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Chemical Synthesis and Biological Properties of Novel Fluorescent Antifolates in Pgp- and MRP-Overexpressing Tumour Cell Lines

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ABSTRACT. We have synthesised a series of fluorescent analogues of methylbenzoprim, a diaminopyrimidine antifolate which we have previously shown to exhibit *in vivo* antitumour activity in a methotrexate (MTX) "transport-resistant" tumour cell line. The analogues bear the dansyl, nitrobenzoxodiazole or methoxycoumarin fluorophores. The cytotoxicity of the compounds was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay against two human lung cancer cell lines, together with their multidrug resistant (MDR) sublines. H69/P is a small cell line and its multidrug resistant subline H69/LX4 overexpresses P-glycoprotein (Pgp). COR-L23/P is a large cell line and its multidrug resistant subline COR-L23/R overexpresses the multidrug resistance associated protein (MRP). IC_{50} values for the compounds (i.e. concentration to reduce cell growth by 50%) in the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay ranged from 0.20 to 0.81 μM in the H69 parental line and from 0.83 to 5.10 μM in the COR-L23 parent line. The MDR sublines both showed clear cross-resistance to each of the compounds, with resistance factors (ratio of IC_{50} value in resistant vs parental cell line) ranging from 16 to 137 in H69/LX4 and from 5 to 16 in COR-L23/R. For compounds (**10**) and (**11**) where drug accumulation was studied using flow cytometry, resistance was associated with an approximately 10-fold reduction in cellular drug accumulation over a period of 30 min. The drug resistance modifiers verapamil (used at 6.6 μM) and cyclosporin A (used at 4.2 μM) were tested for their ability to sensitise the resistant lines. Whereas verapamil showed little activity, cyclosporin A partially restored the activity of compound (**10**), and fully restored the activity of compound (**11**) in H69/LX4 cells. This sensitisation of H69/LX4 by cyclosporin A was associated with a partial restoration of the drug accumulation deficit in this line. Hence, these novel lipophilic antifolates appear to be substrates for both the P-glycoprotein and MRP resistance mechanisms. Therefore, although they have been designed to overcome one mechanism of methotrexate resistance, namely impaired drug transport, this has been achieved only at the cost of rendering them susceptible to alternative mechanisms. BIOCHEM PHARMACOL **56**;7:807–816, 1998. Crown Copyright © 1998. Published by Elsevier Science Inc.

KEY WORDS. drug transport; lipophilic antifolates; multidrug resistance; MRP; fluorescent probes; flow cytometry

Nonclassical inhibitors of DHFR‖ (EC 1.5.1.3), as exemplified by PTX (**1**) and TMQ (**2**) (Fig. 1), are of therapeutic utility in the treatment of tumours resistant to MTX (**3**) and other classical antifolates [1, 2]. These lipid-soluble agents, which lack the polar glutamate side chain of MTX, do not require a carrier-mediated active transport mechanism to gain ingress to cells, but rather enter by passive or facilitated diffusion, and are active against MTX "transport-resistant" tumours and CNS malignancies inaccessible to the more hydrophilic MTX [3, 4]. More recently, TMQ has also gained approval for the treatment of infections by the opportunistic pathogens *Pneumocystis carinii* and *Toxoplasma gondii* in patients with AIDS [5, 6].

PTX and TMQ are substrates for the membrane-bound Pgp, which effluxes a diverse range of hydrophobic weakly basic antitumour agents from cells, and is responsible for the MDR phenotype [7]. An alternative mediator of MDR, the MRP has also been characterised in several resistant tumour cell lines [8–10], although little is known regarding

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‖ *Abbreviations:* DNS-Cl, 5-dimethylamino-1-naphthalenesulphonyl chloride; DHFR, dihydrofolate reductase; MDR, multidrug resistance; MRP, multidrug-resistance-associated protein; methylbenzoprim, 2,4-diamino-5-[*N*-benzyl-*N*-methyl)amino]-6-ethylpyrimidine; MMC-Br, 4-(bromomethyl)-7-methoxycoumarin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; MTX, methotrexate; NBD-Cl, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; Pgp, P-glycoprotein; PTX, piritrexim (2,4-diamino-6-(2,5-dimethoxybenzyl)-5-methylpyrido[2,3-D]pyrimidine); TMQ, trimetrexate (5-methyl-6-[[(3,4,5-trimethoxyphenyl)amino]methyl]-2,4-quinazolinediamine).

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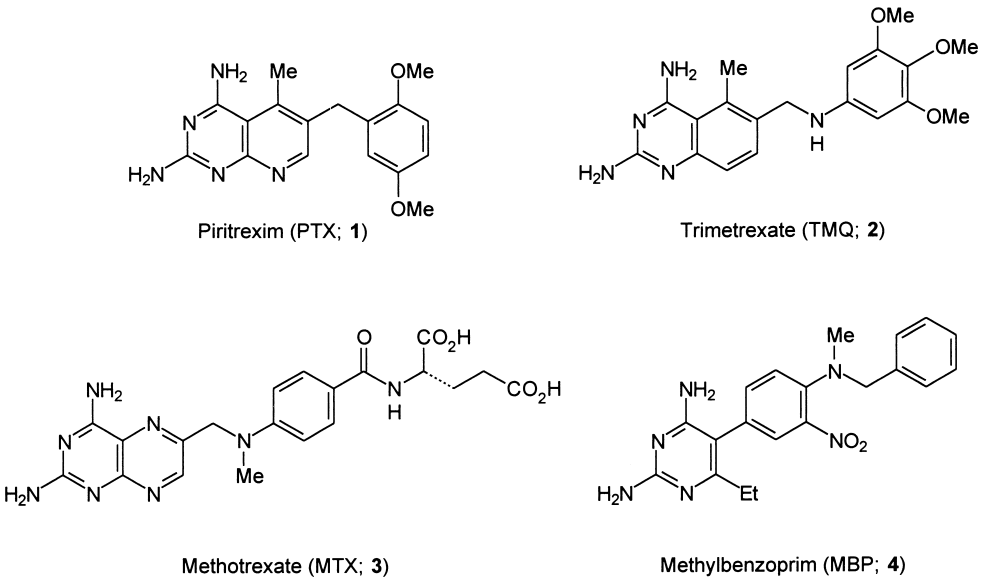


FIG. 1. Chemical structures of antifolates.

the interaction of this transporter with lipophilic antifolates. A number of cell lines resistant to PTX and TMQ, as a consequence of the MDR phenotype, have been identified [11, 12]. Moreover, since the endothelial cells of the blood-brain barrier are also known to overexpress Pgp, by preventing intracellular accumulation of drug, MDR represents a potential mechanism of resistance to these agents in the therapy of CNS tumours [13–15]. More recently, the expression of Pgp in astrocytes has also been reported [16].

A number of lipophilic antifolates are reportedly not subject to MDR [3], suggesting that there may be critical structural determinants of substrate activity for Pgp. A better understanding of the interactions between antifolates and membrane transporters would enable the design of novel analogues which are not susceptible to MDR or MRP or, alternatively, which may function as inhibitors of these glycoproteins. Currently available MDR-reversing agents, which include verapamil (VRP) and cyclosporin A (CsA), restore sensitivity to antitumour drugs but lack potency and specificity [17].

We have previously described the results of studies with methylbenzoprim (MBP, **4**), the most interesting of a novel series of diaminopyrimidine antifolates (benzoprims), which exhibit promising *in vivo* activity in an MTX-resistant tumour cell line [18, 19]. In this paper we describe the synthesis and preliminary biological evaluation of a series of fluorescent analogues of MBP, bearing the DNS, NBD or MMC fluorophores. Such compounds were designed to serve as fluorescent probes in studies to characterise the cellular transport, accumulation and distribution of lipophilic antifolates in parental and Pgp- or MRP-overexpressing tumour cell lines. A preliminary account of part of this work has been published previously [20].

MATERIALS AND METHODS

Chemistry

GENERAL METHODS. Reagents were purchased from Aldrich Chemical Company, and used as received. Melting points were obtained on an electrothermal melting point apparatus and are uncorrected. Mass spectra were recorded on a Kratos MS80 mass spectrometer in electron impact (EI) mode. ^{1}H-NMR spectra were obtained on a Bruker Spectrospin AC 200E (200 MHz) spectrometer, employing TMS as internal standard and [$^2H_6$]-DMSO as solvent. The TLC systems employed aluminium sheets pre-coated with Kieselgel 60F_{254} (0.2 mM) as the adsorbent, and were visualised with light at 254 and 366 nm. Medium pressure column chromatography was conducted on silica gel (Kieselgel 60, 240–400 mesh). The synthesis of 2,4-diamino-5-(4′-chloro-3′-nitrophenyl)-6-ethylpyrimidine (**5**), 2,4-diamino-5-(4′-[2-aminoethylamino]-3′-nitrophenyl)-6-ethylpyrimidine (**6**) and 2,4-diamino-5-(4′-[3-aminopropylamino]-3′-nitrophenyl)-6-ethylpyrimidine (**7**) has been reported elsewhere [18, 21].

PREPARATION OF (**8**) AND (**9**). To a stirred solution of (**6**) (0.50 g, 1.6 mmol) and triethylamine (0.2 mL, 1.6 mmol) in anhydrous DMF (15 mL), was added dansyl chloride (0.43 g, 1.6 mmol) in DMF (5 mL) dropwise over 0.5 hr. The reaction mixture was stirred under N_2, in the dark, at ambient temperature for 12 hr. The orange solid remaining after evaporation of the solvent *in vacuo* was purified by chromatography on silica gel, employing CH_2Cl_2:MeOH (8:2) as eluent, to give **8** (0.73 g, 84%). Recrystallisation from ethyl acetate-petrol afforded the analytical sample, m.p. 160–162°C; ^{1}H NMR δ 1.07 (3H, t, J = 7.5 Hz, CH_2CH_3), 2.20 (2H, q, J = 7.3 Hz, CH_2CH_3), 2.88 (6H, s, N[CH_3]$_2$) 3.18 (2H, m, NHCH_2CH_2NH), 3.47 (2H, m,

$NHCH_2CH_2NH$), 5.89 (2H, br s, NH_2), 6.09 (2H, br s, NH_2), 7.05 (1H, d, J_{ortho} = 8.9 Hz, C(5′)H), 7.33 (2H, m, C(6′)H, C(6″)H), 7.62 (2H, m, C(3″)H, C(7″)H), 7.81 (1H, d, J_{meta} = 1.9 Hz, C(2′)H), 8.21 (1H, dd, J_{ortho} = 8.6 Hz, C(2″)H), 8.34 (1H, dd, J_{ortho} = 8.4 Hz, C(4″)H), 8.49 (1H, dd, J_{ortho} = 8.5 Hz, C(8″)H). ^{13}C-NMR δ (50 MHz) 135.9, 129.8, 128.5, 128.1, 125.6, 123.8, 123.0, 119.3, 115.1, 41.1, 40.7, 40.3, 39.9, 38.6. High resolution electron impact mass spectrometry calculated for $C_{26}H_{30}N_8O_4S$: *m/z* 550.2111. Found 550.2121.

The analogous reaction of (**7**) (0.50 g, 1.51 mmol) with dansyl chloride (0.41 g, 1.51 mmol) afforded (**9**) (0.75 g, 88%). m.p. 122–124°C; ^{1}H NMR δ 1.09 (3H, t, *J* = 7.5 Hz, CH_2CH_3), 1.81 (2H, m, $NHCH_2CH_2CH_2NH$), 2.23 (2H, q, *J* = 7.5 Hz, CH_2CH_3),), 2.91 (6H, s, $N[CH_3]_2$), 3.05 (2H, m, $NHCH_2CH_2CH_2NH$), 3.39 (2H, m, $NHCH_2CH_2CH_2NH$), 5.88 (2H, br s, NH_2), 6.12 (2H, br s, NH_2), 7.22 (1H, d, J_{ortho} = 8.9 Hz, C(5′)H), 7.35 (2H, m, C(6′)H, C(6″)H), 7.68 (1H, br s, NH), 7.72 (2H, m, C(3″)H, C(7″)H), 7.91 (1H, d, J_{meta} = 1.9 Hz, C(2′)H), 8.10–8.30 (2H, m, C(2″)H), NH), 8.46 (1H, dd, J_{ortho} = 8.6 Hz, C(4″)H), 8.51 (1H, dd, J_{ortho} = 8.4 Hz, C(8″)H). ^{13}C-NMR (50 MHz) δ 136.3, 129.8, 128.7, 128.1, 125.6, 123.9, 122.8, 119.3, 115.3, 41.1, 40.7, 40.3, 39.9, 38.6. High resolution electron impact mass spectrometry calculated for $C_{27}H_{32}N_8O_4S$: *m/z* 564.2267. Found 564.2250.

PREPARATION OF (**10**) AND (**11**). 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (0.32 g, 1.58 mmol) in DMF (6 mL), was added dropwise over 15 min to a solution of (**6**) (0.5 g, 1.58 mmol) and triethylamine (0.2 mL, 1.6 mmol) in DMF (15 mL). The reaction mixture was stirred under N_2 at room temperature for 15 hr, and the product was purified by chromatography on silica gel using CH_2Cl_2:MeOH (9:1) as eluent. Recrystallisation from ethanol furnished (**10**) (0.58 g, 77%), m.p. 204–205°C; ^{1}H-NMR δ 1.08 (3H, t, *J* = 7.4 Hz, CH_2CH_3), 2.17 (2H, q, *J* = 7.4 Hz, CH_2CH_3), 3.47 (2H, m, $NHCH_2CH_2NH$), 3.89 (2H, m, $NHCH_2CH_2NH$), 5.85 (2H, br s, NH_2), 6.02 (2H, br s, NH_2), 6.61 (1H, d, *J* = 9.01 Hz, C(2″)H), 7.37 (2H, m, C(5′)H, C(6″)H), 7.87 (1H, d, J_{meta} = 2.0 Hz, C(2′)H), 8.57 (1H, d, J_{ortho} = 9.16 Hz, C(3″)H), 8.61 (1H, m, NH). ^{13}C NMR (50 MHz) δ 164.2, 145.1, 139.1, 131.7, 128.6, 115.9, 106.2, 41.1, 40.5, 40.1, 39.8, 39.4, 38.6. High resolution electron impact mass spectrometry calculated for $C_{20}H_{20}N_{10}O_5$: *m/z* 480.1618. Found 480.1618.

Compound (**11**) was similarly prepared from (**7**) (0.50 g, 1.51 mmol) and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (0.31 g, 1.51 mmol) as described above (0.61 g, 82%). m.p. 150–152°C; ^{1}H-NMR δ 1.07 (3H, t, *J* = 7.4 Hz, CH_2CH_3), 2.21 (4H, m, $NHCH_2CH_2CH_2NH$, CH_2CH_3), 3.45 (2H, m, $NHCH_2CH_2CH_2NH$), 3.66 (2H, m, $NHCH_2CH_2CH_2NH$), 5.92 (2H, br s, NH_2), 6.11 (2H, br s, NH_2), 6.56 (1H, d J_{ortho} = 9.04 Hz, C(2″)H), 7.24 (1H, d, J_{ortho} = 8.9 Hz, C(5′)H), 7.44 (2H, dd, J_{ortho} = 8.90 Hz, C(6′)H), 7.90 (1H, d, J_{meta} = 1.9 Hz, C(2′)H), 8.43 (1H, t, NH), 8.63 (1H, d, J_{ortho} = 8.9 Hz, C(3″)H). ^{13}C NMR δ 164.2, 145.1, 139.3, 131.7, 128.6, 115.9, 106.2, 40.7, 40.5, 40.3, 39.8, 39.4, 39.0, 38.6, 13.0. High resolution electron impact mass spectrometry calculated for $C_{21}H_{22}N_{10}O_3$: *m/z* 494.1775. Found 494.1775.

PREPARATION OF (**12**) AND (**13**). To a solution of (**6**) (0.50 g, 1.58 mmol) in DMF (15 mL), containing triethylamine (0.2 mL, 1.60 mmol), was added 4-bromomethyl-7-methoxycoumarin (0.42 g, 1.58 mmol) in DMF (5 mL) over 20 min, and the reaction mixture was stirred under N_2 at room temperature for 15 hr. The product was purified by chromatography on silica gel with CH_2Cl_2:MeOH (8:2) as eluent, to afford (**12**) as a bright orange powder, after recrystallisation from ethanol, (0.59 g, 74%), m.p. 170–172°C; ^{1}H NMR δ 1.08 (3H, t, *J* = 7.3 Hz, CH_2CH_3), 2.27 (2H, q, *J* = 7.2 Hz, CH_2CH_3), 3.04 (2H, m, $NHCH_2CH_2NH$), 3.56 (2H, m, $NHCH_2CH_2NH$), 3.99 (3H, s, OCH_3), 4.05 (2H, m, $NHCH_2$), 5.86 (2H, br s, NH_2), 6.10 (2H, br s, NH_2), 6.44 (1H, s, J_{meta} = 1.98 Hz, C(3″)H), 7.19 (2H, m, C(6″)H, C(8″)H), 7.23 (1H, d, J_{ortho} = 9.00 Hz, C(5′)H), 7.42 (1H, dd, J_{ortho} = 8.94 Hz, C(6′)H), 7.81 (1H, d, J_{ortho} = 8.70 Hz, C(5″)H), 7.94 (1H, d, J_{meta} = 2.03 Hz, C(2′)H), 8.60 (1H, m, NH). ^{13}C NMR (50 MHz) δ 163.2, 162.5, 155.4, 144.9, 139.6, 128.2, 126.3, 121.8, 115.8, 112.3, 105.3, 101.0, 56.2, 48.6, 42.2, 40.7, 40.2, 39.8, 39.4, 39.0, 38.6, 27.1, 12.8. High resolution electron impact mass spectrometry calculated for $C_{25}H_{27}N_7O_5$: *m/z* 505.2074. Found 505.2075.

A similar reaction of (**7**) (0.50 g, 1.51 mmol) with 4-bromomethyl-7-methoxycoumarin (0.41 g, 1.51 mmol) furnished (**13**) (0.66 g, 84%). m.p. 185–187°C; ^{1}H NMR δ 1.07 (3H, t, *J* = 7.4 Hz, CH_2CH_3), 2.20 (2H, m, $NHCH_2CH_2CH_2NH$), 2.22 (2H, q, *J* = 7.5 Hz, CH_2CH_3), 3.45 (2H, m, $NHCH_2CH_2CH_2NH$), 3.56 (2H, m, $NHCH_2CH_2CH_2NH$), 3.94 (3H, s, OCH_3), 4.02 (2H, m, $NHCH_2$), 5.90 (2H, br s, NH_2), 6.06 (2H, br s, NH_2), 6.47 (1H, s, C(3″)H), 7.05 (2H, m, C(6″)H, C(8″)H), 7.20 (1H, d, J_{ortho} = 8.67 Hz, C(5′)H), 7.43 (1H, dd, J_{ortho} = 8.91 Hz, C(6′)H), 7.84 (2H, m, C(2′)H, C(5″)H), 8.44 (1H, m, NH). ^{13}C NMR δ 163.2, 162.5, 155.2, 144.9, 139.6, 128.2, 126.3, 121.8, 115.6, 112.4, 105.2, 101.1, 56.2, 49.0, 46.8, 41.0, 40.7, 39.8, 39.4, 39.0, 38.6, 27.2, 13.3. High resolution electron impact mass spectrometry, calculated for $C_{26}H_{29}N_7O_5$: *m/z* 519.2230. Found 519.2215.

Cell Lines

The human small cell lung cancer cell line NCI-H69/P and its Pgp-overexpressing MDR subline H69/LX4, and the human large cell lung cancer cell line COR-L23/P and its MRP overexpressing subline COR-L23/R, were used in these studies [22]. Cells were maintained as stock cultures in plastic tissue culture flasks (Falcon), and incubated in an atmosphere of 8% CO_2 and 92% air. All cells were maintained in RPMI 1640 medium supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), glutamine (0.5 mM) and 10% foetal calf serum (Sigma). The

drug-resistant cell lines were maintained in doxorubicin (0.4 μg/mL for H69/LX4; 0.2 μg/mL for COR-L23R) until 48 hr prior to the experiments. The COR-L23 cell lines grew as attached monolayers and were disaggregated using a solution of trypsin (0.5%) and Versene (0.02% EDTA) in PBS, whilst the NCI-H69 lines grew as floating aggregates. All cell lines were screened regularly for mycoplasma contamination and found to be negative throughout the period of this study.

Drugs and Chemicals

Stock solutions (10 mg/mL) of the fluorescent antifolates (**8** & **10–13**) were prepared in DMSO, stored at 4°, and diluted in medium immediately prior to use. Doxorubicin (DOX) (kindly provided by Pharmitalia) was dissolved in sterile water to 500 μg/mL and stored at −20°. For working stock, aliquots were diluted in water and stored at 4° for up to 1 week. Cyclosporin A (CsA), kindly donated by Sandoz, Basle, was dissolved in ethanol at 5 mg/mL, stored at 4°, and diluted in medium immediately before use. Verapamil hydrochloride (VRP; Abbot Labs) was obtained as an aqueous solution (2.5 mg/mL), and was stored at 4° and diluted immediately before use. MTT (Thiazoyl blue; Sigma) was diluted in PBS at 5 mg/mL, sterilised by millipore filtration, and stored as aliquots at 4°.

MTT Assay

The response of the cell lines to the compounds was determined using the MTT colorimetric assay [23] as adapted for use in our laboratory [24]. Briefly, cell suspensions were prepared from exponentially growing cultures and inoculated into 96-well tissue culture plates (Falcon) at the following cell numbers per mL: L23/P 1.5×10^3, L23/R 5×10^3, H69/P 2×10^4 and H69/LX4 4×10^4, in a volume of 200 μL per well. Where appropriate, chemical modifiers were added in a volume of 10 μL, following an initial 1-hr incubation of the cells. Cytotoxic agents, and the appropriate solvent controls, were added 2 hr later in a volume of 20 μL, and the plates were incubated for 6 days. At the end of the incubation period 20 μL of a 5 mg/mL MTT solution was added to each well, and the plates were re-incubated for 5 hr, after which time the medium was aspirated from each well and 200 μL of DMSO was added. The plates were shaken on a Titretek plate shaker for 10 min, and the optical densities were read on a Titretek Multiskan MCC340 plate reader at a wavelength of 540 nm, and a reference wavelength of 690 nm. Results are expressed as IC_{50} values, where the IC_{50} is defined as the dose of drug required to reduce the final optical density to 50% of control.

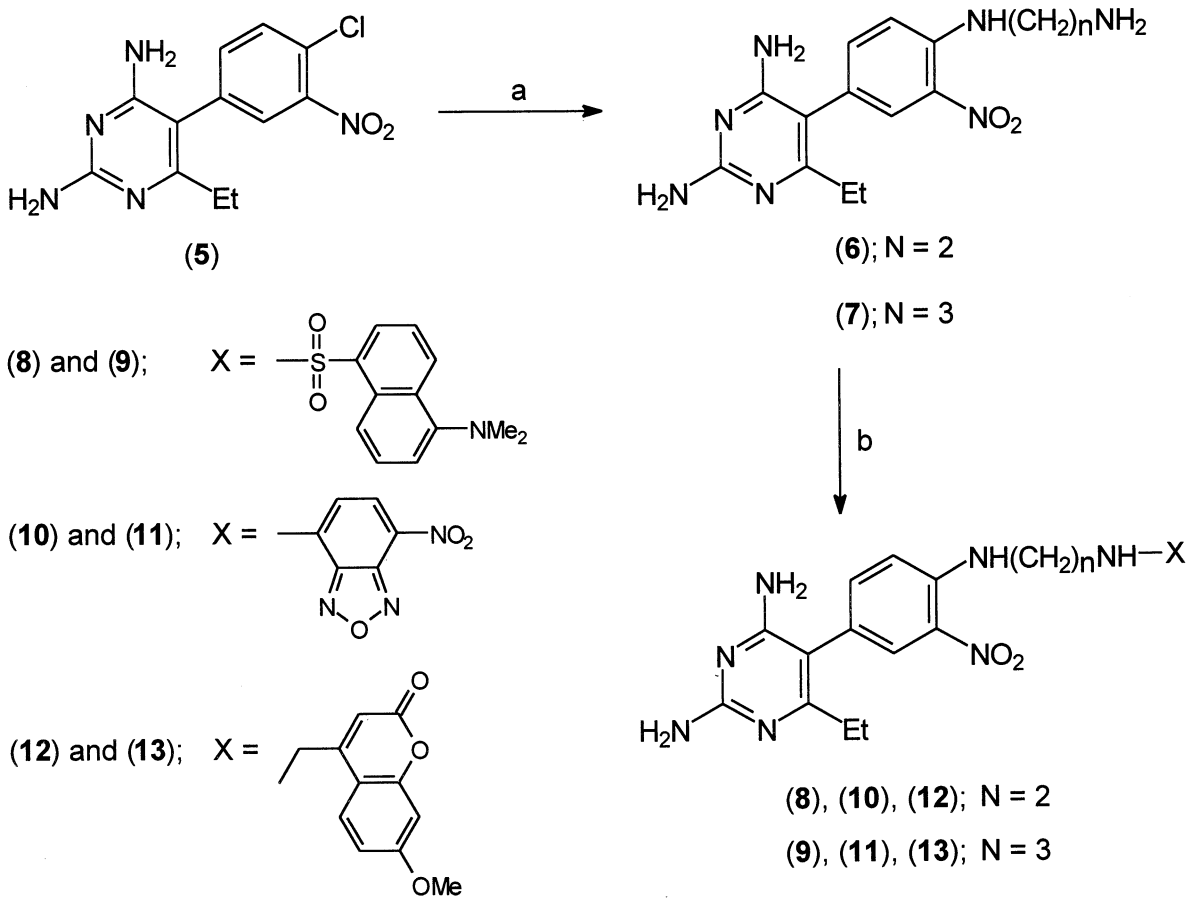
Flow Cytometry

The intracellular accumulation of the fluorescent compounds, in the presence or absence of chemical modifiers, was measured using the Cambridge flow cytometer [25] at an excitation wavelength of 488 nm, with fluorescence emission being collected above 630 nm. Briefly, cell suspensions were prepared at a concentration of 2×10^5 cells/mL. Cell suspensions were transferred to plastic tubes 30 min before the start of experiments, and placed on a roller machine at 37°. At time zero, drugs were added and 500-μL samples of the suspension were removed at timed intervals. The remaining solution was mixed at 37°. The samples were pelleted rapidly using a microcentrifuge (1 min at 3000 *g*), resuspended in ice-cold PBS, vortexed, and kept on ice until analysed (within 5 min). The results are expressed as arbitrary units of fluorescence.

RESULTS

Crystallographic and molecular modelling studies conducted with MBP (**4**) bound to DHFR indicate that the pendant *N*-methylbenzylamino group occupies a large hydrophobic domain capable of accommodating other relatively bulky aromatic substituents (Rachedi A, Sansom CE, Groom CR, Thillet J, Griffin RJ and Geddes AJ, unpublished results, [26]). This implied that derivatives of (**4**) which retained the essential 2,4-diamino-5-(3-nitroaryl)-6-ethylpyrimidine pharmacophore, but which encompassed a suitable fluorophore in the position formerly occupied by the *N*-methylbenzylamino substituent, should combine excellent fluorescence properties with a high affinity for the target enzyme DHFR. The DNS, NBD and MMC fluorophores were selected because they have previously been used successfully in the preparation of fluorescent derivatives, and all three are planar aromatic compounds of relatively low molecular mass [27–30]. The target compounds were readily synthesised in two steps starting from 2,4-diamino-5-(4′-chloro-3′-nitrophenyl)-6-ethylpyrimidine (**5**) as shown in Scheme 1. Thus, treatment of (**5**) with 1,2-diaminoethane or 1,3-diaminopropane afforded the aminopyrimidine derivatives (**6**) and (**7**), respectively, and coupling of (**6**) and (**7**) with the fluorigenic reagents DNS-Cl, NBD-Cl or MMC-Br in DMF gave the required fluorescent derivatives (**8–13**) in good overall yields. The structures of these compounds were confirmed by ^1H and ^{13}C NMR spectroscopy, and high resolution mass spectrometry. Samples of the fluorescent compounds stored in the dark at 4° showed no evidence of decomposition after 12 months.

In order to determine the effect, upon DHFR-inhibitory activity, of replacing the *N*-methylbenzylamino group of MBP by fluorophoric groups, compounds (**8–13**) were screened against rat liver enzyme in comparison with the parent MBP (**4**) (Table 1). The IC_{50} value of 10 nM obtained for (**4**) is consistent with the previously reported value for the compound against rat liver DHFR [18]. The introduction of a DNS or MMS substituent is tolerated with no loss of potency against DHFR, regardless of the size of the spacer group, and the MMC-substituted derivatives (**12** and **13**) are marginally more active than the parent



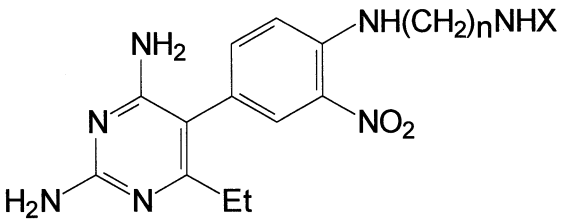
SCHEME I. Synthesis and chemical structures of fluorescent lipophilic antifolates. Reagents used were: (a) $H_2N(CH_2)_2NH_2$ (N = 2) or $H_2N(CH_2)_3NH_2$ (N = 3); (b) DNS-Cl (8 and 9), NBD-Cl (10 & 11) or MMC-Br (12 & 13), NEt_3, DMF. Additional details are provided in Materials and Methods.

antifolate (Table 1). Interestingly, while an NBD group attached *via* an aminoethylamino spacer (**10**) confers DHFR-inhibitory activity comparable to (**4**), the aminopropylamino analogue (**11**) is markedly less potent with an IC_{50} of 30 nM.

Growth Inhibition by Antifolates

The MTT assay was used to determine the sensitivity of the cell lines to five of the antifolates (**8** and **10–13**) and hence to determine the resistance levels. Figure 2 shows a typical set of response curves, i.e. for H69/P and H69/LX4 cells exposed to (**11**) in the presence or absence of cyclosporin A. Table 2 shows the IC_{50} values from a series of experiments. The resistance factors were calculated from the IC_{50} values, (IC_{50} resistant line/IC_{50} parent line), and are summarised in Table 3. A range of resistance was observed in the two MDR sublines, with H69/LX4 generally showing higher resistance factors for the different compounds than L23/R. In H69/P, COR-L23/P and COR-L23/R cells, there was little or no (less than two-fold) sensitisation by VRP or CsA to any of the compounds in the MTT assay (data not shown). This was also true for VRP in H69/LX4. Clear sensitisation of H69/LX4 by CsA was, however, seen as

TABLE 1. Inhibition of rat liver DHFR* by lipophilic antifolates



Compound†	Fluorophore(X)	N	IC_{50}(nM)‡
MBP (**4**)	—	—	10
8	DNS	2	10
9	DNS	3	10
10	NBD	2	9
11	NBD	3	30
12	MMC	2	2
13	MMC	3	3

*Partially purified rat liver DHFR was prepared by a literature procedure [38], and assayed spectrophotometrically at 340 nm as reported previously [39]. Control experiments showed there to be no interference of the assay by the fluorescent compounds at this wavelength.

†Inhibitor solutions were prepared employing ethanol as solvent, at a final concentration (10 mM) which did not interfere with the assay.

‡Defined as the concentration of inhibitor required to reduce DHFR activity to 50% of control. IC_{50} values are the means of duplicate assays conducted at four inhibitor concentrations estimated to reduce DHFR activity by approximately 20, 40, 60 and 80% of control.

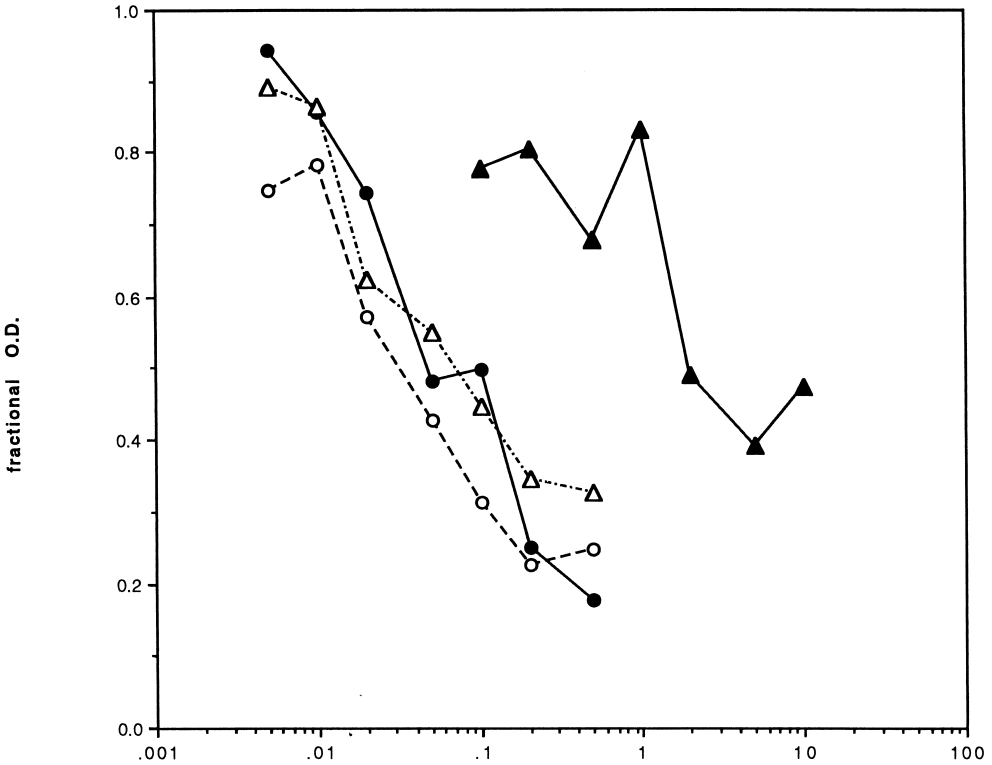


FIG. 2. Dose response curve for (11) in H69 cells. H69/P exposed to (11) alone (●) or in the presence of 4.2 μM CsA (○), or H69/LX4 exposed to (11) alone (▲) or in the presence of 4.2 μM CsA (△). Data are representative of three independent experiments.

shown in Fig. 2. The compound for which sensitisation was least was (**13**), where values of 9-fold or 10-fold were obtained in two independent experiments. For compound (**10**), values of greater than 17-fold and 29-fold were obtained and, for compound (**11**), the values were greater than 22-fold and greater than 500-fold.

Drug Accumulation

Of the six fluorescent antifolates prepared, only compounds (**10**) and (**11**) could be adequately excited by the 488-nm excitation wavelength available on the Cambridge flow cytometer. The intracellular accumulation of these two fluorescent compounds was examined using flow cytometry. There was a large differential in accumulation between both pairs of parent and resistant cell lines over a 30 min to 1-hr time course (Fig. 3). Thus, mean accumulation ratios (ratio of fluorescence in the resistant cell line/parent cell line ± SE) of 0.09 ± 0.05 (N = 6) and 0.15 ± 0.06 (N = 6) were observed for **10** and **11**, respectively, with H69/LX4 vs H69/P cells. This compared with values of 0.08 ± 0.04 (N = 4) and 0.11 ± 0.05 (N = 4) for **10** and **11**, respectively, with L23/R vs L23/P cells. However, only modest effects were seen on the addition of the modifiers

TABLE 2. IC_{50} values* (μM) for lipophilic antifolates in human lung cancer cells as determined by the MTT colorimetric assay

	Compounds				
Cell line	**8**	**10**	**11**	**12**	**13**
H69/P					
Mean	0.20†	0.81	0.36	0.26	0.33
SE (N)	0.05 (5)	0.35 (8)	0.22 (7)	0.04 (6)	0.15 (6)
H69/LX4					
Mean	4.72	20.17‡	15.05‡	9.40	5.20
SE (N)	0.54 (6)	0.58 (8)	2.20 (8)	0.61 (6)	0.79 (6)
L23/P					
Mean	0.87	5.10	2.49	0.93	0.83
SE (N)	0.47 (6)	1.73 (8)	1.15 (7)	0.34 (6)	0.35 (6)
L23/R					
Mean	3.18	10.95	14.97‡	6.19	3.12
SE (N)	0.80 (6)	2.10 (7)	2.51 (8)	1.27 (6)	0.75 (5)

*IC_{50} defined as the concentration of drug required to reduce the final absorbance in the MTT assay to 50% of control (see Materials and Methods).
†Data are given as means (SE) from N independent experiments, in each of which triplicate wells were used at each dose point.
‡Four values of >10 have been included and assumed to be 10.

TABLE 3. Resistance factors* for cell lines exposed to lipophilic antifolates

Cell line	Compounds				
	8	10	11	12	13
H69/LX4 vs H69/P					
Mean	29.6	49.3†	137†	45.8	15.6
SE (N)	6.6 (5)	9.3 (8)	67 (7)	7.9 (6)	5.8 (6)
L23/R vs L23/P					
Mean	6.7	4.9	15.9†	9.8	5.4
SE (N)	2.7 (6)	1.0 (8)	5.8 (7)	2.8 (6)	2.1 (5)

*Resistance factor = IC_{50} resistant line/IC_{50} parent line. These values are derived from the means of the resistance factors for individual experiments.
†Underestimated (see Table 2).

(Fig. 4 and Table 4). Even CsA at 4.2 μM failed to restore drug accumulation in H69/LX4 cells to the levels observed in the parental line H69/P.

DISCUSSION

Although fluorigenic derivatives of MTX, most notably fluorescein-MTX (FMTX), have been directly utilised for the characterisation of MTX-resistant tumour cell lines [31, 32], and indirectly to characterise resistance to TMQ [33], to the best of our knowledge, the analogous use of fluorescent analogues of lipophilic antifolates is without precedent. We have developed a facile two-step synthetic route to multigram quantities of fluorescent lipophilic antifolates starting from nitropyrimethamine (**5**), which is also readily prepared by nitration of the commercially available and inexpensive pyrimethamine [18]. Treatment of (**5**) with the appropriate diamine facilitated the introduction of a 2- or

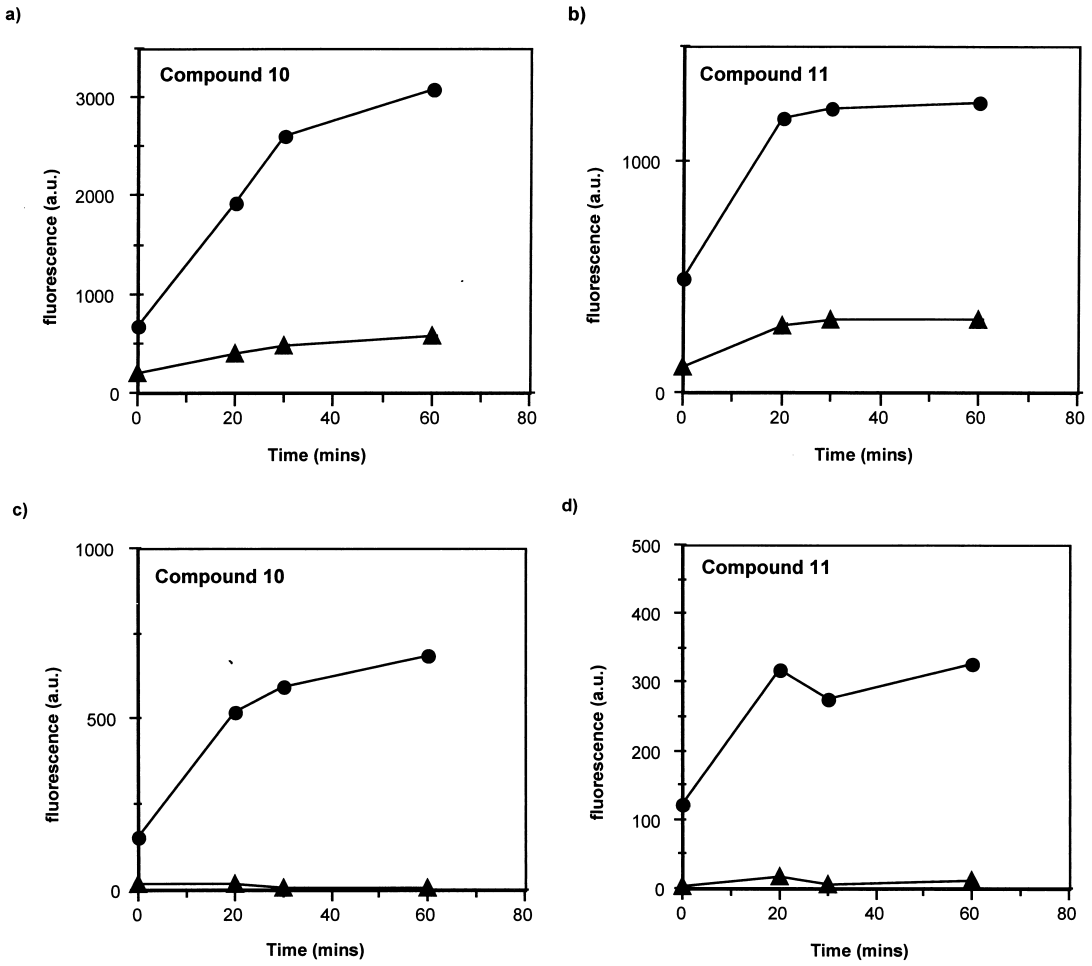


FIG. 3. Accumulation of (10) and (11) in L23 and H69 cells. Panels (a) and (b) are L23/P (●) and L23/R (▲). Panels (c) and (d) are H69/P (●) and H69/LX4 (▲). Note the variation in the scale between the four panels. Data are representative of three independent experiments.

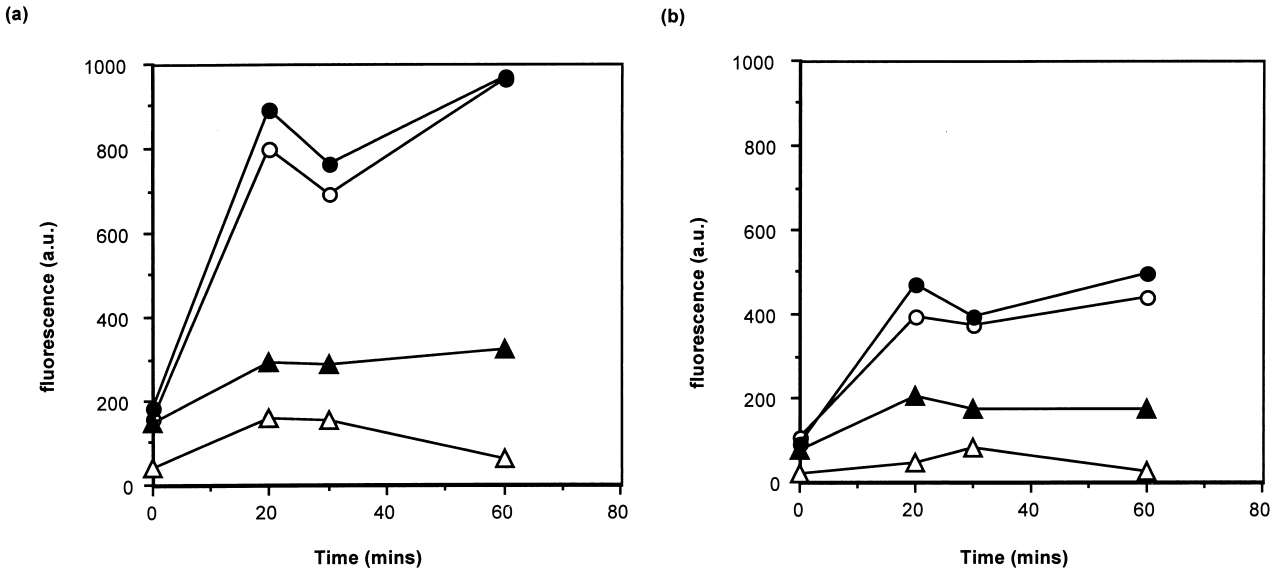


FIG. 4. Accumulation of (10) (a) or (11) (b) in H69/P (circles) and H69/LX4 (triangles) cells in the absence (open symbols) or presence (closed symbols) of CsA at 4.2 μM. Data are representative of three independent experiments.

3-carbon spacer group with a pendant amino function, to which the required fluorophore was subsequently coupled in good yield. Fluorescence studies directly relating to the binding of the compounds to the target enzyme DHFR were not conducted. However, perhaps with the exception of (**11**), it is evident from the inhibitory activities observed against the enzyme that the fluorescent derivatives retain the potency of the parent inhibitor MBP (**4**). Thus, these compounds represent potentially useful alternatives to FMTX, and although only the NBD-substituted derivatives (**10**) and (**11**) proved suitable at an excitation wavelength of 488 nm, it is likely that adequate fluorescence would be observed with the other compounds at alternative excitation wavelengths.

It is clear that both resistant sublines which we have used show moderate to high levels of cross-resistance to this series of lipophilic antifolates. These vary from 15-fold to over 100-fold in H69/LX4 (expresses PgP) and from 5-fold to over 15-fold in L23/R (expresses MRP). Hence, it seems likely that these compounds are substrates for both transporters. Previous studies have shown that expression of Pgp does not normally confer resistance to MTX [12], although Pgp-mediated resistance to MTX has been observed in carrier-deficient cells, where passive diffusion becomes the primary mode of drug uptake [34]. It has also very recently been shown that methotrexate, being a polar organic anion, is a substrate for MRP [35]. By contrast, the lipid soluble compounds TMQ and PTX do have reduced activity in Pgp-expressing cells, although we are not aware of data for their effects in cells which express MRP at high levels. In common with TMQ and PTX, MBP and its fluorescent derivatives are non-polar compounds which lack the glutamate residue of MTX and are thus not anionic at physiological pH.

TABLE 4. Sensitisation ratios* for cell lines exposed to lipophilic antifolates in the presence of the resistance modifiers verapamil (VRP) or cyclosporin A (CsA)

	Compound 10		Compound 11	
Cell Line	**VRP (6.6 μM)**	**CsA (4.2 μM)**	**VRP (6.6 μM)**	**CsA (4.2 μM)**
H69/P	0.90	0.94	1.75	2.12
	0.85	0.89	1.26	0.89
		1.09		1.02
		1.01		0.95
H69/LX4	1.54	2.10	1.85	3.00
	0.46	6.60	1.31	5.20
		3.52		2.15
		1.88		2.09
L23/P	0.92	1.22	0.92	1.09
	1.07	1.25	1.04	1.47
L23/R	3.41	5.80	2.24	1.70
	1.52	2.27	1.51	1.29

*Sensitisation ratio is defined as the ratio of the accumulation of the fluorescent compound in presence/absence of the modifier. Values given are for individual experiments.

For the fluorescent compounds (**10**) and (**11**), resistance in the MTT assay is associated with dramatically reduced accumulation of cellular fluorescence as determined by flow cytometry. This seems likely to be also true for the compounds which we were not able to study by flow cytometry, and hence where we have not been able to measure accumulation *per se*, but for which similar resistance factors are seen. Reduced accumulation can result from both decreased drug influx and increased efflux. We have not carried out experiments intended to differentiate between these two and hence cannot reach firm conclusions. However, for drugs such as doxorubicin and vincristine, both the Pgp and MRP mechanisms are almost always associated with increased efflux in resistant cells [7, 9, 10]. Efflux studies would also be useful in defining the binding capacity of these compounds, and their possible utility as functional probes of Pgp or MRP activity.

Verapamil (6.6 μM) and CsA (4.2 μM) have generally been observed to be highly effective modifiers of MDR arising from overexpression of Pgp, whereas they are less effective as modifiers of MRP [36, 37]. Our finding of sensitisation by CsA only in H69/LX4 is therefore in line with a mechanism of action *via* inhibition of Pgp. The lack of effectiveness of verapamil even in H69/LX4 is, however, surprising and we currently have no explanation for this interesting observation.

Discrepancies between results obtained in chemosensitivity assays (such as the MTT assay as used here) and results of drug accumulation assays are not unusual. Our chemosensitivity assay uses continuous drug exposure over a period of 6 days, whereas the drug accumulation experiments are complete within 1 hr. Furthermore, the concentrations of drug used for flow cytometry are generally much higher than the IC_{50} values obtained in chemosensitivity assays. Hence, the relatively modest restoration of cellular accumulation of compounds (**10**) and (**11**) by cyclosporin A in comparison with the greater effects on chemosensitivity, does not necessarily mean that other mechanisms need be invoked.

If, therefore, these novel compounds are able to overcome methotrexate resistance where this is due to a failure of carrier-mediated active transport, this advantage is likely to be lost where efflux "pump" mechanisms such as PgP or MRP are overexpressed. As both these mechanisms have been shown to be components of the blood-brain barrier, this is likely to preclude their use against brain tumours where the barrier is intact.

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