) and sodium hydroxide (8 mmol) were heated under reflux in 2-ethoxyethanol (5 mL) for 5 h. On cooling, the reaction mixture was diluted with water and neutralized with 0.5 M HCl. The precipitated product was collected by filtration and dried. Yields and melting-points of new compounds are assembled in Table 1. IR, ^{1}H NMR, ^{13}C NMR, and mass spectroscopic data are included in Supporting Information.

2,4-Diamino-5-(4-ethoxyethyloxy-3-nitrophenyl)-6-ethylpyrimidine (32). (i) 2,4-Diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine (1.005 g, 3.42 mmol), potassium carbonate

(0.95 g, 6.84 mmol), and 2-ethoxyethanol (10 mL) were heated under reflux for 42 h. The reaction mixture was cooled to room temperature and neutralized with 0.5 M HCl. The yellow solid was collected by filtration and dried (65.2%), mp 206−207 °C.

(ii) 2,4-Diamino-5-(4-chloro-3-nitrophenyl)-6-ethylpyrimidine (1.00 g; 3.42 mmol), 4-fluorobenzylamine [or 4-(trifluoromethyl)benzylamine] (3.42 mmol), and potassium carbonate (0.95 g; 6.84 mmol) were heated under reflux in 2-ethoxyethanol (10 mL) for 60 h. The reaction mixture was cooled to room temperature and then neutralized with 0.5 M HCl. The precipitate which formed was collected by filtration and dried (50%) and was identical (IR, ^{1}H NMR) to the sample of 2,4-diamino-5-(4-ethoxyethyloxy-3-nitrophenyl)-6-ethylpyrimidine **32**, prepared above.

Biological Investigations. Drug Solutions. Drug stock solutions were all made up to10 mM with compounds dissolved in the following vehicles: MTX, TMX, methylbenzoprim (MBP), and all new compounds in DMSO; hypoxanthine (H) in 10 mM NaOH; thymidine (T) in distilled water. All solutions were sterile filtered and stored at −20 °C.

Cell Culture. Colon HCT-116 (ATCC No. CCL-247) and HT29 (ATCC No. HTB-38) cell lines were cultured in RPMI 1640 medium with L-glutamine (Gibco BRL Life Technologies) supplemented with 10% heat-inactivated (55 °C for 1 h) fetal bovine serum (FBS). All cells were maintained at 37 °C in an atmosphere of 5% CO_2 and 95% air. Cells were mycoplasma free and passaged 2−3 times weekly; the medium aspirated off, 1−2 mL of trypsin/EDTA added and left for 2−5 min until the cells had become detached before addition of 5 mL medium to inactivate the trypsin. Cells were split into T25 flasks (Costar) containing 7−10 mL medium at the desired concentration. Cells were maintained in a Leec incubator and all cell culture processes were carried out in a MDH class II microbiological safety cabinet with a laminar flow system swabbed before each use with 70% ethanol in distilled water.

Growth Inhibition Assays. Following trypsinisation cells were placed in 5 mL of nutrient medium and syringed to remove cell clumps. Cells were counted using a Neubauer haemocytometer and plated in 96-well plates (Nunclon) at appropriate densities for the rate of cell division and the duration of the experiment. Cells were allowed to attach (4 to 24 h) prior to addition of drug to the appropriate concentration bringing the final volume in each well up to 200 μL. Where present H was added to a final concentration of 100 μM and T to 20 μM. A time zero plate was made up to 200 μL in each well by the addition of medium to provide the initial optical density reading. Following incubation at 37 °C in an atmosphere of 5% CO_2 and 95% air for the duration of the experiment (4 days), 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 2 mg/mL in PBS) was added to each well containing 200 μL of medium. Following a 4 h incubation period, the MTT/medium was aspirated from each well and 125 μL of DMSO:glycine buffer (4:1) was added to each well. The plates were then shaken to dissolve all the formazan crystals formed and read at 550 nm on an Anthos plate reader (Anthos Labtech Instruments). Results, expressed as GI_{50} values, were analyzed using the Deltasoft 3 computer program (BioMetallics Inc., PO Box 2251, Princetown, NJ 08543).

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Supporting Information Available: C, H, N analyses for compound **34**; IR, ^{1}H NMR, ^{13}C NMR, and mass spectra for compounds **7**, **9**, **13**, **15**, **17**, **19-32**, **34-48**, **50-55**; dose−response curves for compounds **1**, **26**, **42**, **53** against human HCT-116 cells in the absence or presence of hypoxanthine (H) and thymidine (T) rescue. This material is available free of charge via the Internet at http://pubs.acs.org.

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