In Vitro and in Vivo Properties of Novel Nucleoside Transport Inhibitors with Improved Pharmacological Properties That Potentiate Antifolate Activity\textsuperscript{1,2}

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ABSTRACT

The activity of antimetabolite inhibitors of de novo deoxynucleotide biosynthesis can be compromised by the salvage of extracellular preformed nucleosides and nucleobases. Dipyridamole (DP) is a nucleoside transport inhibitor that has been used clinically in an attempt to increase antimitabolite activity; however, DP binds tightly to the serum protein \(\alpha\)-acid glycoprotein (AGP) thereby rendering this therapeutic strategy largely ineffective. Four novel DP analogues (NU3076, NU3084, NU3108, and NU3121) have been developed with substitutions at the 2,6- and 4,8-positions of the pyrimidopyrimidine ring. The novel DP analogues inhibit thymidine (dTd) uptake into L1210 cells in vitro (NU3076 IC\textsubscript{50} 0.25 \(\mu\)M; NU3084 IC\textsubscript{50} 0.27 \(\mu\)M; NU3108 IC\textsubscript{50} 0.31 \(\mu\)M; NU3121 IC\textsubscript{50} 0.26 \(\mu\)M; and DP IC\textsubscript{50} 0.37 \(\mu\)M), but, unlike DP, their activity remains largely unaffected in the presence of 5 mg/ml AGP. The four DP analogues inhibit dTdh and hypoxanthine rescue from Alimta (multitargeted antifolate)-induced growth inhibition in A549 and COR L23 human lung carcinoma cell lines in the presence of 2.5 mg/ml AGP, whereas the activity of DP is completely abolished. i.p. administration of 10 mg/kg NU3108, NU3121, and DP produced peak plasma concentrations of 4.4, 2.1, and 6.7 \(\mu\)M, respectively, and levels were sustained above 1 \(\mu\)M for \(\sim\)45 min (DP) and 120 min (NU3108 and NU3121). \[^{[3]}\]H]thymidine incorporation into COR L23 xenografts grown in CD1 nude mice was reduced by 64\% (NU3108), 44\% (NU3121), and 65\% (DP) 2 h after administration of the nucleoside transport inhibitors. In conclusion, two novel DP analogues (NU3108 and NU3121) have been identified that do not bind to AGP and that display superior pharmacokinetic profiles in comparison to DP and inhibit \[^{[3]}\]H]thymidine incorporation into human tumor xenografts in vivo.

INTRODUCTION

Antifolate antimitabolites remain components of regimens for the treatment of solid tumors and leukemias. Recently, the TS\textsuperscript{2} inhibitor raltitrexed has been developed for the treatment of colorectal cancer (1) and several other novel antifolates are being investigated [e.g., the multigated antifolate Alimta (pemetrexed disodium, LY231514; Ref. 2); GARFT inhibitors such as AG2034 (3) and LY309887 (4); dihydrofolate reductase (DHFR) inhibitors such as trimetrexate (5) and edatrexate (6); and TS inhibitors such as ZD9331 (7) and BW 1843U89 (8)]. The clinical activity of antifolates may be improved by identifying and attempting to overcome acquired or inherent mechanisms of resistance. One mechanism of resistance to antifolates is circumvention of the inhibition of de novo nucleotide biosynthesis via the salvage of extracellular preformed nucleosides and bases. For example, the cytotoxic effects of TS inhibitors can be reduced by the salvage of the nucleoside, dTdh, and those of GARFT inhibitors by the salvage of the nucleobase HPX (9–11). The salvage pathway is important in tumor cells because salvage pathway enzymes can have higher activities than the rate-limiting enzymes of de novo synthesis (12) and may increase in parallel with neoplastic transformation (13).

The cardiovascular agent DP inhibits nucleoside transport via the equilibrative transporters ENT1 and ENT2 (the principle mechanisms operating at physiological nucleoside concentrations) in all human cell lines tested (14) and has been used successfully to inhibit salvage and, thereby, to potentiate the activity of antifolates in vitro. For example, DP can increase the activity of Alimta (15), the TS inhibitor CB3717 (16), and methotrexate (17, 18) through inhibition of dTdh transport. DP has also been shown to selectively potentiate the activity of lometrexol and LY309887 through inhibition of HPX transport in some cell lines (termed DP sensitive or “ds” cells) but not in other cell lines (termed DP insensitive or “di” cells; 19, 20).

\(^{[3]}\)H]thymidine incorporation into human tumors was reduced by 64\% (NU3108), 44\% (NU3121), and 65\% (DP) 2 h after administration of the nucleoside transport inhibitors. In conclusion, two novel DP analogues (NU3108 and NU3121) have been identified that do not bind to AGP and that display superior pharmacokinetic profiles in comparison to DP and inhibit \(^{[3]}\)H]thymidine incorporation into human tumor xenografts in vivo.
However, successful translation of the promising in vitro activity of DP into in vivo preclinical and clinical activity has not been achieved. DP only slightly increased the activity of methotrexate in mice bearing a human bladder tumor xenograft (21). Clinical trials with oral and i.v. DP in combination with methotrexate (22, 23), N-(phosphonacetyl)-L-aspartate (PALA; Ref. 24), and acivicin (25) have not yielded increased activity when compared with treatment in the absence of DP.

DP binds with high affinity to the serum protein AGP (26), the levels of which are 0.3 to 1 mg/ml in healthy individuals but are elevated 1.5- to 3.8-fold in cancer patients (27). Thus, although the maximum steady-state total plasma concentration of DP achievable by i.v. infusion was 12 \( \text{mM} \) and 16 \( \text{mM} \) after oral administration, free (unbound) DP concentrations were only 27 nM and 38 nM, respectively (28, 29), concentrations that would be insufficient to inhibit nucleoside transport. Additional evidence for the role of AGP in reducing DP activity has come from in vitro studies. For example, 1 mg/ml AGP was sufficient to significantly reduce DP-mediated prevention of dThd rescue from CB3717-induced growth inhibition (30).

In an attempt to overcome the clinical limitations of DP, a large number of DP analogues have been synthesized and evaluated; these studies initially identified NU3076 [2,6-di(ethanolamino)-4,8-bis(3',4'-dimethoxybenzylamino) pyrimidopyrimidine] as an inhibitor of nucleoside transport, with reduced AGP binding, that augmented the activity of TS inhibitors (31). Subsequently, more potent analogues have been developed, e.g., NU3084 [2,6-di(ethanolamino)-4,8-bis(3',4'-dimethoxybenzylamino) pyrimidopyrimidine], NU3108 [2,6-di(2'-hydroxypropylamino)-4,8-bis(3',4'-dimethoxybenzylamino) pyrimidopyrimidine; Ref. 32], and NU3121 [2,6-di-(2'-hydroxypropylamino)-4,8-di-(methyleneoxybenzylamino) pyrimidopyrimidine]; see Table 1 for structures. The aim of the investigations reported here was to compare these novel inhibitors with DP as resistance-modifying agents when used in combination with Alimta and LY309887 in vitro in the presence and absence of AGP. Two lung cancer cell lines, A549 and COR L23, which have previously been characterized as having ds- and di-HPX rescue and transport were used in this study (15, 20). The most active analogues, NU3108 and NU3121, were evaluated in preclinical pharmacokinetic and pharmacodynamic studies.

### MATERIALS AND METHODS

**Materials.** Alimta and LY309887 were gifts from Eli Lilly and Co. (Indianapolis, IN) and were dissolved in dH\(_2\)O and stored at 4°C. dThd, HPX, and DP (Sigma Chemical Co., Poole, United Kingdom) were dissolved in dH\(_2\)O, 0.1 M NaOH, and 100% DMSO, respectively, and stored in the dark at 4°C for a maximum of 4 weeks. Novel nucleoside transport inhibitors (NU3076, NU3084, NU3108, and NU3121) were synthesized in the Department of Chemistry, University of Newcastle upon Tyne. All of the other reagents were obtained from Sigma Chemical Co. unless otherwise stated.

**Cell Culture.** COR L23 (a gift from Dr. P. Twentyman, MRC Clinical Oncology and Radiotherapeutics Unit, Cam-
bridge, United Kingdom) and A549 (National Cancer Institute, NIH, Bethesda, MD) non-small cell lung carcinoma cells were adapted to growth in RPMI 1640 supplemented with 1000 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc., Paisley, United Kingdom) and 10% (v/v) FCS that had been dialyzed (four changes of nine volumes PBS + 1 g/liter activated charcoal) prior to use to remove salvageable nucleosides and bases. The growth of the cells was not compromised by culture in medium containing dialyzed serum.⁵ COR L23 cells (for in vivo studies) and L1210 murine leukemia cells (European Collection of Animal Cell Cultures, Wiltshire, United Kingdom) were grown in RPMI 1640 supplemented with 1000 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc., Paisley, United Kingdom) and 10% (v/v) FCS (nondialyzed). All of the cells were maintained as exponentially growing cultures and were tested every month for mycoplasma contamination using a Hoechst 33258 DNA fluorescence-based technique (34) and shown to be negative.

**dThd Transport Assays.** To determine [³H]thymidine uptake into L1210 murine leukemia cells a modified rapid-mixing technique (34), combined with an inhibitor-stop method (35), was used as described previously (15). dThd transport was measured over 12 s, during which time >95% of intracellularly transported dThd remained unphosphorylated.⁶ To determine IC₅₀ values for the inhibition of [³H]thymidine transport for each compound, a range of inhibitor concentrations (0.03–3 μM) was used. All of the compounds were also tested at a final concentration of 1 μM in the absence and presence of 5 mg/ml AGP, and the percentage reduction in inhibitory potency of the compounds by AGP was calculated.

**Growth-inhibition Assays.** A549 and COR L23 cells were incubated in 96-well plates (Nunc, Roskilde, Denmark; supplied by Life Technologies, Inc.) and grown at 37°C in a humidified atmosphere at 5% CO₂ for a period equivalent to three population doublings (72 h for A549 cells and 96 h for COR L23 cells). After incubation, the plates were washed with PBS, fixed with methanol:acetic acid (3:1 v/v), washed, air-dried and stained with SRB, as described previously (36). The absorbance relative to an air blank was measured on a Dynatech MR7000 96-well microtiter plate reader (Dynatech, Billinghamurst, West Sussex, United Kingdom) using a 570 nm filter.

For each growth-inhibition assay, cells in logarithmic phase growth were harvested with trypsin, seeded at 1 × 10⁵ cells/well in 100 μl of medium in 96-well plates and allowed to attach to the plate overnight. End product reversal experiments (dThd, HPX) and investigations of the effect of AGP on the activity of nucleoside transport inhibitors were conducted with a fixed concentration of Alimta at the 10 × IC₅₀ value. Thus, A549 and COR L23 cells were exposed to 7 μM and 200 nm Alimta, respectively, ± 1 μM dThd, 10 μM HPX and/or 1 μM inhibitor in 1% (v/v) DMSO in the absence and presence of AGP (2.5 mg/ml) for 3 cell doublings after which, cell growth was measured by the SRB assay as described above. To investigate whether, like DP, the analogues could potentiate antipyrine antifolate activity in A549 but not COR L23 cells, the cells were exposed to 1 μM LY309887 ± 30 μM HPX in the absence and presence of 3 μM inhibitor in 1% (v/v) DMSO for 3 cell doublings prior to measuring growth inhibition by the SRB assay.

**Pharmacokinetic Studies.** Experiments were performed using female Balb/C mice (8–10 weeks old) supplied by Charles River (Ramsgate, Kent, United Kingdom). DP and NU3108 were dissolved in a vehicle of 40% polyethylene glycol₃₄₀₀ in sterile saline; NU3121 was dissolved in 10% ethanol/cremophor EL (1:1) in sterile saline and administered at doses of 2 mg/kg and 10 mg/kg (0.1 ml/10 g) by i.p. injection to mice (three per time point). Mice were bled under terminal anesthesia (0.75 mg/kg fentanyl citrate, 25 mg/kg fluanisone, and 12.5 mg/kg midazolam, i.p.) at selected time points posttreatment (5–360 min). Blood was collected into heparinized tubes, centrifuged at 6700 × g for 5 min, and the plasma was removed and stored at −20°C prior to analysis for drug by HPLC. At selected time points (10, 30, 90, and 240 min) the plasma was also removed and frozen in liquid nitrogen prior to storage at −80°C until analysis by HPLC with fluorescence detection. Aliquots of plasma (0.05–ml) were vigorously vortexed with 0.1 ml of acetonitrile, and the precipitate was removed by centrifugation at 6700 × g for 5 min. Ten μl of the resultant supernatant were applied to a 10 × 0.46-cm Genesis C18 4-μm column (Jones Chromatography, Glamorgan, United Kingdom) fitted with an in-line filter. DP and NU3108 were eluted with 0.02 M sodium acetate (pH 5.0):acetonitrile 40:60 (v/v), and NU3121 was eluted with 0.02 M sodium acetate (pH 5.0):acetonitrile 50:50 (v/v) at 1 ml/min. DP, NU3108, and NU3121 were detected by fluorescence at 450 nm after excitation at 292 nm. Plasma concentrations were determined using linear standard curves of DP, NU3108, and NU3121 (0.05–10 μM; r² > 0.98 in all cases) generated by extracting compounds from human plasma. The area under the plasma drug concentration versus time curve (AUC) was calculated using the trapezoidal rule with extrapolation to infinity, the total plasma clearance was calculated as dose/AUC and the drug half-life was calculated using the equation 0.693/kᵰ, where kᵰ is the terminal elimination rate constant derived by fitting a monoeponential decay equation using unweighted nonlinear least-squares regression analysis to the terminal concentration/time data (37). Livers taken from posttreatment mice were homogenized in three volumes of 150 μM NaCl:1 volume of liver. Fifty μl of liver homogenate were extracted with 950 μl of acetonitrile, and drug concentrations were determined using the chromatographic method as described above with quantification being achieved by the method of addition (38). The limit of detection for DP, NU3108, and NU3121 was 2 nmol/g wet liver weight.

**Estimation of [³H]Thymidine Incorporation in Vivo.** The protocol for the estimation of [³H]thymidine incorporation into COR L23 tumors was adapted from a previously described method (39). Female athymic CD1 nude mice (Charles River) were maintained and handled in isolators under specific pathogen-free conditions. The human lung adenocarcinoma xenograft was induced by injection of 1 × 10⁷ COR L23 cells in 50 μl of PBS s.c. into the right flank, and experiments commenced when average tumor size was 0.5 cm × 0.5 cm (usually 10 days after implantation). Mice (5 per group) received i.p. injections of

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⁵ Unpublished observations.

⁶ E. Marshman, personal communication.
Table 2  Inhibition of dThd + HPX rescue from Alimta in A549 and COR L23 cells

Growth of A549 and COR L23 cells exposed to 7 μM Alimta and 200 nM Alimta, respectively, ± 1 μM dThd (T) + 10 μM HPX (H) ± 1 μM NU3076, NU3084, NU3108, or DP in the absence or presence of 2.5 mg/ml AGP for three cell doublings.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% control cell growth</th>
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<td>No AGP</td>
</tr>
<tr>
<td></td>
<td>A549</td>
</tr>
<tr>
<td>Alimta</td>
<td>21 ± 7</td>
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<td>Alimta + T + H</td>
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<td>Alimta + T + H + NU3084</td>
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<tr>
<td>Alimta + T + H + NU3108</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>Alimta + T + H + NU3121</td>
<td>36 ± 4</td>
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</table>

* Significantly different from Alimta + T + H (P < 0.05, paired Student’s t test).

10 mg/kg nucleoside transport inhibitor for 1 or 2 h (DP), 1, 2, or 4 h (NU3108), and 2 h (NU3121) prior to termination of the experiment. Forty-five min prior to the end of the experiment, mice received an i.v. bolus dose into a tail vein of 1 μCi/kg [3H]thymidine (specific activity, 41 Ci/mmol, 5μCi/ml diluted to 0.1 μCi/ml in saline) ~25 nmol/kg. At the end of the experiment (i.e., 45 min later) mice were bled under terminal anesthesia. The tumor was then removed and placed, in foil, in liquid nitrogen and stored at −80°C prior to analysis for [3H]thymidine incorporation and determination of drug concentrations.

Blood samples were treated as described previously to determine drug concentrations.

To analyze for [3H]thymidine incorporation, tumor xenografts were thawed and homogenized in three volumes of saline:1 volume of tumour, One ml of ice-cold 1 M perchloric acid was added to 500 μl of the resultant homogenate, and the samples were mixed and stored on ice. After 30 min, perchloric acid precipitates were centrifuged at 1850 × g for 10 min at 4°C, the supernatant discarded, and the pellet washed with 1 ml of ice-cold 0.2 M perchloric acid and, after centrifugation, the supernatant was discarded. The resultant pellet was digested in 1 ml of 1 M NaOH at 37°C for 30 min, after which the sample was neutralized by the addition of 1 ml of 1 M acetic acid. To determine the tritium present in each sample a 1-ml aliquot was removed and placed in 10 ml of Optiphase Hisafe 2 scintillation fluid (Pharmacia Wallac, Milton Keynes, United Kingdom). The samples were then counted on a Wallac 1410 liquid scintillation counter (Pharmacia Wallac).

[3H]Thymidine incorporation was expressed as pmol/g tumor wet weight and as a percentage of incorporation into control (untreated) tumor samples.

Statistical Analyses. Throughout the study, values in tables and figures are given as the mean ± the SD. Differences between groups were investigated using unpaired, two-tailed, Student’s t test analysis unless otherwise stated.

RESULTS

The chemical structures and dThd transport inhibitory potencies of the compounds in the presence and absence of 5 mg/ml AGP as determined using [3H]thymidine uptake assays in L1210 cells are given in Table 1. Structure-activity relationship studies with DP analogues including NU3076, NU3084, and NU3108 have been described previously (32). NU3121 was synthesized to overcome potential metabolic O-demethylation at the 3- and/or 4- positions on the dimethoxybenzylamino groups of NU3108. At a concentration of 1 μM, NU3108 was equipotent to DP whereas NU3076, NU3084, and NU3121 were significantly less potent than DP (P < 0.05). In the presence of 5 mg/ml AGP, all of the compounds produced significant inhibition of dThd uptake, unlike DP which was rendered essentially inactive. The concentration of inhibitor required to inhibit dThd transport by 50% (IC50) was also determined. All of the analogues tested were significantly more active than DP (P < 0.05) with the exception of NU3084 (P > 0.1); however, NU3084 did show a nonsignificant trend toward being more active than DP. NU3076 was significantly more potent than NU3108 (P < 0.05), whereas comparisons between the other DP analogues did not indicate any significant differences in activity (P > 0.1).

Previously, it has been shown that dThd and HPX can reverse Alimta-induced growth inhibition, and that DP can prevent end-product rescue (15). The ability of the novel compounds, compared with DP, to block end-product reversal in the presence and absence of AGP was determined. Both A549 and COR L23 cells were exposed to an Alimta concentration equal to 10 × the approximate IC50 (7 μM and 200 nM, respectively) ± 1 μM dThd and 10 μM HPX ± 1 μM DP or DP analogue, in the absence or presence of 2.5 mg/ml AGP (Table 2). The concentration of AGP used in cell culture studies was the maximum soluble concentration. DP and the DP analogues (at 1 μM) were not growth inhibitory per se (96–114% of control cell growth). In the absence of AGP, all of the compounds (at 1 μM) significantly inhibited end-product rescue and ranked in the order: DP > NU3108 > NU3084 ≥ NU3121 ≥ NU3076 (Table 2). However, in the presence of AGP, the rank order for prevention of dThd and HPX rescue in A549 cells changed markedly, i.e., NU3108 > NU3084 ≥ NU3121 ≥ NU3076 > DP, with the activity of DP being completely abolished, such that there was no significant inhibition of rescue (Table 2). Notably, the inhibition of rescue by NU3076 was completely unaffected by the presence of 2.5 mg/ml AGP, whereas inhibition of rescue was reduced but not abolished for NU3084, NU3121, and NU3108; reductions were significant in all cases (P < 0.05, paired, two-tailed Student’s t test). In COR L23 cells in the presence of AGP, the rank order for the ability of the compounds to inhibit end-product reversal was slightly...
different from that in A549 cells, i.e., NU3108 ≥ NU3121 ≥ NU3084 > NU3076 ≥ DP (Table 2). Again, DP activity was completely abolished by AGP. AGP significantly reduced the activity of NU3076, NU3084, and NU3108 (P < 0.05, paired, two-tailed Student’s t test), whereas NU3121 activity was unaffected by AGP in this cell line. There was a linear correlation between dThd uptake inhibition by DP and the DP analogues in L1210 cells and prevention of dThd and HPX reversal in both A549 (r² = 0.699; P < 0.05) and COR L23 (r² = 0.972; P < 0.05) cells (data not shown). Similarly, there was also a linear correlation between the inhibition of dThd uptake in L1210 cells in the presence of AGP and prevention of dThd and HPX reversal in the presence of AGP by DP and the DP analogues in A549 cells (r² = 0.939; P < 0.05; data not shown).

DP has previously been shown to block rescue by the nucleobase, HPX, from antipurine antifolate-induced growth inhibition in A549 cells but not COR L23 cells by cell-specific inhibition of HPX uptake (20, 40). In these studies, 10 μM DP was required to completely block HPX rescue from lometrexol- and LY309887-induced cell growth inhibition; however, NU3076 is insoluble at this concentration, and, thus, a concentration of 3 μM DP or DP analogue was used in the present study (Table 3). Complete reversal of the growth-inhibitory effects of 1 μM LY309887 was achieved by the addition of 30 μM HPX in both cell lines. The DP analogues inhibited HPX rescue in A549 cells with the same rank order (DP > NU3108 > NU3084 > NU3076; Table 3), as observed for the prevention of dThd + HPX rescue from Alimta-induced growth inhibition (Table 2). NU3108 was approximately equipotent with DP, and, although all of the compounds significantly reduced HPX rescue (P < 0.05), none blocked it completely at the concentration tested. In COR L23 (df) cells, none of the analogues significantly inhibited HPX rescue (P > 0.1; Table 3).

Because NU3108 was the most potent DP analogue and NU3121 was designed as a more metabolically stable form of NU3108, these two compounds were selected for in vivo evaluation in comparison with DP. The plasma and liver concentrations of DP, NU3108, and NU3121 were compared in Balb/C mice after doses of 2 mg/kg and 10 mg/kg. Data are mean and SD of three animals per time point for each drug.

**Table 3  Inhibition of HPX rescue from LY309887 in A549 and COR L23 cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% control cell growth</th>
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<td>A549</td>
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<td>LY309887</td>
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<td>LY309887 + H</td>
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<tr>
<td>LY309887 + H + NU3108</td>
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* Significantly different from LY309887 + H (P < 0.05, paired Student’s t test).

Fig. 1  Plasma pharmacokinetics of (A) DP, (B) NU3108, and (C) NU3121. Plasma drug levels in Balb/C mice after i.p. administration of 2 mg/kg and 10 mg/kg. Data are mean and SD of three animals per time point for each drug.

![](clincancerres.aacrjournals.org)
was achieved at 10 min, whereas the $C_{\text{max}}$ of NU3108 (4.4 ± 0.1 $\mu$M) was reached at 15 min, and the $C_{\text{max}}$ of NU3121 (2.1 ± 0.5 $\mu$M) at 30 min. The clearance of DP at 2 mg/kg and 10 mg/kg (Fig. 1A) was greater than that of NU3108 at 10 mg/kg (Fig. 1B) and NU3121 at 2 and 10 mg/kg (Fig. 1C), respectively. The half-lives of DP and NU3108 were similar at both 2 and 10 mg/kg (11 and 15 min), whereas the half-life of NU3121 at 2 mg/kg was 58 min and at 10 mg/kg was 199 min. Plasma concentrations of DP at both dose levels were below the limit of detection (<0.05 $\mu$M) at 120 min, whereas after NU3108 (10 mg/kg), they were 1.2 ± 0.6 $\mu$M at 120 min and <0.05 $\mu$M at 240 min. Notably, NU3121 was still readily detectable (0.37 ± 0.36 $\mu$M) 360 min after a 10-mg/kg dose.

In addition to differences between the three compounds in terms of plasma concentrations, there were also differences in liver levels (Table 4). After a dose of 2 mg/kg, DP and NU3108 were detectable in the liver only at 10 min. However, 2 mg/kg NU3121 achieved higher liver concentrations and was still detectable at 90 min. At the higher dose of 10 mg/kg, the peak liver concentration of DP was observed at 10 min, followed by a rapid decline to undetectable levels (<2 nm/g) at 90 min. Peak NU3108 liver concentrations were reached at 30 min and then declined more slowly, thereafter, to 2.5 ± 1.6 $\mu$g/g at 240 min. In marked contrast, NU3121 accumulated rapidly in the liver to reach levels of 15.2 ± 1.0 $\mu$g/g, which remained constant for at least 240 min.

The ability of DP, NU3108, and NU3121 to inhibit [3H]thymidine incorporation into tumor cells in vivo was investigated using COR L23 xenograft-bearing CD1 nude mice. [3H]thymidine was administered by i.v. bolus injection 45 min prior to the end of the experiment to mice treated with drug vehicle (40% v/v) polyethylene glycol1000 in saline or 10% ethanol/cremophor EL (1:1 in saline) or to mice treated with 10 mg/kg NU3108 (1, 2, or 4 h previously), DP (1 or 2 h previously) or NU3121 (2 h previously). In drug vehicle-treated control mice, [3H]thymidine incorporation remained constant regardless of the time interval between drug vehicle administration and [3H]thymidine incorporation determination, i.e., 1 h (2.2 ± 1.1 pmol/g tumor), 2 h (2.3 ± 0.8 pmol/g tumor), and 4 h (2.3 ± 0.6 pmol/g tumor). One and 2 h after the administration of NU3108, [3H]thymidine incorporation into COR L23 xenografts was decreased by 63% ($P < 0.05$) and 64% ($P < 0.05$) compared with control levels, respectively (Fig. 2). However, at 4 h, [3H]thymidine incorporation had returned to control levels (2.6 ± 1.2 pmol/g tumor). One h after DP administration, [3H]thymidine incorporation was significantly reduced by 43% ($P < 0.05$), a reduction that was maintained at 2 h (65%; $P < 0.05$). The effect of NU3121 on [3H]thymidine incorporation was measured only at 2 h, at which time [3H]thymidine incorporation was decreased by 44% ($P = 0.07$). At 2 h, there was no difference among the activity of the three compounds after a dose of 10 mg/kg ($P > 0.1$, one-way ANOVA). The plasma drug concentrations of DP, NU3121, and NU3108 in CD1 nude mice were also measured at 1, 2, and 4 h (data not shown), and the concentrations were not significantly different from those observed in non-tumor-bearing Balb/C mice (Fig. 1).

**DISCUSSION**

The aim of the study reported in this paper was to evaluate the ability of four novel nucleoside transport inhibitors to prevent nucleoside and base rescue from antifolate activity in cell culture and to examine their preclinical pharmacokinetics and pharmacodynamics in tumor-bearing mice. Three DP analogues from a previously published series (NU3076, NU3084, and NU3108; Ref. 32) and a new analogue (NU3121) have been identified that inhibit [3H]thymidine uptake with approximately
equal potency to DP in the absence of AGP but, unlike DP, retain activity in the presence of AGP. NU3076 and DP have also been shown to increase the cytotoxicity of 5-fluorouracil and the novel TS inhibitor, nolatrexed in L1210 cells (31). The four analogues were compared with DP as modulators of Alimta-induced growth inhibition in A549 and COR L23 lung cancer cell lines in the presence of dThd and HPX. All of the analogues inhibited rescue by dThd + HPX from Alimta-induced growth inhibition, with NU3108 being the most potent and causing a complete inhibition of rescue. The activity of the analogues was only marginally reduced by the presence of AGP in contrast to the activity of DP, which was completely abolished by AGP.

The ability of the analogues at 1 μM to block rescue from Alimta was related to their activity at 1 μM as inhibitors of [3H]thymidine uptake into L1210 cells. This result confirms previous studies with less potent inhibitors (31) demonstrating that transport inhibitory potency is a good indicator of the ability to overcome salvage-mediated antifolate resistance. Consistent with the effect of AGP on DP inhibition of nucleoside transport, DP activity in both of the cell lines used here was completely abolished by the addition of AGP presumably as a result of the well-characterized binding of the drug to this protein (41). Although AGP reduced the activity of the novel analogues in both cell lines, they all retained the ability to significantly inhibit rescue in the presence of AGP, which suggests that the affinity of AGP for the analogues is lower than in the case of DP.

It has previously been shown that DP inhibits HPX uptake and HPX rescue from lometrexol and LY309887-induced growth inhibition in A549 (ds) cells but not from COR L23 (dt) cells (15, 20, 40). NU3076, NU3084, and NU3108 were also able to inhibit HPX rescue from LY309887-induced growth inhibition in A549 (ds) but not COR L23 (dt) cells, suggesting that they are acting on the same transporters as DP. HPX rescue may be somewhat less sensitive to inhibition by the DP analogues than dThd rescue because a concentration of 3 μM analogue did not completely inhibit HPX rescue, and previous studies have shown that 10 μM DP is required for complete inhibition of HPX rescue from lometrexol-, LY309887-, and methotrexate-induced growth inhibition (19, 20, 42). Again, the rank order for the ability of the inhibitors to prevent HPX rescue (DP > NU3108 > NU3084 > NU3076) was the same as that observed for prevention of dThd + HPX reversal from Alimta-induced growth inhibition, and inhibition of dThd uptake into L1210 cells. Together, these data indicate that the HPX transporter in A549 cells is closely related to ENT1 and ENT2 nucleoside transporters as has been suggested previously (19, 20, 40).

Earlier studies with DP have demonstrated the ability of DP to prevent HPX reversal of antipurine antifolate-induced cell growth inhibition in ~30% of human tumor cell lines but, importantly, not in drug-sensitive normal tissues, viz., bone marrow and gastrointestinal tract epithelium (20). Thus, DP may be able to selectively enhance the antitumor activity of antipurine antifolates without increasing host toxicity. If, as indicated by the data presented here, the novel analogues display similar cell type-selective prevention of HPX rescue to DP, then they may also be useful in combination with antipurine antifolate chemotherapy.

In vivo studies were conducted with NU3108, the most potent of the new inhibitors, and NU3121 which was selected because it was designed to avoid potential O-demethylation of the methoxy groups on the 4,8-dimethoxybenzylamino substituents of NU3108. Of the three inhibitors tested, DP clearance occurred most rapidly, followed by NU3108, whereas NU3121 was cleared less efficiently. However, NU3121 was dissolved in a vehicle of containing cremophor EL, which may contribute to a reduced elimination rate because this vehicle has been shown to cause nonlinear pharmacokinetics in mice (43). In vitro an extracellular DP or DP analogue concentration of 1 μM inhibited [3H]thymidine (100 μM) uptake into L1210 cells and dThd (1 μM) + HPX (10 μM) rescue from Alimta-induced growth inhibition in A549 and COR L23 cells. Plasma dThd levels have been reported to be slightly higher in human cancer patients (0.8 μM: Ref. 44) than in healthy individuals (0.2 μM; Ref. 45), the former being similar to concentrations in mice (46). Thus, it is anticipated that plasma DP, NU3108, and NU3121 levels would need to be maintained at concentrations ≥1 μM to prevent dThd rescue from Alimta activity or toxicity in mice bearing human tumor xenografts. After a dose of 10 mg/kg (the highest dose of NU3108 and NU3121 that could be administered because of solubility limitations), DP concentrations remained above 1 μM for only 45 min, whereas NU3108 and NU3121 concentrations remained above 1 μM for ~120 min.

To determine whether NU3108 and NU3121 could inhibit the salvage pathway in vivo, [3H]thymidine incorporation into COR L23 xenografts was estimated after administration of the inhibitor at a dose of 10 mg/kg. NU3108 inhibited [3H]thymidine incorporation by 64% for up to 2 h, at which time, plasma concentrations of NU3108 were still detectable but below 1 μM. Four h after NU3108 administration, [3H]thymidine incorporation was not inhibited if it coincided with levels of NU3108 being undetectable (<0.05 μM) in the plasma. Similarly, although DP concentrations were below 1 μM at 1 h and not detectable at 2 h, [3H]thymidine incorporation was inhibited by 43–65% at these time points. Of the three inhibitors, NU3121 plasma levels at 2 h were the highest (0.87 μM) and, although these concentrations were also below 1 μM, NU3121 inhibited [3H]thymidine incorporation by 44% at this time point. Thus, it appears that the inhibition of [3H]thymidine incorporation into COR L23 xenografts does not correlate directly with the plasma concentrations of the three nucleoside transport inhibitors tested here. Determination of the concentration of inhibitors in tumor samples was prevented by the low sensitivity of the HPLC detection method used in this study.

The duration of inhibition of dThd uptake may be critical, because previous studies in TS-negative GC/c1 colon carcinoma cells (which require extracellular dThd for survival) have shown that a 50% decrease in survival was observed at 55 h after dThd withdrawal (47). In contrast, initiation of thymineless death in TS negative mouse FM3A mammary carcinoma cells resulted in a 50% decrease in survival observed within 6 h of dThd withdrawal (48). On the basis of the results described here, repeated dosing or prolonged release formulations of NU3108 and NU3121 would be required to maintain nucleoside transport inhibitor plasma concentrations of >1 μM for a time period sufficient to potentiate antifolates in vivo.
In conclusion, four novel DP analogues (NU3076, NU3084, NU3108, and NU3121) have been identified that are potent inhibitors of nucleoside transport in vitro and whose activity is maintained in the presence of AGP. These four analogues can prevent salvage-mediated rescue from the growth-inhibitory activity of Alimta even in the presence of AGP, which renders DP ineffective. Furthermore, cell line-specific potentiation of the growth-inhibitory effects of the antipurine antifolate LY309887 was observed with the three analogues tested. NU3108, NU3121, and DP inhibited [3H]thymidine incorporation into COR L23 xenografts with similar potency, although the extent of inhibition was not directly related to plasma drug concentrations. Further analogue development is continuing to identify compounds with pharmacokinetic and pharmacodynamic properties that would allow acceptable scheduling for use in combination with antimitobolites in vivo studies.

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REFERENCES


In Vitro and In Vivo Properties of Novel Nucleoside Transport Inhibitors with Improved Pharmacological Properties That Potentiate Antifolate Activity,

Peter G. Smith, Huw D. Thomas, Hannah C. Barlow, et al.


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