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# Metabolism and Metabolic Actions of 6-Methylpurine and 2-Fluoroadenine in Human Cells

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**ABSTRACT.** Activation of purine nucleoside analogs by *Escherichia coli* purine nucleoside phosphorylase (PNP) is being evaluated as a suicide gene therapy strategy for the treatment of cancer. Because the mechanisms of action of two toxic purine bases, 6-methylpurine (MeP) and 2-fluoroadenine (F-Ade), that are generated by this approach are poorly understood, mechanistic studies were initiated to learn how these compounds differ from agents that are being used currently. The concentration of F-Ade, MeP, or 5-fluorouracil required to inhibit CEM cell growth by 50% after a 4-hr incubation was 0.15, 9, or 120 μM, respectively. F-Ade and MeP were also toxic to quiescent MRC-5, CEM, and Balb 3T3 cells. Treatment of CEM, MRC-5, or Balb 3T3 cells with either F-Ade or MeP resulted in the inhibition of protein, RNA, and DNA syntheses. CEM cells converted F-Ade and MeP to F-ATP and MeP-ribonucleoside triphosphate (MeP-R-TP), respectively. The half-life for disappearance of HeP-ribonucleoside triphosphate from CEM cells was approximately 48 hr, whereas the half-lives of F-ATP and ATP were approximately 5 hr. Both MeP and F-Ade were incorporated into the RNA and DNA of CEM cells. These studies indicated that the mechanisms of action of F-Ade and MeP were quite different from those of other anticancer agents, and suggested that the generation of these agents in tumor cells by *E. coli* PNP could result in significant advantages over those generated by either herpes simplex virus thymidine kinase or *E. coli* cytosine deaminase. These advantages include a novel mechanism of action resulting in toxicity to nonproliferating and proliferating tumor cells and the high potency of these agents during short-term treatment. BIOCHEM PHARMACOL **55**;10:1673–1681, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** 6-methylpurine; 2-fluoroadenine; gene therapy; *E. coli* purine nucleoside phosphorylase; adenosine triphosphate analogs

Expression of foreign genes in tumor cells is an attractive strategy to alter their phenotype, making them sensitive to otherwise nontoxic agents [1–5]. Selective delivery of the HSV§ dThd kinase or the *Escherichia coli* Cyt deaminase genes has received the greatest experimental attention. We have developed a gene therapy strategy that is based on the selective expression of the *E. coli* PNP gene in tumor cells [6–9]. *E. coli* PNP, unlike mammalian PNP, accepts Ado and certain Ado analogs as substrates, and hence can be used to cleave nontoxic purine nucleosides to very toxic Ade analogs. The toxic purine analogs generated by *E. coli* PNP readily diffuse across cell membranes and have high bystander activity [6, 7]. Because very little is known about the mechanism of action of two prototype agents that can be liberated by *E. coli* PNP (MeP and F-Ade), studies were done to characterize their metabolism and biochemical effects in human cells to aid the rational development of this suicide gene therapy strategy. A preliminary report of this work has been presented [9].

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§ *Abbreviations:* Ade, adenine; Ado, adenosine; AIA-labile, acid-insoluble/alkali-labile; AIA-stable, acid-insoluble/alkali-stable; APRT, adenine phosphoribosyl transferase; Cyt, cytosine; dThd, thymidine; F-Ade, 2-fluoroadenine; F-Ado, 2-fluoroadenosine; F-araA, arabinofuranosyl-2-fluoroadenine; F-ATP, 2-fluoroadenosine 5′-triphosphate; F-cAMP, 2-fluoroadenosine 3′,5′-monophosphate; F-dATP, 2-fluoro-2′-deoxyadenosine 5′-triphosphate; F-dUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; F-dUTP, 5-fluoro-2′-deoxyuridine 5′-triphosphate; FUra, 5-fluorouracil; FUTP, 5-fluorouridine 5′-triphosphate; GCV, ganciclovir; GCV-MP, ganciclovir 5′-monophosphate; GCV-TP, ganciclovir 5′-triphosphate; HSV, herpes simplex virus; $IC_{50}$, concentration of compound required to inhibit cell growth by 50%; MeP, 6-methylpurine; MeP-dR, 6-methylpurine-2′-deoxyribonucleoside; MeP-dR-TP, 6-methylpurine-2′-deoxyribonucleoside 5′-triphosphate; MeP-R, 6-methylpurine-riboside; MeP-R-DP, 6-methylpurine-ribonucleoside 5′-diphosphate; MeP-R-TP, 6-methylpurine-ribonucleoside 5′-triphosphate; PNP, purine nucleoside phosphorylase; SAX HPLC, strong anion exchange high pressure liquid chromatography; and Urd, uridine.

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## MATERIALS AND METHODS

### *Materials*

[8-$^3$H]F-araA, [2,8-$^3$H]MeP-dR, [*methyl*-$^3$H]dThd, [5-$^3$H]Urd, [8-$^{14}$C]Ade, [2,8-$^3$H]Ade, and [4,5-$^3$H]leucine were obtained from Moravek Biochemicals. [$^3$H]MeP and [$^3$H]F-Ade were obtained by cleaving [2,8-$^3$H]MeP-dR and [8-$^3$H]F-araA with *E. coli* PNP [6], and were purified by reverse-phase

HPLC before use. MeP, FUra, and cycloheximide were obtained from the Sigma Chemical Co. F-Ade was synthesized in our laboratories as described [10]. All other materials used were of standard analytical grade.

### *Cell Culture*

CEM cells, obtained from the American Type Culture Collection, were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. Human diploid embryonic lung cells (MRC-5) were obtained from Bio-Whittaker and cultured in Modified Eagle's Medium containing 9% heat-inactivated fetal bovine serum. Balb-3T3 cells were obtained from Dr. W. J. Pledger (Moffett Cancer Center) and were maintained in Dulbecco's Modified Eagle's Medium supplemented to 10% with iron-supplemented newborn calf serum (Hyclone) without antibiotics. The Balb-3T3 cells were kept in a non-transformed state by twice weekly passage at low culture density. Cells were routinely checked for the presence of mycoplasma and were discarded if contaminated.

### *Measurement of DNA, RNA, and Protein Syntheses in Intact Cells*

The effect of compounds on the incorporation of radiolabeled precursors ([8-$^{14}$C]Ade, [5-$^{3}$H]Urd, [*methyl*-$^{3}$H]dThd, or [4,5-$^{3}$H]leucine) into RNA, DNA, or protein was determined as described [11, 12]. The incorporation of Urd or Ade into RNA is determined by subtracting the incorporation of radiolabel into the alkali-stable/acid precipitable fraction (DNA) from the total acid precipitable fraction (DNA plus RNA). Urd and Ade are primarily incorporated into RNA, but can also be incorporated into DNA as dCyd or dAdo, respectively. The incorporation of dThd and leucine into acid-precipitable material is a measure of their incorporation into DNA and protein, respectively.

Measurements of DNA, RNA, and protein syntheses in Balb-3T3 cells were performed in 96-well microplates using confluent and mitotically quiescent monolayers. Cells were seeded in 180 μL of culture medium containing 2–3 × $10^4$ cells and incubated for 7–9 days (37°, 5% $CO_2$). Cells were treated with MeP or F-Ade in the presence (mitotically stimulated cells) or absence (quiescent cells) of newborn calf serum. To measure DNA synthesis, cells were pulsed for 20 hr with 1 μCi of [$^{3}$H]dThd/well added 10 hr after the addition of MeP or F-Ade. To measure RNA or protein synthesis, cells were pulsed for 6 hr with 1 μCi of [$^{3}$H]Urd or 1 μCi of [$^{3}$H]leucine, respectively, added to replicate wells 24 hr after the addition of MeP or F-Ade. After 30 hr of incubation with MeP or F-Ade, the cells were removed from the wells with a solution containing 0.1% trypsin in 10% glycerol, and the cell suspension was filtered through scintillant-impregnated glass fiber filters (Ready-filter No. 586303, Beckman Instruments, Inc.). The cells on the filters were lysed and washed with 3% acetic acid, and the filters were dried and counted for radioactivity.

### *Extraction and Analysis of the Acid-Soluble Nucleotide Pool* [13]

Cells were collected by centrifugation and resuspended in ice-cold 0.5 M of perchloric acid. The samples were centrifuged at 12,000 *g* for 20 min, and the supernatant fluid was removed and neutralized with 1 M of potassium phosphate (pH 7.4) and 4 M of KOH. $KClO_4$ was removed by centrifugation, and a portion of the supernatant fluid was injected onto a Partisil-10 SAX column (Keystone Scientific Inc.). Elution of the nucleotides was accomplished with a 50-min linear gradient from 5 mM of $NH_4H_2PO_4$ (pH 2.8) to 750 mM of $NH_4H_2PO_4$ (pH 3.7) buffer with a flow rate of 2 mL/min. The natural nucleotides were detected by measurement of the UV absorbance at 260 nm, and the radioactive acid-soluble metabolites were detected by counting 1-min fractions that eluted from the column. The radioactivity in the acid-insoluble material was determined as described [11, 12].

### *Incorporation of MeP and F-Ade into RNA and DNA*

CEM cells incubated with [2,8-$^{3}$H]MeP, [8-$^{3}$H]F-Ade, [5-$^{3}$H]Urd, [*methyl*-$^{3}$H]dThd, or [2,8-$^{3}$H]Ade were collected by centrifugation and resuspended in 0.5 mL of 10 mM of Tris (pH 8.0), 40 mM of EDTA, 0.5% SDS, and 200 μg/mL of proteinase K. The mixture was incubated at 37° overnight, mixed with CsCl, and centrifuged to equilibration as described previously [14]. The gradients were fractionated, and the RNA and DNA in each sample were precipitated onto glass fiber filters with a 5% trichloroacetic acid solution containing 10 mM of pyrophosphate. These filters were washed three times with this 5% trichloroacetic acid solution followed by two washes with 95% ethanol, dried, and counted for radioactivity. To verify that the radioactivity associated with the DNA fractions was due to the incorporation of [$^{3}$H]MeP or [$^{3}$H]F-Ade, the RNA and DNA samples were degraded to their constituent nucleosides, which were separated by reverse-phase HPLC as described [14, 15].

## RESULTS

### *Cytotoxicity of MeP and F-Ade to Proliferating CEM Cells*

The $IC_{50}$ of MeP, F-Ade, or FUra against CEM cells was 9 ± 4, 0.15 ± 0.07, or 120 ± 44 μM, respectively (mean ± SD of 3 determinations). In this experiment cells were exposed to drug for only 4 hr, and the effect on cell growth was determined 72 hr after the removal of drug. If cells were exposed continuously to these compounds (72 hr), the $IC_{50}$ for each compound was decreased to 1.2, 0.10, and 7.2 μM, respectively (mean of 2 determinations). It is of interest that the duration of the exposure had less of an effect on the ability of F-Ade to inhibit cell growth (1.5-fold) than it had on both MeP and FUra (7.5- and 17-fold, respectively). This result indicated that the cytotoxic actions of F-Ade

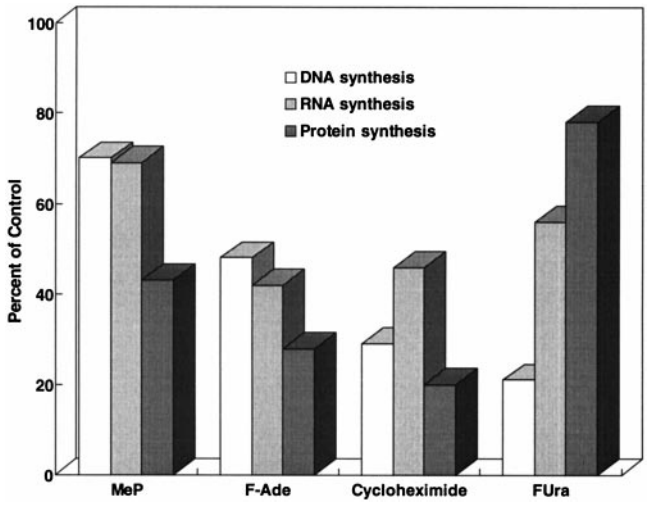


**FIG. 1. Effects of MeP, F-Ade, cycloheximide, and FUra on macromolecular synthesis. CEM cells (approximately 300,000 cells/mL) were incubated with 100 μM of MeP, 2 μM of F-Ade, 1 μM of cycloheximide, or 1000 μM of FUra. Radiolabeled precursors of DNA ([*methyl*-$^3$H]dThd), RNA ([5-$^3$H]Urd), or protein ([4,5-$^3$H]leucine) were added at 2 μCi/mL 4 hr after the addition of the compounds. Samples were taken 1, 2, 3, and 4 hr after the addition of radiolabel to determine their incorporation into RNA, DNA, or protein, as described in Materials and Methods. Because treatment with FUra inhibits thymidylate synthetase, the effect of FUra on DNA and RNA syntheses was determined by measuring the incorporation of [8-$^{14}$C]Ade into the alkali-stable/acid-insoluble fraction and the total acid-insoluble fraction. The amount of incorporation of [$^3$H]leucine into protein, [$^3$H]dThd into DNA, [$^3$H]Urd into RNA, or [$^{14}$C]Ade into DNA in a 4-hr period was 8, 444, 229, or 1.1 dpm/10$^3$ cells, respectively. This experiment was repeated, and similar results were obtained.**

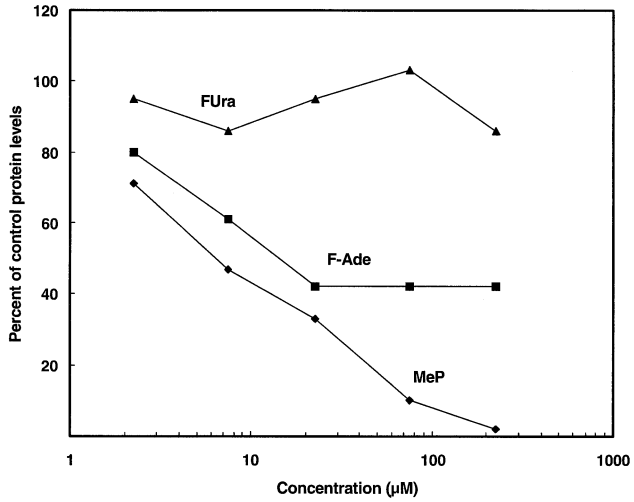


**FIG. 2. Cytotoxicity of MeP, F-Ade, and FUra to nonproliferating MRC-5 cells. Confluent, nonproliferating MRC-5 cells were treated with various concentrations of MeP, F-Ade, or FUra. After incubation for 96 hr, the effects of the compound on the MRC-5 cells were determined by measuring the amount of protein that was attached to the culture flask. The protein concentration in control cells was approximately 35 μg/flask. The effect of MeP on MRC-5 cells was repeated one time with similar results. FUra and F-Ade experiments were done only one time.**

that result in the inhibition of cell growth occur early after addition of the compound. The concentration of cycloheximide that inhibited CEM cell growth by 50% after 72 hr of continuous exposure to cycloheximide was 0.1 μM. A 4-hr incubation of CEM cells with 0.3 μM of cycloheximide did not affect cell growth (data not shown).

### *Effect of MeP, F-Ade, Cycloheximide, or FUra on DNA, RNA, and Protein Syntheses in CEM Cells*

Both MeP and F-Ade inhibited protein, DNA, and RNA syntheses (Fig. 1). This pattern of inhibition of macromolecular syntheses was similar to that observed with cycloheximide, a known inhibitor of protein synthesis [16]. Inhibition of protein synthesis causes an inhibition of DNA and RNA syntheses, presumably due to the fact that some of the enzymes involved in these processes have very short half-lives. Therefore, with inhibitors of protein synthesis such as cycloheximide, it is difficult to find a concentration of compound that selectively inhibits protein synthesis. Unlike MeP, F-Ade, or cycloheximide, FUra primarily inhibited DNA synthesis and had only a lesser effect on RNA or protein synthesis, which is consistent with the known mechanism of action of FUra [17, 18]. The concentrations of the compounds used in this experiment were approximately ten times the concentration of drug that resulted in 50% inhibition of cell growth after a 4-hr incubation. These results indicated that the initial target of MeP and F-Ade that results in the inhibition of cell growth was not an enzyme involved in DNA synthesis, but instead was one or more enzymes involved in either RNA or protein synthesis.

### *Effects of MeP and F-Ade on Nonproliferating Cells*

The previous results indicated that the cytotoxicities of MeP and F-Ade were due to their inhibition of protein and/or RNA synthesis. Because all cells, regardless of their proliferative state, require protein and RNA syntheses, these results suggested that MeP and F-Ade would be toxic to nonproliferating as well as proliferating cells. Therefore, experiments were done to evaluate the effects of MeP and F-Ade on nonproliferating human cells. As seen in Fig. 2, both MeP and F-Ade were toxic to nonproliferating MRC-5 cells, whereas treatment with FUra did not affect these cells. MRC-5 cells are a nontransformed human diploid fibroblast cell line derived from embryonic lung cells. These cells stop growth upon reaching confluence, and they can be maintained indefinitely in a nonproliferative state by regularly changing the medium. In these cultures, the protein concentration in control flasks did not change over the 96-hr incubation period, which indicated that cell numbers did not increase during the experiment.

In a separate experiment, the rates of DNA, RNA, and

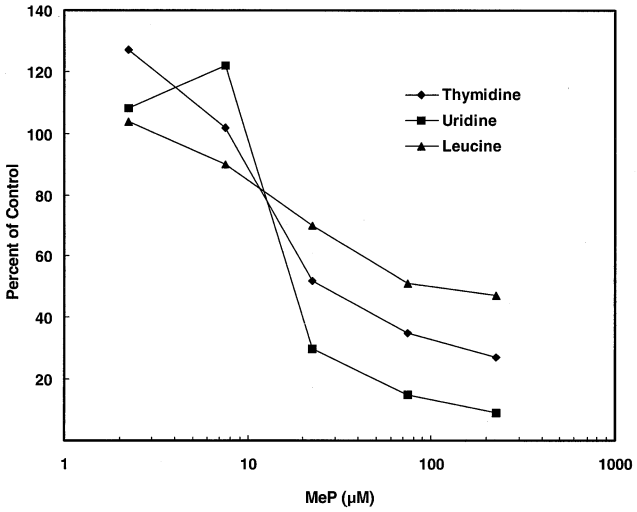


**FIG. 3. Effect of MeP on DNA, RNA, and protein syntheses in nonproliferating MRC-5 cells. Confluent, nonproliferating MRC-5 cells were treated with various concentrations of MeP. After 10 hr of incubation, radiolabeled precursors of DNA ([*methyl*-$^3$H]dThd), RNA ([5-$^3$H]Urd), or protein ([4,5-$^3$H]leucine) were added to the cultures, and DNA, RNA, and protein syntheses were determined as described in Materials and Methods. The incorporation of [$^3$H]dThd into DNA, [$^3$H]Urd into RNA, or [$^3$H]leucine into protein in control cultures was 18, 4.1, or 39 dpm/$10^3$ cells, respectively. Similar results were obtained in cells exposed to MeP for 24 hr.**

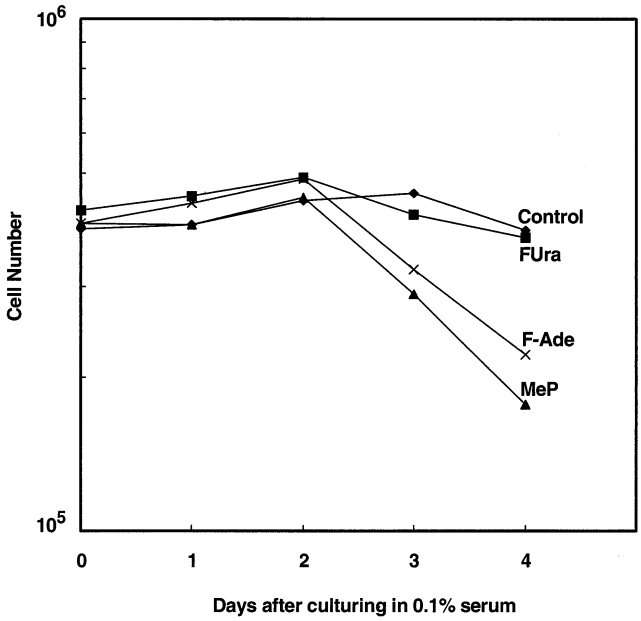


**FIG. 4. Cytotoxicity of MeP, F-Ade, and FUra to nonproliferating CEM cells. CEM cells were cultured in culture medium containing 0.1% serum to induce a nonproliferative state. Forty-eight hours after initiation of the cell cultures in 0.1% serum medium, MeP (10 μg/mL), F-Ade (1 μg/mL), or FUra (10 μg/mL) was added to separate cell cultures. Cell numbers were counted with a Coulter Counter at 24-hr intervals from the time the cells were cultured in the 0.1% serum. This experiment was repeated one time with similar results.**

protein syntheses were determined in MRC-5 cell cultures as they grew into the confluent state to characterize their proliferative status (data not shown). The incorporation of dThd into DNA increased during the first 5 days of culture, but had decreased to less than 10% of that seen at its peak by day 10 of culture (when the experiments shown in Fig. 2 were initiated). In contrast, the rates of RNA and protein syntheses 10 days after initiation of the cell culture were greater than or equal to their rates of synthesis after day 1 of culture. These results indicated that the MRC-5 cells at the time of treatment were in a relatively nonproliferative state.

The effect of MeP on DNA, RNA, and protein syntheses in nonproliferating MRC-5 cells was determined to obtain a different measure of the cytotoxicity of MeP to these cells. Incubation of nonproliferating MRC-5 cells with MeP for 4 hr at concentrations as high as 120 μM had little effect on DNA, RNA, or protein synthesis (data not shown). However, 10 hr (Fig. 3) and 24 hr (data not shown) of treatment with MeP decreased DNA, RNA, and protein syntheses by a similar degree. These results confirm that MeP is toxic to the nonproliferating MRC-5 cells and support the conclusion from the studies in CEM cells that the toxicity of MeP is due to its inhibition of protein and/or RNA synthesis.

In another experiment, CEM cells were cultured in 0.1% serum to induce a nonproliferative state and then were treated with MeP, F-Ade, or FUra. The compounds were added to CEM cell culture 48 hr after the CEM cells were placed in the 0.1% serum to allow time for the cells to stop growing. As can be seen in Fig. 4, treatment with MeP and F-Ade resulted in a decline in cell numbers within 24 hr of addition of compound. In contrast, treatment with FUra did not affect cell numbers. The incorporation of dThd into DNA 48 hr after initiating culture in 0.1% serum was 10–15% of the incorporation of dThd into DNA in proliferating cells, which indicated that CEM cells incubated in 0.1% serum were in a quiescent state.

Experiments were also done to determine the effects of MeP and F-Ade on DNA, RNA, and protein syntheses in nontransformed Balb-3T3 cells (Table 1). Addition of 5% newborn calf serum to the Balb-3T3 cell cultures resulted in a 2.5-fold increase in [$^3$H]dThd incorporation into DNA, which indicated that these cultures were in a moderately quiescent state (12–15% cycling). Consistent with the results observed with CEM and MRC-5 cells, MeP and F-Ade inhibited protein, RNA, and DNA syntheses by a similar amount in Balb-3T3 cells incubated in serum-free medium (Table 1). These results support the conclusion that both MeP and F-Ade are toxic to quiescent cells.

### *Metabolism of MeP and F-Ade in CEM cells*

The predominant metabolite in CEM cells treated with either 3 μM of MeP or 0.03 μM of F-Ade for 4 hr eluted in fractions 35 and 36 from the SAX HPLC column (Fig. 5). Their retention times on the SAX HPLC were 2–3 min longer than the retention time of ATP, which eluted at approximately 32 min from this column. These metabolites

**TABLE 1. Effects of MeP and F-Ade on DNA, RNA, and protein syntheses in quiescent Balb-3T3 cells**

| | Concn (μM) | DNA synthesis | RNA synthesis | Protein synthesis |
|---|---|---|---|---|
| | | (% of Control Synthesis) | | |
| MeP | 25 | 34 ± 17 | 37 ± 4 | 15 ± 2 |
| | 2.5 | 62 ± 22 | 90 ± 13 | 37 ± 12 |
| | 0.25 | 84 ± 26 | 85 ± 2 | 78 ± 11 |
| F-Ade | 22 | 8 ± 3 | 17 ± 3 | 4 ± 1 |
| | 2.2 | 76 ± 24 | 64 ± 10 | 66 ± 15 |
| | 0.22 | 120 ± 23 | 86 ± 15 | 76 ± 27 |

Balb-3T3 cells were incubated with the indicated concentrations of MeP and F-Ade in serum-free medium for 30 hr, and the effects of the compounds on the incorporation of dThd, Urd, and leucine into acid-precipitable material were determined as described in Materials and Methods. The incorporation of [$^3$H]dThd, [$^3$H]Urd, and [$^3$H]leucine into acid-precipitable material in control cells was 8,400 ± 1,800, 162,000 ± 24,000, and 12,200 ± 1,800 dpm/well, respectively. Each value represents the mean ± SD from 4 replicate samples. Similar results were obtained in mitotically stimulated cells. This experiment was repeated with similar results.

were collected and degraded to their respective nucleosides by treating with phosphodiesterase and alkaline phosphatase. The nucleosides generated in this manner were then identified as MeP-R and F-Ado by reverse-phase HPLC, confirming that these metabolites eluting in fractions 35 and 36 from the SAX HPLC column were ribonucleotides, not deoxyribonucleotides. Based on these results and those of others [19–28], the metabolites eluting at 35 min were identified as MeP-R-TP and F-ATP, respectively. At higher concentrations of these compounds, the metabolites of MeP and F-Ade could also be detected by their absorbance at 254 nm as they eluted from the SAX column (data not shown).

The concentration of F-ATP generated in a 4-hr period from 0.3 μM of F-Ade was 62 pmol/$10^6$ cells, and the concentration of MeP-R-TP generated in a 4-hr period from 20 μM of MeP was 85 pmol/$10^6$ cells (data not shown). Incubation of CEM cells for 4 hr with either 20 μM of MeP or 0.3 μM of F-Ade resulted in approximately 70% inhibition of CEM cell growth. Since the ATP concentrations in these experiments were approximately 700 nmol/$10^6$ cells, these results indicated that the cytotoxicity of these agents occurred when only a relatively small amount of the triphosphate analog was formed in cells (13% of the ATP pool). It is of interest that similar amounts of MeP-R-TP and F-ATP were formed in CEM cells treated with equitoxic concentrations of MeP and F-Ade. In addition, incubation of CEM cells with either 0.3 μM of F-Ade or 20 μM of MeP did not affect ATP, CTP, UTP, or GTP levels (data not shown), which indicated that inhibition of purine or pyrimidine nucleotide biosynthesis was not responsible for the cytotoxicity of these two agents. ATP levels were decreased in cells treated with higher concentrations of F-Ade (10 μM). However, the total ATP/F-ATP pool in cells treated with F-Ade was equal to the ATP pool in untreated cells.

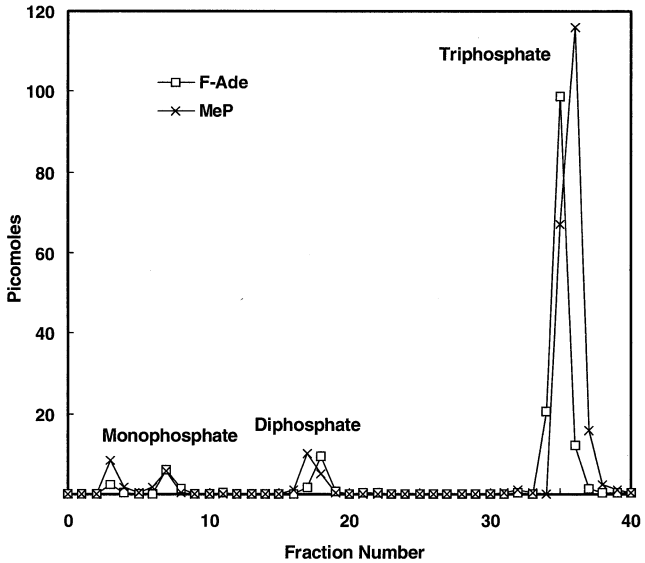


**FIG. 5. Metabolism of MeP and F-Ade in CEM cells. CEM cells were incubated with 3 μM of MeP (94 Ci/mol) or 0.03 μM of F-Ade (6300 Ci/mol). After 4 hr the acid-soluble metabolites were collected and analyzed by SAX HPLC as described in Materials and Methods. The radioactive acid-soluble metabolites of MeP and F-Ade were detected by counting 1-min fractions that eluted from the column. This experiment was repeated with similar results.**

The half-lives of MeP-R-TP and F-ATP in proliferating CEM cells were determined and compared to the half-life of ATP (Fig. 6). Because the concentrations of MeP and F-Ade used in this experiment had a minimal effect on cell growth, the cell numbers in all treatment groups increased over the 48-hr period of the experiment. Therefore, the amount of triphosphate remaining after removal of Ade, MeP, or F-Ade was normalized to the volume of culture medium to correct for the decrease in triphosphate that would occur due to the proliferation of the cells. This correction is only necessary due to the long half-life observed with MeP-R-TP. For example, 48 hr after the removal of MeP, the CEM cell numbers had doubled, and the amount of MeP-R-TP per cell had decreased by 75%. Therefore, half of the decrease in MeP-R-TP levels in these cells is due to the increase in cell number. The calculation of the half-lives of ATP and F-ATP was determined from the first three measurements (2, 4, and 10 hr) when negligible cell growth had occurred. Regardless, it is clear from this experiment that the half-life of MeP-R-TP in CEM cells is much greater than that for either ATP or F-ATP.

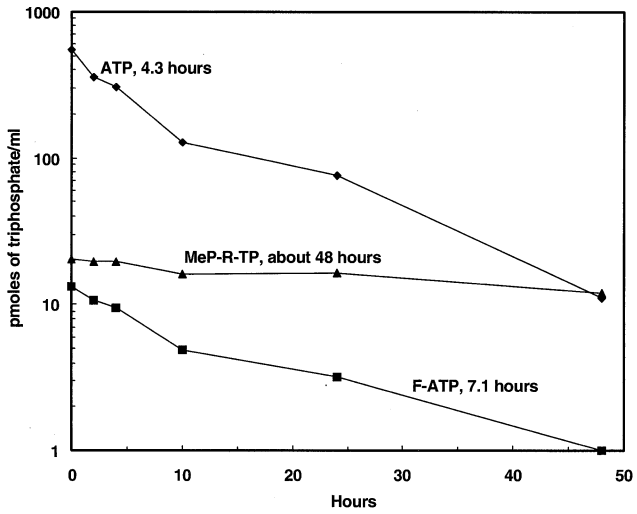


**FIG. 6. Half-lives of ATP, MeP-R-TP, and F-ATP in CEM cells. After incubation of CEM cells with 3.6 μM of [$^{14}$C]Ade (56 Ci/mol), 3.0 μM of [$^{3}$H]MeP (94 Ci/mol), or 0.03 μM of [$^{3}$H]F-Ade (6300 Ci/mol) for 4 hr, the cells were collected, washed with fresh medium, and resuspended in fresh medium that did not contain radiolabeled purines. Samples were collected at various times after resuspending the cells in fresh medium, and the amount of label in the ribonucleoside triphosphate peak was determined using SAX HPLC as described in Materials and Methods. This particular experiment was done only one time, but similar results were obtained when the half-lives of MeP-R-TP and F-ATP were determined using nonradioactive compounds.**

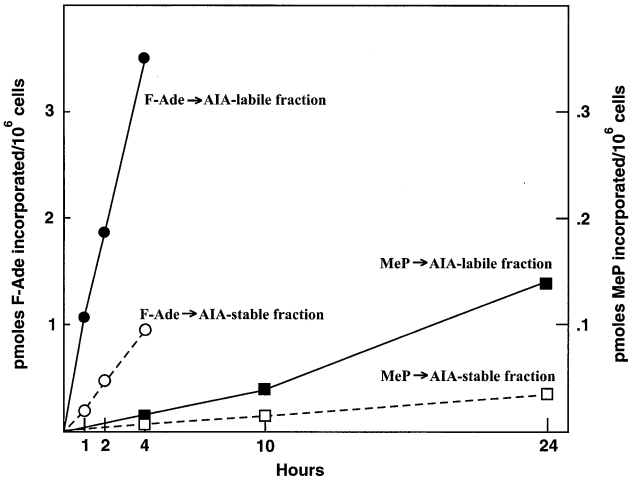


**FIG. 7. Incorporation of MeP and F-Ade into RNA and DNA. CEM cells were incubated with either 20 nM of [$^{3}$H]F-Ade (6,300 Ci/mol, ●, ○) or 77 nM of [$^{3}$H]MeP (10,000 Ci/mol, ■, □). Cell samples (6–8 × 10$^6$ total cells) from cells treated with F-Ade were removed 1, 2, and 4 hr after the addition of F-Ade, and the incorporation of radiolabel into the AIA-labile fraction (RNA, closed symbols) and the AIA-stable fraction (DNA, open symbols) was determined as described in Materials and Methods. Because of the small amount of incorporation of MeP into these fractions, cell samples were taken at 4, 10, and 24 hr from cells treated with MeP. This experiment was repeated one time with similar results.**

The metabolism of MeP to MeP-R-TP in 4 hr was much less than that seen with Ade, even though the concentrations of MeP and Ade were similar (Fig. 6). This result indicated that MeP was a poor substrate for human APRT activity, as has been observed with the APRT from *Leishmania donovani* [29]. Approximately 400 pmol/10$^6$ cells of F-ATP was formed in cells treated with 2 μM of F-Ade for 4 hr (data not shown), which was comparable to the amount of ATP formed from 3.6 μM of Ade in a 4-hr period (821 pmol/10$^6$ cells) and indicated that F-Ade was metabolized as effectively as Ade in CEM cells.

### *Incorporation of MeP and F-Ade into RNA and DNA*

During the 4-hr incubation period, there was a linear increase in the incorporation of F-Ade into both the AIA-labile (RNA) and AIA-stable (DNA) fractions in CEM cells treated with 20 nM of [$^{3}$H]F-Ade (Fig. 7), and more than 60% of the F-Ade in the cell culture medium was taken up by the CEM cells. A much smaller amount of MeP was incorporated into these fractions (note the 10-fold difference in the scale for MeP vs F-Ade in Fig. 7). However, the incorporation of 77 nM of [$^{3}$H]MeP into both the AIA-labile and AIA-stable fractions increased linearly with time for 24 hr, and in contrast to that seen with F-Ade, less than 10% of the MeP had been taken up by the CEM cells during the 24-hr incubation.

Even though [$^{3}$H]MeP and [$^{3}$H]F-Ade were purified using reverse-phase HPLC before use in these experiments, it is still important to verify the identity of the radioactivity in these crude fractions as being associated with MeP or F-Ade in RNA or DNA. Therefore, DNA and RNA were isolated from CEM cells treated with [*methyl*-$^{3}$H]dThd, [5-$^{3}$H]Urd, [2,8-$^{3}$H]Ade, [$^{3}$H]MeP, or [$^{3}$H]F-Ade using CsCl gradients (data not shown). The location of the DNA band in the CsCl gradients was identified by the incorporation of [$^{3}$H]dThd into acid-insoluble material, and the fractions that contained RNA were identified by the incorporation of [$^{3}$H]Urd into acid-insoluble material (the RNA was found in the pellet because RNA is more dense than the CsCl gradient that is formed during the centrifugation). The DNA and RNA fractions were collected, dialyzed twice against water, and degraded to their component nucleosides. In CEM cells treated with [$^{3}$H]F-Ade, [$^{3}$H]F-Ado was detected in the RNA and [$^{3}$H]F-dAdo was detected in the DNA, which confirmed that F-Ade was incorporated into both RNA and DNA of CEM cells. Likewise, [$^{3}$H]MeP-R was detected in the RNA and MeP-dR was detected in the DNA of CEM cells treated with [$^{3}$H]MeP, which confirmed that MeP was also incorporated into both RNA and DNA. The incorporation of F-Ade and MeP into DNA indicated that ribonucleotide reductase recognized the ribonucleoside diphosphates composed of either MeP or F-Ade as a substrate and that the dATP analogs of these compounds were utilized as substrates by human DNA polymerases. F-dATP has been shown to be a good substrate for DNA polymerase α [30]. No studies have been done to evaluate MeP-dR-TP as a

substrate for human DNA polymerases. Even though MeP and F-Ade are incorporated into DNA, the data in the rest of this manuscript indicate that their incorporation into DNA is not the primary reason for the cytotoxicity of these two agents to CEM cells.

## DISCUSSION

A considerable amount of information is available concerning the mechanism of action of GCV-MP [31–34] and FUra [17, 18], which are products of HSV dThd kinase and *E. coli* Cyt deaminase, respectively. The selective delivery to cancer cells of the gene for these two enzymes currently represents the bulk of suicide cancer gene therapy studies under way. Once GCV is phosphorylated by HSV dThd kinase, GCV-MP is then further phosphorylated by human nucleotide kinases to GCV-TP, which is a substrate for human DNA polymerases. Incorporation into the DNA of GCV-MP results in inhibition of DNA chain elongation and thereby inhibits DNA synthesis, which results in cell death. FUra is converted in human cells to three metabolites (FUTP, F-dUMP, and F-dUTP) that are believed to be responsible for the cytotoxicity of this agent. Although FUra is incorporated into RNA, the inhibition of thymidylate synthetase by F-dUMP and the subsequent incorporation of FUra into DNA is believed to be the primary effect of this agent that contributes to its anticancer activity. Therefore, like GCV-MP, FUra is primarily toxic to proliferating cells [35]. One of the reasons that antitumor therapy with conventional agents has only modest activity against solid tumors is that most antitumor agents primarily target proliferating cells, even though many solid tumors have a low fraction of actively dividing cells [36].

Conversely, relatively little is known about the mechanism of action of either MeP or F-Ade, two prototype toxic purines that could be generated by *E. coli* PNP in a gene therapy strategy. The present studies were done to obtain information about the biochemical effects of these agents, so that comparisons could be made with the other suicide gene therapy strategies. In addition, such information is critical for the rational development of this particular gene therapy strategy. The results indicated that both of these agents are converted to ATP analogs, which then inhibit some reaction that involves ATP and results in cell death. Therefore, the mechanisms of action of these two agents are quite different from that of GCV-MP and FUra in that they target one or more enzymes that are not related to DNA synthesis and can kill cells that are not in a proliferative state. The mechanism of action of these two agents is also quite different from all of the drugs currently used to treat cancer and therefore represents a novel approach to killing tumor cells. The precise mechanism of action of these two compounds is not known and may be difficult to determine, because of the many enzyme reactions that utilize ATP. However, our results indicate that the mechanism of action of these agents is likely to involve the inhibition of protein and/or RNA synthesis. It is possible that the incorporation of these agents into RNA results in the disruption of its secondary structure (possibly by inhibiting dsRNA deaminase, see [37]), which could explain the inhibition of both RNA and protein synthesis seen with these agents.

An interesting finding in our studies was the long half-life of MeP-R-TP in CEM cells. The reason for the difference in half-life between MeP-R-TP and F-ATP is not known. In this and other respects, F-Ade is much more similar to Ade than is MeP. This information could be important in determining the appropriate treatment schedules of these two agents. Prodrugs liberating F-Ade may need to be given more frequently than those that generate MeP.

Because MeP and F-Ade were first synthesized many years ago, there has been considerable study of their metabolism and cytotoxicity in various organisms that is relevant to the conclusions of this study. Both of these agents have been evaluated as antitumor agents and were found to have no selectivity for tumor versus normal cells in intact animals [38]. This observation is consistent with the results presented in the current work, which indicate that these agents inhibit protein and/or RNA synthesis, two metabolic processes important to all cells. In addition, MeP and F-Ade are known to be converted to their respective ribonucleoside triphosphate analogs [19–28, 39]. Indeed, in cells treated with high concentrations of F-Ado, the F-ATP pool can replace the ATP pool [20, 21, 25–27], which suggests that F-ATP is recognized as ATP by the enzymes involved in maintaining the ATP pool [40]. 2-F-*S*-Adenosylmethionine and F-cAMP have been detected in cells treated with F-Ado [25–27] and would also be expected in cells treated with F-Ade. We did not specifically look for these metabolites in our experiments. However, the majority (>90%) of the radioactivity from either [$^3$H]MeP or [$^3$H]F-Ade in the acid-soluble pool was in the triphosphate fraction (Fig. 5), which indicated that at best 2-F-*S*-adenosylmethionine and F-cAMP were relatively minor metabolites of F-Ade in CEM cells. Cells lacking APRT activity are resistant to the cytotoxic effects of both MeP and F-Ade [28], which indicates that the conversion of these agents to nucleotide analogs is required for their cell killing action. F-Ade has been shown to be incorporated into a fraction that was insoluble in alcohol (mixture of RNA and DNA) in *Tetrahymena pyriformis* [19]. There have been some studies that have evaluated F-Ade-containing metabolites as substrates for various enzymes. Where tested, these metabolites have been found to be good substrates for their respective enzymes: RNA polymerase isolated from *Micrococcus lysodeikticus* [23]; catechol-*O*-methyltransferase [25]; activation of protein kinase isolated from rat brain [25]; adenylate cyclase [26, 27]; and DNA polymerase α [30].

Less work has been done to characterize the metabolic effects of MeP. The studies most relevant to the current work have been presented only in the form of an abstract [39]. Regardless, this work indicated that 1) MeP-R-DP was a good substrate of pyruvate kinase; 2) MeP-R-TP was

a good substrate for hexokinase; and 3) MeP-R-TP was capable of replacing ATP in cell-free protein synthesis reactions and muscle contraction experiments. However, MeP-R-TP was not able to reverse thyroxin-induced mitochondrial swelling, and MeP-R-DP was not used as a substrate for phosphoglycerate kinase. MeP has been shown to equally inhibit RNA, DNA, and protein syntheses in *Collybia maculata* [41], which is similar to our results in CEM, MRC-5, and Balb-3T3 cells. Other studies have been done with MeP, but no clear mechanistic details were elucidated. References of these studies are included for the interested reader [42–44].

Another characteristic of these compounds that distinguishes MeP and F-Ade from FUra is their potent cytotoxicity. F-Ade and MeP were 1000- and 10-fold more potent against CEM cells than was FUra, respectively. It is possible that F-Ade and MeP could cause tumor regressions in animals under conditions (equal expression of activating enzymes) where FUra and GCV-MP would have little effect. Given the difficulty of selectively delivering genes to tumor cells in an intact animal, the potency of these agents (particularly F-Ade) may allow suicide gene therapy strategies to work when only a small amount of gene expression in the tumor is achieved. In addition, these agents were potent inhibitors of cell growth after only a short duration of treatment.

A major concern with agents such as F-Ade and MeP that target nonproliferating as well as proliferating cells is that the bystander activity may be too great, and that normal nonproliferating host cells will also be killed by these agents. However, we have shown that MeP-dR is curative against gliomas in the flanks of animals that express *E. coli* PNP [8], which indicates that this potential problem is manageable. This problem is not a concern with the HSV dThd kinase strategy, because as GCV nucleotides leak out of the tumor mass, they would be quickly inactivated by serum phosphatases back to GCV. We believe that this rapid deactivation of GCV nucleotides and the inability of cells to take up nucleotides are serious disadvantages of the HSV dThd kinase strategy. Although the Cyt deaminase strategy would result in the release of FUra from the tumor mass, similar to that seen with MeP and F-Ade, it is unlikely to cause systemic toxicity because FUra is much less potent than both MeP and F-Ade, and it is primarily toxic to proliferating cells. It is possible that the attributes of the compounds generated by *E. coli* PNP (high potency of toxin, activity against nonproliferating cells, and high bystander activity) will be necessary for significant antitumor activity to occur in a gene therapy scenario.

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## References


1. Moolten FL, Drug sensitivity ("suicide") genes for selective cancer chemotherapy. *Cancer Gene Ther* **1:** 279–287, 1994.
2. Mullen CA, Metabolic suicide genes in gene therapy. *Pharmacol Ther* **63:** 199–207, 1994.
3. Culver KW and Blaese RM, Gene therapy for cancer. *Trends Genet* **10:** 174–178, 1994.
4. Conners TA, The choice of prodrugs for gene directed enzyme prodrug therapy of cancer. *Gene Ther* **2:** 702–709, 1995.
5. Freeman SM, Whartenby KA, Freeman JL, Abboud CN and Marrogi AJ, *In situ* use of suicide genes for cancer therapy. *Semin Oncol* **23:** 31–45, 1996.
6. Sorscher EJ, Peng S, Bebok Z, Allan PW, Bennett LL Jr and Parker WB, Tumor cell bystander killing in colonic carcinoma utilizing the *Escherichia coli* DeoD gene to generate toxic purines. *Gene Ther* **1:** 233–238, 1994.
7. Hughes BW, Wells AH, Bebok Z, Gadi VK, Garver RI Jr, Parker WB and Sorscher EJ, Bystander killing of melanoma cells using the human tyrosinase promoter to express the *Escherichia coli* purine nucleoside phosphorylase gene. *Cancer Res* **55:** 3339–3345, 1995.
8. Parker WB, King SA, Allan PW, Bennett LL Jr, Secrist JA III, Montgomery JA, Gilbert KS, Waud WR, Wells AH, Gillespie GY and Sorscher EJ, *In vivo* gene therapy of cancer using *E. coli* purine nucleoside phosphorylase. *Hum Gene Ther* **8:** 1649–1656, 1997.
9. Allan PW, Shaddix SC, Gillespie GY, Bennett LL Jr and Parker WB, Mechanism of action of 6-methylpurine and 2-fluoroadenine. *Proc Am Assoc Cancer Res* **38:** 379, 1997.
10. Montgomery JA and Hewson K, Synthesis of potential anticancer agents, XX. 2-Fluoropurines. *J Am Chem Soc* **82:** 463–468, 1960.
11. Hershko A, Mamont P, Shields R and Tompkins GM, Pleiotropic response. *Nature New Biol* **232:** 206–211, 1971.
12. Bennett LL Jr, Smithers D, Hill DL, Rose LM and Alexander JA, Biochemical properties of the nucleoside of 3-amino-1,5-dihydro-5-methyl-1,4,5,6,8-pentaazaacenaphthylene (NSC-154020). *Biochem Pharmacol* **27:** 233–241, 1978.
13. Parker WB, Shaddix SC, Chang C-H, White EL, Rose LM, Brockman RW, Shortnacy AT, Montgomery JA, Secrist JA III and Bennett LL Jr, Effects of 2-chloro-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenine on K562 cellular metabolism and the inhibition of human ribonucleotide reductase and DNA polymerases by its 5′-triphosphate. *Cancer Res* **51:** 2386–2394, 1991.
14. Parker WB, Shaddix SC, Bowdon BJ, Rose LM, Vince R, Shannon WM and Bennett LL Jr, Metabolism of carbovir, a potent inhibitor of human immunodeficiency virus type 1, and its effect on cellular metabolism. *Antimicrob Agents Chemother* **37:** 1004–1009, 1993.
15. Parker WB, Shaddix SC, Allan PW, Arnett G, Rose LM, Shannon WM, Shealy YF, Montgomery JA, Secrist JA III and Bennett LL Jr, Incorporation of the carbocyclic analog of 2′-deoxyguanosine into the DNA of herpes simplex virus and of HEp-2 cells infected with herpes simplex virus. *Mol Pharmacol* **41:** 245–251, 1992.
16. Bennett LL Jr, Smithers D and Ward CT, Inhibition of DNA synthesis in mammalian cells by actidione. *Biochim Biophys Acta* **87:** 60–69, 1964.
17. Myers CE, The pharmacology of the fluoropyrimidines. *Pharmacol Rev* **33:** 1–15, 1981.
18. Parker WB and Cheng Y-C, Metabolism and mechanism of action of 5-fluorouracil. *Pharmacol Ther* **48:** 381–395, 1990.
19. Hill DL, Straight S and Allan PW, Use of *Tetrahymena pyriformis* to evaluate the effects of purine and pyrimidine analogs. *J Protozool* **17:** 619–623, 1970.
20. Parks RE Jr and Brown PR, Incorporation of nucleosides into the nucleotide pools of human erythrocytes. Adenosine and its analogs. *Biochemistry* **12:** 3294–3302, 1973.
21. Agarwal KC and Parks RE, Adenosine analogs and human

platelets. Effects on nucleotide pools and the aggregation phenomenon. *Biochem Pharmacol* **24:** 2239–2248, 1975.
22. Parks RE Jr, Crabtree GW, Kong CM, Agarwal RP, Agarwal KC and Scholar EM, Incorporation of analog purine nucleosides into the formed elements of human blood, erythrocytes, platelets, and lymphocytes. *Ann NY Acad Sci* **255:** 412–434, 1975.
23. Shigeura HT, Boxer GE, Sampson SD and Meloni ML, Metabolism of 2-fluoroadenosine by Ehrlich ascites cells. *Arch Biochem Biophys* **111:** 713–719, 1965.
24. Avramis VI and Plunkett W, 2-Fluoro-ATP: A toxic metabolite of 9-β-D-arabinosyl-2-fluoroadenine. *Biochem Biophys Res Commun* **113:** 35–43, 1983.
25. Zimmerman TP, Deeprose RD, Wolberg G and Duncan GS, Metabolic formation of nucleoside-modified analogues of S-adenosylmethionine. *Biochem Biophys Res Commun* **91:** 997–1004, 1979.
26. Zimmerman TP, Rideout JL, Wolberg G, Duncan GS and Elion GB, 2-Fluoroadenosine 3′:5′-monophosphate. A metabolite of 2-fluoroadenosine in mouse cytotoxic lymphocytes. *J Biol Chem* **251:** 6757–6766, 1976.
27. Zimmerman TP, Wolberg G, Duncan GS, Rideout JL, Beachman LM III, Krenitsky TA and Elion GB, Inhibition of lymphocyte-mediated cytolysis by 2-fluoroadenosine—Evidence for two discrete mechanisms of drug action. *Biochem Pharmacol* **27:** 1731–1737, 1978.
28. Bennett LL Jr, Vail MH, Chumley S and Montgomery JA, Activity of adenosine analogs against a cell culture, line resistant to 2-fluoroadenine. *Biochem Pharmacol* **15:** 1719–1728, 1966.
29. Tuttle JV and Krenitsky TA, Purine phosphoribosytransferases from *Leishmania donovani*. *J Biol Chem* **255:** 909–916, 1980.
30. Parker WB, Bapat AR, Shen J-X, Townsend AJ and Cheng Y-C, Interaction of 2-halogenated dATP analogs (F, Cl, and Br) with human DNA polymerases, DNA primase, and ribonucleotide reductase. *Mol Pharmacol* **34:** 485–491, 1988.
31. Cheng YC, Grill SP, Dutschman GE, Nakayama K and Bastow KF, Metabolism of 9-(1,3-dihydroxy-2-propoxymethyl)guanine, a new anti-herpes virus compound, in herpes simplex virus-infected cells. *J Biol Chem* **258:** 12460–12464, 1983.
32. Reid R, Mar E-C, Huang E-S and Topal MD, Insertion and extension of acyclic, dideoxy, and ara nucleotides by herpesviridae, human α and human β polymerases. A unique inhibition mechanism for 9-(1,3-dihydroxy-2-propoxymethyl)guanine triphosphate. *J Biol Chem* **263:** 3898–3904, 1988.
33. Balzarini J, Bohman C and DeClercq E, Differential mechanism of cytostatic effect of (*E*)-5-(2-bromovinyl)-2′-deoxyuridine, 9-1(1,3-dihydroxy-2-propoxymethyl)guanine, and other antiherpetic drugs on tumor cells transfected by the thymidine kinase gene of herpes simplex virus type 1 or type 2. *J Biol Chem* **268:** 6332–6337, 1993.
34. Ilsley DD, Lee SH, Miller WH and Kutcha RD, Acyclic guanosine analogs inhibit DNA polymerases α, δ, and ε with very different potencies and have unique mechanisms of action. *Biochemistry* **34:** 2504–2510, 1995.
35. Bruce WR and Meeker BE, Comparison of the sensitivity of hematopoietic colony-forming cells in different proliferative states to 5-fluorouracil. *J Natl Cancer Inst* **38:** 401–405, 1967.
36. Tannock IF, Principles of cell proliferation: Cell kinetics in cancer. In: *Principles and Practice of Oncology* (Eds. Devita VJ, Hellman S and Rosenberg S), pp. 3–13. L.B. Lippincott, Philadelphia, 1989.
37. Bass BL, The dsRNA unwinding/modifying activity: Fact and fiction. *Semin Dev Biol* **3:** 425–433, 1992.
38. Skipper HE, Montgomery JA, Thomson JR and Schabel FM Jr, Structure–activity relationships and cross-resistance observed on evaluation of a series of purine analogs against experimental neoplasms. *Cancer Res* **19:** 425–437, 1959.
39. Davidson IWF and Fellig J, Cellular conversion of methylpurine to an active analog of ATP. *Fedn Proc* **21:** 160, 1962.
40. Bennett LL Jr and Smithers D, Feedback inhibition of purine biosynthesis in H. EP. #2 cells by adenine analogs. *Biochem Pharmacol* **13:** 1331–1339, 1964.
41. Leonhardt K and Anke T, 6-Methylpurine, 6-methyl-9-β-D-ribofuranosylpurine, and 6-hydroxymethyl-9-β-D-ribofuranosylpurine as antiviral metabolites of *Collybia maculata* (Basidiomycetes). *Z Naturforsch* **42C:** 420–424, 1987.
42. Benson CE, Love SH and Remy CN, Inhibition of *de novo* purine biosynthesis and interconversion by 6-methylpurine in *Escherichia coli*. *J Bacteriol* **101:** 872–880, 1970.
43. Miller JH and Kempner ES, Effects of an adenine analog on yeast metabolism. *Biochim Biophys Acta* **76:** 333–340, 1963.
44. Dewey VC, Heinrich MR, Markees DG and Kidder GW, Multiple inhibition by 6-methylpurine. *Biochem Pharmacol* **3:** 173–180, 1960.