Pergamon

Biochemical Pharmacology, Vol. 48, No. 11, pp. 2117–2126, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00399-8

CYTOTOXICITY AND METABOLISM OF 4-METHOXY-8-(β-D-RIBOFURANOSYLAMINO)PYRIMIDO[5, 4-*d*]PYRIMIDINE IN HCT 116 COLON CANCER CELLS

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(*Received* 18 *March* 1994; *accepted* 15 *July* 1994)

Abstract—We examined the cytotoxicity, biochemical effects and metabolism of 4-methoxy-8-(β-D-ribofuranosylamino)pyrimido[5, 4-*d*]pyrimidine (MRPP), a synthetic nucleoside inhibitor of phosphoribosylpyrophosphate synthetase, in HCT 116 human colorectal cancer cells. A 4-hr exposure to 1 and 10 μM MRPP inhibited cell growth over a 72-hr period by 76 and 89%, and inhibited clonogenic capacity by 36 and 65%, respectively. MRPP was avidly metabolized to the 5′-monophosphate derivative (MRPP-MP), and MRPP-MP formation increased with increasing MRPP exposure (μM·hr). MRPP-MP was stable, and the intracellular half-life was in excess of 48 hr. A 4-hr exposure to 10 μM MRPP resulted in significant decreases in ATP, UTP, GTP, CTP, dATP, dTTP, and PRPP pools. Near maximal ribonucleotide triphosphate depletion was achieved with ≥24 μM·hr MRPP, and growth inhibition as a function of MRPP μM·hr closely reflected the biochemical effects. Ribonucleotide triphosphate pools remained depleted for up to 48 hr after drug removal, apparently as a consequence of the prolonged retention of MRPP-MP. MRPP (10 μM) inhibited the salvage of [^{3}H]guanine, [^{3}H]-adenine and [^{3}H]guanosine, and concurrent exposure to MRPP and either 100 μM adenine, hypoxanthine, or guanine did not reverse ATP or GTP depletion. Concurrent exposure to 10 μM MRPP and either 10 μM adenosine, uridine or thymidine was accompanied by repletion of ATP, UTP, and dTTP pools, respectively, but depletion of other nucleotide pools was not corrected. In contrast, 10 μM guanosine did not correct GTP depletion in the presence of MRPP. The combination of 10 μM each of thymidine, uridine, adenosine and guanosine during and following a 24-hr exposure to MRPP provided partial protection against 0.1 or 1 μM MRPP, but did not affect the cytotoxicity associated with 10 μM MRPP. MRPP is a novel antimetabolite that inhibits both *de novo* and salvage pathways for purine synthesis and *de novo* pyrimidine synthesis.

Key words: antimetabolites; purines; pyrimidines; experimental therapeutics

MRPP§ (NSC D-630635-M), synthesized by Sanghvi and coworkers, has *in vitro* toxicity against murine L1210 leukemia, human B-lymphoblast WI-L2 and human colon cancer LoVo/L, and also has activity *in vivo* against L1210 leukemia [1–4]. MRPP is resistant to deamination. WI-L2 B lymphoblast cells deficient in adenosine kinase (EC 2.7.1.20) were resistant to MRPP, suggesting that phosphorylation by this enzyme is required for MRPP cytotoxicity [2]. Upon activation to the 5′-monophosphate form, MRPP inhibits the synthesis of PRPP [2]. With inorganic phosphate (K_m = 2 mM) as the variable substrate, MRPP is a competitive inhibitor of PRPP synthetase (EC 2.7.6.1; K_i = 40 μM); however, with ATP (K_m = 18 μM) as the variable substrate, MRPP is a non-competitive inhibitor (K_i = 190 μM)[2]. PRPP is a required substrate in the following pathways (enzyme reaction): *de novo* purine synthesis (amidophosphoribosyltransferase, EC 2.4.2.14); purine salvage (adenine phosphoribosyltransferase, EC 2.4.2.7 and hypoxanthine / guanine - phosphoribosyltransferase, EC 2.4.2.8); *de novo* pyrimidine synthesis (orotate phosphoribosyltransferase, EC 2.4.2.10); and pyridine synthesis (nicotinate phosphoribosyltransferase, EC 2.4.2.11). Thus, PRPP synthetase activity and the availability of PRPP play a central role in nucleic acid biosynthesis.

In preclinical models, some tumors have elevated levels of PRPP synthetase, and enzyme content appears to be linked to transformation and progression [5, 6]. The ability of cancer cells to salvage purines and pyrimidines may influence their sensitivity to a variety of antimetabolites, and strategies to circumvent salvage have been explored in both the preclinical and clinical setting. Because of its reported preclinical activity and its potential to inhibit *de novo* purine and pyrimidine synthesis as well as purine salvage, we examined the cytotoxicity, biochemical effects and metabolism of MRPP in the HCT 116 human colorectal cancer cell line. We herein characterize the toxicity of MRPP in cell growth and clonogenic assays, and correlate the biochemical effects with metabolite formation.

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§ Abbreviations: MRPP, 4-methoxy-8-(β-D-ribofuranosylamino)pyrimido[5, 4-*d*]pyrimidine; MRPP-MP, 5′-monophosphate derivative of MRPP; PRPP, 5-phospho-α-D-ribosyl-1-pyrophosphate; and MTT, 3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide.

MATERIALS AND METHODS

Materials. MRPP was provided by Dr. Roland Robins (ICN Pharmaceuticals, Costa Mesa, CA). Moravek Biochemicals (Brea, CA) supplied the [5-^{3}H]uridine (20 Ci/mmol), [2, 8-^{3}H]adenosine (50 Ci/mmol), [*methyl*-^{3}H]thymidine (25 Ci/mmol), [8-^{3}H]-guanosine (15 Ci/mmol), [2, 8-^{3}H]adenine (23 Ci/mmol), [8-^{3}H]guanine (10 Ci/mmol) and [2, 8-^{3}H]-hypoxanthine (20 Ci/mmol). The [^{3}H]nucleosides and bases were over 97% pure as documented by reversed-phase HPLC analysis (C8 μ-Bondapak column, Waters Millipore, Milford, MA) with water as the mobile phase (HPLC method 1); elution of cold standard and tritium were monitored at 260–280 nm by a Waters photodiode array detector and an in-line scintillation detector (Radiomatic, Tampa, FI). Unless otherwise specified, chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO) or were J. T. Baker products (North-Strong Scientific, Phillipsburg, NJ). Phosphate-buffered isotonic saline (PBS) with calcium and magnesium was from Biofluids, Inc. (Rockville, MD).

Cell culture. HCT 116 cells [7] were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine (both from Biofluids, Inc.), and 7% dialyzed fetal bovine serum (Gibco, Inc., Grand Island, NY). For cell growth experiments, 20,000 cells were replicately plated in 6-well tissue-culture plates. The following day, the cells were exposed to various concentrations of MRPP. After the 4-hr exposures, the cells were gently washed twice with RPMI medium; then drug-free medium was added. At 72 hr, the cells were trypsinized and enumerated with a Coulter Counter. The control cell number at the end of the incubation period averaged 250,000 ± 110,000 (mean ± SD). For clonogenic assays, 800 cells were replicately plated in 6-well plates [8]. MRPP was added the following day; after a 4-hr exposure, the cells were gently washed as described above, and the cells were incubated in fresh, drug-free medium for 7 days. The colonies were stained with 0.25% methylene blue in 50% methanol, and enumerated. Clonogenic efficiency for control cells in dialyzed medium was 20 ± 4% (mean ± SD). MTT assays were performed using previously described techniques [9, 10].

Determination of ribonucleotide and deoxyribonucleotide triphosphate pools. Cells (500,000) were plated in 80 cm^2 or 175 cm^2 tissue culture flasks. Forty-eight hours later, the cells were exposed to either diluent (dH_2O) or various concentrations of MRPP for 4 and 24 hr. The cells were washed once with iced PBS, and then extracted with 0.5 N perchloric acid for 20 min at 4°. The acid-soluble fraction was neutralized with 2 vol. of trichlorotrifluoroethane/tri-*n*-octylamine (3.4:1.5); after adjusting the pH to 7 with 0.5 N sodium hydroxide, the sample was frozen on dry ice, lyophilized, and stored at −70°. Immediately prior to analysis, the residue was resuspended in dH_2O, and analyzed according to a previously published anion-exchange HPLC method, which will be referred to as HPLC method 2 [8]. To determine the duration of biochemical abnormalities, the cells were washed gently twice with RPMI medium after a 4-hr exposure to 10 μM MRPP and then placed in drug-free medium. The cells were harvested at 24-hr intervals thereafter, as described above. A DNA polymerase assay (EC 2.7.7.7) with poly d[AT] as the template was used for measurement of dATP and dTTP pools [11–13].

Analysis of MRPP nucleotide formation. Exponentially growing cells were exposed to 1, 10 or 50 μM MRPP or diluent for 1 and 4 hr, then washed three times with iced PBS, and extracted with 0.5 N perchloric acid. The acid-soluble fraction was separated, neutralized and lyophilized as described above. An aliquot of the resuspended residue was analyzed by HPLC directly or following treatment with alkaline phosphatase (EC 3.1.3.1) in alkaline phosphatase buffer (Promega Inc., Madison, WI) for 60 min at 37°. The latter samples were centrifuged through a 0.22 μm Amicon® Centrifree micropartition system (W. R. Grace & Co., Beverly, CT) to remove protein prior to HPLC analysis. A protracted gradient was employed (termed HPLC method 3) to separate mono-, di- and triphosphates of guanosine and adenosine. An SAX Radial-Pak column (Waters) was developed using two buffers as the mobile phase: buffer A was 0.001 M ammonium phosphate, pH 3.0, and buffer B was 0.75 M ammonium phosphate, pH 4.5. An isocratic gradient of 100% buffer A was run at 2.4 mL/min for 10 min, followed by a linear gradient to 85% buffer A/15% buffer B over 20 min; a 5-min isocratic gradient followed. The following linear gradients were then run in series (% buffer A/% buffer B): to 70%/30% over 16 min; 0%/100% over 35 min. The column was allowed to equilibrate for 15 min at initial conditions prior to the next run. The retention times of standards were as follows (min): AMP, 28; GMP, 31; ADP, 42; GDP, 56; UTP, 64; CTP, 68; ATP, 72; GTP, 78.

Effects on [^{3}H]nucleoside and [^{3}H]purine base metabolism. Cells were exposed to either diluent (dH_2O) or MRPP. After the desired interval, the medium was aspirated, and the cells were washed twice with RPMI medium and then placed in drug-free medium. Next, the cells were pulse-labeled with either 1 μM [^{3}H]nucleoside or base, and then extracted with iced 60% methanol. After quantitating the total radioactivity, an aliquot of the methanol-soluble fraction was analyzed by anion-exchange HPLC (method 4) as previously described [14]. Incorporation into methanol-precipitable material was determined by washing the pellet three times with iced 60% methanol; the pellet was then dissolved in 0.5 N sodium hydroxide, neutralized with hydrochloric acid, and counted in a liquid scintillation counter. Tritium incorporation into methanol-precipitable material was used as an index of DNA (thymidine) or RNA (uridine) synthesis.

Cell-free assays for nucleoside phosphorylation. Flasks containing exponentially growing cells were washed once with PBS, incubated in 20 mM EDTA (pH 7.5) for 10 sec, then incubated in PBS at 37° until the cells detached. The dislodged cells were washed once with 10 vol. of iced PBS and collected by centrifugation at 800 *g* for 12 min. The cell pellet was then frozen at −70° until the day of the assay.

The cells were resuspended in 300–400 μL of 50 mM Tris–HCl buffer (pH 7.0) and sonicated, and then the cell mixture was centrifuged at 8000 *g* for 30 min. An aliquot of the supernatant was added to a microfuge tube containing the following constituents in a total volume of 200 μL (final concentration): 50 mM Tris–HCl, pH 7.0; 10 mM ATP; 10 mM $MgCl_2$; 10 mM sodium fluoride; 0.1 or 1 mM [^{3}H]-nucleoside precursor (500,000 dpm). For adenosine kinase experiments, the assay mixture contained the following in 200 μL (final concentrations): 50 mM Tris–HCl, pH 7.0; 5 mM each of ATP, GTP, and UTP; 10 mM $MgCl_2$; 10 mM sodium fluoride; 10 μM deoxycoformycin; 0.1 mM [^{3}H]nucleoside precursor (500,000 dpm). In some experiments, the lysate was preincubated with all reagents except nucleoside precursor and either diluent or MRPP at final concentrations of 0.1, 1.0 or 10 mM; 0.1 or 1 mM [^{3}H]nucleoside was added 1 hr later. The sample was incubated in a 37° shaking water bath for 5–60 min. The time over which the reaction remained linear was determined in initial experiments. The reaction was terminated by adding an equal volume of ice-cold methanol; after incubation on ice for 20 min, the samples were centrifuged, and the methanol-soluble supernatant was frozen until analysis. The formation of nucleotides during the linear portion of the reaction was determined by HPLC (method 4). Protein concentrations were determined by the method of Bradford [15].

Assay for PRPP. Cellular levels of PRPP in control and MRPP-treated cells were determined by PRPP-dependent release of [^{14}C]carbon dioxide from [*carboxyl*-^{14}C]orotic acid according to a previously described method [16].

Cell cycle analysis. Exponentially growing cells were exposed either to diluent or to 0.1, 1 or 10 μM MRPP for 24 hr. The viability of adherent control cells and drug-treated cells was assessed by trypan blue exclusion. Single cell suspensions were prepared from duplicate flasks. The cells were fixed in 50% ethanol, treated with RNase A (EC 3.1.26.2), stained with propidium ioidide, and analyzed on a Becton-Dickinson FACStar flow cytometer to determine the fraction of cells in each DNA cycle phase [12].

pH Step alkaline elution of nascent DNA. Exponentially growing cells were exposed to either no drug, or 1 and 10 μM MRPP for 24 hr. [^{3}H]-Thymidine (10 μCi) was added for the final 2 hr of exposure. The radiolabeled flasks were washed three times with ice-cold PBS, and then the cells were detached by incubation in ice-cold 20 mM Na_2-EDTA (adjusted to pH 7.0 with sodium hydroxide). An equal number of cells were loaded onto Nuclepore® filters (25 mm, 1 μm pore size; Costar Corp., Cambridge, MA), held in alkaline elution funnels (Millipore Corp., Milford, MA) and were then lysed in the dark with 5 mL of buffer containing 2 M sodium chloride, 0.3% *N*-lauroyl sarcosine sodium salt, pH 7.0 (ICN Biochemicals Inc., Cleveland, OH) and 20 mM Na_2-EDTA, pH 10.0 [17, 18]. The lysed cells were washed with 3 mL of 20 mM Na_2-EDTA (pH 10), followed by sequential 1-hr elutions with 20 mM EDTA (free acid form) adjusted to pH 11.0, 11.3, 11.5, 11.7 and 12.1 with 1 M tetrapropylammonium hydroxide (RSA Corp., Ardsley, NY) as described by Ross *et al.* [18].

RESULTS

Cytotoxicity of MRPP. A 4-hr exposure to 1 and 10 μM MRPP potently inhibited HCT 116 cell growth to 24 ± 8 and 11 ± 5% of control (mean ± SD, N = 3), respectively, and the IC_{50} was 0.3 μM. Extending the duration of exposure to 72 hr produced slightly greater growth inhibition with lower MRPP concentrations (0.05 and 0.1 μM), but the IC_{50} (0.2 μM) was similar. In clonogenic assays, the IC_{50} for a 4-hr MRPP exposure was 5 μM; 1 and 10 μM MRPP reduced colony formation by 36 ± 4 and 65 ± 7% (mean ± SEM, N = 5), respectively.

Metabolism of MRPP. After exposure of HCT 116 cells to concentrations of MRPP ranging from 1 to 50 μM for 1 and 4 hr, two apparent MRPP metabolites were identified by anion-exchange HPLC analysis; both eluted in the monophosphate region. The two peaks were present at a 4:1 ratio over the concentration range of 1 to 50 μM MRPP, and appeared to be authentic MRPP metabolites as suggested by UV absorbance maxima at 289 and 320 nm; a 260 nm/270 nm ratio identical to parent compound (0.59); and similarity of the UV absorbance spectrum compared with parent drug. That these metabolites represented phosphorylated forms was suggested by their elution times and disappearance when treated with alkaline phosphatase. These metabolites were assumed to represent the α- and β-enantiomers of MRPP [3]. No MRPP metabolite peaks were evident in the regions where diphosphates and triphosphates elute. HPLC analysis of the cellular extract after destruction of ribonucleotides by incubation with sodium periodate followed by methylamine, as previously described, failed to reveal any MRPP deoxyribonucleotide metabolites [19].

The effect of the nucleoside transport inhibitor dipyridamole on MRPP metabolism was determined by incubating cells for 1 hr in the presence of 1 μM MRPP alone or with increasing concentrations of dipyridamole. MRPP-MP formation was inhibited in a concentration-dependent manner by dipyridamole (IC_{50} = 10 nM), providing indirect evidence that MRPP enters human colon cancer cells via the facilitated nucleoside transport mechanism. Following a 1-hr exposure to 1 and 10 μM MRPP, the total phosphorylated metabolite pool was 1 and 5.9 nmol/10^6cells, respectively. Increasing the duration of exposure to 4 hr increased the total MRPP-MP pool about 2-fold. Figure 1 demonstrates the increase in MRPP-MP formation as a function of MRPP concentration·time (C·T). An inverse relationship was noted between clonogenic survival and MRPP-MP formation.

The intracellular stability of MRPP-MP was determined by comparing the MRPP-MP levels at 24-hr intervals after drug removal with that immediately after a 4-hr exposure to 10 μM. MRPP-MP was relatively stable: the levels were (mean ± SEM) 60 ± 12 and 58 ± 13% of baseline 24 and 48 hr after drug removal, respectively. After a 4-hr exposure to 1 μM MRPP, MRPP-MP levels

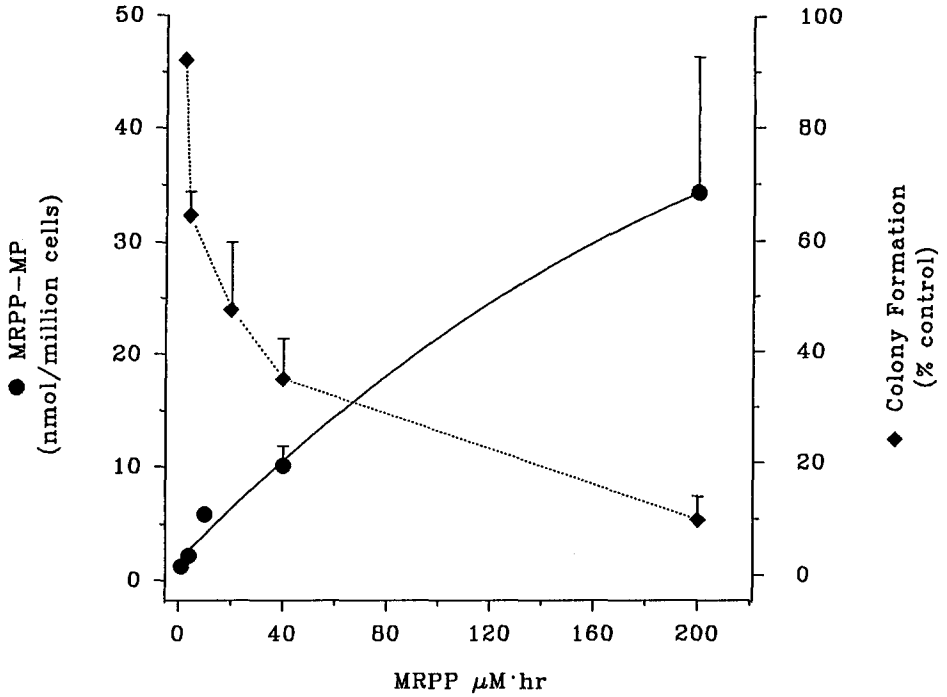

Fig. 1. MRPP-monophosphate formation as a function of MRPP concentration·time. Cells were exposed to MRPP for 1 hr (1 and 10 μM) or 4 hr (1, 10 and 50 μM). MRPP-MP formation was determined by anion exchange HPLC as described in Materials and Methods, and is shown on the left Y-axis. The data for 1 and 10 μM, presented as mean ± SEM, are from 5 experiments; the data for 50 μM, presented as means ± SD, are from 3 experiments. Clonogenic survival after a 4-hr exposure to MRPP, determined in separate experiments, is shown on the right Y-axis as the mean ± SEM (N = 4 experiments, each done in duplicate).

(mean ± range) had decreased by only 13% after 72 hr.

Biochemical effects of MRPP. PRPP-dependent [^{14}C]carbon dioxide release from [^{14}C]orotic acid with PRPP as the rate-limiting substrate was used to determine intracellular PRPP pools before and after drug exposure. A 4-hr exposure to 10 μM MRPP produced significant depletion of PRPP pools from 0.47 ± 0.08 to 0.04 ± 0.04 nmol/mg (mean ± SD, N = 3; P = 0.006).

MRPP was associated with a concentration- and time-dependent depletion of all four physiologic ribonucleotide triphosphate pools. After a 4-hr exposure to 1 μM MRPP, ATP, UTP and CTP pools were decreased by 11–26%, while GTP pools were decreased by 45%; when the duration of exposure was extended to 24 hr, all four ribonucleotide triphosphate pools were decreased by 42–65% compared with control. The change in ribonucleotide triphosphate pools as a function of MRPP C·T is shown in Fig. 2. Nucleotide pools decreased sharply with increasing MRPP C·T; near maximal effects were observed with ≥24 μM·hr. The inset demonstrates that inhibition of cell growth paralleled the ribonucleotide depletion. After a 4-hr exposure to 10 μM MRPP, ATP, UTP, GTP and CTP pools remained depressed for up to 48 hr after drug removal (Fig. 3), presumably a consequence of the stable retention of MRPP-MP after drug removal.

Baseline dATP and dTTP pools were 27 ± 3 and 71 ± 13 pmol/10^6 cells, respectively (mean ± SEM, N = 10). With a 4-hr exposure to 1 μM MRPP, dATP and dTTP pools were decreased by 52 and 25% to 13 ± 4 and 53 ± 12 pmol/10^6 cells, respectively (mean ± SD, N = 3). Extending the duration of exposure to 1 μM for 24 hr produced further depletion of dTTP pools to 42 ± 6 pmol/10^6 cells, but dATP pools were not depleted further. With a 4-hr exposure to 10 μM MRPP, the decrease in dATP pools was comparable to that produced by 1 μM, but dTTP pools were decreased by 69% (22 ± 5 pmol/10^6 cells).

Purine salvage in intact cells was compared in cells exposed to diluent or to 10 μM MRPP for 2 hr with 1 μM [^{3}H]purine added for the final hour. HCT 116 cells were capable of salvaging purine bases and [^{3}H]guanosine in the absence of MRPP (Table 1). Following exposure to [^{3}H]adenine in the absence of MRPP, [^{3}H]ATP accounted for 89% of the metabolites; MRPP reduced the total [^{3}H]adenine nucleotide pool by 89%. [^{3}H]Hypoxanthine was also avidly metabolized in the absence of MRPP, and ATP and GTP accounted for 81 and 7% of the total [^{3}H]metabolite pool, respectively. MRPP reduced the total [^{3}H]nucleotide pool by 80%. These data are consistent with the ability of MRPP to deplete the PRPP cofactor required for salvage of purine bases. In addition, 10 μM MRPP potently inhibited [^{3}H]guanosine salvage by 86%.

In control cells, the cell cycle distribution was as

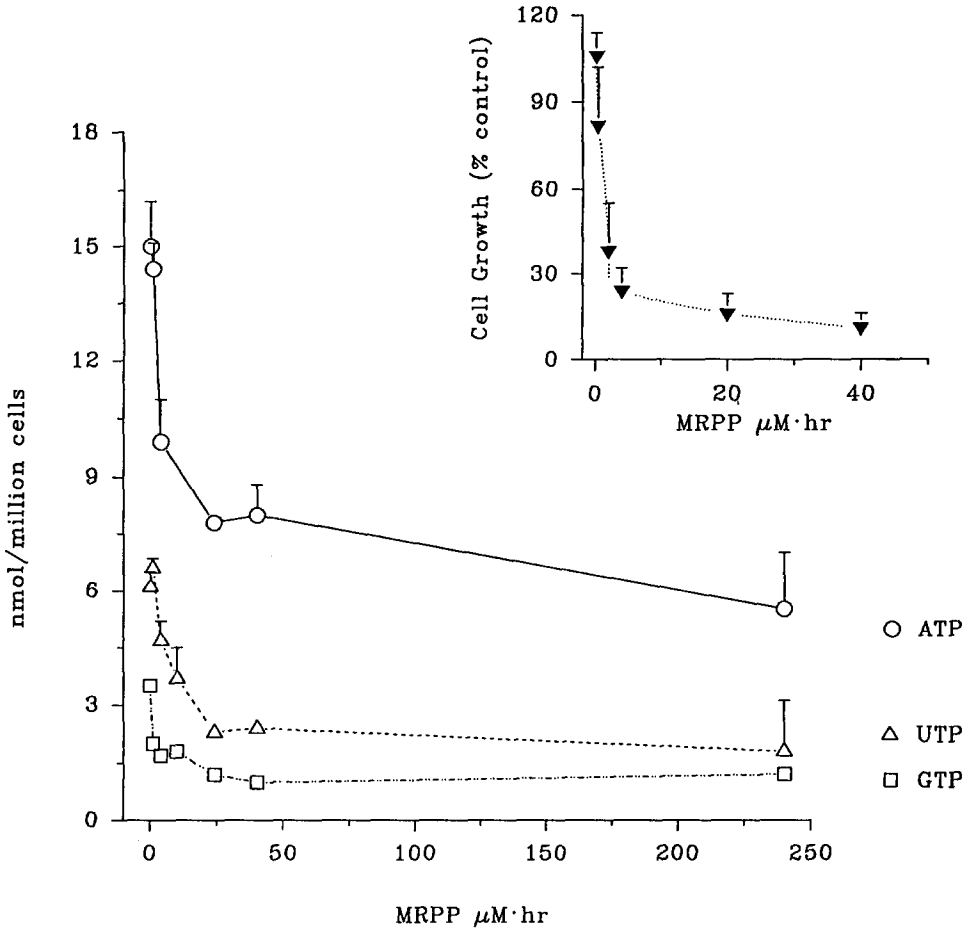

Fig. 2. Ribonucleotide triphosphate levels as a function of MRPP concentration-time. Cells were exposed to 1 and 10 μM MRPP for 1, 4 or 24 hr; then the cells were extracted with dilute perchloric acid as described in Materials and Methods, and ribonucleotide triphosphate pools were determined by HPLC method 2. The inset shows inhibition of cell growth following a 4-hr exposure to MRPP. The data, expressed as percent control cell number (mean ± SD) as a function of MRPP μM·hr, are from 3 experiments done in duplicate.

follows; G_1, 50%; S, 34%; G_2 + M, 16%. A 24-hr exposure to 0.1 and 1.0 μM MRPP did not appreciably alter cell cycle distribution. With 10 μM, however, an accumulation of cells in S phase (49%) was apparent, accompanied by a decrease in the G_1 fraction (35%); the proportion of cells in G_2 + M phase was not altered (16%).

Effect of concurrent nucleosides and nucleobases on MRPP toxicity. The profound effect on nucleotide pools appeared to contribute to MRPP-associated toxicity. MRPP cytotoxicity was not reversed appreciably by exposure in medium supplemented with non-dialyzed fetal bovine serum. We then determined whether cells could be protected from MRPP toxicity by co-incubation with various nucleosides (10 μM) including thymidine, uridine, adenosine and guanosine. Concurrent exposure to both MRPP and each nucleoside individually failed to protect HCT 116 cells from MRPP cytotoxicity (data not shown). To understand the basis for the lack of protection, ribonucleotide triphosphate pools were measured after concurrent exposure for 4 hr to 10 μM MRPP and either 10 μM uridine, adenosine, or guanosine. Uridine (10 μM) partially repleted UTP pools from 40% of control with MRPP alone to 77% of control, but ATP, GTP and CTP pools remained depleted (Table 2). With 10 μM adenosine, ATP pools were repleted from 53 to 87% of control, but GTP, UTP and CTP pools remained depressed. In contrast, 10 μM guanosine failed to replete GTP pools in the presence of MRPP. Concurrent exposure to 10 μM MRPP and 10 μM thymidine fully repleted dTTP pools. The multiple biochemical abnormalities produced by MRPP appear to explain the lack of protection by coadministration of a single nucleoside.

We then examined the effect of combined nucleosides on MRPP toxicity. A 96-hr exposure to 1 and 10 μM MRPP reduced MTT staining to 4 ± 1 and 2 ± 1% of control, respectively. Continuous exposure to 10 μM each of adenosine, thymidine and uridine in the absence of MRPP was associated with 81 ± 14% of control MTT staining. Concurrent

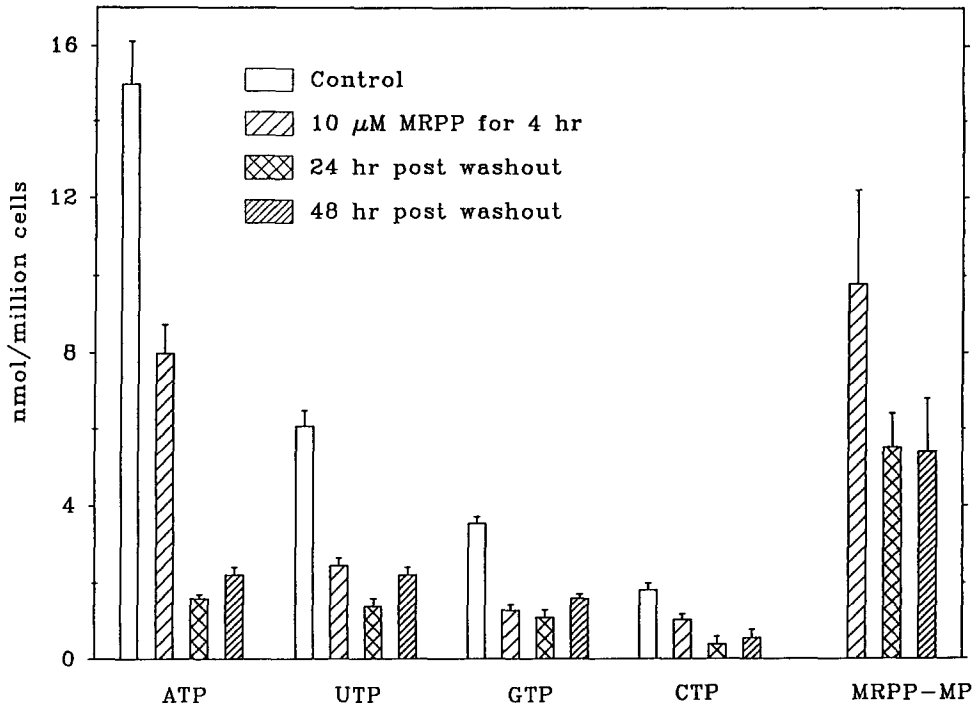

Fig. 3. Duration of ribonucleotide pool depletion following a 4-hr exposure to 10 μM MRPP. After drug exposure, the medium was aspirated, and the cells were washed three times with PBS. The cells were then incubated in fresh, drug-free medium. At the indicated times, the cells were harvested and nucleotide pools were determined as described in Materials and Methods. The ribonucleotide triphosphate data, presented as mean (±SEM for control and MRPP for 4 hr; ±SD for 24 and 48 hr post-MRPP samples), are from the following number of experiments: control, N = 9; 10 μM MRPP for 4 hr, N = 8; 24 and 48 hr post removal of MRPP, N = 3. The pool sizes at 4 hr were significantly different from control: CTP, P = 0.03; ATP, UTP, GTP, P < 0.001, *t*-test); 48 hr after drug removal, the ATP, UTP, and GTP pool sizes remained significantly depressed (P ≤ 0.005; CTP pools, P = 0.06). The MRPP-MP data are shown on the far right as mean ± SD (N = 3).

Table 1. Inhibition of purine salvage by MRPP in HCT 116 cells

	pmol/10^6 cells						
	AMP	GMP	ADP	GDP	ATP	GTP	Total
[^{3}H]Adenine, 1 μM							
Control	53		271		2916		3240
10 μM MRPP	2		78		890		970
[^{3}H]Hypoxanthine, 1 μM							
Control	28	29	188	0	1917	156	2317
10 μM MRPP	0	14	31	2	326	86	458
[^{3}H]Guanosine, 1 μM							
Control		39		150		1615	1804
10 μM MRPP		0		8		191	199

HCT 116 cells in exponential growth phase were exposed to no drug or to 10 μM MRPP for 1 hr; then 1 μM [^{3}H]purine was added. After 1 hr, the cells were extracted with 60% methanol, and the soluble fraction was analyzed by HPLC method 4 as described in Materials and Methods. The data are from a single experiment and are shown as the average of duplicate samples.

exposure to MRPP with the three nucleosides was associated with only minor protection (MRPP concentration, % control): 1 μM, 12 ± 6%; 10 μM, 15 ± 10%. The addition of 10 μM guanosine to the other three nucleosides provided partial protection against the toxicity associated with 1 μM MRPP (31 ± 17% of control), but did not reverse the toxicity associated with 10 μM MRPP (1 ± 1%).

Table 2. Ability of pyrimidine and purine bases and nucleosides to replete nucleotide pools

10 μM MRPP	Nucleoside or base	nmol/million cells dTTP	UTP	CTP	ATP	GTP
No	None	0.06 ± 0.01	6.1 ± 0.4	1.8 ± 0.2	15 ± 1.2	3.5 ± 0.2
Yes	None	0.02 ± 0.01	2.4 ± 0.2	1.1 ± 0.1	8.0 ± 0.8	1.3 ± 0.1
Yes	Uridine		4.7 ± 0.6	1.3 ± 0.4	7.2 ± 0.9	0.6 ± 0.2
Yes	Adenosine		1.0 ± 0.1	0.2	13.0 ± 1.1	1.2 ± 0.2
Yes	Guanosine		2.5 ± 0.3	1.6 ± 0.2	8.6 ± 0.2	1.6 ± 0.2
Yes	Thymidine	0.08 ± 0.01				
Yes	Adenine		1.9 ± 0.1	0.3 ± 0.1	11.6 ± 0.6	1.5 ± 0.2
Yes	Hypoxanthine		2.6 ± 0.6	0.8 ± 0.3	11.0 ± 2.6	1.7 ± 0.6
Yes	Guanine		2.3 ± 0.3	0.7 ± 0.3	7.4 ± 0.7	1.7 ± 0.4

Exponentially growing cells were exposed to no drug or to 10 μM MRPP alone or with either 10 μM nucleoside or 100 μM nucleobase. After 4 hr, the cells were extracted with 0.5 N perchloric acid, and nucleotide pools were determined as described in Materials and Methods. The data for control, 10 μM MRPP (N = 10), MRPP plus either adenine, hypoxanthine, guanine, thymidine or guanosine (N ≥ 4) are shown as mean ± SEM; the data for MRPP plus either uridine or adenosine (N = 2) are shown as mean ± range.

Failure to replete guanine nucleotide pools with either the three or four nucleoside combination may account for the incomplete protection against MRPP toxicity.

The ability of HCT 116 cells to salvage purine bases during MRPP exposure was also examined (Table 2). Measurement of ribonucleotide pools after a concurrent 4-hr exposure to 10 μM MRPP and either 100 μM adenine, hypoxanthine, or guanine indicated that ATP and GTP pools were not repleted, presumably because of MRPP-associated PRPP depletion. We then tested whether continuous exposure to 10 μM each of adenosine, thymidine, uridine and 100 μM guanine starting 24 hr prior to the addition of MRPP was associated with protection from MRPP-associated cytotoxicity. The combination of the three nucleosides plus guanine partially antagonized the growth inhibition resulting from a 4-hr exposure to 1 and 10 μM MRPP, but were ineffective if MRPP was present continuously for 72 hr (data not shown). In clonogenic assays, the combination of adenosine/uridine/thymidine/guanine in the absence of MRPP did not affect clonogenic capacity, whereas continuous exposure to ≥0.05 μM MRPP alone for 144 hr resulted in 100% lethality. Partial protection was seen when the three nucleosides plus guanine were given with 0.05 and 0.1 μM MRPP (colony formation 36 and 29% of control, respectively), but not with MRPP concentrations ≥0.5 μM.

Effect of MRPP on RNA and DNA synthetic rates. Incorporation of [^{3}H]uridine and [^{3}H]thymidine into RNA and DNA during brief incubations is commonly used to assess the effects of a drug on RNA and DNA synthetic rates. In preliminary experiments, we found that pre-exposure to 1 and 10 μM MRPP for 4 and 24 hr inhibited the metabolism of 1 μM [^{3}H]uridine and 1 μM [^{3}H]thymidine to [^{3}H]UTP and [^{3}H]dTTP by ≥75%, even after the cells were washed to remove MRPP prior to the [^{3}H]nucleoside pulse. Therefore, changes in pulse incorporation of these [^{3}H]nucleoside precursors into DNA and RNA would overestimate the inhibitory effect of MRPP on net DNA and RNA synthetic rates. Since concurrent exposure to MRPP and 10 μM uridine or thymidine for at least 4 hr was accompanied by repletion of UTP and dTTP, respectively, but not by repletion of purine triphosphate pools, we reasoned that an estimate of the effect of MRPP on RNA and DNA synthesis might be made under these conditions. Following a 4- or 24-hr exposure to 1 or 10 μM MRPP and 10 μM [^{3}H]uridine, methanol-precipitable incorporation of [^{3}H]uridine was inhibited by 47–56%. Simultaneous exposure to 10 μM [^{3}H]thymidine and either 1 or 10 μM MRPP for 24 hr decreased [^{3}H]thymidine incorporation by 54%. These data suggest that both RNA and DNA syntheses are inhibited by MRPP exposure to a level commensurate with nucleotide depletion.

Effect of MRPP and MRPP-MP on cell-free phosphorylation of nucleosides. To determine whether MRPP or its monophosphate could directly inhibit phosphorylation of nucleosides, we studied the activity of uridine, thymidine and adenosine kinase in a cell-free assay using 0.1 or 1 mM substrate under the following conditions: (1) no exogenous MRPP; (2) with exogenous MRPP; and (3) preincubation with MRPP and a phosphate donor for 1 hr prior to the addition of the substrate of interest. HPLC analysis of the methanol-soluble fraction of the cytosolic extract after the 2-hr incubation confirmed that MRPP-MP was formed when MRPP was preincubated with 10 mM ATP; MRPP-MP was not formed, however, in the absence of exogenous ATP. In the absence of MRPP, uridine and thymidine kinase activities (1 mM substrate, mean ± SEM) were 8.5 ± 1.2 nmol/min/mg protein (N = 6) and 0.84 ± 0.04 nmol/min/mg protein (N = 8), respectively. MRPP (1 and 10 mM) added either 1 hr prior to or concurrently with the [^{3}H]nucleosides did not interfere with uridine or thymidine kinase activities (data not shown). In contrast, MRPP inhibited adenosine phosphorylation in a concentration-dependent manner, with an IC_{50} of 32 μM. In the absence of MRPP, adenosine kinase activity was 1.06 ± 0.15 nmol/min/mg protein (N = 4). With

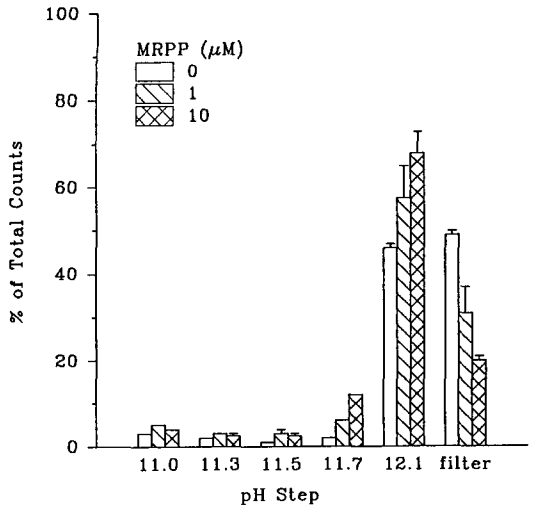

Fig. 4. Effect on MRPP on the pH step alkaline elution profile of newly synthesized DNA. Exponentially growing cells were exposed to either 1 or 10 μM MRPP for 24 hr, and then were pulsed with 10 μCi [^{3}H]thymidine for 2 hr. The cells were harvested and lysed on a polycarbonate filter as described in Materials and Methods. The size distribution of single-stranded DNA species was determined by pH step alkaline elution. The data are presented as radioactive counts eluting with each pH step (mean ± range) as a percentage of the total counts, and represent the average of two separate experiments.

0.1 and 1 mM MRPP, however, adenosine kinase activity was reduced to 0.23 ± 0.14 (22% of control, N = 3, P = 0.052) and 0.063 ± 0.024 nmol/min/mg protein (6% of control, N = 4, P = 0.012), respectively.

HCT 116 cells were capable of phosphorylating guanosine in this cell-free system provided that the substrate concentration was at least 1 mM: 0.63 ± 0.08 nmol/min/mg protein (N = 14). To determine if the apparent phosphorylation of guanosine might have resulted from conversion to guanine with subsequent conversion to GMP by PRPP synthetase, 1 mM [^{3}H]guanine was substituted for guanosine in the cytosolic assay. Only minimal conversion to nucleotides, however, was apparent, with guanine as the substrate: 0.06 nmol/min/mg (N = 2), suggesting that direct phosphorylation of guanosine occurred. MRPP inhibited guanosine phosphorylation: 1 and 10 mM MRPP, 0.54 ± 0.08 (86% of control, N = 14, P = 0.04) and 0.37 ± 0.08 nmol/min/mg (59% of control, N = 14, P < 0.001), respectively.

Effect of MRPP on pH step alkaline elution of nascent DNA. pH Step alkaline elution was used to assess the effects of MRPP on DNA chain elongation. In the pH transition zone, molecular weight influences the selective denaturation of newly replicated DNA exposed to alkali [15, 16]. Therefore, stepwise elution of DNA with EDTA (anhydrous) adjusted with 1 M tetrapropylammonium hydroxide to pH values ranging from 11.0 to 12.1 may show relative differences in the single-strand length of newly synthesized DNA. A 24-hr exposure to 1 and 10 μM MRPP altered the elution profile (Fig. 4). An accumulation of lower molecular weight DNA single-stranded species in the fractions eluting with pH steps 11.7 and 12.1 was noted with MRPP compared with control, accompanied by a decreased proportion of DNA retained on the polycarbonate filter: 31 and 20% retained vs 49% for control. These results suggest interference with DNA chain elongation and/or DNA damage during drug exposure, presumably as a result of substrate (e.g. dATP) depletion.

DISCUSSION

The major objectives of this study were to determine the cytotoxicity of MRPP in HCT 116 colon cancer cells, to characterize its intracellular metabolism, and to correlate the biochemical effects with the formation of active metabolites. Metabolism to the 5′-monophosphate form increased in proportion to the MRPP C·T. MRPP-MP was extremely stable intracellularly, with a half-life in excess of 48 hr. Further phosphorylation to the diphosphate and triphosphate forms was not observed. The monophosphate of MRPP is presumably a poor substrate for the nucleotide kinases, as has been reported for other exocyclic aminoribonucleosides such as 4-amino-8-(D-ribofuranosylamino)pyrimido[5,4-*d*]pyrimidine (ARPP, NSC 283867) and tricyclic nucleoside (NSC 154020) [3, 20, 21].

MRPP was associated with depletion of purine and pyrimidine nucleotide pools; near maximal biochemical effects were achieved with 24 μM·hr MRPP; growth inhibition as a function of MRPP C·T closely reflected the biochemical effects. Following a 4-hr exposure to 10 μM MRPP, persistent depletion of the four ribonucleotide triphosphate pools was noted up to 48 hr after drug removal, consistent with the stable retention of MRPP-MP. The duration of these biochemical effects may explain the impact of a brief exposure to MRPP on the viability of HCT 116 cells. MRPP markedly inhibited metabolism of [^{3}H]adenine and [^{3}H]-hypoxanthine to the nucleotide level, presumably as a consequence of PRPP depletion. MRPP interfered with [^{3}H]adenosine salvage in a cell-free assay in a concentration-dependent manner; an equimolar concentration of MRPP inhibited adenosine kinase by over 80%. Our results are consistent with those of Willis *et al.* [3], who reported that MRPP is a substrate for adenosine kinase and competitively inhibits adenosine phosphorylation. Our observation that 10 μM adenosine could replete ATP pools during a 4-hr incubation with 10 μM MRPP, however, suggests that adenosine is a much better substrate for adenosine kinase than MRPP in intact cells. In contrast, concurrent exposure to 10 μM guanosine did not replete GTP pools. HCT 116 cells were capable of metabolizing [^{3}H]guanosine to the nucleotide level; a pre-exposure to 10 μM MRPP, however, potently inhibited guanosine salvage. Cell-

free assays documented that HCT 116 cells were capable of directly phosphorylating guanosine, and that MRPP inhibited guanosine phosphorylation. In intact cells, guanosine is presumably a poor substrate for the enzyme mediating its direct phosphorylation.

Pretreatment with MRPP inhibited [^{3}H]UTP and [^{3}H]dTTP formation *in situ* after a 1-hr exposure to 1 μM [^{3}H]uridine and [^{3}H]thymidine. Since uridine and thymidine kinase activities were not appreciably affected in a cell-free assay, MRPP-associated depletion of phosphate donors such as ATP, which are required for pyrimidine salvage, may account for decreased nucleoside metabolism during brief exposures. Changes in pulse incorporation of these [^{3}H]nucleoside precursors into DNA and RNA would overestimate the inhibitory effect of MRPP on net DNA and RNA synthetic rates. Concurrent exposure of intact cells to 10 μM MRPP and either 10 μM uridine or thymidine for 4 hr, however, did lead to repletion of UTP and dTTP pools, respectively, presumably because a higher substrate concentration was used and sufficient time had elapsed to permit equilibration. Simultaneous exposure to 10 μM [^{3}H]thymidine or 10 μM [^{3}H]-uridine and MRPP for 4 and 24 hr decreased incorporation of these radioactive nucleoside precursors into DNA and RNA, respectively, to a comparable degree (about 50% of control), a level commensurate with the extent of nucleotide depletion.

Protection of HCT 116 cells was not achieved with continuous exposure to a single nucleoside. In addition, a combination of thymidine, adenosine and uridine at concentrations sufficient to replete the respective triphosphate pool size provided only partial protection against MRPP. Neither 10 μM guanosine nor 100 μM guanine was capable of repleting GTP pools in the presence of MRPP. Because MRPP produces multiple biochemical defects, neither individual nucleosides nor a combination of nucleosides afforded protection, most likely because not all of the affected nucleotide pools could be restored. The inability to replete GTP pools with either guanosine or guanine suggests that guanine nucleotide depletion alone can mediate MRPP cytotoxicity.

Fry *et al.* [21] recently described the metabolism and biochemical effects of 10 μM ARPP in WI-L2 human lymphoblastoid cells. ARPP differs from MRPP by virtue of a 4-amino group in place of a 4-methoxy group on the pyrimido[5,4-*d*]pyrimidine ring. ARPP was phosphorylated to the monophosphate form, and the levels after 1- to 2-hr exposures (3 and 4.6 nmol/10^6 cells) were about one-half of those we measured for MRPP-MP in HCT 116 cells. The biochemical effects were similar to that produced by MRPP: depletion of both purine and pyrimidine nucleotide pools occurred quickly; PRPP levels decreased to 40% of control within 2 hr [21]. The duration of biochemical effects in WI-L2 cells was not described. Partial protection from the growth inhibition association with a 72-hr exposure to 1.5 μM MRPP has been observed with concurrent exposure to 20 μM adenosine, 100 μM uridine, and 2 μM 2′-deoxycoformycin [21]. Willis reported that when the cytotoxicity of MRPP and ARPP were directly compared, MRPP was 4–10 times more active against three lymphoblastoid cell lines; MRPP was also more active on a per mg basis *in vivo* in mice bearing i.p.-implanted L1210 leukemia [2, 3]. The activities of these two analogs against human epithelial malignancies have not been directly compared.

In conclusion, MRPP is a novel antimetabolite that can inhibit both *de novo* and salvage pathways for purine synthesis and *de novo* pyrimidine synthesis, and may be of use in clarifying the regulation of PRPP synthetase and the dependent nucleotide biosynthetic pathways in intact cells. MRPP may be of particular use against tumors with elevated PRPP synthetase activity. Preliminary studies in our laboratory suggest that MRPP has comparable activity against a parental and doxorubicin-resistant human breast cancer line with the multidrug-resistant phenotype. MRPP may be a reasonable candidate for testing in the National Cancer Institute's *in vitro* human cancer cell line panel. Given the central role of GTP in regulating cellular anabolism and differentiation, the combination of MRPP with other nucleosides may offer a tool to selectively deplete GTP pools. Growth inhibition and biochemical effects were seen at MRPP exposures lower than those resulting in frank lethality (the IC_{50} for a 4-hr exposure was 0.3 vs 5 μM, respectively), and it is possible that biochemically active but non-lethal exposures to MRPP may be capable of modulating the activity of other antimetabolites. Additional studies are planned to determine the therapeutic effects of MRPP against human solid tumor xenografts.

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