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***In vivo* antitumor activity of intratumoral fludarabine phosphate in refractory tumors expressing *E. coli* purine nucleoside phosphorylase**

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Abstract

Purpose—Systemically administered fludarabine phosphate (F-araAMP) slows growth of human tumor xenografts that express *E. coli* purine nucleoside phosphorylase (PNP). However, this treatment has been limited by the amount of F-araAMP that can be administered *in vivo*. The current study was designed to 1) determine whether efficacy of this overall strategy could be improved by intratumoral (IT) administration of F-araAMP, 2) test enhancement of the approach with external beam radiation, and 3) optimize recombinant adenovirus as a means to augment PNP delivery and bystander killing *in vivo*.

Methods—The effects of systemic or intratumoral F-araAMP in mice were investigated with human tumor xenografts (300 mg) in which 10% of the cells expressed *E. coli* PNP from a lentiviral promoter. Tumors injected with an adenoviral vector expressing *E. coli* PNP (Ad/PNP; 2×10^{11} viral particles, 2×/day × 3 days) and the impact of radiotherapy on tumors treated by this approach were also studied. Radiolabeled F-araAMP was used to monitor prodrug activation *in vivo*.

Results—Intratumoral administration of F-araAMP in human tumor xenografts expressing *E. coli* PNP resulted in complete regressions and/or prolonged tumor inhibition. External beam radiation significantly augmented this effect. Injection of large human tumor xenografts (human glioma, non-small cell lung cancer, or malignant prostate tumors) with Ad/PNP followed by intratumoral F-araAMP resulted in excellent antitumor activity superior to that observed following systemic administration of prodrug.

Conclusions—Activation of F-araAMP by *E. coli* PNP results in destruction of large tumor xenografts *in vivo*, augments radiotherapy, and promotes robust bystander killing. Our results indicate that intratumoral injection of F-araAMP leads to ablation of tumors *in vivo* with minimal toxicity.

Keywords

tumor sensitization; low growth fraction malignancy; fludarabine; viral gene transfer; *E. coli* PNP

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Introduction

Refractoriness of tumors with low mitotic index. Poor *in vivo* efficacy against quiescent or slowly dividing cancer cells represents a major barrier to effective chemotherapy of refractory solid tumors [1-3]. The inability to destroy nondividing cells (including a putative tumor stem cell compartment) is viewed as a fundamental cause of therapeutic failure against common human malignancies, including low-growth fraction tumors such as glioma, prostate, breast, lung, and colon, **as well as** others with less than 40 % proliferating cancer cells [4-9]. Even in the case of a malignancy with mitotic index of 40%, and assuming that all dividing cancer cells were completely destroyed by a cumulative exposure to conventional chemotherapy, a solid tumor would still be less than one doubling time away from achieving pretreatment dimensions. The same considerations are relevant to clinical scenarios managed by local radiation therapy, including treatments with curative, adjuvant, neoadjuvant, or palliative intent. Non-metastatic cancers of breast, prostate, larynx, and brain treated with preoperative radiation therapy (XRT) as a debulking measure [10], and inoperable malignancies that obstruct a viscous (e.g., esophagus, larynx, colon, or airway) administered radiotherapy for palliation [11] invariably exhibit a low growth fraction, and at some point become unresponsive to the best available radiotherapy, which specifically targets DNA replication in dividing cancer cells. Although DNA damage can elicit a measure of necrosis and/or apoptosis within quiescent cancer cells, better methods for ablating the non-dividing tumor compartment remain an important priority [1-5, 10, 11].

Experimental strategies directed against locally invasive malignancy

Several modalities have been advanced in an attempt to improve treatment of locally destructive, non-metastatic tumors [12-14]. Experimental gene therapies, such as GDEPT (gene directed enzyme prodrug therapy), have been extensively tested, but have not succeeded in the clinic for at least two reasons [15-17]. First, efficiency of cancer cell transduction is low, and a sufficiently robust bystander effect against untransduced cells has not been achieved. Second, herpes simplex virus thymidine kinase (HSV-tk) or prokaryotic cytosine deaminase (CD) GDEPT-based strategies release intratumoral chemotherapeutic compounds (gancyclovir monophosphate and 5-fluorouracil (FUra), respectively) that are primarily active only against dividing tumor cells.

Intratumoral liberation of F-Ade by purine nucleoside phosphorylase

The *E. coli* purine nucleoside phosphorylase (PNP) gene has been shown to generate potent compounds such as 2-fluoroadenine (F-Ade) or 6-methylpurine (MeP) intratumorally [18-22]. F-Ade diffuses freely between *E. coli* PNP transduced and neighboring (bystander) cells via facilitated diffusion pathways ubiquitous in all cells and confers a strong bystander killing effect [23,24, see also below]. The compound works by a unique mechanism that disrupts RNA and protein synthesis and therefore ablates both dividing and nondividing (quiescent) tumor cells [25]. Direct comparisons between *E. coli* PNP and first generation strategies (HSV-tk and CD) indicate substantial augmentation of GDEPT by a PNP based mechanism [26,27,28]. In this study, we describe the conceptual framework for a means to concentrate very potent chemotherapy by direct infiltration of a tumor mass, establish feasibility *in vivo*, demonstrate usefulness as an adjunct to conventional XRT for treating refractory cancer, and illustrate proof of principle for the approach in solid tumors.

Methods

Studies with human tumor xenografts in mice

Parental and *E. coli* PNP expressing D54MG (human glioma) tumor cells (2×10^7 cells) were injected subcutaneously into the flanks of nude mice (*nu/nu*) purchased from Charles

River Laboratories (Wilmington, MA). D54 tumor cells stably transduced with *E. coli* PNP were prepared as described previously [29]. Tumors were measured with calipers and an estimate of the weight calculated using the equation, $(\text{length} \times \text{width}^2)/2 = \text{mm}^3$, and converted to mg assuming unit density. Unless stated otherwise, therapeutic drugs and the adenoviral vector expressing *E. coli* PNP (Ad/PNP), or vehicle controls were injected into D54 tumors in 150 μl volumes by 8 separate injections of approximately 20 μl each in an effort to evenly distribute the administered agent. At least 6 mice were studied in each treatment group. Mice were monitored daily and body weights and tumor dimensions collected twice weekly. T-C (tumor growth delay) was determined as the difference in median days to 2 doublings (median days to 600 mg for the D54 and DU145 (human prostate cancer) analysis) between drug-treated and vehicle-treated groups. For the NIH-H322M (human non-small cell lung cancer) study, because of tumor proliferation characteristics, total growth inhibition (TGI) was used as the evaluation point. TGI is equal to the control group mean delta minus the treated group mean delta divided by the control group mean delta, where delta is the change in tumor weight for each animal between day 36 and day 59. The time to the evaluation point for each animal was used as the end point for the student's t-test, Mann-Whitney rank sum test, or a life table analysis in order to statistically compare growth data between treatment groups. All key results were repeated under similar conditions and findings confirmed. Procedures were performed in accordance with a protocol approved by the IACUC of Southern Research Institute. F-araAMP was obtained from Schering A.-G. (Berlin, Germany). F-Ade was obtained from General Intermediates of Canada, Inc. (Edmonton, Alberta Canada). Treatments were initiated when tumors were 250 to 300 mg (~1-1.5% of total animal weight).

Measurement of *E. coli* PNP activity

The proportion of lentiviral transduced cells in a tumor mass was verified by measuring *E. coli* PNP activity in representative cancers removed from mice on the first day of drug treatment. Crude extracts were prepared as described previously [22] after tumor excision from the flanks of mice. The extracts were incubated with 50 mM potassium phosphate, 100 μM 6-methylpurine-2'-deoxyriboxide (MeP-dR), and 100 mM HEPES buffer (pH 7.4) at a concentration of extract that resulted in a linear reaction over the incubation period. The formation of MeP was monitored using reverse phase HPLC. One unit of PNP activity was defined as the amount of extract necessary to cleave 1 nmole of MeP-dR per mg protein in a 1 hour period.

Monitoring intratumoral metabolism of F-araAMP

Total radioactivity was determined after injection of 3 mg [$8\text{-}^3\text{H}$]F-araAMP (10 μCi) into 300 mg D54 flank tumors. [$8\text{-}^3\text{H}$]F-araAMP was obtained from Moravsek Biochemicals Inc. (Brea, CA). Tumors were removed from the mice at 10 minutes or 4 hours after injection and dissolved in 1 ml of Soluene 350 (Packard Instrument, Meriden, CT) by incubating at 55° C for 4 hours. A portion of each extract was mixed with scintillation fluid and radioactivity was determined.

Results

A D54 human glioma model was selected to initially test safety and efficacy of *E. coli* PNP/F-araAMP. D54 tumors in mice are refractory to conventional chemotherapeutic agents, including compounds such as BCNU that are clinically approved for human glioma treatment. In addition, the D54 model is relatively slow growing in mice (doubling time of approximately 7 days), and therefore provides a means to test whether an approach destroys both dividing and nondividing tumor cells. Because F-araAMP administration schedules described below were given over a two day period, complete regression or cure establishes

destruction of the non-proliferative compartment of a tumor mass. Human gliomas have also been a target for clinical testing of GDEPT type strategies in the past, and are highly resistant to interventions of this type. Finally, it should be noted that although a glioma model was selected for the present analysis, the established mechanism of cell killing by purine bases such as F-Ade (disruption of RNA and protein synthesis) [23] is anticipated to be active against diverse malignant cell types. The experiments below describe ablation of quiescent tumor cells following either lentiviral transduction or adenoviral gene delivery. However, the same approach would be expected to have relevance to numerous gene transfer vectors under development by others.

Figure 1 depicts the anti-tumor activity of *E. coli* PNP plus F-araAMP when 5% of tumor cells (transduced with the gene prior to implantation) express the recombinant enzyme. Intraperitoneal (systemic) administration of F-araAMP (100 mg/kg given 15 times, 167 mg/kg given 9 times, or 250 mg/kg given 3 times) led to complete regressions of all tumors and cures of all mice. Parental D54 tumors (i.e. without *E. coli* PNP) are not sensitive to treatment with F-araAMP. Tumors in which 100% of cells are transduced with *E. coli* PNP and non-transduced (parental) tumors grow at similar rates, suggesting that a ~5% level of transduction would be maintained throughout expansion of this tumor model. To verify the assertion, extracts were prepared from three representative tumors taken from mice on the first day of drug treatment and levels of *E. coli* PNP were determined to be $2,500 \pm 400$ units. *E. coli* PNP activity of 126,000 units is present in tumors comprised of 100% PNP expressing cells from this line. The findings therefore suggest that the parental (no PNP-expression) cells grew slightly more rapidly than the PNP transduced line *in vivo*, and confirm that transduction percentage described in Figure 1 is 5%, and perhaps closer to 2-3%.

The results shown in Figure 1 establish that complete regressions or cures of large tumors (approximately 1% of the total body weight of the animal) can be safely accomplished by PNP-based GDEPT. The findings also demonstrate excellent *in vivo* bystander activity. As few as three intraperitoneal (IP) injections of F-araAMP led to destruction of large tumors, although 5% of cells expressed the activating gene. Moreover, treatment with F-araAMP resulted in only a 10 to 20% decrease in body weight, which was regained after the F-araAMP schedule was completed. There was no gross tissue damage in the region immediately surrounding the tumor or other evidence of undesired sequelae despite a substantial prolongation of life.

Since only a fraction of F-araAMP injected IP actually perfuses a malignant tumor *in vivo*, we next tested whether injecting F-araAMP directly into the tumor mass could augment efficacy. An initial experiment is shown for tumors in which 10% of cells expressed *E. coli* PNP (Figure 2A). Intratumoral injection of 3 mg of F-araAMP per injection (3 daily injections) conferred significant antitumor effects ($p < 0.001$) with little or no weight loss (less than 4%), whereas F-araAMP had no effect when injected into parental (no *E. coli* PNP expressing) tumors (Figure 2B). Because the weight of a mouse is approximately 0.025 kg, injection of 3 mg F-araAMP is equivalent to a dose of 120 mg/kg F-araAMP. The total amount administered (360 mg/kg) was 25 to 50 percent of the total systemic amount studied in Figure 1. Injection of F-Ade (the active metabolite of F-araAMP) into tumors failed to elicit antitumor activity when tested at the highest possible solubility in saline (Figure 2B). F-Ade was also dissolved in DMSO, and three daily injections of 1.26 mg F-Ade (the approximate molar equivalent of F-Ade in a 3 mg injection of F-araAMP) into the tumor resulted in minimal antitumor activity (Figure 2B; $p = 0.011$ with respect to mice injected with DMSO vehicle), but caused a 10% decrease in body weight. Three intratumoral injections of either 2.5 or 5 mg of F-Ade (dissolved in DMSO) led to death in 3 of 6 and 4 of 6 mice, respectively, indicating that 1.26 mg is very near the maximally tolerated dose

(MTD). The results therefore demonstrate that unlike IT F-araAMP following intratumoral expression of *E. coli* PNP, direct IT injection of F-Ade (even at MTD dosing) has minimal antitumor effect. Negligible activity using IT F-Ade may be due to poor intratumoral uptake, rapid extracellular release, systemic escape of the compound, or a combination of these factors. As noted above, the amount of F-araAMP given intratumorally (in Figure 2A) was less than the total intraperitoneal dose described in Figure 1. In order to investigate the impact of higher doses of F-araAMP, the compound was dissolved in DMSO, and given at concentrations well tolerated IT, but above the maximally-tolerated dose if administered IP. Three daily doses of 6 or 24 mg were administered by IT injection to tumors in which 10% of cells expressed *E. coli* PNP (Figure 3). In this experiment, 3 of the 6 PNP tumors treated with 24 mg of F-araAMP became ulcerated (days 17, 21, and 24), requiring euthanasia of the study animals. The remaining 3 tumors completely regressed and mice remained tumor-free until the experiment was ended on day 70. There were no ulcerations in tumors treated with 6 mg of F-araAMP, and a single course of this treatment led to robust tumor regressions and a prolonged antitumor effect. Injection of F-araAMP at the highest dose resulted in a modest (7%) decrease in body weight, which recovered rapidly following completion of drug treatment. As expected, injection of F-araAMP at these doses into parental tumors (no PNP-expression) did not result in antitumor activity. Locally advanced solid tumors often become resistant to radiation therapy. As a test of adjuvant *E. coli* PNP in this setting, we investigated F-araAMP together with external beam radiation. In Figure 4, tumors in which 10% of cells expressed *E. coli* PNP were administered 4 Gy of radiation (determined previously to confer a measurable effect on D54 tumor growth) with or without three IT injections of 3 mg of F-araAMP (a dose that resulted in tumor suppression but no tumor free-survivors, Figure 2A). Combining *E. coli* PNP/F-araAMP with radiation therapy resulted in pronounced antitumor activity, which was much greater than either treatment alone. Ten to fourteen percent body weight loss was observed in all treatment groups (which resolved after termination of treatment), indicating that toxicities of the two interventions were not additive.

Previous studies from our laboratory have shown that treatment with a replication-deficient adenoviral vector encoding *E. coli* PNP, followed by systemic F-araAMP administration led to significant antitumor activity, but no tumor regression or cures in mice. This was attributable in part to inadequate delivery of F-araAMP to tumor parenchyma [25]. Based on the excellent activity demonstrated in Figure 3 when high doses of F-araAMP were administered directly to the tumor mass, we evaluated activity of Ad/PNP plus F-araAMP when both adenovirus and prodrug were inoculated directly into growing xenografts (Figure 5A). In the experiment shown, 2×10^{11} VP (virus particles) of Ad/PNP were administered IT twice per day (at 6 hour intervals) for 3 days (days 15, 16, and 17) for a total of 6 injections. The tumors were subsequently inoculated with 24 mg of F-araAMP once per day on Days 20, 21, and 22. *E. coli* PNP activity in the tumors on day 20 (the first day of F-araAMP treatment) was $7,500 \pm 2,000$ units, which is similar to that observed for tumors in which 5 to 10% of cells stably expressed the enzyme (2,500, 14,000, 8,600, or 12,000 units; Figures 1-4) and within a range for which robust antitumor activity attributable to IT F-araAMP could be expected. Although 2 of 10 mice treated with Ad/PNP plus F-araAMP died, there was an excellent antitumor effect in the 8 surviving mice who experienced 17% body weight loss during treatment that later resolved. Because of the evidence of toxicity noted in the experiment shown in Figure 5A, we repeated the study at a reduced dose of F-araAMP (18 mg/ injection). With this schedule, there were no drug-related deaths in the treatment group, and although murine body weights did not decrease appreciably (maximum of 5% on day 22), prolonged antitumor activity was noted through conclusion of the experiment on day 71 post implant (Figure 5B). In order to test the general efficacy of this approach, the same protocol was applied to human NCI-H322M (non-small cell lung) and DU145 (prostate) solid tumors (Figures 5C and 5D), and strong tumor regressions were

observed following a single cycle of therapy in each case. These results establish that intratumoral injection of Ad/PNP followed by IT F-araAMP can elicit a substantial regressive effect on otherwise refractory solid tumors in a fashion substantially superior to viral PNP transduction followed by systemic prodrug administration [18-22].

In an effort to understand the pharmacodynamics of IT F-araAMP, the levels of prodrug activation in parental (D54) tumors and tumors in which 10% of cells expressed *E. coli* PNP were monitored (Table 1). Tumor tissue was collected 10 minutes or 4 hours after IT injection of 3 mg [³H]-F-araAMP and the amount of radioactivity remaining in the tumor mass was determined. Ten minutes after injection with F-araAMP, there were no differences between parental and D54 tumors in which 10% of cells express *E. coli* PNP. By 4 hours, radioactivity in D54 tumors that expressed *E. coli* PNP was substantially higher than parental tumors, representing the amount of F-araAMP converted to F-Ade metabolites. The experiment indicated that 190 nmoles of F-Ade metabolites were generated and retained per gram of tumor tissue after intratumoral injection of 3 mg F-araAMP. Three mg of F-araAMP is equal to 8200 nmoles, and since tumors in this experiment were ~ 0.3 grams, approximately 57 nmoles of F-Ade were retained in the tumor tissue, derived from 0.7% of total F-araAMP injected into the tumor mass.

Discussion

These experiments describe generation of a potent anticancer agent within tumor parenchyma. The strategy is safe in preclinical models based on the limited radius of intratumoral F-Ade diffusion and extensive dilution (to unmeasurable F-Ade levels in serum, data not shown) after release from dying tumor cells. The mechanism of antitumor activity differs fundamentally from all other approaches to GDEPT. F-Ade destroys malignant cells by disrupting RNA and protein synthesis, which causes ablation of both dividing and non-dividing tumor compartments [25]. The finding that relatively slow growing D54 tumors (doubling time of approximately 7 days) can be completely destroyed by IP or IT treatment over a period of two days indicates activity against both cycling and noncycling tumor cells *in vivo*.

Intratumoral injection of F-araAMP was pursued as a way to minimize systemic prodrug exposure and maximize drug levels within tumor tissue. We provide evidence of pronounced and safe antitumor activity following intratumoral injection of F-araAMP in the setting of *E. coli* PNP expression (Figures 2-5). Our findings suggest a comparable strategy could be useful in human subjects without the need for modification of vector tropism, enhanced bystander killing, or viral oncolysis. The doses of F-araAMP given as local therapy to treat 300 mg tumors in mice (3 to 18 mg administered 3 times) are much less than the amount of F-araAMP routinely administered as part of standard clinical care in humans (~ 40 mg per dose × 5 daily doses given every 4 weeks). Intratumoral dose intensities of F-araAMP significantly greater than those shown in Figures 2-5 should therefore be well-suited for clinical testing. We also note that first generation adenoviral constructs (similar to the vector described here but without PNP expression) have been extensively characterized in earlier clinical trials of gene delivery to tumors, and are an approved cancer therapeutic in Asia.

A primary barrier to GDEPT results from inability of available vectors to deliver therapeutic genes efficiently *in vivo*. This limitation can be partially overcome if a transgene mediates destruction of neighboring cancer cells that are not transduced (i.e. bystander killing). A bystander effect has been observed with the HSV-thymidine kinase which is attributable to passage of ganciclovir nucleotides through gap junctions (for example, see Ref 30). Cytotoxic metabolites generated by *E. coli* PNP also partition between cells via gap junctions, but bystander activity of the PNP approach is greatly augmented by diffusion of

purine bases such as F-Ade across cell membranes with the aid of *es* and *ei* equilibrative nucleoside transporters (hENT1, hENT2, and hENT3) which are expressed in most, if not all, cell types [23-24]. Results from the present study demonstrate that tumors with 5% of cells expressing *E. coli* PNP can be eradicated following treatment with F-araAMP. We are not aware of this level of *in vivo* bystander activity with any other GDEPT approach. Moreover, because equilibrative transporters are ubiquitously expressed, the high level of bystander killing described here should be effective against many other tumor types *in vivo*.

Cancer cells expressing *E. coli* PNP that are mixed prior to implantation provide a homogeneous distribution of transgene throughout a growing tumor mass. This is not the case when *E. coli* PNP is administered by an adenoviral vector inoculated intratumorally. Following IT delivery, transgene expression occurs primarily in a compartment surrounding the needle track [22]. Treatment with F-araAMP results in a large number of cells in the injected region - together with a fraction of bystander cells in close proximity - to be ablated. Based on data described in this report, bystander killing with *E. coli* PNP appears suitable for reaching portions of a tumor mass several cell volumes away from the injection site. The results therefore demonstrate strong anti-tumor activity following IT injection of adenoviral vectors *in vivo*, and indicate a very robust bystander effect.

In summary, we envision a modality in which tumors resistant to available agents are selectively infiltrated with vector, followed by direct injection of F-araAMP to destroy tumor parenchyma. Such an approach might be useful as a neo-adjuvant (preoperative, debulking) measure for needle-accessible tumors such as breast or prostate, or as a palliative modality for obstructing cancers of the esophagus or airways, for example. The robust bystander effect could be titrated by decreasing either vector or prodrug, in order to achieve the desired effect. In addition, based on the very low doses of vector and prodrug that are required following IT inoculation, the intervention could be given repeatedly, and tested in combination with radiotherapy against low growth fraction and otherwise untreatable tumors *in vivo*. Use of *E. coli* PNP has recently been approved by the Food and Drug Administration for clinical testing in the United States (IND #14271).

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References

1. Takimoto, CH.; Calvo, E. Cancer Management Handbook. 11th. UBM Medica; London: 2009. Chapter 3: Principles of Oncologic Pharmacotherapy.
2. Sang L, Roberts JM, Collier HA. Hijacking HES1: How tumors co-opt the anti-differentiation strategies of quiescent cells. Trends Mol Med. 2010; 16(1):17–26. [PubMed: 20022559]
3. Mellor HR, Ferguson DJP, Callaghan R. A model of quiescent tumor microregions for evaluating multicellular resistance to chemotherapeutic drugs. Br J Cancer. 2005; 93(3):302–9. [PubMed: 16052217]
4. Vessella RL, Pantel K, Mohla S. Tumor cell dormancy: an NCI workshop report. Cancer Biol Ther. 2007; 6(9):1496–504. [PubMed: 17881897]
5. Kusumbe AP, Bapat SA. Cancer stem cells and aneuploid populations within developing tumors are the major determinants of tumor dormancy. Cancer Res. 2009; 69(24):9245–53. [PubMed: 19951996]
6. Sal S, Takashi M, Miyake K, Koshikawa T. Study of growth fraction on fine needle aspirated prostatic tissue smear using monoclonal antibody Ki-67. Hinyokika Kyo Acta Urologic Japonica. 1991; 37(8):881–6.

7. Sadi MV, Barrack ER. Determination of growth fraction in advanced prostate cancer by Ki-67 immunostaining and its relationship to the time to tumor progression after hormonal therapy. *Cancer*. 1991; 67:3065–3071. [PubMed: 1710538]
8. Tay DLM, Bhathal PS, Fox RM. Quantitation of G₀ and G₁ phase cells in primary carcinomas. *J Clin Invest*. 1991; 87:519–527. [PubMed: 1991836]
9. Giangaspero F, Doglioni C, Rivano MT, Pileri S, Gerdes J, Stein H. Growth fraction in human brain tumors defined by the monoclonal antibody Ki-67. *Acta Neuropathologica*. 1987; 74(2):179–182. [PubMed: 3673508]
10. DeVita, VT.; Lawrence, TS.; Rosenberg, SA. Principles and Practice of Oncology. 8th. Depinho, Ronald A.; Weinbert, Robert A., editors. Lippincott Williams & Wilkins; Philadelphia: 2008.
11. Washington, CM.; Leaver, DT. Principles and Practice of Radiation Therapy. 3rd. Mosby; Philadelphia: 2009.
12. Osada S, Yoshida K, Saji S. A novel strategy by cryoablation for advanced hepatoma. *Anticancer Res*. 2009; 29(12):5203–9. [PubMed: 20044637]
13. Krishnan S, Diagaradjane P, Cho SH. Nanoparticle-mediated thermal therapy: Evolving strategies for prostate cancer therapy. *Int J Hyperthermia*. 2010; 26(8):775–89. [PubMed: 20858069]
14. Margreiter M, Marberger M. Focal therapy and imaging in prostate and kidney cancer: High-intensity focused ultrasound ablation of small renal tumors. *J Endourol*. 2010; 24(5):745–6. [PubMed: 20380511]
15. Both GW. Recent progress in gene-directed enzyme prodrug therapy: an emerging cancer treatment. *Curr Opin Mol Ther*. 2009; 11(4):421–32. [PubMed: 19649987]
16. Altaner C. Prodrug gene therapy. *Cancer Lett*. 2008; 270(2):191–201. [PubMed: 18502571]
17. Dachs GU, Hunt MA, Syddall S, Singleton DC, Patterson AV. Bystander or no bystander for gene directed enzyme prodrug therapy. *Molecules*. 2009; 14(11):4517–45. [PubMed: 19924084]
18. Tai CK, Wang W, Lai YH, Logg CR, Parker WB, Li YF, Hong JS, Sorscher EJ, Chen TC, Kasahara N. Enhanced efficiency of prodrug activation therapy by tumor-selective replicating retrovirus vectors armed with the *Escherichia coli* purine nucleoside phosphorylase gene. *Cancer Gene Ther*. 2010; 17(9):614–23. [PubMed: 20467451]
19. Xie X, Guo J, Kong Y, Xie GX, Li L, Lv N, Xiao X, Tang J, Wang X, Liu P, Yang M, Xie Z, Wei W, Xie X. 2011 Targeted expression of *E. coli* purine nucleoside phosphorylase and Fludara for prostate cancer therapy. *J Gene Med* 2011. 2011 Oct 18. Epub ahead of print.
20. Parker WB, Allan PW, Waud WR, Hong JS, Sorscher EJ. Effect of expression of adenine phosphoribosyltransferase on the *in vivo* anti-tumor activity of prodrugs activated by *E. coli* purine nucleoside phosphorylase. *Cancer Gene Ther*. 2011; 18(6):390–8. [PubMed: 21394111]
21. Bossow S, Grossardt C, Temme A, Leber MF, Sawall S, Rieber EP, Cattaneo R, von Kalle C, Ungerechts G. Armed and targeted measles virus for chemovirotherapy of pancreatic cancer. *Cancer Gene Ther*. 2011; 18(8):598–608. [PubMed: 21701532]
22. Hong JS, Waud WR, Levasseur DN, Townes TM, Wen H, McPherson SA, Moore BA, Bebok Z, Allan PW, Secrist JA 3rd, Parker WB, Sorscher EJ. Excellent *in vivo* bystander activity of fludarabine phosphate against human glioma xenografts that express the *Escherichia coli* purine nucleoside phosphorylase gene. *Cancer Res*. 2004; 64:6610–6615. [PubMed: 15374975]
23. Yao SY, Ng AM, Cass CE, Baldwin SA, Young JD. Nucleobase transport by human equilibrative nucleoside transporter 1 (hENT1). *J Biol Chem*. 2011; 286:32552–32562. [PubMed: 21795683]
24. Zhang J, Visser F, King KM, Baldwin SA, Young JD, Cass CE. The role of nucleoside transporters in cancer chemotherapy with nucleoside drugs. *Cancer Metastasis Rev*. 2007; 26:85–110. [PubMed: 17345146]
25. Parker WB, Allan PW, Shaddix SC, Rose LM, Speegle HF, Gillespie GY, Bennett LL Jr. Metabolism and metabolic actions of 6-methylpurine and 2-fluoroadenine in human cells. *Biochem Pharmacol*. 1998; 55:1673–1681. [PubMed: 9634004]
26. Lockett LJ, Molloy PL, Russell PJ, Both GW. Relative efficiency of tumor cell killing *in vitro* by two enzyme-prodrug systems delivered by identical adenovirus vectors. *Clin Cancer Res*. 1997; 3:2075–80. [PubMed: 9815599]
27. Nestler U, Heinkelein M, Lucke M, Meixensberger J, Scheurlen W, Kretschmer A, Rethwilm A. Foamy virus vectors for suicide gene therapy. *Gene Ther*. 1997; 4:1270–77. [PubMed: 9425452]

28. Puhlmann M, Gnant M, Brown CK, Alexander HR, Bartlett DL. Thymidine kinase-deleted vaccinia virus expressing purine nucleoside phosphorylase as a vector for tumor-directed gene therapy. *Human Gene Ther.* 1999; 10:649–657. [PubMed: 10094208]
29. Parker WB, Allan PW, Waud WR, Hong JS, Sorscher EJ. Effect of expression of adenine phosphoribosyltransferase on the *in vivo* anti-tumor activity of prodrugs activated by *E. coli* purine nucleoside phosphorylase. *Cancer Gene Ther.* 2011; 18(6):390–8. [PubMed: 21394111]
30. Mesnil M, Piccoli C, Tiraby G, Willecke K, Yamasaki H. Bystander killing of cancer cells by herpes simplex virus thymidine kinase gene is mediated by connexins. *Proc Natl Acad Sci USA.* 1996; 93:1831–1835. [PubMed: 8700844]

Figure 1. Effect of F-araAMP schedule on D54 tumors that express *E. coli* PNP in 5% of the cells Parental D54 tumor cells were mixed with D54 tumor cells stably transduced with *E. coli* PNP so that 5% of the mixture expressed the PNP transgene. This 95/5 mixture was injected sc into the flanks of nude mice. Intraperitoneal treatment with F-araAMP (250 mg/kg once per day for 3 consecutive days (○); 167 mg/kg 3 times per day (4 hour intervals) for 3 consecutive days (□); 100 mg/kg 5 times a day (2 hour intervals) for 3 consecutive days (Δ); or vehicle control (○) 5 times a day (2 hour intervals) × 3 days) began on day 14 when tumors were approximately 250 mg. The effect of 160 mg/kg F-araAMP 3 times per day (4 hour intervals) for 3 consecutive days (■) on parental (no *E. coli* PNP expression) D54 tumors is also shown indicating that F-araAMP is not active against tumors that do not express *E. coli* PNP. The activity of *E. coli* PNP in the 5% D54 tumors at the time of treatment (day 14) was $2,500 \pm 400$ units. No *E. coli* PNP activity is detectable in parental tumors. Growth in all F-araAMP treatment groups for tumors with PNP expression was significantly different than that in vehicle treated controls ($P < 0.001$). Tumor means $\pm$ standard error of the mean (SEM) are shown.

Figure 2. Effect of intratumoral injection of F-araAMP on D54 tumors

In Panel 2a, parental D54 tumor cells were mixed with D54 tumor cells that had been transduced with *E. coli* PNP to a final proportion in which 10% expressed the transgene. This 90/10 mixture was injected sc into the flanks of nude mice. Tumors were injected once per day for 3 consecutive days starting on day 14 with 150 μ l of saline containing 0.05% Tween-80, 1.5 mg of F-araAMP dissolved in saline containing 0.05% Tween-80, or 3 mg of F-araAMP dissolved in saline containing 0.05% Tween-80. The activity of *E. coli* PNP in the tumors at the time of treatment (day 14) was $14,300 \pm 2,600$ units. Tumor growth in the 3 mg treatment group was significantly different than that in the vehicle treated group ($P < 0.001$) but was not significantly different than that in the 1.5 mg treatment group ($P = 0.458$). In Panel 2b, parental D54 tumor cells (no *E. coli* PNP expression) were injected sc into the flanks of nude mice. Tumors were inoculated once per day for 3 consecutive days starting on day 14 with 150 μ l of saline containing 0.05% Tween-80, 3 mg F-araAMP dissolved in saline containing 0.05% Tween-80, 0.15 mg of F-Ade dissolved in saline containing 0.05% Tween-80, 1.26 mg of F-Ade dissolved in DMSO, 0.63 mg of F-Ade dissolved in DMSO, or DMSO. Tumor growth in mice treated with 0.63 or 1.26 mg F-Ade dissolved in DMSO was significantly different than DMSO vehicle treatment ($P = 0.011$ and 0.002 , respectively). Tumor means $\pm$ SEM are shown.

Figure 3. Effect of intratumoral injection of F-araAMP at high concentrations on D54 tumors Parental D54 tumor cells or a mixture of D54 tumor cells in which 10% of the cells expressed *E. coli* PNP were injected sc into the flanks of nude mice. Tumors were injected once per day for 3 consecutive days starting on day 14 with 150 μ l of DMSO, 24 mg F-araAMP dissolved in DMSO, or 6 mg F-araAMP dissolved in DMSO. The activity of *E. coli* PNP in the D54 tumors in which 10% of the cells expressed *E. coli* PNP (on day 14) was $8,600 \pm 620$ units. The tumor growth in the mice bearing 10% PNP tumors and treated with 6 or 24 mg of F-araAMP was significantly different than the vehicle treated group ($P = 0.014$ and < 0.001 , respectively). Tumor means $\pm$ SEM are shown.

Figure 4. Effect of intratumoral injection of F-araAMP plus radiation on D54 tumors
 D54 tumor cells in which 10% of cells expressed *E. coli* PNP were injected sc into the flanks of nude mice. Starting on day 13 the tumors were treated with radiation, F-araAMP, or F-araAMP plus radiation. In two treatment groups, tumors were injected once per day for 3 consecutive days with 150 μ l of either saline containing 0.05% Tween-80 (filled circles) or 3 mg F-araAMP dissolved in saline containing 0.05% Tween-80 (filled triangles). In two other treatment groups radiation (4 Gy) was administered once per day for 3 consecutive days 3 hours after injection of 150 μ l of either saline containing 0.05% Tween-80 (open circles) or 3 mg F-araAMP dissolved in saline containing 0.05% Tween-80 (open triangles). The activity of *E. coli* PNP in the D54 tumors (on day 13) was $11,900 \pm 3,100$ units. The tumor growth in mice treated with radiation plus F-araAMP was significantly different than in mice treated with F-araAMP or radiation only ($P < 0.001$ and $= 0.004$, respectively). Tumor means $\pm$ SEM are shown.

Figure 5. Effect of Ad/PNP plus F-araAMP on D54 tumors

In Panel A, parental D54 human glioma tumors were treated IT with vehicle (closed circles), Ad/PNP (open circles), F-araAMP (filled squares), or Ad/PNP plus F-araAMP (open squares). Ad/PNP (2×10^{11} VP) was injected twice a day (6 hour intervals) for 3 consecutive days starting on day 15. Twenty four mg F-araAMP dissolved in DMSO was injected into tumors once per day for 3 consecutive days starting on day 20. The tumors in the vehicle treated mice were injected 6 times with saline followed by 3 injections of DMSO (otherwise as described above). The activity of *E. coli* PNP in the D54 tumors (on day 20) was $7,500 \pm 2,000$ units. Each treatment arm contained 10 mice. In the combined treatment arm, two of ten mice died as a consequence of therapy with a maximum loss in body weight of 17%. Four of the eight surviving mice were tumor-free on day 65, two mice had small tumors (63 and 288 mg), and two mice had growing tumors (1,666 and 1,584 mg). The tumor growth in mice treated with Ad/PNP plus F-araAMP was significantly different than that in mice treated with F-araAMP or Ad/PNP only ($P = 0.010$ and 0.002 , respectively). In Panel B, mice were treated as described in Panel A except that the dose of F-araAMP was 18 mg per injection. The activity of *E. coli* PNP on day 18 in this experiment was $1,900 \pm 1,500$ units. With regard to the combination of Ad/PNP with F-araAMP, four mice were tumor-free on day 71, two mice had very small tumors (72 and 108 mg), two mice had larger tumors (1,437 and 4,332 mg), and two mice died by day 71 due to unknown causes temporally unrelated to F-araAMP toxicity (days 13 and 43). The maximum loss in body weight (5%) occurred on day 22. Tumor growth in mice treated with Ad/PNP plus F-araAMP was significantly different than that in mice treated with F-araAMP or Ad/PNP only ($P = 0.001$ and 0.011 , respectively). In Panel C, human non-small cell lung (H322M) tumors were treated by the same protocol. Tumor growth for all treatment groups was significantly different than that of the vehicle control ($P < 0.011$), but there was no significant difference between tumor growth in any of the different treatment groups. *E. coli* PNP activity in these tumors (224 ± 123 units) was lower than in D54, but in a range shown previously to mediate robust antitumor effects *in vivo* [22]. In Panel D, human prostate (DU145) tumors were treated by the same protocol. Tumor growth in mice treated with Ad/PNP and F-araAMP was significantly different than that of the other treatment groups ($P < 0.034$). *E. coli* PNP activity in these tumors (441 ± 348 units) was lower than in D54, but in a range shown previously to mediate robust antitumor effects *in vivo* [22]. Tumor means $\pm$ SEM are shown.

Table 1
Radioactivity in D54 tumors after intratumoral injection of F-araAMP

	nmoles F-araAMP metabolites/gram of tissue	
	10 min	4 hours
D54 tumors	980 ± 730	31 ± 6
10% PNP D54 tumors	1100 ± 990	220 ± 75 ^a

Three mg (8,200 nmoles) of [³H]F-araAMP (10 µCi/injection) were injected into D54 tumors or D54 tumors in which 10% of the cells expressed *E. coli* PNP as described in Figure 2. Tumors (approximately 300 mg) were removed 10 minutes and 4 hours after injection with F-araAMP and the amount of radioactivity in each tumor was determined. There were 4 tumors per group. This experiment has been repeated with similar results.

^a Significantly different from D54 tumors: $p < 0.02$, paired Student's *t* test.