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### Gene Therapy of Cancer: Activation of Nucleoside Prodrugs with *E. coli* Purine Nucleoside Phosphorylase

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## GENE THERAPY OF CANCER: ACTIVATION OF NUCLEOSIDE PRODRUGS WITH *E. COLI* PURINE NUCLEOSIDE PHOSPHORYLASE

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**ABSTRACT:** During the last few years, many gene therapy strategies have been developed for various disease targets. The development of anticancer gene therapy strategies to selectively generate cytotoxic nucleoside or nucleotide analogs is an attractive goal. One such approach involves the delivery of herpes simplex virus thymidine kinase followed by the acyclic nucleoside analog ganciclovir. We have developed another gene therapy methodology for the treatment of cancer that has several significant attributes. Specifically, our approach involves the delivery of *E. coli* purine nucleoside phosphorylase, followed by treatment with a relatively non-toxic nucleoside prodrug that is cleaved by the enzyme to a toxic compound. This presentation describes the concept, details our search for suitable prodrugs, and summarizes the current biological data.

### Preamble

Over the years, research on nucleic acid components has resulted in many important and useful discoveries. All of this research has had as its long-term goal the development of new therapeutics for the treatment of human diseases, especially cancer and viral diseases. In recent years, the boundaries of what can be achieved with nucleic acid components have been considerably expanded. Examples of this expansion include the pursuit of antisense oligonucleotide analogs, the development of certain L-nucleosides with therapeutic potential, and the utilization of prodrugs and phosphate analogs such as phosphonates to bypass enzymic blocks. It appears that we will continue to discover new and exciting structural diversity, and certainly new therapeutic uses will

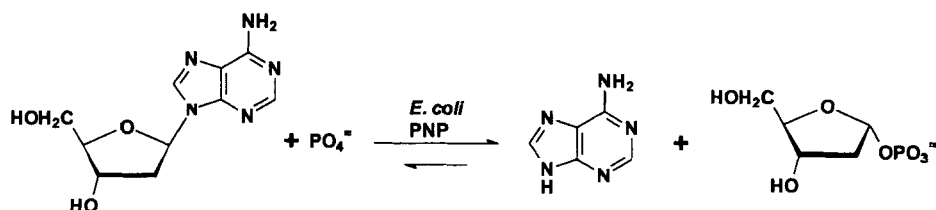
derive from this effort. The future of the area holds continued promise for small molecule nucleosides as well as for oligonucleotide analogs. In order to facilitate such development, we must continue to broaden our understanding of the metabolism of these compounds as well as their interactions with other biological entities at the molecular level. The types of new directions that the future holds will very likely include non-life-threatening diseases where toxicity issues can be controlled and molecules can be tailored for very specific purposes.

## Introduction

Over the past few years we have been conducting research on a multidisciplinary project designed to develop a new approach for the treatment of cancer utilizing suicide gene therapy. This project certainly fits the above description of a new concept for the therapeutic application of nucleosides. The progress of our research has been described in several publications.<sup>1-6</sup> This paper will provide an overview of the project, in the process presenting data that has not appeared in our other publications.

The specific approach to suicide gene therapy being pursued is the utilization of a nucleoside as a prodrug of a nitrogen base, which itself is serving as a prodrug of a nucleotide. The gene to be delivered to tumor cells is one encoding a non-human protein, the *E. coli* DeoD gene, whose product is purine nucleoside phosphorylase (PNP). This protein, once produced in a cell, would then act on the nucleoside, liberating an active cytotoxic agent. An example of a reaction catalyzed by *E. coli* PNP is presented in Scheme 1. From a medicinal chemistry standpoint, there are two key challenges facing us. First, a nitrogen base must be selected that when liberated in a transfected cell, will be converted to a toxic nucleoside metabolite. Second, a nucleoside prodrug must be selected that will itself be as non-toxic as possible to the body, but will be a suitable substrate for *E. coli* PNP. This nucleoside must be a substrate for neither human PNP nor any other possible cleavage enzyme, such as methylthioadenosine phosphorylase. *E. coli* PNP, unlike human PNP, accepts adenosine as a substrate. This difference is used to selectively activate nucleoside analogs.

There are two approaches that are well known currently for the activation of a nucleic acid component in a gene therapy approach.<sup>7,8</sup> The first utilizes herpes simplex



**SCHEME 1.** A Typical Reaction Catalyzed by *E. coli* PNP.

virus thymidine kinase to activate the known antiviral agent ganciclovir, and the second uses *E. coli* cytosine deaminase to convert 5-fluorocytosine to 5-fluorouracil (FUra). Our approach has advantages over both of these systems.

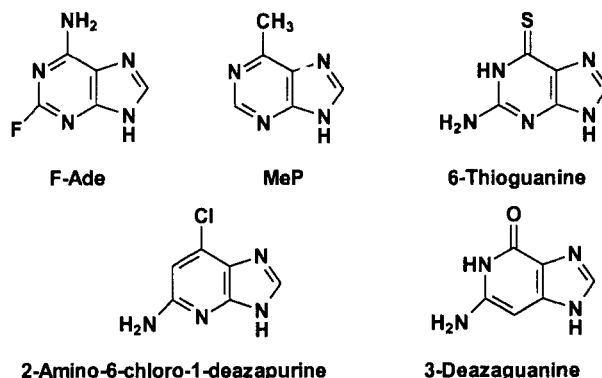
### ***In Vitro* Sensitization with the *E. coli* PNP Gene**

Several approaches have been taken to the introduction of a foreign gene into human tumor cells. In this research, we have utilized a retroviral vector with the PNP gene appropriately inserted under the control of an SV-40 early promoter.<sup>3</sup> Transfections into various cell lines, including human colon carcinoma T84 cells and human malignant D54MP glioma cells, have been accomplished with the aid of lipofectin.<sup>1,2</sup> Both transfected cancer cells and transduced cell lines have been shown to be sensitive to the agents generated from prodrug cleavage by *E. coli* PNP, while the parent cell lines are not sensitive to the prodrugs.<sup>1-3,6</sup> The magnitude of tumor cell sensitization is impressive in that even when less than one in a thousand cells expresses the *E. coli* PNP gene, elimination of entire populations of cells can be accomplished by prodrug administration. Other gene transfer vectors, including replication-deficient adenovirus, vaccinia virus, as well as a variety of plasmid-based systems for the expression of *E. coli* PNP, all potentially augment nucleoside prodrug toxicity as intended in this strategy (data not shown).

### **Nucleoside Prodrug Selection**

A solid body of literature exists on substrate specificities of various nucleosides toward *E. coli* PNP.<sup>9-16</sup> This work, mainly from the laboratories of Shugar and Holy, provided the foundation upon which our medicinal chemistry considerations were based. Some key conclusions derived from this work include: 1) The bacterial enzyme is much more permissive about the substitution at the purine C-6 than the mammalian enzyme,

accepting amino, oxo, and other substituents at that position, while the human enzyme accepts only on oxo (or thioxo) group; 2) *E. coli* PNP is not very tolerant of changes in the carbohydrate moiety, strongly preferring a ribofuranosyl or 2'-deoxyribofuranosyl group in the  $\beta$  configuration. A few carbohydrates, all close analogs of these two, had modest substrate activities when contained in an adenine nucleoside.



Our initial challenge was to identify purine analogs that appeared to have a suitable toxicity profile, and could be incorporated into nucleosides that might be sufficiently good substrates for *E. coli* PNP. In examining cytotoxicity data on a variety of bases, five compounds initially appeared to be suitable choices. These compounds were 2-fluoroadenine (F-Ade), 6-methylpurine (MeP), 6-thioguanine, and 2-amino-6-chloro-1-deazapurine, and 3-deazaguanine. These five bases all demonstrate cytotoxicity with  $IC_{50}$ 's in the range of 0.1-10  $\mu M$ ,<sup>5,17,18</sup> with F-Ade being the most potent (*ca.* 0.1  $\mu M$ ) and 3-deazaguanine being the least potent (*ca.* 10  $\mu M$ ). Of these the first two were particularly attractive because they are toxic to both proliferating and non-proliferating cells,<sup>5</sup> while the latter three act mainly on proliferating cell populations. In addition, we had several different nucleosides incorporating F-Ade and MeP that had been prepared in our laboratories.

In a search for suitable prodrugs, we embarked on an evaluation of all relevant nucleosides that we could obtain, including some compounds that had already been evaluated in earlier publications, in order to obtain a direct comparison under identical conditions. Those data are presented in Table 1.

These data confirm the earlier observations as well as expand our knowledge about substrate activity of a variety of nucleosides. In order to understand how the table

data reflects the potential utility of a compound, it should be noted that 2-fluoro- $\beta$ -D-arabinofuranosyladenine, with a cleavage rate of 1000-1500 nmoles/mg/hr, does have activity in animal systems. Potential utility obviously is influenced not only by the cleavage rate but also by the nature and potency of the liberated base. Some conclusions that can be drawn upon inspection of Table 1 data are noted below.

- *E. coli* PNP is very permissive in terms of substituents allowed at C-6 of the purine, as noted by previous researchers.
- With nucleosides containing either a ribofuranose or 2'-deoxyribofuranose moiety, excellent cleavage is seen to generate adenine, hypoxanthine, 2-fluoroadenine, 2-methyladenine, 6-methylpurine, 6-methoxypurine, 6-methylthiopurine, 6-mercaptopurine, and 6-thioguanine.
- Neither L-nucleosides nor  $\alpha$ -anomers are cleaved significantly by the enzyme. The only exceptions are 9- $\alpha$ -L-lyxofuranosyladenine, which actually closely resembles a more standard nucleoside, with only the 5'-hydroxymethyl changed in configuration from adenosine, and 9-(2,3-dideoxy-3-hydroxymethyl- $\alpha$ -O-erythio-pentofuranosyl)-6-thioguanine, which also resembles a standard nucleoside by virtue of the disposition of its two hydroxymethyl groups.
- Nucleosides containing six-membered ring carbohydrates, whether hexopyranosyl or pentopyranosyl, regardless of substitution pattern, are not significantly cleaved.
- With the same nitrogen base, 2'-deoxy- $\beta$ -D-ribofuranosyl nucleosides are consistently somewhat better substrates than the  $\beta$ -D-ribofuranosyl nucleosides.
- Other  $\beta$ -D-furanosides with low but significant substrate activity include arabinofuranosyl, 5-deoxyribofuranosyl, and 6-deoxy-*allo/talo*-hexofuranosyl nucleosides.
- Other  $\beta$ -D-furanosides with little or no substrate activity include 4-thioribofuranosyl, xylofuranosyl, 3-deoxyribofuranosyl, 2,3-dideoxyribofuranosyl, 2-deoxy-2-fluoroarabinofuranosyl, 2-deoxy-2,2-difluororibofuranosyl, and ribofuranuronamidyl nucleosides.

**TABLE 1.** Substrate Activity of Various Nucleosides with Purified *E. coli* PNP.

Compound	Activity (SD) nmoles/mg/hr	No. of Expts.
6-methyl-9- $\beta$ -D-ribofuranosylpurine	92,000	1*
6-methyl-9-(2-deoxy- $\beta$ -D- <i>erythro</i> -pentofuranosyl)purine (MeP-dR)	461,000 (206,000)	10
6-methyl-9-(2-deoxy- $\alpha$ -D- <i>erythro</i> -pentofuranosyl)purine	<5	1*
6-methyl-9- $\beta$ -D-xylofuranosylpurine	<5	1*
9-(6-deoxy- $\beta$ -D- <i>allo</i> -hexofuranosyl)-6-methylpurine	52	2
9-(6-deoxy- $\alpha$ -L- <i>talo</i> -hexofuranosyl)-6-methylpurine	772	2
9-(5-deoxy-5-phenylthio- $\beta$ -D-ribofuranosyl)-6-methylpurine	<5	1
2-fluoroadenosine	215,000 (70,000)	3
2-fluoro-2'-deoxyadenosine	435,000 (219,000)	4
2-fluoro-9- $\beta$ -D-arabinofuranosyladenine	1308 (406)	4
2-fluoro-9-(2-deoxy-2-fluoro- $\beta$ -D-arabinofuranosyl)adenine	30	2
5'-deoxy-2-fluoroadenosine	29,000	2
Adenosine	398,000 (87,000)	5
$\alpha$ -adenosine	<5	1*
2'-deoxyadenosine	338,000 (95,000)	4
4'-thio-2'-deoxyadenosine	<5	1
3'-deoxyadenosine	<5	2
2',3'-dideoxyadenosine	<5	2
9- $\beta$ -D-arabinofuranosyladenine	621 (299)	5
9- $\alpha$ -D-arabinofuranosyladenine	<5	1*
9- $\beta$ -D-xylofuranosyladenine	<5	1*
6'-deoxy-9-( $\beta$ -D- <i>allo</i> / <i>talo</i> -hexofuranosyl)adenine	2,400	2
9-( $\alpha$ -L-lyxofuranosyl)adenine	2,600	2
1-(aden-9-yl)-1-deoxy- $\beta$ -D-ribofuranuronamide	<5	1
2'-O-methyladenosine	<5	2
2-methyladenosine	166,000	2
2-chloro-2'-deoxyadenosine	39,000 (26,000)	3
2-chloro-9-(2-deoxy- $\beta$ -L- <i>erythro</i> -pentofuranosyl)adenine	<5	1*
2-chloro-9-(2-deoxy- $\alpha$ -L- <i>erythro</i> -pentofuranosyl)adenine	<5	1*
2-chloro-9-(2-deoxy-2-fluoro- $\beta$ -D-arabinofuranosyl)adenine	73	1
2-chloro-9-(2-deoxy-2,2-difluoro- $\beta$ -D-ribofuranosyl)adenine	<5	1

**TABLE 1.** (Cont.) Substrate Activity of Various Nucleosides with Purified *E. coli* PNP.

Compound	Activity (SD) nmoles/mg/hr	No. of Expts.
2-amino-6-chloro-9- $\beta$ -D-ribofuranosyl-1-deazapurine	68,000	2
7-deaza-8-azaadenosine	<5	1
Inosine	342,000 (143,000)	9
6-methoxy-9- $\beta$ -D-ribofuranosylpurine	164,000	1*
2'-deoxyinosine	664,000	2
3'-deoxyinosine	<5	2
2',3'-dideoxyinosine	<5	2
9- $\beta$ -D-arabinofuranosylhypoxanthine	61	1
6-methylthio-9- $\beta$ -D-ribofuranosylpurine	114,000 (56,000)	3
6-mercapto-9- $\beta$ -D-ribofuranosylpurine	83,000 (24,000)	3
9-(2-deoxy- $\alpha$ -L- <i>erythro</i> -pentofuranosyl)guanine	<5	1*
9-(2-deoxy- $\alpha$ -L- <i>erythro</i> -pentofuranosyl)-2,6-diaminopurine	<5	1*
9-(2-deoxy- $\beta$ -L- <i>erythro</i> -pentofuranosyl)-2,6-diaminopurine	<5	1*
6-thioguanosine	74,000	1*
6-thio-2'-deoxyguanosine	71,000	1*
9-(2-deoxy- $\alpha$ -D- <i>erythro</i> -pentofuranosyl)-6-thioguanine	<5	1*
9-(2,3-dideoxy-3-hydroxymethyl- $\alpha$ -O- <i>erythro</i> -pentofuranosyl)-6-thioguanine	242	2
9- $\beta$ -D-allopyranosyladenine	<5	2
9- $\beta$ -D-fructopyranosyladenine	<5	2
9- $\alpha$ -D-mannopyranosyladenine	<5	2
9- $\alpha$ -D-talopyranosyladenine	<5	2
9- $\beta$ -L-galactopyranosyladenine	<5	2
1-(aden-9-yl)-1-deoxy- $\beta$ -D-glucopyranuronamide	<5	2
6-(aden-9-yl)tetrahydropyran-2-methanol (racemic)	<5	2
9-(6-O- $\alpha$ -D-galactopyranosyl- $\beta$ -D-glucopyranosyl)adenine	<5	2
9- $\beta$ -D-ribopyranosyladenine	<5	2
9-(2-deoxy- $\alpha$ -D- <i>erythro</i> -pentopyranosyl)adenine	<5	2
9-(2-deoxy- $\beta$ -D- <i>erythro</i> -pentopyranosyl)adenine	<5	2
9-(3-azido-3,4-dideoxy- $\beta$ -D- <i>erythro</i> -pentopyranosyl)adenine	<5	2

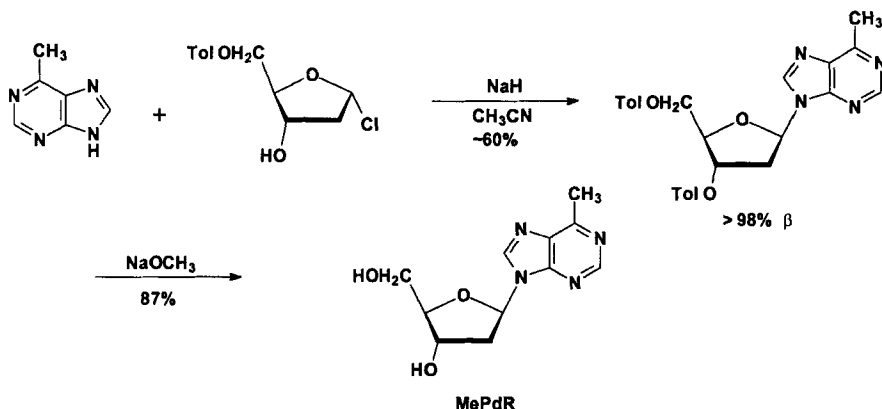
\*These experiments have been confirmed using a crude *E. coli* preparation.



The structures of a number of complexes of *E. coli* PNP with various substrates and inhibitors have been determined in Dr. Ealick's laboratory.<sup>6</sup> We are using the combination of that information along with the enzymic cleavage data as presented in Table 1 to develop new target nucleoside prodrugs. These compounds would obviously have attributes as described above, and would under the best circumstances not be subject to phosphorylation or glycosidic cleavage by other human enzymes, and would be hydrolytically stable through oral administration.

The data in the table certainly support further evaluations of appropriate nucleosides containing both 6-methylpurine and 2-fluoroadenine. We selected 9-(2-deoxy-*erythro*-pentofuranosyl)-6-methylpurine (MePdR) and 2-fluoro-9- $\beta$ -D-arabinofuranosyladenine monophosphate (F-ara-AMP), an FDA-approved anticancer drug, for more detailed evaluations. Preparation of MePdR in gram quantities has been accomplished using a modification of the literature procedure, shown in Scheme 1. F-ara-AMP has been kindly provided by Berlex, Inc., a subsidiary of Schering AG. Our initial focus has been on MePdR, so the discussion below will generally focus on this agent. In parallel, we continue to search for other suitable prodrugs of MeP and F-Ade, as well as other desirable nitrogen bases.

For the two compounds selected for detailed evaluation, a comparison of their cell culture cytotoxicities is presented in Table 2. The at least 100-fold selectivity seen with



**SCHEME 2.** Synthesis of MePdR.

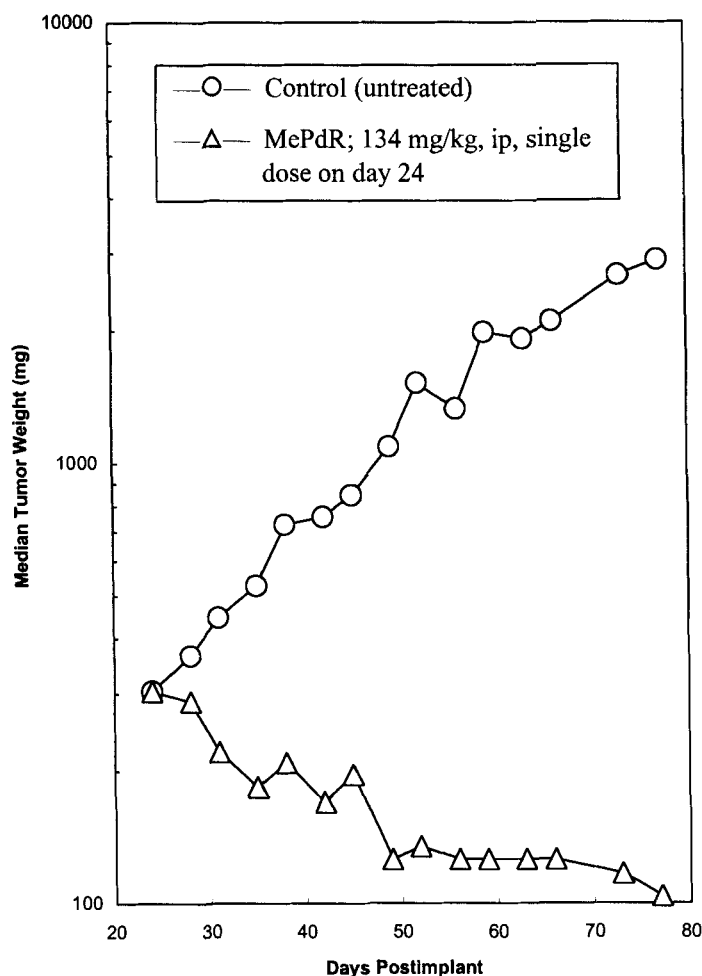
Compound	Cytotoxicity <sup>a</sup> (μM)
6-methylpurine	1
MePdR	>160
2-fluoroadenine	0.1
F-ara-A	0.1 – 2

<sup>a</sup>Survey of literature data.

MePdR made it an attractive candidate for *in vivo* evaluation.<sup>5</sup> F-ara-AMP was not initially considered to be a good prodrug, because of the small differences in the potency of F-Ade and F-ara-AMP, as seen in Table 2. Furthermore, F-ara-AMP is currently used in the treatment of hematologic malignancies and is known to be toxic. However, as we evaluated the two compounds in animals, we realized that mice could tolerate approximately ten times more F-ara-AMP than they could MePdR, and that F-Ade was 100-fold more potent than MeP in its ability to kill cells. Comparison of these parameters suggested that F-araAMP may have a good affect against tumors expressing *E. coli* PNP even though there was little difference in cytotoxicity between F-araA and F-Ade.

### ***In Vivo* Data**

Detailed animal evaluations of both MePdR and F-ara-AMP have been undertaken.<sup>3</sup> We have examined both compounds in a nude mouse model involving subcutaneously injected cells with intraperitoneal administration of drug. We have used D54MG cells (a malignant glioma cell line) and D54-PNP cells, a transduced cell line expressing *E. coli* PNP. Against mice bearing the parent cell line, neither drug had any effect on tumor growth. With mice bearing the D54-PNP tumor, however, tumors weights were rapidly reduced to below the limit of detection. These results involved multiple treatments of each drug (daily for 3 days for MePdR, and five doses given every two hours for F-ara-AMP). In a recent experiment with a single dose of MePdR, we have found significant activity in this same model system, as shown in Figure 1. A single dose schedule appears more relevant in a gene therapy approach to cancer treatment because it would be expected that cells containing the *E. coli* PNP gene would be destroyed early, and later treatments might therefore be less effective or ineffective. Further experiments expanding upon this observation are currently under way.



**FIG. 1.** Response of Subcutaneous D54 CNS Tumors Transfected with *E. coli* PNP to Treatment with MePdR.

### Biochemical Pharmacology

Because of the *in vivo* activity of MePdR and F-ara-AMP, we have studied the mechanism of toxicity of F-Ade and MeP.<sup>5</sup> Our results indicate that both compounds are metabolized to ATP analogs that then inhibit one or more reactions involved in protein or RNA synthesis, thereby resulting in cell death.

Biochemical studies into the mechanism of action of the prodrugs and their toxins used with *E. coli* PNP have revealed three main differences between this gene therapy

approach and the HSV-TK and *E. coli* CD approaches. First, we have demonstrated a much higher bystander activity than that achieved with HSV-TK. When as few as 0.1% of cells in a culture dish express *E. coli* PNP, all of the cells are killed after treatment with MeP-dR. Cell to cell contact is not required for this cell killing. Sufficient 6-methylpurine is generated in cells containing *E. coli* PNP that when the cell dies from effects derived from the conversion of 6-methylpurine to its ribonucleoside triphosphate, this remaining 6-methylpurine is released and taken up by nearby cells, thereby causing their death. Second, MeP and F-Ade are toxic to nonproliferating as well as proliferating cells, and should therefore be active against tumors with a low growth fraction. Agents, such as ganciclovir and FUra that mostly target proliferating cells, would not be expected to have much effect on these tumors. Third, F-Ade and MeP are much more potent toxins than are either ganciclovir or FUra. Because of the potency of these two agents, it is possible that they will cause tumor regression in animals under conditions (equal expression of activating enzyme) where ganciclovir and FUra would have little effect. These results indicate that the mechanisms of cytotoxicity of MeP and F-Ade are quite different from those of conventional antitumor agents. We believe that the attributes of these compounds generated by *E. coli* PNP will be necessary for significant antitumor activity to occur in a gene therapy scenario.

### Summary

We have demonstrated that it is possible to utilize the *E. coli* PNP gene, introduced into tumor cells by means of a retroviral vector, as a means of generating significant quantities of the enzyme in those cells. The enzyme can be used in tandem with either MePdR or F-ara-AMP, both of which are subject to cleavage by the bacterial enzyme but not by mammalian PNP, to generate the corresponding nitrogen bases, which are highly cytotoxic compounds. Focusing on MePdR, which generates MeP, we have found that it is possible to obtain a high rate of bystander killing without cell-to-cell contact, and that good activity is seen against non-proliferating cells. These attributes offer significant advantages over other related strategies, as mentioned above. In several different tumor systems in nude mice, our approach has been used quite successfully not only with a cell population consisting of all transduced cells, but also with a transduced cell fraction as low as 20%.

Our future goals include the development of relevant new methods to deliver the *E. coli* PNP gene to tumors in animals, the identification of new prodrugs with even greater selectivity than those described herein, and the advancement of these prodrugs to the level of clinical trials.

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