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# EFFECTS OF *BIS*(6-MERCAPTOPURINE-9-β-D-RIBOFURANOSIDE)-5′,5‴-PHOSPHATE AND ITS BUTYRYL DERIVATIVE ON MOUSE LEUKAEMIA L1210 AND A 6-MERCAPTOPURINE-RESISTANT SUBLINE IN CULTURE

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**Abstract**—*Bis*(6-mercaptopurine-9-β-D-ribofuranoside)-5′-5‴-monophosphate (*bis*(MPR)P) and its butyryl derivative, *bis*($O^2$′,$O^3$′-dibutyryl-6-mercaptopurine-9-β-D-ribofuranoside)-5′,5‴-monophosphate (*bis*(dibutyrylMPR)P) were synthesized from 6-mercaptopurine-9-β-D-ribofuranoside (MPR). *Bis*(MPR)P ($EC_{50}$ = 0.014 μM) and MPR ($EC_{50}$ = 0.022 μM) were essentially equivalent in their growth inhibitory activities against L1210/0 cell cultures, whilst *bis*(dibutyrylMPR)P ($EC_{50}$ = 1.1 μM) was considerably less effective. L1210/MPR cells grew normally in the presence of 1 mM MPR but were inhibited by *bis*(MPR)P ($EC_{50}$ = 580 μM) and *bis*(dibutyrylMPR)P ($EC_{50}$ = 42 μM). *Bis*(dibutyrylMPR)P was less readily broken down to MPR by enzymes in the serum component of the culture medium than was *bis*(MPR)P, and leukaemia cells did not contribute to the extracellular degradation of the acylated derivative. The delayed cytotoxic effects of *bis*(MPR)P and *bis*(dibutyrylMPR)P on L1210/0 cells were those of the MPR breakdown product. Exposure to *bis*(MPR)P resulted in delayed cytotoxicity in L1210/MPR cultures whilst *bis*(dibutyrylMPR)P produced only acute growth inhibition and no delayed effect on the MPR-resistant subline. MPR was incorporated into DNA of L1210/0 cells as 6-thioguanine deoxyribonucleotide whilst *bis*(MPR)P was not incorporated into L1210/MPR cell DNA. These results suggested that the ultimate mechanisms of action of *bis*(MPR)P and *bis*(dibutyrylMPR)P in L1210/MPR cells may have been different from that of MPR in sensitive L1210/0 cells and therefore might not represent true circumvention of resistance to MPR.

6-Mercaptopurine (MP)† has been widely used in the clinical treatment of acute leukaemia and there have been extensive investigations of the biochemical mechanism of action of this purine anti-metabolite and of the bases of cellular resistance to it. The drug is converted intracellularly to the nucleotide, 6-thioinosine 5′-monophosphate (MPRP) as the first obligatory step in its cytotoxic action [1]. MPRP has been shown to induce a number of biochemical perturbations in proliferating cells including inhibition of purine nucleotide biosynthesis *de novo* and inhibition of purine nucleotide interconversions. However, it is the further metabolism of MPRP to 6-thioguanine nucleotides and the subsequent incorporation of 6-thioguanine deoxyribonucleotide into internucleotide linkage in DNA that is responsible for the antileukaemic activity of MP [2–4].

A major drawback in the use of MP and similar drugs is that cellular resistance is often encountered or readily develops during the course of prolonged treatment. Although resistance to MP could conceivably result from a number of alterations at enzyme loci along the path to its incorporation into DNA, it has generally been found that low intracellular concentrations of MPRP or short persistence of the nucleotide once formed are most commonly responsible for insensitivity to the drug [5]. This is presumably because MPRP is formed from MP through the 'purine salvage' system which, since it is not vital to cell survival, may undergo alteration without affecting cell viability. Reduction in the nett intracellular accumulation of MPRP may result from one or more of several mechanisms. These have been shown to include: reduction in or loss of the enzyme activity responsible for nucleotide formation, IMP-GMP: pyrophosphate phosphoribosyltransferase (EC 2.4.2.8) (trivial name, hypoxanthine–guanine phosphoribosyltransferase; abbreviation, HGPRT); increase in dephosphorylating enzyme activity, orthophosphoric monoester phosphohydrolase (EC 3.1.3.1), notably particulate bound alkaline phosphohydrolases, which degrade MPRP to the drug riboside, 6-thioinosine (MPR); decreased availability of 5-phosphoribosyl pyrophosphate, the second substrate of HGPRT; increased dethiolation of MPRP to produce the normal nucleotide inosinate [5].

Resistance to MP involving reduced intracellular accumulation of MPRP may not be circumvented by treatment with performed MPRP since cell membranes are relatively impermeable to nucleotides and in the case of MPRP extracellular dephosphorylation at the cell membrane readily converts the drug nucleotide to its nucleoside. MPR may be taken up

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† Abbreviations used: MP, 6-mercaptopurine; MPR, 6-mercaptopurine-9-β-D-ribofuranoside; MPRP, 6-mercaptopurine-9-β-D-ribofuranoside-5′-monophosphate; *bis*(MPR)P, *bis*(6-mercaptopurine-9-β-D-ribofuranoside)-5′,5‴-monophosphate; *bis*(dibutyrylMPR)P, *bis*($O^2$′,$O^3$′-dibutyryl-6-mercaptopurine-9-β-D-ribofuranoside)-5′,5‴-monophosphate; HGPRT, IMP-GMP: pyrophosphate phosphoribosyltransferase.

rapidly by cells but a kinase capable of phosphorylating the nucleoside directly is present at very low levels, if at all, in animal cells. However, in some cases MPR has been shown to be somewhat more effective than MP against MP-resistant cells (for example, see [6]) presumably either because the nucleoside is phosphorylated directly by a low activity of kinase present in the cells, or because ribose-1-phosphate released by phosphorolytic cleavage of MPR may stimulate 5-phosphoribosyl pyrophosphate synthesis through its conversion to ribose-5-phosphate by phosphoribomutase. However, the activity of MPR has never been sufficiently different from that of MP to make this drug a useful alternative when resistance to the latter is encountered [5]. For all practical purposes intracellular MPRP is produced from the extracellular drug nucleotide via dephosphorylation, uptake and phosphorolysis of MPR and conversion of the resulting MP base to nucleotide by HGPRT. A similar state of affairs has been found with other purine and pyrimidine antimetabolites although there are reports that less readily hydrolyzed nucleotides such as 9-β-D-arabinofuranosyl-adenine 5′-monophosphate may penetrate cells slowly as intact molecules [5].

So-called 'prodrugs' of drug nucleoside 5′-monophosphates have been developed in attempts to circumvent mechanisms of resistance to MP and similar agents. These are derivatives of the drug nucleotides which are designed to be relatively resistant to extracellular degradation whilst at least theoretically possessing the ability to enter cells and undergo intracellular modification with release of the free 5′-monophosphates at cytotoxic concentrations. Reduction of the negative charge on the phosphate group has been a general feature of such prodrug design since it is thought that charge repulsive effects at the cell membrane are largely responsible for the poor uptake of nucleotides by cells. Esterification or derivatization of the phosphate group also protects the nucleotides from the action of extracellular phosphohydrolases. In addition, the lipophilicity of prodrug molecules has been increased in attempts to enhance diffusion into and across cell membranes. This has been achieved through derivatization with non-polar groups which are susceptible to spontaneous or enzyme catalysed removal within the cell.

Montgomery *et al.* [7] synthesized a series of phosphodiesters of MPR on the basis that these might be cleaved by cellular phosphodiesterases to liberate MPRP. Simple alkyl esters of MPRP were found to have minimal ability to inhibit an HGPRT-deficient, MP-resistant human epidermoid cell subline, HEp No. 2/MP in culture. However, *bis*(6-mercaptopurine-9-β-D-ribofuranoside)-5′,5‴-monophosphate [trivial name, *bis*(thioinosine)-5′,5‴-phosphate; abbreviation *bis*(MPR)P] exhibited considerable activity against the resistant cells in contrast to MP, MPR and MPRP which were ineffective. In discussing these results, they suggested that the presence of a nucleoside group on each side of the phosphodiester bond was a requisite for cleavage by cellular phosphodiesterase. *Bis*(MPR)P was shown to be a good substrate for phosphodiesterases present in cell-free extracts of HEp No. 2 cells, and on the grounds of the apparent circumvention of resistance to MP it was postulated that the intact derivative might have penetrated HEp No. 2/MP cells and undergone intracellular hydrolysis to generate cytotoxic concentrations of MPRP. However, no further data on *bis*(MPR)P were published and other antimetabolite dinucleoside phosphates were found to be no more effective than the parent drugs against sensitive and resistant neoplasms [8].

3′,5′-Cyclic phosphates of purine and pyrimidine nucleoside antimetabolites have also been investigated as prodrugs of the 5′-monophosphates in which the charge on the phosphate is reduced by internal esterification [5, 8]. Meyer *et al.* [6] demonstrated that although 6-thioinosine 3′,5′-cyclic monophosphate was not significantly more effective than MP against HGPRT-deficient lymphoma cells, the addition of a removable lipophilic group by acylation of the sugar 2′-hydroxyl gave rise to compounds which were more active than MP against the MP-resistant cells. They observed a progressive increase in activity of these derivatives with increasing carbon chain length of the acyl group up to a maximum effect with the palmitoyl ($C_{16}$) compound, 2′-*O*-palmitoyl-6-thioinosine 3′,5′-cyclic phosphate. The activity of the derivatives decreased with increase in carboxylic acid chain length beyond $C_{16}$. Meyer *et al.* [6] suggested that the effects of the acylated cyclic nucleotides on MP-resistant cells represented true circumvention of the resistance mechanism through cellular uptake of the intact prodrugs and subsequent intracellular release of MPRP.

Following from our interest in the mechanism of action of purine and pyrimidine antimetabolites, we have initiated an investigation of 5′-monophosphate prodrugs which have been reported to circumvent biochemical mechanisms of cellular resistance. An aim of this work is to establish whether or not the effects of the prodrugs upon resistant cells are mediated through the same ultimate mechanisms of action as those of the parent drugs in sensitive cells (i.e. true circumvention of resistance). We have also embarked upon a programme of chemical syntheses to introduce further modifications into prodrug molecules in an attempt to enhance their activity against resistant cells. In the present paper we describe the effects of *bis*(MPR)P on thiopurine-sensitive and -resistant L1210 cell cultures in relation to the action of MPR. In addition, the effects of a new derivative, *bis*($O^{2'}$, $O^{3'}$-dibutyryl-6-mercaptopurine-9-β-D-ribofuranoside)-5′,5‴-monophosphate (abbreviation, *bis*(dibutyrylMPR)P) on these cell lines are reported.

## MATERIALS AND METHODS

*Chemical syntheses.* *Bis*(6-mercaptopurine-9-β-D-ribofuranoside)-5′,5‴-monophosphate (*bis*(MPR)P) and its butyryl derivative, *bis*($O^{2'}$,$O^{3'}$-dibutyryl-6-mercaptopurine-9-β-D-ribofuranoside)-5′,5‴-monophosphate [*bis*(dibutyrylMPR)P] were synthesized from MPR (Sigma (London) Chemical Co., Poole, U.K.). The chemical structures of the compounds are presented in Fig. 1. *Bis*(MPR)P was synthesized by a modification of the procedure of Thomas and Montgomery [9]. In the first step of the synthesis

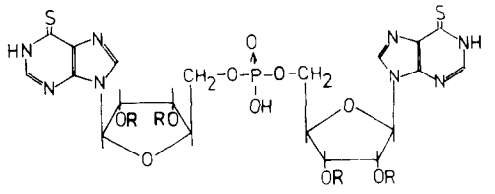

Fig. 1. Chemical structures of *bis*(6-mercaptopurine-9-β-D-ribofuranoside)-5′,5‴-monophosphate (R = H), and *bis*($O^{2'}$, $O^{3'}$-dibutyryl-6-mercaptopurine-9-β-D-ribofuranoside)-5′,5‴-monophosphate (R = $C_3H_7 \cdot CO$).

2′,3′-isopropylidene 6-thioinosine was prepared by the perchloric acid-catalysed condensation of MPR and acetone described by Zderic *et al.* [10]. Two equivalents of this derivative were allowed to react over a period of 24 hr at room temperature with one equivalent of *p*-nitrophenylphosphorodichloridate in anhydrous pyridine in the presence of 10 equivalents of 1,2,4-triazole catalyst. The *p*-nitrophenyl group was removed from the resulting phosphotriester by alkaline hydrolysis at room temperature and the isopropylidene groups were displaced during chromatographic purification of the product on Dowex-50W resin, hydrogen form (Sigma). The product was eluted with water supplemented with 0.01% (v/v) β-mercaptoethanol and fractions containing *bis*(MPR)P free acid were pooled and lyophilized. The derivative was converted to the sodium salt and lyophilized for cell culture investigations. Analysis of *bis*(MPR)P free acid, $C_{20}H_{23}N_8O_{10}PS_2.2H_2O$ as calculated was: C, 36.03; H, 4.09; N, 16.81; S, 9.61; and as found: C, 36.35; H, 3.92; N, 16.22; S, 9.77. The u.v. spectrum of *bis*(MPR)P at pH 4.5 was identical to that of MPR with an absorbance maximum at 322 nm.

The 25 MHz carbon-13 NMR spectrum of MPR consisted of 10 singlet peaks whilst in the spectrum of *bis*(MPR)P the C4′,C5′-resonances were a doublet and a partially resolved doublet respectively due to spin coupling with phosphorus. Chemical shifts (ppm from TMS) in the $^{13}C$ NMR spectra of *bis*(MPR)P in $D_2O$ were: C5′ 65.58, C3′ 70.68, C2′ 75.80, C4′ 84.28, C1′ 89.56, C5 135.45, C8 142.26, C4 144.30, C2 147.15, C6 175.69. Chemical shifts (ppm from TMS) in the corresponding $^{13}C$ NMR spectra of MPR in $D_2O$ were: C5′ 58.87, C3′ 68.03, C2′ 70.76, C4′ 82.94, C1′ 85.76, C5 133.13, C8 138.00, C4 142.39, C2 147.84, C6 175.13.

*Bis*(MPR)P was analysed by high performance liquid chromatography (HPLC) on a strong anion exchange column. The elution time of the compound was intermediate between that of MPR and MPR 5′-monophosphate and corresponded to the region of the chromatograms where compounds bearing a single negative charge are normally detected.

*Bis*(dibutyrylMPR)P was synthesized by direct acylation of *bis*(MPR)P. *Bis*(MPR)P free acid was shaken with a 64-fold molar excess of *n*-butyric anhydride in anhydrous pyridine at room temperature for 4 days. The reaction was terminated by addition of excess water with ice cooling of the reaction flask and the mixture was allowed to stand for 1 hr at room temperature. The bulk of the solvent was removed *in vacuo*. The product was extracted with chloroform from an aqueous methanolic suspension of the residue at pH 2. The organic phase was washed with water and *bis*(dibutyrylMPR)P was precipitated by addition of ethyl acetate. The precipitate was collected by filtration, washed with chloroform and dried *in vacuo*. *Bis*(dibutyrylMPR)P was converted to its sodium salt for experiments with cell cultures. Analysis of *bis*(dibutyrylMPR)P free acid, $C_{36}H_{47}N_8O_{14}PS_2.H_2O$ as calculated was: C, 46.53; H, 5.32; N, 12.06; S, 6.90; and as found: C, 46.31; H, 5.06; N, 11.81; S, 6.80. The u.v. spectrum of *bis*(dibutyrylMPR)P in methanol:25 mM potassium phosphate buffer, pH 4.5, 1:1 by volume, was the same as that of MPR with an absorbance maximum at 322 nm.

*Chromatography.* Chemical syntheses, purification and drug metabolism were monitored by HPLC using an Altex gradient chromatograph (Anachem Ltd, Luton, U.K.) fitted with a Whatman Partisil-10 SAX, strong anion exchange analytical column (Uniscience Ltd, Cambridge, U.K.). Separation conditions for nucleotides were as described by Tidd and Dedhar [11], comprising a 15 min concave gradient from 0.005 M potassium phosphate, pH 3.5, to 0.25 M potassium phosphate/0.5 M potassium chloride, pH 4.5, at a flow rate of 3 ml/min. The u.v. absorbance of the column effluent was measured simultaneously at 254 nm and 320 nm.

The course of butyration reactions was followed by thin-layer chromatography on Merck 5 × 10 cm silica gel 60 $F_{254}$ pre-coated plates (BDH Chemicals Ltd, Enfield, U.K.) developed with chloroform/methanol/water, 62:25:4 by volume.

*Cell cultures.* The cell culture line of parent thiopurine-sensitive L1210 cells, L1210/0, was kindly provided by Dr. K. R. Harrap, The Institute of Cancer Research (Sutton, U.K.). The highly resistant subline L1210/MPR was derived in our laboratory from an L1210/0 culture by repetitive exposure to increasing concentrations of MPR. The basis of thiopurine resistance of L1210/MPR cells was apparently their inability to accumulate significant intracellular concentrations of thiopurine nucleoside 5′-monophosphate relative to L1210/0 cells. The L1210 cell lines were maintained in continuous culture in Fischer's medium containing 10% horse serum and penicillin/streptomycin [100 units/100 μg per ml (Gibco Europe Ltd, Paisley, U.K.)]. L1210/MPR stock cultures were passaged in the presence of 1 mM MPR. Cells were enumerated with a Model ZB Coulter Counter (Coulter Electronics Ltd, Luton, U.K.). For determination of dose–response curves replicate 2.5 ml cultures of leukaemia cells were prepared in 100 × 14 mm Nunc, sterile, plastic, disposable culture tubes (Gibco) and were treated with a range of drug concentrations. Cell numbers in each culture were determined after 3 days of incubation at 37° in an atmosphere of 5% $CO_2$/95% air. Untreated control cultures were still growing exponentially at the end of the 3-day incubation period. Cultures of thiopurine-sensitive V79 chinese hamster lung cells, CH/O, and a thiopurine-resistant subline deficient in HGPRT, CH/TG, were kindly donated by Dr. M. Fox, The Paterson Laboratories, Christie Hospital and Holt Radium Institute (Manchester, U.K.). The cells were maintained in continuous culture in Eagles Minimal Essential medium with

Earle's salts containing 10% foetal calf serum and penicillin/streptomycin, 100 units/100 μg per ml (Gibco). For determination of dose–response curves replicate 5 ml cultures of chinese hamster cells were prepared in 25 $cm^2$ Nunc, sterile, plastic, disposable tissue culture flasks (Gibco). Flasks were gassed with 5% $CO_2$/95% air and the cultures were incubated at 37° for 3 days. Drugs were added as 0.1 ml of an appropriate stock solution in 0.9% sodium chloride and the cultures were incubated for a further 3 days, after which cells were detached by trypsinization and counted. Untreated control cultures were still growing exponentially at the end of the 6-day incubation period.

For cell culture investigations stock solutions of MPR and *bis*(MPR)P sodium salt were prepared in 0.9% sodium chloride solution and were sterilized by filtration through Millex sterile, disposable, 0.22 μm membrane filter units (Millipore U.K. Ltd, London, U.K.).

*Bis*(dibutyrylMPR)P was sparingly soluble in water even in the form of its sodium salt. However, the drug was considerably more soluble in complete Fischer's cell culture medium presumably as a result of hydrophobic interactions with proteins of the serum component of the culture fluid. In preparing stock solutions of *bis*(dibutyrylMPR)P for cell culture studies homogeneous suspensions of the sodium salt were prepared in 0.9% sodium chloride by ultrasonication. Measured volumes of the gelatinous suspensions were dissolved in culture medium at 37° and the solutions were sterilized by filtration through Nalgene, sterile, disposable, 0.20 μm membrane filter units (Fisons Scientific Apparatus Ltd, Loughborough, U.K.). The concentrations of drug in the sterile solutions were checked spectrophotometrically following dilution of samples in a solvent consisting of methanol:25 mM potassium phosphate buffer, pH 4.5, 1:1 by volume, containing 0.1% (v/v) β-mercaptoethanol ($E_{322}$ = 51,800).

*Drug metabolism and degradation.* Degradation of *bis*(MPR)P in culture media at 37° was determined by HPLC analysis. Media samples (0.5 ml) were removed at various times, cooled to 0° and the serum proteins precipitated by addition of 0.05 ml 42% (w/v) perchloric acid. The precipitates were removed by centrifugation and the supernatants neutralized with 6 N potassium hydroxide at 0°. Potassium chlorate precipitates were removed by centrifugation prior to analysis. The degradation of *bis*(dibutyrylMPR)P in culture media in the presence and absence of cells was also measured by HPLC. The deproteination procedure was similar to that for the *bis*(MPR)P experiments except that cells were removed by centrifugation and 0.5 ml of methanol was added to the samples prior to addition of the perchloric acid to prevent precipitation of *bis*(dibutyrylMPR)P free acid.

Incorporation of 6-mercaptopurine compounds into internucleotide linkage in newly replicated cell DNA as 6-thioguanine deoxyribonucleotide was measured as previously described [11]. Briefly, drug-treated cells were washed and acid-soluble constituents were removed by extraction with perchloric acid. RNA was solubilized by alkaline hydrolysis and DNA in the acid insoluble residue was digested with DNase I plus venom phosphodiesterase (Sigma). The acid soluble components of the DNA hydrolyzate were subjected to mild oxidation with alkaline potassium permanganate to convert 6-thioguanine deoxyribonucleotide to the highly fluorescent guanine-6-sulphonate derivative. The latter was analysed by HPLC using the standard nucleotide separation conditions with flow-fluorescence monitoring of the column effluent.

## RESULTS

The effects of MPR, *bis*(MPR)P, and *bis*(dibutyrylMPR)P on the growth of thiopurine-sensitive L1210/0 cell cultures are presented in the dose–response curves of Fig. 2. MPR and *bis*(MPR)P were essentially equivalent in terms of their growth inhibitory activity whereas *bis*(dibutyrylMPR)P was considerably less effective than the former two compounds. The concentrations of the drugs required to inhibit growth by 50% ($EC_{50}$ values) estimated by interpolation from these data are: MPR, $EC_{50}$ = 0.022 μM; *bis*(MPR)P, $EC_{50}$ = 0.014 μM; *bis*(dibutyrylMPR)P, $EC_{50}$ = 1.1 μM. MPR rather than the free base MP is included as a control in determinations of the effects of the phosphorylated derivatives, since the nucleoside would be the immediate product of their dephosphorylation. The dose–response curves in Fig. 3 represent the effects of MPR, *bis*-(MPR)P, *bis*(dibutyrylMPR)P, and sodium butyrate on the growth of cultures of the highly MPR-resistant, L1210/MPR subline. It can be seen that MPR had no effect on these cells even at a concentration of 1 mM. *Bis*(MPR)P at high concentrations inhibited cell proliferation ($EC_{50}$ = 580 μM) whilst butyration of the dinucleoside phosphate resulted in an approximately 14-fold increase in activity (*bis*(dibutyrylMPR)P, $EC_{50}$ = 42 μM) over

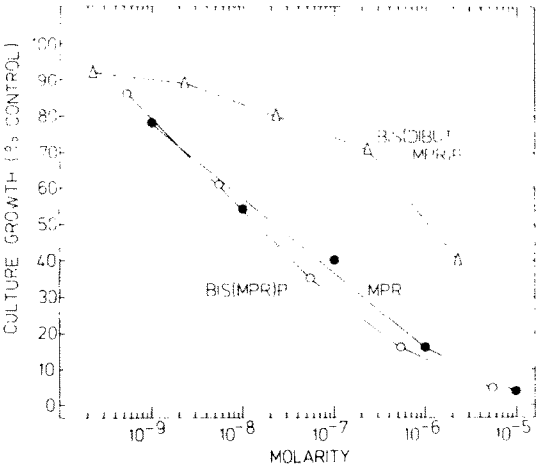


Fig. 2. Dose–response curves for treatment of thiopurine-sensitive L1210/0 cell cultures with MPR, *bis*(MPR)P and *bis*(dibutyrylMPR)P. Drugs were added immediately after the cells were subcultured and cell numbers were determined following incubation for 3 days at 37°. Each point represents the mean value for two cultures. ●, MPR; ○, *bis*(MPR)P; △, *bis*(dibutyrylMPR)P.

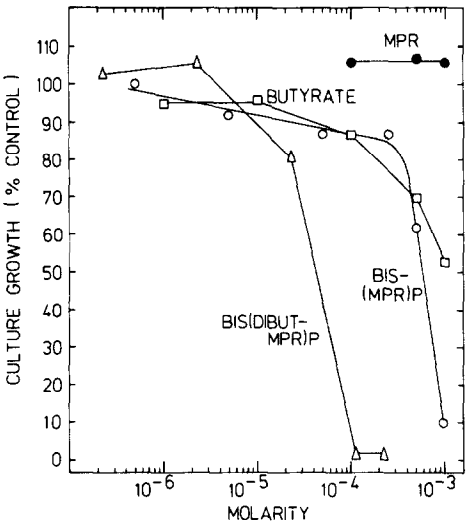


Fig. 3. Dose–response curves for treatment of thiopurine-resistant L1210/MPR cell cultures with MPR, *bis*(MPR)P, *bis*(dibutyrylMPR)P, and sodium butyrate. Drugs were added immediately after the cells were subcultured and cell numbers were determined following incubation for 3 days at 37°. Each point represents the mean value for two cultures. ●, MPR; ○, *bis*(MPR)P; △, *bis*(dibutyrylMPR)P; □, sodium butyrate.

that of *bis*(MPR)P itself. Sodium butyrate, included as a control for the acylated compound, inhibited cell proliferation at high concentrations; an $EC_{50}$ of approximately 1 mM was observed.

*Bis*(MPR)P was also equivalent to MPR ($EC_{50}$ = 1.7 μM) in terms of its growth inhibitory effects on cultures of thiopurine-sensitive V79 chinese hamster lung cells, CH/O (Fig. 4). However, in contrast to the results with L1210/MPR cells, *bis*(MPR)P was no more effective than MPR ($EC_{50}$ > 1mM) against the thiopurine-resistant chinese hamster subline, CH/TG (Fig. 4). It was apparent that this difference

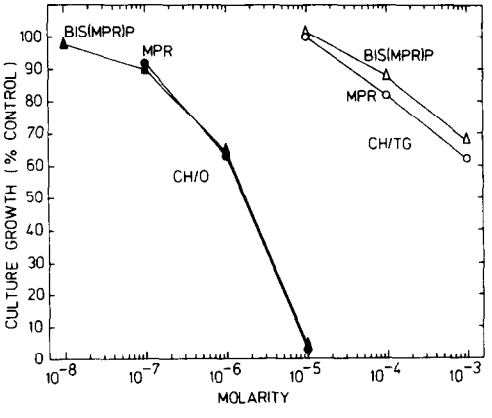


Fig. 4. Dose–response curves for treatment of thiopurine-sensitive CH/O and thiopurine-resistant CH/TG cell cultures with MPR and *bis*(MPR)P. Drugs were added 3 days after the cells were subcultured and cell numbers were determined following further incubation for 3 days at 37°. Each point represents the mean value for two cultures. Filled symbols, CH/O cells; open symbols, CH/TG cells; ● ○, MPR; ▲ △, *bis*(MPR)P.

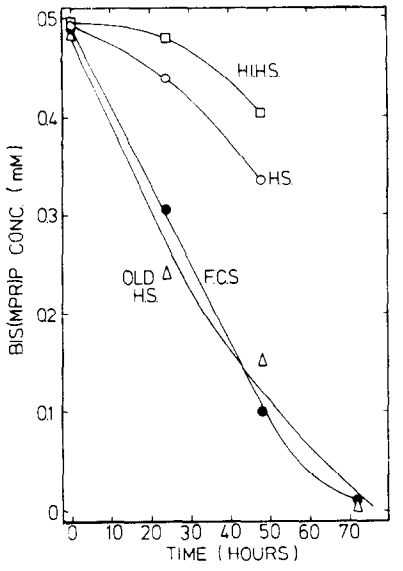


Fig. 5. Degradation of *bis*(MPR)P in culture media in the absence of cells during incubation at 37°. Solutions of *bis*(MPR)P (0.5 mM) were prepared in cell culture media and were analysed by high performance liquid chromatography. ○, Fischer's medium containing 10% horse serum (HS); □, Fischer's medium containing 10% heat inactivated horse serum (HIHS); △, Fischer's medium previously stored for 6 weeks at 2° following addition of 10% horse serum; ●, Eagle's Minimal Essential medium containing 10% foetal calf serum (FCS).

in activity of *bis*(MPR)P on the two resistant cell lines might be related to differences in the stability of the derivative in the two cell culture media. In order to investigate this possibility, the degradation of *bis*(MPR)P (0.5 mM) was monitored by HPLC during incubation at 37° in the absence of cells in Fischer's medium supplemented with 10% horse serum (L1210 culture medium) and in Eagle's Minimal Essential medium supplemented with 10% foetal calf serum (chinese hamster cell culture medium). Samples of the media were removed at various times and deproteinated with perchloric acid prior to HPLC analysis. MPR was the major breakdown product of *bis*(MPR)P whilst small amounts of MPRP (<10% total thiopurines) were also detected during the course of this experiment, the results of which are presented in Fig. 5. It can be seen that *bis*(MPR)P was readily degraded in Eagle's medium and hydrolysis of the compound was complete within three days. The dinucleoside phosphate was comparatively more stable during incubation at 37° in freshly prepared Fischer's medium and hydrolysis of the derivative was even slower in Fischer's medium containing heat inactivated horse serum. However, rapid degradation of the drug was observed when *bis*(MPR)P was incubated at 37° in Fischer's medium that had previously been stored for 6 weeks at 2° following addition of horse serum from the frozen stock. It is noteworthy that the activity of *bis*(MPR)P against L1210/MPR cells was abolished when the cells were cultured in such 'old' Fischer's medium (data not shown). These data demonstrated that the sera used in preparation of the culture media contained enzymes, presumably a phosphodiesterase

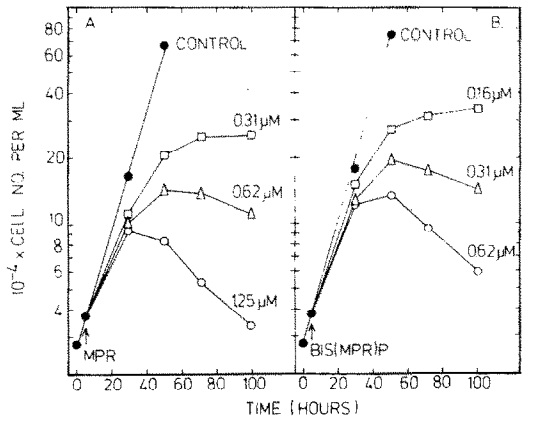


Fig. 6. Effects of MPR and *bis*(MPR)P on the growth curves of L1210/0 cultures. Replicate cultures of L1210/0 cells were prepared at 0 hr and the drugs were added at 5 hr. (A) MPR. □, 0.31 μM; △, 0.62 μM; ○, 1.25 μM; ●, untreated control. (B) *Bis*(MPR)P. □, 0.16 μM; △, 0.31 μM; ○, 0.62 μM; ●, untreated control.

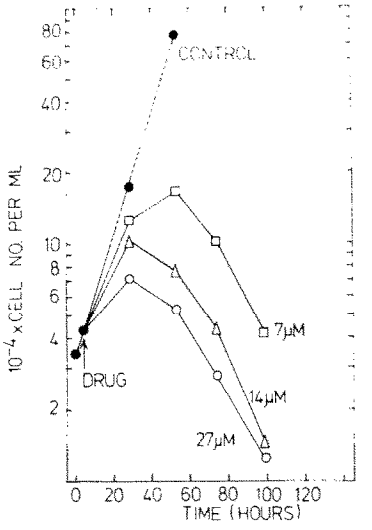


Fig. 7. Effects of *bis*(dibutyrylMPR)P on the proliferation of L1210/0 cells. Replicate cultures of L1210/0 cells were prepared at 0 hr and the drug was added at 4.25 hr. □, 7 μM; △, 14 μM; ○, 27 μM; ●, untreated control.

and a phosphohydrolase, capable of degrading *bis*(MPR)P to MPR. The presence of a phosphohydrolase was indicated by the fact that MPR was by far the major breakdown product whilst equimolar amounts of MPRP and MPR would be produced by the action of phosphodiesterase alone. Higher activities of these enzymes were present initially in foetal calf serum than in horse serum, but the horse serum enzymes appeared to increase in activity during storage of the medium at 2°.

MP and MPR induce a characteristic delayed cytotoxic effect in exponentially proliferating cultures of sensitive leukaemia cells [2, 3, 12]. Therefore, an investigation of the effects of *bis*(MPR)P and *bis*(dibutyrylMPR)P on culture growth curves, particularly those of thiopurine-resistant L1210/MPR cells was pertinent to an understanding of the mode of action of these derivatives. A prodrug of MPRP capable of true circumvention of the biochemical mechanisms of resistance to MPR would be expected to elicit qualitatively similar effects on growth curves of MPR-resistant cells as those of MPR on thiopurine-sensitive cells, since the ultimate mechanism of action would be the same in both instances. The delayed cytotoxic effect of MPR on thiopurine-sensitive L1210/0 cells is illustrated in the growth curves of Fig. 6A. The cells completed 1–2 divisions after the drug was added to the cultures before cell proliferation was finally arrested. Flow-fluorescence measurements of cell DNA content indicated that MP-treated cells became blocked and accumulated in the G2 phase of the cell cycle at the time of the delayed cytotoxic effect*. *Bis*(MPR)P produced similar effects on L1210/0 cells (Fig. 6B) at concentrations which were equivalent to those of MPR in Fig. 6A (NB, one molecule of *bis*(MPR)P contains two molecules of MPR). However, the data of Fig. 6B suggest that *bis*(MPR)P was slightly slower acting than MPR. *Bis*(dibutyrylMPR)P also induced delayed cytotoxic effects in exponentially growing cultures of L1210/0 cells (Fig. 7) but at concentrations that were considerably higher than those of MPR (Fig. 6A) and *bis*(MPR)P (Fig. 6B). In apparent agreement with its proposed mechanism of action as a prodrug of intracellular MPRP, *bis*(MPR)P was also shown to elicit a delayed cytotoxic reaction in cultures of MPR-resistant L1210/MPR cells (Fig. 8).

We have previously shown that the mechanism of the antileukaemic action of MP involves intracellular metabolism of MPRP to 6-thioguanine nucleotides and its subsequent incorporation into DNA as 6-thioguanine deoxyribonucleotide [3]. A specific and sensitive method has been developed for detection of such incorporation of MP into DNA as TG [11]. This involves extraction of acid solution components from cell samples with perchloric acid, removal of

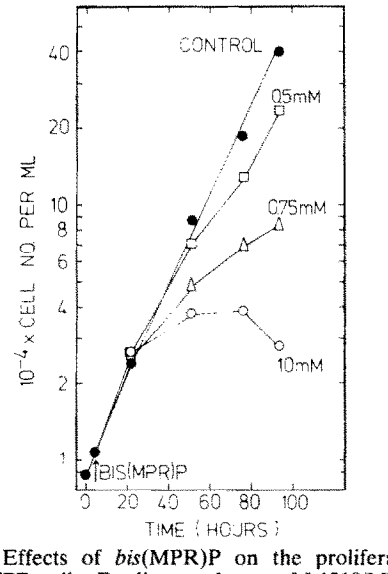


Fig. 8. Effects of *bis*(MPR)P on the proliferation of L1210/MPR cells. Replicate cultures of L1210/MPR cells were prepared at 0 hr and the drug was added at 4.75 hr. □, 0.5 mM; △, 0.75 mM: ○, 1.0 mM; ●, untreated control.

* D. M. Tidd, unpublished observations.

RNA by alkaline hydrolysis and digestion of DNA present in the acid insoluble residues with DNase I plus venom phosphodiesterase. The soluble DNA digests are then subjected to mild oxidation with alkaline potassium permanganate which converts any 6-thioguanine deoxyribonucleotide present to its highly fluorescent 6-sulphonate derivative. The components of the resulting mixtures are separated by HPLC and analysed for guanine-6-sulphonate deoxyribonucleotide by flow-fluorescence monitoring of the column effluent. In the present work incorporation into DNA was measured in L1210/0 cell cultures, exposed to 10 $\mu$M MPR for 15 hr and in L1210/MPR cells treated with 1 mM *bis*(MPR)P for 15 hr. This experiment demonstrated that whilst MPR was incorporated into the DNA of L1210/0 cells as 6-thioguanine at a level of 111 nmoles/$10^9$ cells, *bis*(MPR)P was not incorporated into DNA of L1210/MPR cells to any detectable extent. The HPLC method is capable of detecting 0.003 nmoles of oxidized 6-thioguanine derivatives and therefore with DNA hydrolyzates from $3 \times 10^6$ cells applied to the column the limit of sensitivity of the assay for 6-thioguanine incorporation was 1 nmole/$10^9$ cells.

The data of Fig. 9 demonstrate that *bis*(dibutyrylMPR)P did not induce delayed cytotoxic effects in cultures of L1210/MPR cells. Instead the derivative produced acute inhibition of culture growth, the duration of which was related to the concentration of the derivative present in the culture media. *Bis*(dibutyrylMPR)P appeared to have a predominantly cytostatic action, and cells exposed at the lower concentrations at least were able subsequently to recover from the inhibitory effects of the drug. HPLC analysis was employed to determine both the stability of the acylated derivative during incubation in Fischer's medium at 37°, and the contribution if any to extracellular drug breakdown from exponentially growing L1210/MPR cells. Solutions of *bis*(dibutyrylMPR)P (244 $\mu$M) in culture fluid, prepared from the same batch of horse serum as that used in the experiment of Fig. 9, were incubated at 37° in the absence and presence of exponentially proliferating L1210/MPR cells (initial cell density

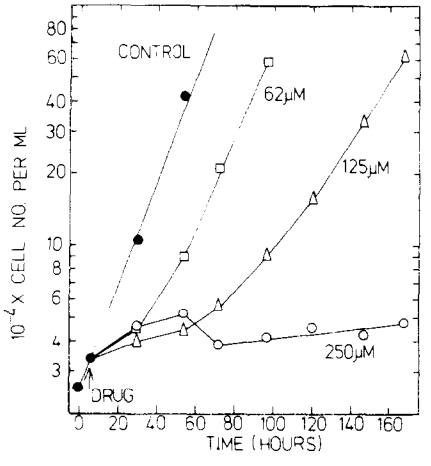


Fig. 9. Effects of *bis*(dibutyrylMPR)P on the proliferation of L1210/MPR cells. Replicate cultures of L1210/MPR cells were prepared at 0 hr and the drug was added at 5.75 hr. □, 62 $\mu$M; △, 125 $\mu$M; ○, 250 $\mu$M; ●, untreated control.

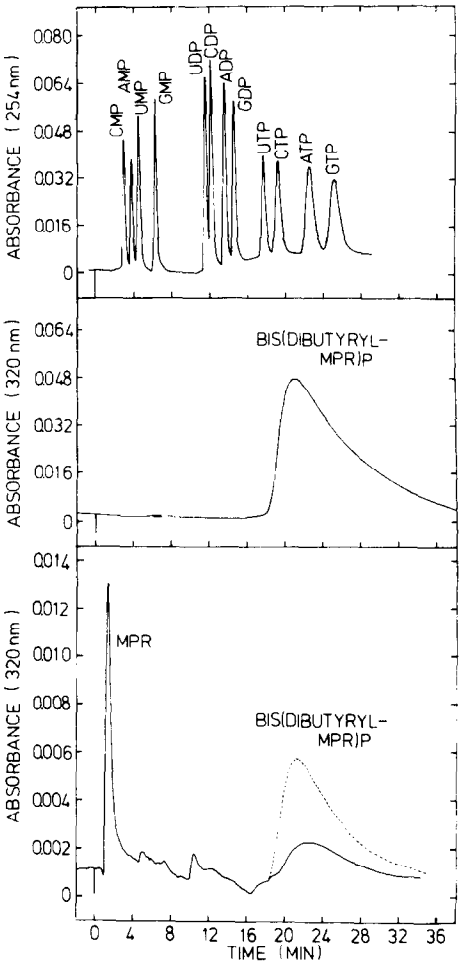


Fig. 10. High performance liquid chromatographic separation of nucleotide standards and of *bis*(dibutyrylMPR)P and its degradation product present in culture medium during incubation of the drug with L1210/MPR cells. (A) Top panel: separation of nucleotide standards mixture. (B) Middle panel: chromatogram of standard *bis*(dibutyrylMPR)P. (C) Lower panel: solid line, chromatogram of deproteinated sample of cell culture medium from 26 hr incubation of L1210/MPR cells with *bis*(dibutyrylMPR)P (244 $\mu$M); broken line, *bis*-(dibutyrylMPR)P peak in chromatogram of corresponding 0 hr sample.

36,000 cells/ml). Samples were taken at various times and cells were removed by centrifugation. The supernatants were deproteinated with perchloric acid in the presence of methanol (50%, v/v) to prevent precipitation of *bis*(dibutyrylMPR)P free acid. A representative chromatogram presented in Fig. 10C corresponds to a 26 hr incubation with L1210/MPR cells. This is compared with the separation of a nucleotide standards mixture (Fig. 10A) and the chromatogram of standard *bis*(dibutyrylMPR)P (Fig. 10B). *Bis*(dibutyrylMPR)P gave a very broad peak with a long retention time unrelated to its single negative charge as a result of a secondary reverse phase interaction between the lipophilic molecule and the alkyl coupling chains between the silica and the quarternary ammonium groups of the column packing. MPR was the only breakdown product of

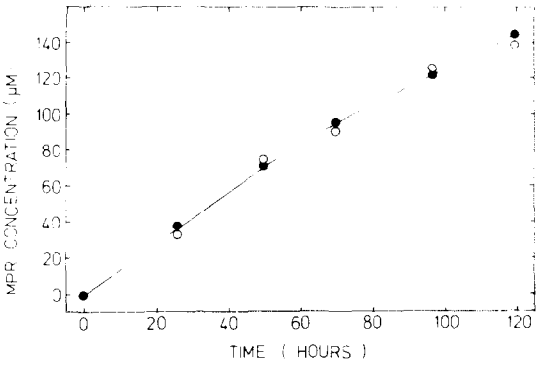


Fig. 11. Degradation of *bis*(dibutyrylMPR)P (244 μM) to MPR during incubation in Fischer's medium at 37° in the absence (●) and presence (○) of L1210/MPR cells (initial count 36,000 cells/ml). The MPR concentration was measured by high performance liquid chromatographic analysis of deproteinated media samples. A representative chromatogram from this experiment is reproduced in Fig. 10.

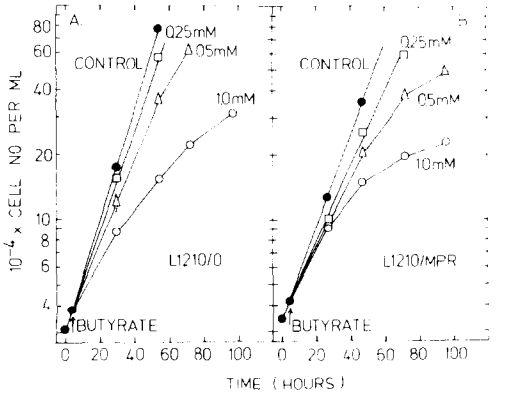


Fig. 12. Effects of sodium butyrate on the proliferation of L1210/0 and L1210/MPR cells. Replicate cultures were prepared at 0 hr and sodium butyrate was added to L1210/0 cells (panel A) at 4.1 hr, and to L1210/MPR cells (panel B) at 4.5 hr. □, 0.25 mM; △, 0.5 mM; ○, 1.0 mM; ●, untreated control.

*bis*(dibutyrylMPR)P detected in the media during incubation of the drug both in the presence and absence of L1210/MPR cells. The minor 320 nm absorbing peaks in the chromatogram of Fig. 10C were components of the tissue culture medium. The results of the complete experiment are presented in Fig. 11 in terms of the rate of increase in the concentration of MPR in the culture media. It can be seen that the rate of degradation of *bis*(dibutyrylMPR)P to MPR was slow and was the same whether cells were present or absent. After five days of incubation, the concentrations of MPR represented breakdown of only approximately 30% of the *bis*(dibutyrylMPR)P, taking into account that one molecule of the latter contains two molecules of the former. Enzymes present in the horse serum component of the Fischer's medium were responsible for the decomposition of *bis*(dibutyrylMPR)P during incubation at 37°. Negligible degradation of the derivative was observed in similar experiments when *bis*(dibutyrylMPR)P (114 and 57 μM) was incubated both in the presence and absence of L1210/MPR cells in Fischer's medium prepared with heat-inactivated horse serum (data not shown). In this case a very small amount of a thiopurine-containing product, tentatively identified as dibutyrylMPR, was detected in the media after 6 days incubation at 37°. Despite the slow breakdown of *bis*(dibutyrylMPR)P to MPR (Fig. 11) the recovery of the former in solution after deproteination fell progressively during the course of incubation at 37°. At 0 hr the acylated *bis*(nucleoside) phosphate was retained quantitatively in solution after addition of perchloric acid plus methanol (see Fig. 10C dotted line, 0 hr *bis*(dibutyrylMPR)P peak), but at 26 hr an appreciable proportion of the derivative precipitated with the protein (Fig. 10C), and beyond 50 hr only very small amounts were seen in the chromatograms. However, MPR continued to accumulate and the presence of the initial total thiopurine content of the media was confirmed from u.v. spectra of non-acidified media samples diluted in methanol:25 mM potassium phosphate buffer, pH 4.5, 1:1 supplemented with β-mercaptoethanol (0.1%). The progressive reduction in the ability to recover *bis*(dibutyrylMPR)P appeared to be temperature dependent since this effect was not observed when the derivative was incubated in culture medium for 6 days at 2°. It was shown that MPR, MPRP and dibutyrylMPRP remained in solution during deproteination of Fischer's medium with perchloric acid alone, and that disulphide-linked polymers of *bis*(thiopurine nucleoside) phosphates were very rapidly reduced to monomeric forms when added to tissue culture medium at 37° and hence would be unlikely to form under such conditions.

Since degradation of *bis*(dibutyrylMPR)P occurred, albeit slowly, with release of butyrate during incubation at 37°, the effects of sodium butyrate on the growth curves of L1210/0 and L1210/MPR cells were examined. The results of these experiments are presented in Fig. 12. The action of sodium butyrate, which was similar for both cell lines, was to produce a progressive time-dependent inhibition of culture growth. A concentration of 1 mM sodium butyrate corresponds to the concentration of butyrate which would result from the total deacylation of 250 μM *bis*(dibutyrylMPR)P. It can be seen by comparison of the data of Fig. 9 and Fig. 12 that *bis*(dibutyrylMPR)P and sodium butyrate produced distinctly different effects on culture growth curves. In addition, the results shown in Fig. 12 demonstrate that 30% release of butyrate from 250 μM *bis*(dibutyrylMPR)P spread over the course of 5 days (Fig. 11) would by itself have a negligible effect on L1210/MPR culture growth.

## DISCUSSION

*Bis*(MPR)P was originally synthesized by Thomas and Montgomery [9] and was shown to have activity ($EC_{50}$ = 13.9 μM) against the HGPRT-deficient HEp No.2/MP subline in culture [7]. We have demonstrated that *bis*(MPR)P also had activity against our MPR-resistant mouse leukaemia L1210 subline, L1210/MPR. However, the concentrations of *bis*(MPR)P that were active against L1210/MPR cells were considerably higher ($EC_{50}$ = 580 μM) than those

reported to inhibit the resistant human cell subline [7]. Differences in the rate of breakdown of *bis*(MPR)P by enzymes present in sera may account at least partially for the contrasting effects of the drug relative to the effects of MPR on thiopurine-resistant L1210/MPR and CH/TG cell cultures. Acylation of the dinucleoside phosphate with butyric acid afforded considerable protection against decomposition by serum enzymes and enhanced activity against L1210/MPR cells. However, it is not clear whether the mechanisms of action of *bis*(MPR)P and *bis*(dibutyrylMPR)P on resistant cells were the same.

*Bis*(MPR)P and *bis*(dibutyrylMPR)P induced delayed cytotoxic effects characteristic of the thiopurines in cultures of thiopurine-sensitive L1210/0 cells. These effects were undoubtedly the result of release of MPR from the derivatives by serum enzymes. The fact that *bis*(dibutyrylMPR)P was approximately 80 times less effective than MPR and *bis*(MPR)P against L1210/0 cells underlines the comparative resistance of the acylated derivative to degradation. It may be that deacylation of *bis*(dibutyrylMPR)P by an esterase was the rate-limiting step in its degradation to MPR since no intermediate breakdown products (e.g. MPRP, *bis*(MPR)P, dibutyrylMPRP) were detected in culture media (Fig. 10). L1210/MPR cells did not contribute to the extracellular degradation of *bis*(dibutyrylMPR)P (Fig. 11) presumably because there was no release of the appropriate rate-limiting degradative enzyme from the cells. In contrast, dephosphorylation of MPRP in culture media was greatly enhanced by the presence of L1210 cells* as a result of the action of extracellular phosphohydrolase(s) produced by the cells.

The L1210/MPR cells were resistant to MP and MPR as a result of their inability to synthesize the drug nucleotide. Therefore, the introduction of preformed MPRP into these cells would be expected to have the same consequences as those resulting from the intracellular synthesis of MPRP from MPR by sensitive L1210/0 cells. MPR induced a characteristic delayed cytotoxic effect in cultures of L1210/0 cells and *bis*(MPR)P at high concentration was shown to have a delayed action on resistant L1210/MPR cultures. However, whilst the previously described biochemical mechanism of the antileukaemic action of MP [3, 4] was demonstrated in L1210/0 cells treated with MPR, i.e. incorporation of the drug into newly replicated DNA as 6-thioguanine deoxyribonucleotide, this mechanism was not observed in L1210/MPR cells exposed to *bis*(MPR)P at growth inhibitory concentration. *Bis*(dibutyrylMPR)P was considerably more active than *bis*(MPR)P against L1210/MPR cells, but unlike the latter this derivative produced acute inhibition of culture growth and no delayed cytotoxic effect was observed. The acylated compound had a predominatly cytostatic action against exponentially proliferating L1210/MPR cells and with the lower concentrations, at least, cells recovered from the inhibition and culture growth resumed after a delay that was related to the concentration of the drug. Of possible relevance to an understanding of this phenomenon was the observation that despite the very slow rate of degradation of *bis*(dibutyrylMPR)P to MPR during treatment of cell cultures at 37° (Fig. 11) an increasing proportion of the compound was co-precipitated with protein by perchloric acid in the presence of 50% methanol. This effect which was also seen during incubation of the drug with Fischer's medium alone at 37°, but not at 2°, may be interpreted in terms of a possible slow, temperature-dependent binding of the lipophilic derivative to a serum component such as a lipoprotein. The progressive removal of free drug in this way could reduce the concentration available to the cells down to a point at which they were no longer inhibited, and consequently cell proliferation would be able to resume after a period of culture growth inhibition.

It is apparent that the ultimate biochemical mechanisms of action of *bis*(MPR)P and *bis*(dibutyrylMPR)P against L1210/MPR may well be different from that of MPR in L1210/0 cells, and that inhibition of L1210/MPR cell proliferation by these agents may not represent true circumvention of the mechanism of resistance to MP. Release of butyrate was not responsible for the effects of *bis*(dibutyrylMPR)P although sodium butyrate at high concentration has been shown to inhibit the growth of neoplastic cells reversibly and to induce a better differentiated or more benign phenotype [13].

Although *bis*(MPR)P and *bis*(dibutyrylMPR)P may possibly not act as prodrugs of intracellular MPRP, it seems likely that their inhibitory effects on L1210/MPR cultures resulted from cellular uptake of the intact molecules. The lipophilic acylated derivative may have been taken up more readily by cells than the non-acylated compound. Meyer *et al.* [6] found that of a series of fatty acid esters of MPR cyclic nucleotide the palmitoyl ($C_{16}$) derivative was most effective against MP-resistant cells. Similarly, Tsuruo *et al.* [14] demonstrated that cellular uptake of $N^4$-acyl derivatives of cytosine arabinoside increased with increasing acyl group chain length up to a maximum for the palmitoyl compound. It is noteworthy that *bis*(dibutyrylMPR)P also contains 16 acyl group carbon atoms; higher fatty acid derivatives of *bis*(MPR)P would be poorly soluble in aqueous media.

*Bis*(dibutyrylMPR)P may possibly have a potential application as a slow release depot form of MPR, and at the same time its lipophilic properties might facilitate passage of the intact molecule across the blood–brain barrier. If confirmed, these characteristics would suggest a possible use for *bis*(dibutyrylMPR)P in the treatment of meningeal leukaemia.

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* D. M. Tidd, unpublished observations.

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