Biochemical Pharmacology, Vol. 40, No. 3, pp. 615–619, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

INHIBITION OF NUCLEOSIDE TRANSPORT BY NITROBENZYLTHIOFORMYCIN ANALOGS

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(*Received* 10 *November* 1987; *accepted* 16 *January* 1990)

Abstract—The formycin analogs of nitrobenzylthioinosine and nitrobenzylthioguanosine were synthesized and evaluated as nucleoside transport inhibitors. These analogs have a potential therapeutic advantage over their parent compounds in that their *C*-nucleosidic linkages prevent them from being degraded to the immunosuppressive agents, 6-mercaptopurine and 6-thioguanine. 7-[(4-Nitrobenzyl)-thio]-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine (NBTF) and 5-amino-7-[(4-nitrobenzyl)thio]-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine (NBTGF) were inhibitors of nucleoside transport in human erythrocytes and HL-60 leukemia cells. The IC_{50} values for nitrobenzylthioinosine, NBTF and NBTGF with 10% erythrocyte suspensions were 18, 18 and 40 nM respectively. Specific binding studies with [^{3}H]NBTF yielded a K_d of 3.4 nM with erythrocytes, approximately 10-fold higher than values reported for nitrobenzylthioinosine. NBTF and nitrobenzylthioinosine bound to HL-60 cells with K_d values of 8.1 and 0.81 nM respectively. The octanol/water partition coefficients of nitrobenzylthioinosine, NBTF and NBTGF were 3.5, 3.2, and 2.8 respectively. NBTF could be expected to be equipotent with nitrobenzylthioinosine in whole blood where inhibitor concentrations of 10^{-7} to 10^{-6} M are required in order to saturate erythrocytic binding sites; hence, it may exhibit the advantages inherent in a *C*-nucleoside.

The formycin analogs of nitrobenzylthioinosine and nitrobenzylthioguanosine have been designed as specific nucleoside transport inhibitors that could have therapeutic advantages over their parent compounds. Nitrobenzylthioinosine and nitrobenzylthioguanosine are highly potent and specific inhibitors of the carrier-mediated diffusion of nucleosides in mammalian erythrocytes [1, 2], leukocytes [3], and many freshly isolated and cultured tumor cells [3–7]. Radiolabeled nitrobenzylthioinosine has been of value in assessing the density of nucleoside carriers on cell surfaces [8, 9] and monitoring the purification of carrier protein [10]. Paterson and his colleagues demonstrated that nitrobenzylthioinosine, or its more soluble 5′-monophosphate form, can serve to protect the host and increase the therapeutic indices of cytotoxic nucleosides such as nebularine and tubercidin against murine tumours [11–13]. Using a similar approach, el Kouni *et al.* [14, 15] demonstrated host protection in the treatment of mice for schistosomal infections.

Although nitrobenzylthioinosine is highly specific in its interaction with the nucleoside transport mechanism, the *p*-nitrobenzyl moiety is labile and 6-thioinosine may be liberated. This analog is readily cleaved by the action of purine nucleoside phosphorylase [16] to yield the immunosuppressive [17] and mutagenic [18] purine base, 6-mercaptopurine. The toxicity of nitrobenzylthioinosine in mice and its potentiation of the antitumor effect of cytosine arabinoside against L1210 cells *in vivo*, when fifteen injections of 100 mg/kg were administered at 8-hr intervals, have been attributed to this mechanism [19]. Although toxicity from nitrobenzylthioinosine has not been observed at the lower doses used in more recent studies [12, 13], potential mutagenic effects during long-term treatment remain a concern.

Nucleosides with a formycin structure possess a C—C bond between the base and ribose moieties. Since this renders them fully resistant to phosphorolytic cleavage [16], they cannot give rise to base analogs. Nitrobenzylthioformycin (NBTF¶) and nitrobenzylthioguanine formycin (NBTGF), which are shown in Fig. 1, were designed as specific, phosphorylase-resistant nucleoside transport inhibitors. The present report describes the syntheses of NBTF and NBTGF and their affinities for the non-concentrative nucleoside carrier systems of human erythrocytes and HL-60 leukemia cells.

MATERIALS AND METHODS

Materials

The syntheses of NBTF and NBTGF are described

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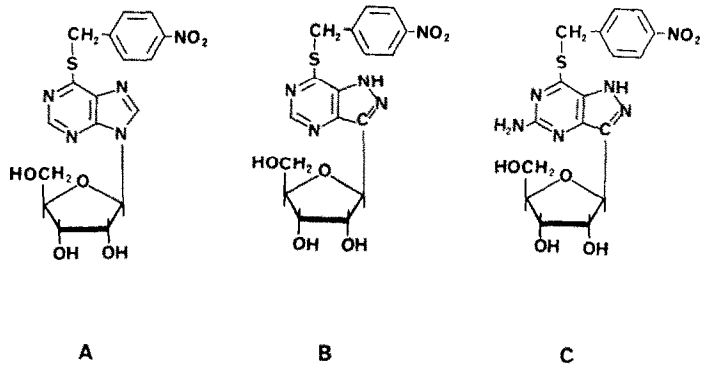
¶ Abbreviations: NBTF, nitrobenzylthioformycin, 7-[(4-nitrobenzyl)thio] - 3 - (β-D-ribofuranosyl)pyrazolo[4,3-d]pyrimidine; NBTGF, nitrobenzylthioguanine formycin, 5 - amino - 7 - [(4 - nitrobenzyl)thio] - 3 - (β - D - ribofuranosyl)pyrazolo[4,3-d]pyrimidine; nitrobenzylthioinosine, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine, also called NBMPR; and Hepes, N-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.



A B C

Fig. 1. Structures of the transport inhibitors (A) nitrobenzylthioinosine, (B) nitrobenzylthioformycin (NBTF), and (C) nitrobenzylthioguanine formycin (NBTGF).

below. [^{3}H]NBTF (13 mCi/μmol) was prepared by Moravek Biochemicals (Brea, CA). Nitrobenzylthioinosine was a gift from A. R. P. Paterson, of the University of Alberta, Edmonton, Alberta, Canada; dilazep was provided by M. Gudenzi, Asta-Werke, AG, Frankfurt/Main, Federal Republic of Germany; and 6-(2-hydroxy-5-nitrobenzyl)thioguanosine was purchased from CalBiochem (San Diego, CA). [U-^{14}C]Adenosine (300 mCi/mmol) was a product of ICN Pharmaceuticals (Irvine, CA). All cell culture materials were obtained from GIBCO Laboratories (Grand Island, NY).

Human erythrocytes from healthy volunteers were washed and suspended in Hanks' balanced salt solution as described [20]. HL-60 cells, originally derived from a patient with acute promyelocytic leukemia [21], were cultured as reported previously [7]. Logarithmically growing cells of unknown passage number were washed and suspended in RPMI 1640 medium buffered with 20 mM Hepes-NaOH, pH 7.2. HL-60 cells were counted on a hemacytometer.

Chemical syntheses

NBTF was synthesized according to a standard procedure, by treating 7-thioformycin B [22] in aqueous ammonium hydroxide solution with *p*-nitrobenzyl bromide. The melting point was determined on a Gallenkamp apparatus and is uncorrected. The UV absorption spectrum was measured on a Perkin–Elmer model 401 spectrophotometer and the ^{1}H-NMR spectrum on a Bruker WM-250 instrument in DMSO-d_6, using trimethylsilane as an internal standard. Analyses were performed by the Baron Consulting Co., Orange, CT, and by Galbraith Laboratories, Knoxville, TN. NBTGF was synthesized from the thioguanosine analog of the formycin series [23].

7 - [(4-*Nitrobenzyl)thio]*-3-(*β*-D-*ribofuranosyl)-pyrazolo*[4,3-d]*pyrimidine (NBTF)*. 3-(*β*-D-Ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine-7-thione [22] (thioformycin B), 0.50 g (1.76 mmol) was dissolved in 4.5 mL of water and 0.4 mL of concentrated ammonium hydroxide. To this solution was added 0.45 g (2 mmol) of *p*-nitrobenzyl bromide in 22 mL of methanol, and the mixture was stirred overnight at room temperature. The solvent was evaporated to dryness under reduced pressure. The residue was dissolved in 6 mL of ethanol and applied to preparative TLC plates (1.5 mL/200 mm band width) and developed once with methylene chloride–ethanol (40:3). The low R_f band which contained the product (UV) was eluted with methanol. The residue of this solution was redissolved in ethanol, replated, and developed with methylene chloride–ethanol (6:1). Elution of the higher R_f band yielded 153 mg of analytically pure but partly amorphous material (21%). Recrystallization from a small amount of ethanol gave a pale yellow crystalline solid, m.p. 181–182°. UV (EtOH): λ_{max} 275 nm (ε 13,900); λ_{max} 315 (16,100), with shoulders at 305 (14,600) and 325 (13,300). NMR (DMSO-d_6): δ 3.36 (br.s-m, 2'-, 3'- and 5'-OH and H_2O), 3.48–3.70 (ddd, 2H, C_5—H, $J_{4',5'a}$ = 3 Hz, $J_{4',5'b}$ = 3 Hz, J_{gem} = 8 Hz), 3.93 (dt, 1H, C_4—H, $J_{3',4'}$ = 3 Hz, $J_{4',5'}$ = 3 Hz), 4.57 (t-dd, 1H, C_2—H, $J_{1',2'}$ = 5 Hz, $J_{2',3'}$ = 3 Hz), 4.14 (t, 1H, C_3—H, $J_{2',3'}$ = 3 Hz, $J_{3',4'}$ = 3 Hz), 4.88 (s, 2H, CH_2 of p-NO_2Bzl), 5.00 (br.s, 1H, N_1—H), 5.06 (d, 1H, C_1—H, $J_{1',2'}$ = 5 Hz), 7.79 (d, 2H, m-H of NO_2Bzl, $J_{o,m}$ = 6 Hz), 8.20 (d, 2H, o-H of NO_2Bzl, $J_{o,m}$ = 6 Hz) 8.80 (s, 1H, C_5—H). Anal. calc. for $C_{17}H_{17}N_5O_6$: C, 48.68; H, 4.09; N, 16.70; S, 7.65. Found: C, 48.83; H, 3.91; N, 16.51; S, 7.38.

5 - *Amino* - 7 - [(4 - *nitrobenzyl)thio]*-3-(*β*-D-*ribofuranosyl)pyrazolo*[4,3-d]*pyrimidine (NBTGF)*. The formycin analog of thioguanosine [23] (20 mg, 0.067 mmol) was dissolved in a solution of 6.0 mg (0.11 mmol) of sodium methoxide in 10 mL of methanol. The solution was chilled, treated with 15 mg (0.069 mmol) of 4-nitrobenzylbromide, and stirred overnight at room temperature. It was neutralized with acetic acid to pH 6 and evaporated to dryness under vacuum. The residue was crystallized from hot water–methanol (10:1) to give 24 mg in two crops (82% yield), 99.9% pure by reverse-phase high pressure liquid chromatography on a C-8 column in 0.003 M KH_2PO_4–methanol (65:35). UV: (MeOH), λ_{max} 270 nm (ε 14,700), 351 (7200); (0.1 M HCl), λ_{max} 288 (20,500), sh 335; (pH 7), λ_{max} 273 (15,900), 347 (9400); (0.1 M NaOH), λ_{max} 292 (15,200), 352 (6200). NMR (DMSO-d_6): δ 8.21 d and 7.77 d (p-substituted C_6H_4), 5.03 d (H-1', J = 7.9 Hz), 4.66 s ($PhCH_2S$). Anal. calc. for $C_{17}H_{18}N_6O_6S \cdot H_2O$: C,

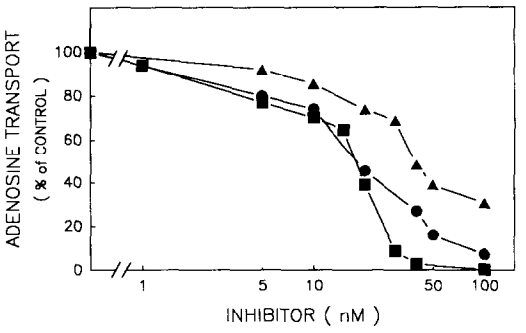


Fig. 2. Inhibition of adenosine transport in human erythrocytes by nitrobenzylthioinosine (■), NBTF (●), and NBTGF (▲). The 10% cell suspensions were preincubated with the inhibitors for 10 min before transport was measured at room temperature. The points shown are the means of results from three separate experiments. Control rates of adenosine influx were 34.1 ± 3.8 pmol/min per 10^6 cells, calculated on the basis of 1.17×10^8 cells/mL at a 10% hematocrit.

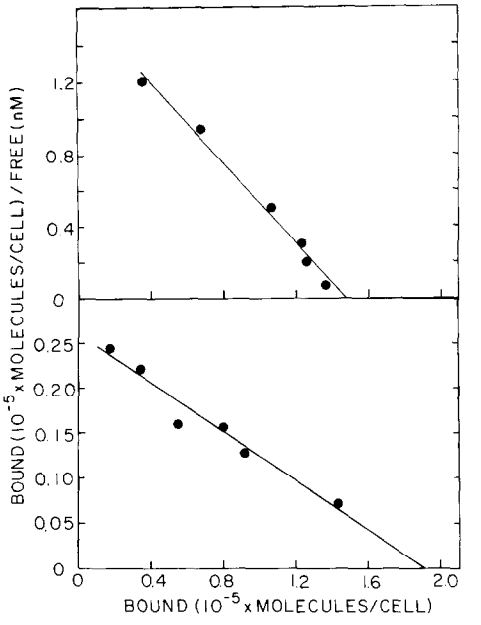


Fig. 3. High-affinity binding of tritiated nitrobenzylthioinosine (upper panel) and NBTF (lower panel) to HL-60 leukemia cells. The Scatchard plots shown yielded K_d values of 0.73 and 7.3 nM and B_{max} values of 140,000 and 190,000 sites/cell, respectively, for specific binding at 37°. The data from separate experiments gave K_d values of 0.81 (average of two) and 8.0 ± 0.9 (mean ± SD of three determinations) for nitrobenzylthioinosine and NBTF, with corresponding B_{max} values of 170,000 and 220,000 ± 30,000 sites/cell.

45.13; H, 4.46; N, 18.57. Found: C, 45.36; H, 4.71; N, 18.67.

Transport and binding assays

The influx of radiolabeled adenosine at room temperature was measured by the inhibitor-stop procedure [7]. Erythrocytes were preincubated with inhibitors for 10 min at room temperature. A 25 μL volume of permeant was added to 175 μL of the cell suspension to initiate influx. Final concentrations were: 10% cell suspension, 10 μM adenosine, and inhibitor concentrations, as shown in Fig. 2. Transport was stopped by the addition of 200 μL of ice-cold medium containing 750 μM dilazep, a potent, readily soluble transport inhibitor [24]. Influx was measured for 0.5 to 3.0 sec, and 0 time points were obtained by adding stop solution first. The initial rates of adenosine influx were not altered when 1 μM 2′-deoxycoformycin was used to prevent deamination (data not shown).

The specific binding of the radiolabeled transport inhibitors at 37° to intact erythrocytes and HL-60 cells was determined as previously described [7, 25]. The nonspecific binding curves were determined in the presence of a saturating concentration (5 μM) of nitrobenzylthioinosine or 6-(2-hydroxy-5-nitrobenzyl)thioguanosine, which were equally effective in eliminating the specific binding of NBTF.

RESULTS AND DISCUSSION

When tested as an inhibitor of adenosine transport into human erythrocytes, NBTF displayed potency similar to that of nitrobenzylthioinosine. Figure 2 shows identical apparent IC_{50} values of 18 nM for these two compounds, although the concentration–response curve for NBTF is less steep. The curve for NBTGF paralleled that for NBTF, but transport inhibition was somewhat weaker with an apparent IC_{50} of 40 nM. At 100 nM incubation concentrations, nitrobenzylthioinosine, NBTF, and NBTGF caused >99, >90 and 70% inhibition, respectively, of the transport of 10 μM adenosine into erythrocytes. The lower potency of NBTGF resembles the 2- to 3-fold lower potency of its parent compound, nitrobenzylthioguanosine, in relation to nitrobenzylthioinosine [26, 27].

The true affinities of radiolabeled NBTF and nitrobenzylthioinosine were determined from binding studies. K_d and B_{max} values of 3.4 ± 0.4 nM and 9500 ± 800 sites/cell (mean ± SD of three experiments) were determined for the high-affinity binding of NBTF to erythrocytes. This K_d is approximately 10-fold higher than the reported value of 0.31 ± 0.29 nM for nitrobenzylthioinosine, whereas the B_{max} value is close to the reported value of 8400 [9]. The affinities of both compounds were somewhat lower in HL-60 cells, as illustrated by the Scatchard plots in Fig. 3. The K_d values of 0.81 nM (average of two determinations) and 8.0 ± 0.9 nM for nitrobenzylthioinosine and NBTF, respectively, again showed a 10-fold difference between the compounds. The higher K_d value of 2–3 nM reported earlier for nitrobenzylthioinosine in HL-60 cells [7] resulted from the overestimation of nonspecific binding in the absence of competing ligands. B_{max} values for nitrobenzylthioinosine and NBTF were 170,000 and 220,000 ± 30,000 sites/cell, respectively, in the present study. Nucleoside transport capacity is subject to variation in HL-60 cells and decreases significantly during serum starvation [28] and differentiation [7].

The large discrepancy between the IC_{50} values and the affinity constants of nitrobenzylthioinosine and NBTF is due to depletion of the inhibitor from the medium by the cells. The apparent IC_{50} values are based on total inhibitor concentrations and are not corrected for loss of inhibitor due to its binding to the cells. In the studies with radiolabeled inhibitors, specific binding, i.e. binding to the nucleoside transport proteins, was the major cause of depletion with both compounds until the specific binding sites were saturated (data not shown). Accordingly, cell concentration accounts for the difference between the IC_{50} value of 18 nM for nitrobenzylthioinosine in the present study, which used 10% (v/v) cell suspensions, and the value of 35 nM reported earlier by this laboratory for experiments on deoxycoformycin transport into erythrocytes suspended at 20% concentrations [29].

The much steeper IC_{50} curve for nitrobenzylthioinosine indicates that the depletion of this inhibitor by the cells has shifted the apparent concentration–response curve to a concentration range that is more than 10-fold higher than the K_d. Lower IC_{50} values may be obtained if the concentration–response curves are based on the final free inhibitor concentration [30]. However, IC_{50} values based on total inhibitor concentration more closely reflect the concentrations required to stop transport *in vivo*.

The fact that nitrobenzylthioinosine and NBTF have similar efficacies indicates that the 10-fold lower K_d of the former compound offers no advantage. Inhibitor depletion by specific binding precludes either inhibitor from being active in the nanomolar concentration range in whole blood where, at a 45% hematocrit, the concentration of nucleoside transport sites can be estimated to be at least 7.5×10^{-8} M [31]. Thus, in whole blood, all transport inhibitors with K_d values in the 10^{-9} M range or lower could be equipotent unless they differed significantly with respect to their nonspecific binding characteristics or possessed unrelated specific binding sites. Octanol/water partition coefficients of 3.47 ± 0.28, 3.16 ± 0.21, and 2.77 were determined for nitrobenzylthioinosine, NBTF, and NBTGF, respectively, at room temperature with 0.1 M potassium phosphate, pH 7.4, as the aqueous phase. Since all are highly lipophilic, they may be expected to display similar pharmacokinetic properties.

The numbers of high-affinity binding sites on HL-60 cells for both agents are similar to the 240,000 sites/cell reported previously [7] for nitrobenzylthioinosine. Specific binding sites with either agent usually have ranged from 180,000 to 300,000/cell. However, HL-60 cells also possess a significant number of nitrobenzylthioinosine-insensitive nucleoside carriers [7, 32]. It was shown previously that, unlike erythrocytes, many cultured tumor cell lines possess a nitrobenzylthioinosine-insensitive component of nucleoside transport that may account for as little as 2% or as much as 100% of the influx of permeants [33]. The greater protection afforded by nitrobenzylthioinosine to normal human and murine bone marrow stem cells than to many tumor cells has been attributed to the larger nitrobenzylthioinosine-insensitive component in the malignant cells [32, 34]. Dipyridamole, a clinically useful vasodilator, which has a similar affinity for the human erythrocyte nucleoside transporter (apparent K_d 0.65 nM [35]) but a much higher IC_{50} [27], does inhibit nitrobenzylthioinosine-insensitive nucleoside influx [36, 37] and may, therefore, protect the tumor cells equally well. Thus, an inhibitor such as NBTF, which possesses the specificity of nitrobenzylthioinosine, would be preferable to dipyridamole when the aim is to increase the therapeutic index of a cytotoxic nucleoside by protecting normal marrow cells.

In conclusion, NBTF and NBTGF are potent and specific inhibitors of nucleoside transport in human erythrocytes and leukemia cells. Both formycin and-logs have lower IC_{50} values in 10% erythrocyte suspensions than those reported for dilazep and dipyridamole [27]. Loss of the nitrobenzyl substituent, as occurs *in vivo* with nitrobenzylthioinosine [19], would yield the parent thioformycin nucleosides, whose resistance to phosphorolysis prevents the generation of 6-thiopurines. The formycin analog of 6-thioguanosine was found to be inactive against cultured murine lymphoma cells when compared to the parent compound.* Therefore, these transport inhibitors are not likely to be myelosuppressive or mutagenic and may be superior protective agents for use in combination with toxic nucleoside analogs.

Acknowledgements—We thank Emily R. Lance for excellent technical assistance in the preliminary studies for this work. This research was supported by Grants CA 37901, 07340 and 47268 and Roger Williams Cancer Center Grants CA 20892 and 13943 awarded by the National Cancer Institute, N.I.H., DHHS. C. G. R. was a Fellow, Division of Pediatric Hematology/Oncology, Rhode Island Hospital.

* Robison BS, Stoeckler JD and Parks RE Jr, unpublished data.

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