

## ORIGINAL ARTICLE

**Effect of expression of adenine phosphoribosyltransferase on the *in vivo* anti-tumor activity of prodrugs activated by *E. coli* purine nucleoside phosphorylase**WB Parker<sup>1</sup>, PW Allan<sup>1</sup>, WR Waud<sup>1</sup>, JS Hong<sup>2</sup> and EJ Sorscher<sup>2</sup><sup>1</sup>*Southern Research Institute, Birmingham, AL, USA and* <sup>2</sup>*University of Alabama at Birmingham, Birmingham, AL, USA*

The use of *E. coli* purine nucleoside phosphorylase (PNP) to activate prodrugs has demonstrated excellent activity in the treatment of various human tumor xenografts in mice. *E. coli* PNP cleaves purine nucleoside analogs to generate toxic adenine analogs, which are activated by adenine phosphoribosyl transferase (APRT) to metabolites that inhibit RNA and protein synthesis. We created tumor cell lines that encode both *E. coli* PNP and excess levels of human APRT, and have used these new cell models to test the hypothesis that treatment of otherwise refractory human tumors could be enhanced by overexpression of APRT. *In vivo* studies with 6-methylpurine-2'-deoxyriboside (MeP-dR), 2-F-2'-deoxyadenosine (F-dAdo) or 9-β-D-arabinofuranosyl-2-fluoroadenine 5'-monophosphate (F-araAMP) indicated that increased APRT in human tumor cells coexpressing *E. coli* PNP did not enhance either the activation or the anti-tumor activity of any of the three prodrugs. Interestingly, expression of excess APRT in bystander cells improved the activity of MeP-dR, but diminished the activity of F-araAMP. *In vitro* studies indicated that increasing the expression of APRT in the cells did not significantly increase the activation of MeP. These results provide insight into the mechanism of bystander killing of the *E. coli* PNP strategy, and suggest ways to enhance the approach that are independent of APRT.

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**Introduction**

The use of *E. coli* purine nucleoside phosphorylase (PNP) to activate deoxyadenosine (dAdo) analogs has demonstrated impressive efficacy against numerous human tumor xenografts in mice.<sup>1–6</sup> This strategy differs fundamentally from earlier generation suicide gene approaches such as those utilizing herpes simplex virus-thymidine kinase or *E. coli* cytosine deaminase because of its unique mechanism of action and its robust bystander activity. To date, three prodrugs have been used in combination with *E. coli* PNP (6-methylpurine-2'-deoxyriboside (MeP-dR); 2-F-2'-dAdo (F-dAdo) and arabinofuranosyl-2-F-adenine monophosphate (F-araAMP, fludarabine phosphate)). These dAdo analogs are not activated in normal human tissues, because human PNP does not recognize adenine-containing nucleosides or their analogs as substrates. F-araAMP is of particular interest as a prodrug, because it is currently approved for use in the treatment of chronic

lymphocytic leukemia, and has demonstrated excellent *in vivo* anti-tumor activity even when 97.5% of tumor cells do not express *E. coli* PNP.<sup>3</sup> F-araAMP is used in *in vivo* studies as a more soluble precursor of F-araA. In the plasma it is quickly converted to F-araA, which is the primary circulating form of the drug<sup>7,8</sup> and a substrate for *E. coli* PNP.

*E. coli* PNP cleaves the glycosidic bond of these dAdo analogs liberating either (MeP) or 2-fluoroadenine (F-Ade), two potent cytotoxic adenine analogs.<sup>9</sup> Both MeP and F-Ade readily diffuse across cell membranes and kill neighboring tumor cells that do not express *E. coli* PNP,<sup>10,11</sup> accounting for the robust bystander activity observed with this strategy. In human cells adenine is salvaged from the extracellular environment by adenine phosphoribosyl transferase (APRT), and this enzyme is responsible for the first and rate-limiting step in the activation of both MeP and F-Ade. Once monophosphates of MeP or F-Ade have been formed, they are rapidly converted by cellular mono and diphosphate kinases to their respective triphosphates (MeP-riboside-TP and F-ATP), which inhibit both RNA and protein synthesis.<sup>9</sup> Because of the unique mechanism of action, these two agents are active against non-proliferating tumor cells, an attribute that could be quite useful in the

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treatment of solid tumors with a low growth fraction. Furthermore, these agents should also be active against the putative tumor 'stem cell' compartment, which is constituted of cells that spend a substantial interval of time in a quiescent state (a factor that contributes to tumor growth and regeneration after treatment with conventional chemotherapy).

Although excellent *in vivo* anti-tumor activity has been observed with three prodrugs, the amount of *E. coli* PNP enzyme activity in tumor cells transfected with the *E. coli* PNP gene is much greater than the endogenous APRT activity. Therefore, it is possible that MeP and F-Ado produced by *E. coli* PNP are not efficiently activated by APRT, but instead diffuse away from the tumor. Therefore, to determine whether or not additional APRT activity in tumor cells that express *E. coli* PNP would improve the anti-tumor effectiveness of these prodrugs, we established tumor cell lines that encode both *E. coli* PNP and excess levels of human APRT, and have used these new cell models to test the hypothesis that treatment of otherwise refractory human tumors could be enhanced by overexpressing APRT.

## Materials and methods

### Cloning of APRT

The APRT gene was amplified from HeLa complimentary DNA pool using AccuPrime Pfx Supermix (Invitrogen; Carlsbad, CA), according to manufacturer's protocol. The sequences of PCR primers used were APRT-f: 5'-AGCTGGATCCACCATGGCCGACTCCGAGCTG-3' and APRT-r: 5'-ACCTCTCGAGTCACTCATACTGCA GGAGAGAGAA-3'. The amplified PCR product was cloned into pCR4-Blunt (Invitrogen) vector and confirmed by DNA sequencing. The APRT gene was cloned into the PmeI site of pWPI lentiviral vector (Addgene, Cambridge, MA). The pWPI vector encodes green fluorescent protein downstream of cloning sites and an internal ribosome entry site allowing coexpression of green fluorescent protein together with the cloned gene of interest.

*Creation of D54 cells expressing excess levels of APRT*  
D54MG cells were obtained from Yancey Gillespie, University of Alabama, Birmingham<sup>12</sup> and grown in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD) containing 10% fetal bovine serum (Gibco-BRL), 10 U ml penicillin, 10 µg ml<sup>-1</sup> streptomycin and 50 µg ml<sup>-1</sup> gentamycin. To produce a lentiviral vector, 40 µg of plasmid DNA was used for calcium-phosphate transfection of one 10 cm dish. The DNA mixture contained 5 µg of envelope-coding plasmid pMD.G; 15 µg of the packaging plasmid pCMVdR8.91, which expresses Gag, Pol, Tat and Rev; and 20 µg of pWPI-APRTase. The tissue culture media was collected and used to infect cells. Twenty-four hours after lentiviral infection, the cells were split into 96-well plates for cloning a homogenous population with APRT expression. Cell clones were visually inspected for green fluorescent protein expression. A final round of clonal isolation was performed to ensure the homogeneity of

each cell population. Cells were analyzed for APRT activity to confirm the expression of the enzyme.

### Measurement of PNP and APRT activity

Crude cell extracts were prepared as described previously from D54MG (human glioma) cells in culture<sup>13</sup> and from D54MG tumors removed from the flanks of mice.<sup>2</sup> Cell extract was incubated with 50 mM potassium phosphate, 100 µM MeP-dR and 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.4) to obtain a linear reaction over the incubation period. The formation of MeP was monitored using reverse phase high pressure liquid chromatography. One unit of PNP activity is equal to the amount of extract that can cleave 1 nmole of MeP-dR per mg protein in a 1-h period.

To measure APRT activity the extracts prepared above were incubated with 100 µM [2,8-<sup>3</sup>H]adenine (2000 Ci mole<sup>-1</sup>, from Moravex Biochemicals; Brea, CA), 1 mM phosphoribosyl pyrophosphate, 5 mM MgCl<sub>2</sub> and 50 mM Tris (pH 7.4). A portion of the extract was applied to DE81 anion exchange disks (Whatman International, Maidstone, England) at various times after starting the reaction. The [<sup>3</sup>H]adenine was removed by washing the disks three times with 1 mM ammonium formate solution and twice with 95% ethanol. After drying, the amount of [<sup>3</sup>H]AMP formed was determined by counting the radioactivity left on the DE81 disks.

### Studies with human tumor xenografts in mice

Mixtures of D54MG tumor cells expressing *E. coli* PNP and/or human APRT ( $2 \times 10^7$  cells) were injected subcutaneously into the flanks of nude mice (*nu/nu*) purchased from Charles River Laboratories (Wilmington, MA). Tumors were measured with calipers and an estimate of the weight was calculated using the equation,  $(\text{length} \times \text{width}^2)/2 = \text{mm}^3$ , which is converted to mg assuming unit density.<sup>14</sup> The proportions of transduced tumor cells were verified by measuring *E. coli* PNP activity in representative tumors removed from the mice on the first day of drug treatment. When tumors reached approximately 300 mg, the mice were treated intraperitoneally with MeP-dR, F-dAdo or F-araAMP at maximally tolerated doses. F-dAdo was administered at a dose of 20 mg kg<sup>-1</sup> five times a day (separated by 2 h) for 3 consecutive days (Q1Dx3-Q2Hx5). MeP-dR was administered at a dose of either 33 or 67 mg kg<sup>-1</sup> once per day for 3 consecutive days (Q1Dx3). F-araAMP was administered at a dose of 160 mg kg<sup>-1</sup> given three times a day (separated by 4 h) for 3 consecutive days (Q1Dx3-Q4Hx3), except in Figure 2 where it was administered at a dose of 100 mg kg<sup>-1</sup> five times a day (separated by 2 h) for 3 consecutive days. There were six mice per treatment group. MeP-dR was synthesized in our laboratories as described previously.<sup>15</sup> F-araAMP was obtained from Schering AG (Berlin, Germany) and F-dAdo was obtained from General Intermediates of Canada (Edmonton, Alberta, Canada). Mice were monitored daily and were evaluated for weight loss and tumor mass twice weekly. T-C (tumor growth delay) is the difference in days to 2 doublings between drug-treated

and saline-treated groups. T-C was used as the end point in a Student's *t*-test, the Mann–Whitney rank sum test or a life table analysis in order to statistically compare growth data between treatment groups. All procedures were performed in accordance with a protocol that was approved by the Institutional Animal Care and Use Committee of Southern Research Institute.

Total radioactivity in tumors was determined after intraperitoneal injection of 20 mg kg<sup>-1</sup> [8-<sup>3</sup>H]F-dAdo (6.7 Ci mole<sup>-1</sup>), 100 mg kg<sup>-1</sup> [8-<sup>3</sup>H]F-araAMP (1.4 Ci mole<sup>-1</sup>) or 33 mg kg<sup>-1</sup> [2,8-<sup>3</sup>H]MeP-dR (3.8 Ci mole<sup>-1</sup>) into mice bearing D54 tumors of approximately 300 mg. Prodrugs labeled with tritium were obtained from Moravek Biochemicals. Tumors were removed from the mice 4 h after injection of compound and were dissolved in 1 ml of Soluene 350 (Packard Instrument, Meriden, CT) by incubating at 55 °C for 4 h and then at room temperature. A portion of each extract was mixed with scintillation fluid and the radioactivity was determined.

## Results

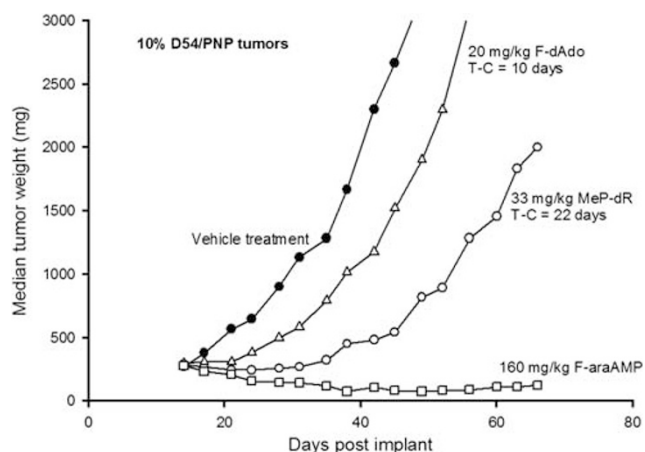
### Creation of D54 cells expressing excess APRT activity

We have expressed *E. coli* PNP in D54 cells (D54/PNP) at very high levels (126 000 nmol per mg per h) and used these cells to evaluate the use of *E. coli* PNP to activate F-araA as a therapy for the treatment of cancer.<sup>3</sup> The endogenous specific activity of APRT in this cell line was determined to be approximately 87 nmol of adenine activated per mg per cellular protein per h, which is 1400-fold lower than the *E. coli* PNP activity in these cells. Because APRT is the first enzyme in the activation pathway for adenine analogs generated by *E. coli* PNP and its activity appeared limiting in these cells, we established D54 cell lines that stably express human APRT and determined whether increased expression of APRT in cells expressing *E. coli* PNP would enhance cell killing activity of F-araA, F-dAdo or MeP-dR. D54/PNP cells were transduced with the human APRT gene resulting in a cell line (D54/PNP/APRT) that stably expresses both *E. coli* PNP (159 000 nmol per mg per h) and excess human APRT activity (6000 nmol per mg per h). Wild-type D54 cells were also transfected with the human APRT gene resulting in a cell line (D54/APRT) that stably expresses excess APRT activity (17 000 nmol per mg per h). The differences in expression levels of *E. coli* PNP and human APRT in the various cell lines are expected, based on the variable genomic integration sites of a particular lentiviral vector, the number of tandem repeats at the site of integration, partial cellular repression of transcription and other factors. D54 clones were selected that had similar activities for comparison. The *in vitro* and *in vivo* rates of proliferation of both of these cell lines were similar to that seen in wild-type D54 and D54/PNP cells.

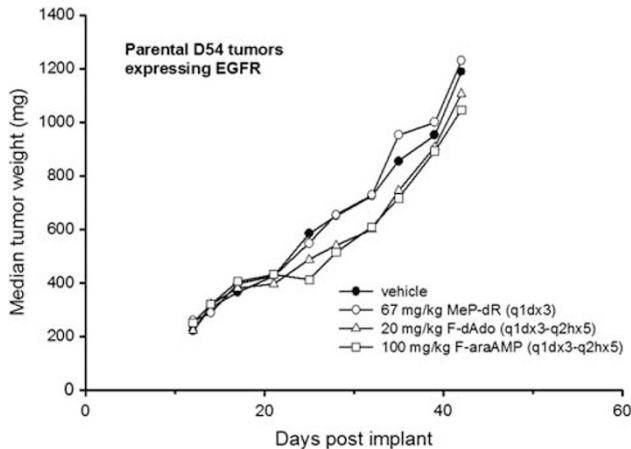
### *In vivo* anti-tumor activity of MeP-dR, F-dAdo or F-araAMP against tumors that express excess APRT activity

In previous studies, we demonstrated excellent *in vivo* anti-tumor activity with F-araAMP against D54 tumors

in which all cells expressed *E. coli* PNP.<sup>3</sup> To evaluate *in vivo* bystander activity of MeP-dR, F-dAdo and F-araAMP, mice bearing D54 tumors in which 10% of the cells expressed *E. coli* PNP were treated with the three prodrugs at maximally tolerated doses. Treatment with F-araAMP cured mice of D54 tumors in which 10% of the cells expressed *E. coli* PNP, whereas treatment with either F-dAdo or MeP-dR had a modest effect on the growth of these tumors. In the experiment shown in Figure 1, the amount of MeP-dR that could be tolerated by the mice bearing tumors expressing *E. coli* PNP was decreased, indicating that a substantial amount of the MeP produced in these tumors was released into the systemic circulation. Unlike MeP-dR, the toxicity of F-dAdo or F-araAMP was not affected by expression of *E. coli* PNP in the tumor xenografts, which suggested that little or no F-Ade escaped from tumor tissues after treatment with these agents. The toxicity of F-dAdo or F-araAMP was not affected even when mice bearing tumors in which 100% of the cells expressed *E. coli* PNP at high levels (data not shown) were treated with these two agents at their maximally tolerated doses. The three prodrugs had no *in vivo* anti-tumor activity against D54 tumors that do not express *E. coli* PNP (Figure 2). Although both F-dAdo and F-araAMP are known to inhibit the growth of parental D54 tumor cells *in vitro* due to their conversion to dATP analogs and subsequent



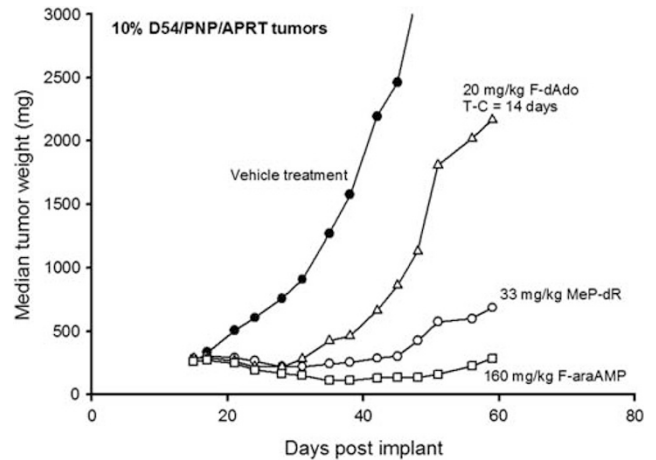
**Figure 1** Effect of prodrugs on tumors that express *E. coli* PNP (purine nucleoside phosphorylase) in 10% of the cells. Wild-type D54 tumor cells were mixed with D54 tumor cells that had been transduced with the *E. coli* PNP gene, so that 10% of the mixture contained cells that expressed *E. coli* PNP. This 90/10 mixture was injected subcutaneously into the flanks of nude mice. Drug treatment began on day 14 when tumors were approximately 300 mg. The activity of *E. coli* PNP and human adenine phosphoribosyl transferase in the tumors at the time of treatment were 11 200 ± 2300 units and 87 ± 30 units, respectively. In F-araAMP-treated mice on day 66 there was one complete regression, two small non-growing tumors and three small (<500 mg) but growing tumors. The growth of tumors in mice treated with drugs was significantly different than that in vehicle treated animals; *P* values ≤ 0.001 in all cases. The experiment has been repeated with similar results.



**Figure 2** Effect of prodrugs on D54 tumors that express EGFR. The D54 tumors cells that had been transduced with the EGFR gene were injected subcutaneously into the flanks of nude mice. Drug treatment began on day 12 when tumors were approximately 250 mg. There was no significant difference in tumor growth between any of the treatment groups.

inhibition of DNA synthesis,<sup>16</sup> these results indicated that *in vivo* activation of both drugs by endogenous nucleoside kinases is not sufficient to have an effect on the growth of parental D54 tumors. In Figure 2 the same total amount of F-araAMP was administered to the mice as in the other *in vivo* experiments, but the schedule of administration was slightly different (five times per day vs three times per day). Similar negative results have been seen in experiments where 160 mg kg<sup>-1</sup> of F-araAMP (Q1Dx3-Q4Hx3) was administered to mice bearing parental D54 tumors (data not shown).

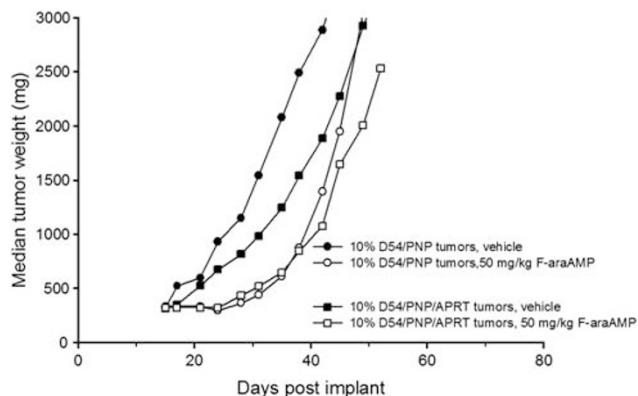
To determine the effect of excess APRT expression on *in vivo* bystander activity, the D54/PNP/APRT cell line was mixed with parental D54 cells and injected into mice to establish xenografts in which 10% of the cells expressed both *E. coli* PNP and excess APRT activity. The effectiveness of F-dAdo, MeP-dR and F-araAMP against these tumors (Figure 3) was similar to the effectiveness of these same drugs against D54 xenografts in which 10% of the tumor cells only expressed *E. coli* PNP (Figure 1). Since treatment with F-araAMP was very active against D54 tumors in which 10% of the cells expressed *E. coli* PNP (Figure 1), it was not possible to determine from the experiment shown in Figure 3 whether F-araAMP was more effective against D54 tumors in which 10% of the cells expressed both *E. coli* PNP and excess APRT. Therefore, mice bearing tumors in which 10% of the cells were either D54/PNP or D54/PNP/APRT were treated with 50 mg kg<sup>-1</sup> F-araAMP (Figure 4), which was approximately 30% of the dose used in Figure 3. At this dose, F-araAMP had a small effect on the growth of both tumor models (T-C values of 7–10 days). Another experiment was also performed to determine the influence of F-dAdo or F-araAMP at dose and schedules used in Figure 3 against D54 tumor xenografts, in which 2.5% of the cells expressed either *E. coli* PNP or *E. coli* PNP



**Figure 3** Effect of prodrugs on tumors that express both *E. coli* PNP (purine nucleoside phosphorylase) and excess human APRT (adenine phosphoribosyl transferase) in 10% of the cells. Wild-type D54 tumor cells were mixed with D54 tumor cells that had been transduced with both the *E. coli* PNP gene and the human APRT gene so that 10% of the mixture contained cells that expressed *E. coli* PNP and excess levels of human APRT. This 90/10 mixture was injected subcutaneously into the flanks of nude mice. Drug treatment began on day 15 when tumors were approximately 300 mg. The activity of *E. coli* PNP and human APRT in the tumors at the time of treatment were  $14\,100 \pm 550$  units and  $173 \pm 45$  units, respectively. In 6-methylpurine-2'-deoxyribose-treated mice on day 59 there were six growing tumors (405–1800 mg). In F-araAMP-treated mice on day 59 there was one complete regression, two small non-growing tumors (48 and 88 mg) and three growing tumors (486, 608, 1029 mg). The growth of tumors in mice treated with drugs was significantly different than that in vehicle treated animals; *P* values 0.001 in all cases. The experiment has been repeated with similar results.

plus excess APRT (data not shown). We observed that F-dAdo had no anti-tumor activity against either tumor, and F-araAMP demonstrated similar, but modest, activity against both tumor models (T-C values of approximately 10 days). These results indicate that increased expression of APRT in cells expressing *E. coli* PNP activity does not enhance the anti-tumor activity of MeP-dR, F-dAdo or F-araAMP.

In order to investigate the mechanisms underlying bystander killing by *E. coli* PNP, tumors were established in which the bystander cells expressed excess levels of APRT. Dramatic differences in the anti-tumor activity of MeP-dR and F-araAMP were seen in tumors in which 10% of the cells expressed *E. coli* PNP, and all the bystander cells expressed excess APRT activity (Figure 5). Treatment with MeP-dR was much more effective against these tumors than it was against tumors that did not express excess APRT in the bystander cells (Figure 1). This result suggested that the increased expression of APRT in the bystander cells enhanced the ability of these cells to activate MeP to toxic metabolites. The expression of excess APRT activity in either the *E. coli* PNP-expressing cells (Figure 3) or the bystander cells (Figure 5) did not reduce the toxicity of MeP-dR, which indicated

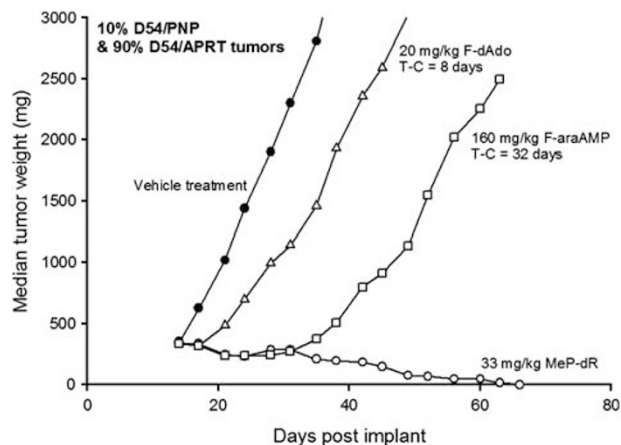


**Figure 4** Effect of low doses of F-araAMP on tumors that express both *E. coli* PNP (purine nucleoside phosphorylase) and excess human APRT (adenine phosphoribosyl transferase) in 10% of the cells. Wild-type (parental, no *E. coli* PNP-expressing) D54 tumor cells were mixed with D54 cells that had been transduced with either *E. coli* PNP (circles) or both the *E. coli* PNP and the human APRT (squares) so that 10% of the mixture contained transduced cells. These 90/10 mixtures were injected subcutaneously into the flanks of nude mice. F-araAMP treatment began on day 15 when tumors were approximately 300 mg. The activities of *E. coli* PNP and human APRT in the D54/PNP tumors at the time of treatment were  $9000 \pm 1900$  units and  $180 \pm 10$  units, respectively. The activities of *E. coli* PNP and human APRT in the D54/PNP/APRT tumors at the time of treatment were  $4800 \pm 650$  units and  $260 \pm 44$  units, respectively. The growth of tumors in mice treated with F-araAMP was significantly different than that in vehicle-treated mice; *P* values  $\leq 0.001$ .

that the increased APRT activity did not prevent significant amounts of MeP from escaping to the systemic circulation. In contrast to the result with MeP-dR, treatment with F-araAMP was much less effective against tumors in which the bystander cells expressed excess APRT activity (Figure 5). The anti-tumor activity of F-dAdo was similar to that seen against D54 tumors in which 10% of the cells express *E. coli* PNP.

#### Measurement of prodrug activation in subcutaneous D54 tumors

Mice bearing D54 tumors of various compositions were treated with  $33 \text{ mg kg}^{-1}$  [ $^3\text{H}$ ]MeP-dR,  $20 \text{ mg kg}^{-1}$  [ $^3\text{H}$ ]F-dAdo or  $100 \text{ mg kg}^{-1}$  [ $^3\text{H}$ ]F-araAMP to determine the effect of excess APRT expression on the activation of the three prodrugs. Tumors were removed 4 h after injection of compound and the total amount of radioactivity was determined. Because the tritium label in the three nucleosides is positioned on the purine base, differences in radioactivity between the parental D54 tumors (which do not express *E. coli* PNP) and D54 tumors that express *E. coli* PNP reflect the amounts of F-Ade or MeP and their metabolites generated by *E. coli* PNP and retained in the tumor cells. The plasma half-life of these agents (MeP-dR, 20 min; F-dAdo, 7 min; F-araAMP, 50 min: reference number 2) is such that there is very little compound still circulating 4 h after injection. The results in Table 1, therefore, indicate that expression of excess APRT



**Figure 5** Effect of prodrugs on tumors that express *E. coli* PNP (purine nucleoside phosphorylase) in 10% of the cells and excess levels of human APRT (adenine phosphoribosyl transferase) in 90% of the cells. D54 tumor cells transduced with the human APRT gene were mixed with D54 tumor cells that had been transduced with the *E. coli* PNP gene, so that 10% of the mixture contained cells that expressed *E. coli* PNP and 90% of the cells expressed excess human APRT activity. This 90/10 mixture was injected subcutaneously into the flanks of nude mice. Drug treatment began on day 14 when tumors were approximately 300 mg. The activity of *E. coli* PNP and human APRT in the tumors at the time of treatment were  $11000 \pm 3600$  units and  $16000 \pm 2400$  units, respectively. In the 6-methylpurine-2'-deoxyriboside-treated mice on day 66 there were four complete regressions and two small persistent tumors (32 and 40 mg) that were not growing. The growth of tumors in mice treated with drugs was significantly different than that in vehicle-treated animals; *P* values  $\leq 0.003$  in all cases. The experiment has been repeated with similar results.

activity in either *E. coli* PNP-expressing cells or in bystander cells, does not increase the amount of F-Ade present in tumors treated with either F-dAdo or F-araAMP. This result is consistent with the observation that the anti-tumor activity of these two compounds was not enhanced in these tumors (Figures 1, 3 and 5). However, there was a significant increase of MeP in tumors with excess APRT activity in bystander cells vs tumors without excess APRT activity, or tumors with recombinant APRT in *E. coli* PNP-expressing cells. This finding is consistent with the enhanced *in vivo* efficacy observed with MeP-dR against this tumor xenograft model (Figure 5), and supports the conclusion that increasing APRT activity in bystander cells allows the cells to more efficiently activate MeP that has diffused from cells expressing *E. coli* PNP.

#### Measurement of prodrug activation in D54 cells in culture

In an effort to understand the reasons that increased expression of APRT in cells did not enhance the anti-tumor activity of F-araAMP, F-dAdo or MeP-dR, the activation of MeP-dR in the various cell lines was determined in cell culture. Cells were incubated with  $100 \mu\text{M}$  [ $^3\text{H}$ ]MeP-dR, and the amount of radioactivity in the medium was determined at various times after the

**Table 1** Incorporation of MeP-dR, F-dAdo or F-araA into D54 tumors expressing *E. coli* PNP with and without excess APRT

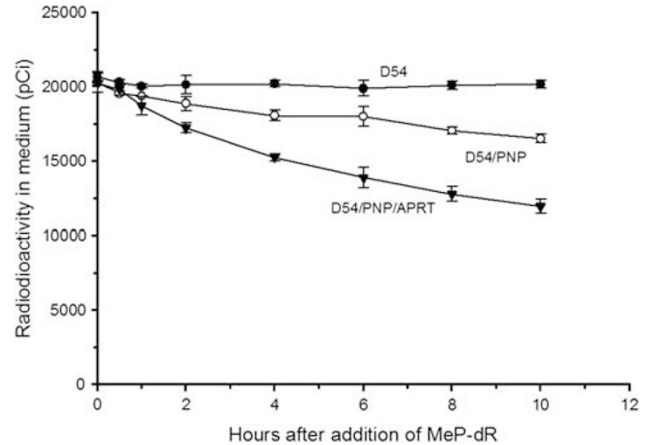
Tumor	MeP-dR	F-dAdo (nmoles per gram of tissue)	F-araAMP
D54 tumors	10 ± 7	4 ± 2	13 ± 9
10% PNP tumors	43 ± 14	14 ± 6	135 ± 62
10% PNP/APRT tumors	49 ± 23	16 ± 8	130 ± 63
10% PNP and 90% APRT tumors	110 ± 48*	15 ± 9	114 ± 68

Abbreviations: APRT, adenine phosphoribosyl transferase; PNP, purine nucleoside phosphorylase.

Mice bearing D54 tumors expressing PNP and/or excess APRT activity as shown in the table were treated with <sup>3</sup>H-MeP-dR (33 mg/kg), <sup>3</sup>H-F-dAdo (20 mg/kg) or <sup>3</sup>H-F-araAMP (100 mg/kg). Tumors were removed 4 h after injection of prodrug and the radioactivity in each tumor was determined. Results are mean plus or minus s.d. from eight mice. Two experiments of four mice each were performed, which were in reasonable agreement. D54 tumors are tumors in which 100% of the cells are wild-type D54 cells; 10% PNP tumors are tumors in which 10% of the cells express *E. coli* PNP; 10% PNP/APRT tumors are D54 tumors in which 10% of the cells express both *E. coli* PNP and excess APRT; 10% PNP and 90% APRT tumors are D54 tumors in which 10% of the cells express *E. coli* PNP and the other 90% of the cells express recombinant APRT. \**P* = 0.0018 with respect to the result seen with 10% PNP tumors (Student's *t*-test).

addition of drug (Figure 6). As expected, there was no change in radioactivity in the medium surrounding D54 cells. In D54/PNP cells the radioactivity in the medium decreased by 20% over a 10-h period, and in D54/PNP/APRT cells the radioactivity had decreased by 40%. Because the volume of the medium far exceeds the cellular volume and because nucleobases and nucleosides are cell permeable and nucleotides are not, the missing radioactivity represents MeP-dR that was cleaved to MeP and activated to MeP nucleotides in the cells. The rate of utilization of MeP-dR in D54/PNP cells was 2.6 nmol per 10<sup>6</sup> cells per h, which was slightly less than that in D54/PNP/APRT cells (4.2 nmol 10<sup>6</sup> cells per h). High-pressure liquid chromatography analysis of the medium surrounding D54/PNP or D54/PNP/APRT cells revealed that no MeP-dR was present 10 h after addition of MeP-dR, and that all radioactivity in the medium was MeP. In contrast, in wild-type D54 cells, the radioactivity in the medium at 10 h was MeP-dR. This result indicated that a large amount of the MeP formed by *E. coli* PNP inside tumor cells was not activated by APRT, but instead diffused out of the cell. Furthermore, a 70-fold enhanced expression of APRT activity in cells expressing *E. coli* PNP, had only a modest effect on the amount of MeP that was activated in cells. Similar results were observed with F-dAdo and F-araA (data not shown), which indicated that addition of APRT also had only a modest effect on the amount of F-Ade that was activated in cells.

While performing these experiments we noticed that the utilization of F-araA was similar to that of F-dAdo. Therefore, we directly compared the utilization of these

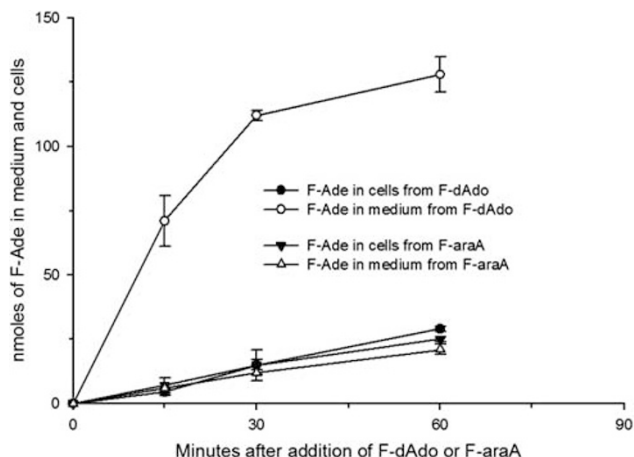


**Figure 6** Removal of MeP-dR (6-methylpurine-2'-deoxyribose) from medium surrounding D54, D54/PNP (purine nucleoside phosphorylase) or D54/PNP/APRT (adenine phosphoribosyl transferase) cells. Confluent cells in T75 flasks were incubated with 100 μM of <sup>3</sup>H-MeP-dR (100 nCi ml<sup>-1</sup>) in 20 ml of culture volume (2 μmol total). Total number of cells in each group were 20 ± 0.35 (D54), 14 ± 0.1 (D54/PNP) and 19 ± 0.4 (D54/PNP/APRT) million cells. Two hundred microliters of medium was removed at various times after the addition of <sup>3</sup>H-MeP-dR and the amount of radioactivity was determined. Results are the mean and standard deviation from three separate incubations.

two agents in D54/PNP cell cultures that were prepared at the same time. As with MeP-dR, there was no change in the concentration of either F-dAdo or F-araA in the medium surrounding D54 cells that did not express *E. coli* PNP over the 1-h incubation period (data not shown), which indicated that the rate of activation of these two compounds by nucleoside kinases in the cells was very low. However, in D54/PNP cells the concentration of both F-araA and F-dAdo decreased rapidly (data not shown). The rate of disappearance of F-araA from the medium was 51 nmol per h, which was only six-fold less than the rate seen in F-dAdo (300 nmol per h). Based on the *K<sub>m</sub>* and *V<sub>max</sub>* values of F-dAdo and F-araA with *E. coli* PNP,<sup>2</sup> we would expect a 2000-fold difference in the rate of cleavage of F-araA and F-dAdo at 10 μM of each compound. Similar amounts of F-Ade metabolites were produced in the D54/PNP cells from both F-araA and F-dAdo (Figure 7). However, most (93%) of the F-Ade generated from F-dAdo diffused out of cells and was detected in the surrounding medium, whereas with F-araA 50% of the F-Ade generated was found in the medium. These results also indicated that excess expression of APRT activity could (at most) increase the activation of F-araA only by a factor of 2.

## Discussion

Based on the limitations inherent in first generation 'suicide gene' strategies for cancer therapy (poor bystander killing, activity only against dividing cells and need for



**Figure 7** Activation of F-dAdo (2-F-2'-deoxyadenosine) and F-araA in D54/PNP (purine nucleoside phosphorylase) cells. Confluent D54/PNP cells were incubated with  $10\ \mu\text{M}$  of  $^3\text{H}$ -F-dAdo or  $^3\text{H}$ -F-araA in 20 ml ( $100\ \text{nCi ml}^{-1}$ ) of culture volume (200 nmol). Two hundred microliter of medium was removed at various times after the addition of the  $^3\text{H}$ -compounds and the total amount of radioactivity in the medium was determined. In addition, the amount of F-araA, F-dAdo or F-Ade (2-fluoroadenine) in the medium was determined by reverse phase high-pressure liquid chromatography. The results from these measurements were used to determine how much F-Ade was in either the medium or the cells. Each point is the mean  $\pm$  the s. d. of three measurements. This experiment has been repeated with similar results.

prolonged schedules of administration), a number of laboratories have pursued *E. coli* PNP. *E. coli* PNP mediates pronounced killing of tumor-bystander cells and has been used in animal models to cure mice of slow-growing tumors refractory to conventional agents.<sup>1-6</sup> Clinical trials of the strategy are under development in the United States and Australia. The foundation for optimizing *E. coli* PNP-based solid tumor therapy includes an understanding of the role of purine metabolism in tumor regressions by this approach. However, very little is known regarding the importance of APRT, the primary enzyme responsible for activating purine bases such as F-Ade and MeP, in the anti-tumor activity of the *E. coli* PNP approach to killing tumor cells. APRT could represent a rate-limiting step for tumor cell killing or bystander-dependent tumor regressions. The present study was, therefore, intended to investigate the relationship of APRT to *in vivo* tumor regression with *E. coli* PNP, and the mechanism of bystander killing responsible for anti-tumor efficacy.

The results reported here clearly indicate that additional APRT activity in tumor cells expressing *E. coli* PNP does not augment the anti-tumor activity of the overall strategy. It is not clear why excess APRT activity in *E. coli* PNP-expressing tumor cells did not result in more pronounced activation of either MeP or F-Ade, although it is possible that the APRT reaction is limited by the concentration of phosphoribosyl pyrophosphate present in the tumor cells. If phosphoribosyl pyrophosphate were rate limiting, increasing the APRT enzyme

concentrations would not result in increased metabolism of these agents. Cell lines were also obtained that expressed higher APRT activity levels (36 000 nmol per mg per h) than described in this manuscript; however, the growth of these cells was impaired, which indicated that modestly greater expression of APRT activity than used in the current studies (6000 and 17 000 nmol per mg per h) was toxic to the cells (data not shown), and suggested that overexpression of APRT may utilize all of the phosphoribosyl pyrophosphate in the cell and limit its availability for other important metabolic reactions. It was of interest that additional expression of APRT in bystander cells enhanced the activity of MeP-dR, but these results do not have practical significance, as it is not possible to reproduce this artificial situation in clinical disease.

A similar approach to the one described here is being pursued to enhance the use of *E. coli* cytosine deaminase (CD) plus 5-fluorocytosine (F-Cyt) as a cancer gene therapy strategy.<sup>17</sup> The product of CD and F-Cyt is 5-fluorouracil, which, similar to the adenine analogs generated by *E. coli* PNP, is activated by a phosphoribosyl transferase. A number of studies have combined CD with uracil phosphoribosyl transferase (UPRT) in an effort to enhance activation of 5-fluorouracil in tumor cells. However, there have not been many reports directly comparing the activity of CD alone with that of CD plus UPRT. Tiraby *et al.*<sup>18</sup> provided evidence in bacterial cells that combination of these two genes is better than CD alone, and Adachi *et al.*<sup>19</sup> demonstrated that treatment with two adenoviral vectors (each containing one of the genes of interest) was superior to treatment with an adenoviral vector expressing only CD. In the latter study, results indicated that UPRT increased incorporation of F-Cyt into RNA by a factor of 3, increased the *in vitro* cytotoxicity of F-Cyt by about 10-fold and enhanced the *in vivo* anti-tumor activity of CD. Therefore, it appears that addition of UPRT to a CD regimen can improve the anti-tumor activity of F-Cyt. Erbs *et al.*<sup>20</sup> have shown that a CD/UPRT fusion gene is superior to treatment with CD alone, but the improved anti-tumor activity of this vector can also be explained by a 100-fold increase in CD activity of the fusion gene.

The issue regarding *E. coli* PNP is not whether addition of excess APRT activity can improve killing of cells that express the enzyme, as all the three prodrugs effectively cure mice of tumors in which all cells express this recombinant transgene.<sup>1-3</sup> Instead, the question posed here is whether expression of excess APRT can improve killing of neighboring bystander cells that do not express *E. coli* PNP. There are two possible mechanisms that could account for the bystander activity observed with *E. coli* PNP. First, there is an 'immediate' bystander effect resulting from diffusion of MeP or F-Ade from cells expressing *E. coli* PNP to neighboring cells. Second, there may be 'delayed' bystander activity due to the release of MeP or F-Ade (and/or their cytotoxic metabolites, MeP-riboside or 2-F-adenosine) following death of cells. The primary metabolites released from dying cells would be MeP-riboside or 2-F-adenosine, unless these compounds interacted with *E. coli* PNP, which would convert them to

MeP and F-Ade. Any nucleotides released from dying cells would not be able to enter bystander cells, but would instead be rapidly degraded to nucleosides. If the immediate mechanism was primarily responsible for *in vivo* bystander activity observed with F-araA, then excess expression of APRT in cells expressing *E. coli* PNP could actually diminish *in vivo* bystander killing by enhancing activation of adenine analogs in cells already destined to die. On the other hand, if the delayed mechanism was primarily responsible for the *in vivo* bystander effect, expression of excess APRT together with *E. coli* PNP would be expected to enhance bystander killing by activating more of the purine base to be released at a later time.

A delayed type bystander activity might be viewed as more likely with MeP-dR, as MeP-riboside is much more potent than MeP in killing tumor cells, whereas the potency of F-adenosine is similar to F-Ade. MeP-riboside is an excellent substrate for its initial activating enzyme, adenosine kinase and it is possible that MeP-riboside released from RNA of dying cells is activated in bystander cells more efficiently than MeP. However, the increased anti-tumor activity of MeP-dR and the enhanced activation of MeP-dR in tumors with excess APRT activity in bystander cells, strongly suggest that augmented APRT in bystander tumor cells leads to improved capture of MeP in these cells shortly after its generation *in vivo*; that is, the immediate bystander mechanism.

F-araAMP is curative in tumors in which 10% of cells express *E. coli* PNP, which indicates that F-Ade is able to access and kill the 90% of cells (that is, bystander) that do not express *E. coli* PNP. However, the overexpression of APRT in bystander cells paradoxically impaired anti-tumor efficacy of F-araA. As there was a similar level of activation of F-araA in tumors with and without excess APRT activity in the bystander cells, this result suggested that expression of APRT in the bystander cells may determine the extent of tumor cell killing by limiting the radius of diffusion of F-Ade away from its site of generation in tumor cell foci expressing *E. coli* PNP.

It is curious that F-araAMP is a more robust anti-cancer prodrug than either MeP-dR or F-dAdo, because F-araA is a relatively poor substrate for *E. coli* PNP. The catalytic efficiency of *E. coli* PNP with F-araA is 2000–3000-fold less than MeP-dR or F-dAdo,<sup>2</sup> and in the case of F-dAdo, the same molecule (F-Ade) is generated by *E. coli* PNP. The unexpectedly strong activity of F-araAMP in D54 tumors can be partially explained by the ability of mice to tolerate much more F-araAMP than either F-dAdo or MeP-dR. In addition, the plasma half-life<sup>2</sup> of F-araA (50 min) is substantially longer than that of either F-dAdo (7 min) or MeP-dR (20 min). An additional consideration with respect to MeP-dR activity is that MeP is a much less potent toxin than F-Ade, because of its poor activity as a substrate for APRT.<sup>9</sup> Moreover, the maximally tolerated dose of MeP-dR is significantly diminished in mice bearing tumors expressing *E. coli* PNP.

The results presented in this work also suggest another factor that may contribute to the robust anti-tumor

activity of F-araAMP in cells expressing *E. coli* PNP. Similar amounts of F-Ade were produced and activated in cells expressing *E. coli* PNP during a 1-h period, regardless of whether the cells were treated with F-dAdo or F-araA. This result indicates that F-araA is cleaved by *E. coli* PNP and activated by APRT at a rate similar to that observed for F-dAdo, despite a catalytic efficiency of F-araA that is 3000-fold less than that of F-dAdo. The reason for this better than expected cleavage of F-araA relative to F-dAdo is not understood, but our results indicate that the cleavage reaction in a cell expressing high levels of *E. coli* PNP does not obey classical Michaelis–Menten kinetics. In summary, superior *in vivo* anti-tumor activity of F-araAMP may be fully explained by (1) similar activation of F-araA and F-Ado in tumor cells, (2) the ability to administer five times the amount of F-araA compared F-dAdo and (3) the seven-fold difference in plasma half-life between these two compounds.

Our results demonstrate that improved anti-tumor activity cannot be achieved simply by high-level co-expression of *E. coli* PNP and APRT. Furthermore, increasing *E. coli* PNP activity beyond the levels accomplished here would not enhance anti-tumor effectiveness of these prodrugs, because most of the purine analog that is created by *E. coli* PNP is not activated by APRT, but diffuses into the extracellular space. Even with F-araA, which is a relatively poor substrate of *E. coli* PNP, about half of the F-Ade produced diffuses out of the cell. Therefore, high-level expression of *E. coli* PNP is not rate limiting for the activation of F-araA, and the level of endogenous APRT is sufficient to activate most of the F-Ade that is generated. This finding points to prodrug delivery as the limiting factor for effective *in vivo* anti-tumor activity with prodrugs activated by *E. coli* PNP. Improved methods for enhancing delivery of the various prodrugs to tumor cells (slow release in depot form, regional administration and so on) should be sought to augment the overall strategy. Moreover, our results with F-araA indicate that high substrate activity with *E. coli* PNP is not the most important parameter in identifying effective prodrugs. At the levels of enzyme expression used in our experiments (similar to levels of enzyme activity achieved with adenoviral vectors), prodrug characteristics such as maximally tolerated dose and plasma half-life are much more important predictors of overall anti-tumor effect than cleavage rate by *E. coli* PNP. Improved anti-tumor activity using *E. coli* PNP might therefore be accomplished with new prodrugs that exhibit less toxicity, higher peak serum levels and prolonged systemic circulation. The ideal agent would be one that is inert in normal tissues but is only activated after contact with *E. coli* PNP. ‘Designer’ nucleosides of this kind have been reported<sup>21,22</sup> as a means to augment specificity in combination with a structurally modified PNP enzyme. However, this approach has not yet been successful in identifying prodrug/enzyme combinations that demonstrate better *in vivo* anti-tumor activity than that seen in *E. coli* PNP and F-araAMP.



**Conflict of interest**

Drs Parker and Sorscher have a significant equity position in PNP Therapeutics, which has licensed the patents concerning the use of *E. coli* PNP for the treatment of cancer.

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