A Long-Acting Suicide Gene Toxin, 6-Methylpurine, Inhibits Slow Growing Tumors after a Single Administration

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ABSTRACT

We have demonstrated antitumor activity against refractory human glioma and pancreatic tumors with 6-methylpurine (MeP) using either a suicide gene therapy strategy to selectively release 6-methylpurine in tumor cells or direct intratumoral injection of 6-methylpurine itself. A single i.p. injection in mice of the prodrug 9-β-D-[2-deoxyribofuranosyl]-6-methylpurine (MeP-dR; 134 mg/kg) caused sustained regression lasting over 70 days of D54 (human glioma) tumors transduced with the Escherichia coli purine nucleoside phosphorylase (PNP), and a single intratumoral injection of 6-methylpurine (5–10 mg/kg) elicited prolonged delays of the growth of D54 tumors and CFPAC human pancreatic carcinoma. Because the D54 tumor doubling time is >15 days, the experiments indicate that prodrug activation by E. coli PNP engenders destruction of both dividing and nondividing tumor compartments in vivo and, therefore, address a fundamental barrier that has limited the development of suicide gene strategies in the past. A prolonged retention time of 6-methylpurine metabolites in tumors was noted in vivo (T½ >24 h compared with a serum half-life of <1 h). By high-pressure liquid chromatography, metabolites of [3H]MeP-dR were 5- to 6-fold higher in tumors expressing E. coli PNP. These experiments point to new endpoints for monitoring E. coli PNP suicide gene therapy, including intratumoral enzymatic activity, in situ (intratumoral) prodrug conversion, and tumor regressions after direct injection of a suicide gene toxin. The findings also help explain the strong in vivo bystander killing mechanism ascribed by several laboratories to E. coli PNP in the past.

Pancreatic, brain, lung, liver, prostate, and other human cancers often invade locally, become inoperable, and cause death even in the absence of distant metastases. These nonmetastatic, locally invasive cancers account for over 100,000 cases per year in the United States alone, and the majority lead to death (DeVita et al., 1997; SEER Cancer Incidence Public Use Database 1973–1996, 1999). Treatment of these types of cancer remains a significant therapeutic challenge. Nonsurgical modalities (chemotherapy and radiotherapy) are ineffective in this setting because these approaches primarily kill proliferating cells. Refractory tumors often have a very low growth fraction (4–40% of cells actively dividing at any time) (Giangaspero et al., 1987; Sadi and Barrack, 1991; Vescio et al., 1990; Dionne et al., 1998; Springer and Niculescu-Duvaz, 2000). Conventional anticancer agents that are selective for rapidly dividing tumor cells fail to eradicate tumors with a low growth fraction. Compounds designed to kill both proliferating and quiescent tumor cells are limited by toxicity following systemic administration.

One proposed solution to this problem is expression of “suicide” genes to generate highly toxic compounds specifically inside growing tumors. An essential question is whether a suicide gene strategy as applied to locally invasive tumors offers an advantage over simply injecting toxins into a tumor mass. Although suicide genes such as herpes simplex virus thymidine kinase (HSV-tk) and Escherichia coli cytosine deaminase have been tested previously for releasing concentrated chemotherapy within tumors, they seem less likely to be useful in exploring the above question (Freeman et al., 1993; Ram et al., 1993; Huber et al., 1994; Beck et al., 1995; Fick et al., 1995; Elshami et al., 1996; Sacco et al., 1996; Dilber et al., 1997; Imaizumi et al., 1998). Ganciclovir monophosphate (the toxin generated from ganciclovir by HSV-tk) cannot be given by direct intratumoral injection because the plasma membrane is impermeant to phosphorylated nucleosides. Direct injection of even very high levels of 5-flourouracil (the toxin liberated by E. coli cytosine deaminase) would seem unlikely to elicit tumor regressions in vivo based upon
the relative inability of the compound to kill nondividing compartments of human tumors and the failure of even concentrated 5-flurouracil to prolong survival as part of regional (hepatic infusion) therapy in human trials (DeVita et al., 1997).

We have suggested an alternative strategy that selectively activates purine analogs by E. coli purine nucleoside phosphorylase (PNP) (Hughes et al., 1998). Specificity results from very inefficient cleavage of the substrates used in this approach by mammalian PNP. Toxins produced by E. coli PNP differ fundamentally from HSV-tk because they kill tumors independent of gap junctions or other cell-to-cell communication. Strong bystander effects in vitro (complete elimination of entire populations of tumor cells when 1 in 100 to 1 in 1000 cells express the PNP gene) and significant antitumor effects in vivo (e.g., when 1 in 1000 cells express PNP) have been observed previously (Hughes et al., 1995, 1998; Parker et al., 1997; Gadi et al., 2000). Although the method has been reported to elicit strong regressions and cures in mouse models of human ovarian, glioma, prostate, and liver cancers (Parker et al., 1997; Martiniello-Wilks et al., 1998; Puhlmann et al., 1999), very little is known regarding in vivo mechanism of action of the bystander effects, or the intermediate endpoints that may be useful in understanding and optimizing this system. One toxin produced by E. coli PNP, 6-methylpurine (MeP), is membrane permeant and, unlike ganciclovir monophosphate or 5-flurouracil, efficiently kills both dividing and nondividing cells in vitro (Parker et al., 1998; Secrist et al., 1999). Whether the same is true in vivo is not known.

In the present study, we therefore examined in vivo antitumor activity and the mechanism of action of 6-methylpurine. First, we tested the ability of 6-methylpurine to ablate PNP-transduced tumor growth after a single administration of the E. coli PNP substrate, 9-β-D-[deoxyribofuranosyl]-6-methylpurine (MeP-dR), which is cleaved by PNP to liberate 6-methylpurine. Next we measured the kinetics of toxin clearance from tumors treated by this regimen. We found that 6-methylpurine had pronounced antitumor effects after a single dose of prodrug and tumor responses and cures against slow growing tumors with a low growth fraction. The mechanism of tumor regression was attributable to a very long half-life (>24 h) of toxic metabolites within tumor tissue. Based on the above results, we also examined antitumor effects after direct injection of 6-methylpurine into growing tumors in vivo.

**Materials and Methods**

**Establishment of Human Glioma Tumors.** pLNSX, a gift of Dr. D. Miller (Fred Hutchinson Cancer Research Center, Seattle, WA) (Miller and Rosman, 1989), was used to generate retrovirus encoding the E. coli PNP gene under control of the SV-40 promoter (Parker et al., 1997). Retrovirus was used to transduce D54MG glioma tumor cells (Andreansky et al., 1996). D54MG cells expressing PNP were isolated with cloning rings after G418 selection. D54MG and D54-PNP cells (2 × 10⁷) were injected s.c. into the flanks of nude mice (nu/nu) purchased from Taconic Farms. The tumors were measured with calipers two times per week, and an estimate of weight (milligrams) was calculated as described (Dykes et al., 1992). MeP-dR was made in our laboratories as previously described (Montgomery and Rosman, 1968). Tumor regression studies were conducted according to NCI protocols.

**Intratumoral PNP Activity and Trapping of 6-Methylpurine Metabolites.** MeP-dR (67 mg/kg) together with 10 μCi of the titrated compound ([2,8-3H]; Moravek Biochemicals, Brea, CA) was administered intraperitoneally, and tumor extracts were analyzed by HPLC for parent compound and its metabolites at the time points shown. Each value was the average of three tumors. MeP-dR and its metabolites were eluted from a Spherisorb ODS1 (5 μm) column (Keystone Scientific, Inc., Bellefonte, PA) with a solvent containing 5 mM ammonium dihydrogen phosphate (95%) and acetonitrile (5%) at a flow rate of 1 ml/min. Fractions were collected as they eluted from the column and were counted for radioactivity. Using this HPLC system, we were able to separate MeP-dR from 6-methylpurine-riboside, 6-methylpurine, and 6-methylpurine-ribose phosphates. Separation of these metabolites was necessary because there was considerable degradation of the nucleotides during the extraction procedure. The amount of 6-methylpurine that was produced and retained in the tumor tissue was determined by adding the radioactivity that eluted as 6-methylpurine, 6-methylpurine-riboside, and 6-methylpurine-ribose phosphates (6-methylpurine-ribose is a metabolite more toxic to tumor cells than 6-methylpurine; 6-methylpurine-ribose phosphates are the active forms of these compounds). Since MeP-dR is a poor substrate for mammalian kinases (W. B. Parker, unpublished observation), the phosphorylated metabolites should be only phosphates of 6-methylpurine-riboside. That these metabolites were retained in the tumors for more than 24 h indicated that the primary metabolites in the tumor cells before extraction were phosphates of 6-methylpurine-riboside because 6-methylpurine-ribose and 6-methylpurine would rapidly equilibrate across the cell membrane and would not be retained in tumor cells. 6-Methylpurine was obtained from Sigma-Aldrich (St. Louis, MO) to modicum of this method was used to follow MeP-dR in mouse plasma. Measurements of PNP enzymatic activity in tumor lysates (by conversion of unlabeled MeP-dR to 6-methylpurine) were as described previously (Gadi et al., 2000).

**6-Methylpurine Injection into Pre-Established Tumors.** The susceptibility of CFPAC-1 cells (Bradbury et al., 1992) to 6-methylpurine was determined in severe combined immunodeficiency (SCID) mice implanted subcutaneously in the flanks with 20 million CFPAC-1 cells. Tumors greater than 75-mm³ tumor volume were injected intratumorally (i.t.) in a delivery volume of 50 μl with water (vehicle) or 6-methylpurine dissolved in water each day for 3 days. The dosing was selected based on the initial studies, which indicated that the approximate amount of 6-methylpurine analogs could tolerate when given i.p. In glioma tumors, 6-methylpurine was given by intratumoral injection in 100 μl of normal saline every day for a total of 3 days (days 18, 19, and 20 postimplantation) and otherwise established as above.

**Tumor Regression Measurements.** Mice were evaluated for weight loss, tumor mass, and overall appearance every 3 days. D54 tumor mass (in cubed millimeters) was determined as described in (Dykes et al., 1992) or for CFPAC tumors by measuring with calipers two dimensions for hemispherical shaped tumors (length (l) and width (w); mass = 0.4lw²) or three dimensions for oval-shaped tumors (length (l), width (w), and the distance between the closest edges (d); mass = 0.52lw²d). Mice died from the natural progression of their disease process or were euthanized by carbon dioxide inhalation when the tumor mass was greater than 1500 mm³, the tumor was ulcerated, or the animal displayed premorbid behavior (imminent death from lethargy, respiratory depression, and/or severe weight loss).

**Results**

Figure 1 shows an experiment in which 6-methylpurine is released within a human (D54 glioma) tumor by virtue of E. coli PNP expression. A single dose of MeP-dR (134 mg/kg, i.p.) caused tumors to regress to 30% of the original volume.
significant (for example, at day 49, comparison to the vehicle-treated control group that was statistically MeP-dR at a dosage of 134 mg/kg effected a reduction in tumor size in the experiment has been repeated with very similar results. Treatment with the T

Results.

were analyzed by reverse phase HPLC for MeP-dR or MeP and its metabolites. Each value is the average of three tumors for the injection of [3H]MeP-dR, only 6-methylpurine and its metabolites. Four and 24 h after injection of [3H]MeP-dR, and an estimate of the weight (milligrams) was calculated as described under Materials and Methods. MeP-dR at these doses does not alter growth of nontransduced D54 (parental) tumors (data not shown). ● control, vehicle-treated; ○, 134 mg/kg/dose MeP-dR. This experiment has been repeated with very similar results. Treatment with MeP-dR at a dosage of 134 mg/kg effected a reduction in tumor size in comparison to the vehicle-treated control group that was statistically significant (for example, at day 49, p = 0.0002; Student's t test).

Although the tumors did not completely disappear, there was no evidence of tumor growth in any of these tumors for prolonged periods after administration of drug.

To examine the in vivo metabolism of MeP-dR, the identity of the radioactivity associated with tumors after i.p. injection of [3H]MeP-dR was determined. Table 1 demonstrates that there was a similar amount of radioactivity in both the D54 and D54-PNP tumors 30 min after injection of [3H]MeP-dR. Most of the radioactivity in the D54-PNP tumors, however, was detected as 6-methylpurine and its metabolites (96%), whereas in D54 tumors only 10% of the radioactivity was 6-methylpurine and metabolites. Four and 24 h after injection of [3H]MeP-dR, only 6-methylpurine and its metabolites were detected in the tumors. Of interest were the similar amounts of 6-methylpurine metabolites in the tumors 4 and 24 h after injection of [3H]MeP-dR. These results indicated that the T1/2 of 6-methylpurine metabolites in tumor tissue was over 24 h. In contrast to the prolonged retention of 6-methylpurine metabolites in tumor tissues, serum half-lives of MeP-dR (approximately 15–20 min) and 6-methylpurine (~60 min) are relatively short (Fig. 2).

The maximally tolerated dose of 6-methylpurine was found to be approximately 1 mg/kg b.wt. given daily (i.p.) for 9 days. Figure 3 demonstrates antitumor effects of 6-methylpurine after direct intratumoral injection in human gliomas. A treatment regimen of 4.5 mg/kg 6-methylpurine given i.t. every day for 3 days led to a growth delay in tumors of approximately 18 days. A single intratumoral dose of 10 mg/kg tested under similar conditions led to a 16-day delay in tumor growth compared with controls (data not shown). Experiments in human pancreatic tumors are shown in Fig. 4. At the highest dose tested (10 mg/kg 6-methylpurine/kg b.wt. given i.t. for 3 days), animals had no tumor growth but were dead by 18 days. 6-Methylpurine (5 mg/kg) elicited significant antitumor effects and cures with no deaths [4% weight loss, 3 of 15 animals in this group and 1 of 10 mice given 1.7 mg/kg were long term survivors (160 days)].

Discussion

These findings establish persistence of 6-methylpurine and its metabolites specifically within tumor tissues following prodrug activation, and provide useful information regarding the mechanism of in vivo bystander killing mediated by E. coli PNP. The long intratumoral half-life of toxic metabolites released by E. coli PNP is likely to facilitate tumor cell killing in this setting, including destruction of nonproliferating tumor cells. The results in Table 1 also suggest intermediate endpoints that could contribute to understanding and optimizing prodrug activation systems in vivo. Previous studies with HSV-tk, cytosine deaminase, and PNP have not emphasized the importance of direct in vivo measurements of prodrug cleavage. The present experiments describe a new assay for understanding the robust in vivo antitumor effects that have been observed previously with E. coli PNP (Parker et al., 1997; Martinello-Wilks et al., 1998; Puhlmann et al., 1999; Gadi et al., 2000) and define threshold levels of prodrug conversion in vivo that may be useful predicting tumor re-gression in the future.

Because the plasma half-life of MeP-dR in mice is on the order of 20 min and the D54 tumor doubling time is approximately 10 to 15 days, the data shown in Fig. 1 supports strong activity of the systemically administered drug against both the dividing and nondividing tumor compartments. The regressions observed with this slow growing tumor (i.e.,

Table 1

<table>
<thead>
<tr>
<th>Tumor</th>
<th>E. coli PNP Activity</th>
<th>Time after Injection</th>
<th>MeP-dR</th>
<th>MeP-Metabolites</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>nmoles/mg/h</td>
<td>h</td>
<td>pCi</td>
<td></td>
<td>gCi</td>
</tr>
<tr>
<td>D54-PNP</td>
<td>200</td>
<td>0.5</td>
<td>500 ± 260*</td>
<td>14,000 ± 4,200</td>
<td>14,500</td>
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<tr>
<td></td>
<td></td>
<td>4</td>
<td>0</td>
<td>8,100 ± 550**</td>
<td>8,600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>0</td>
<td>7,200 ± 1,300*</td>
<td>7,500</td>
</tr>
<tr>
<td>D54</td>
<td>0</td>
<td>0.5</td>
<td>13,000 ± 3,100</td>
<td>1,400 ± 870</td>
<td>14,400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>0</td>
<td>1,800 ± 250</td>
<td>1,800</td>
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<tr>
<td></td>
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<td>24</td>
<td>0</td>
<td>1,300 ± 240</td>
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Compared to D54: *p < 0.02; **p < 0.0005 (Student's t test).

Fig. 1. Response of s.c. D54-PNP tumors to treatment with a single dose of MeP-dR. Twelve (nu/nu) mice were inoculated subcutaneously with D54 (human glioma) tumor cells transduced with a retrovirus mediating stable expression of the E. coli PNP gene, which was under the regulatory control of the SV-40 early promoter. Twenty-four days after inoculation, MeP-dR was given as a single i.p. injection to six of the animals. The tumors were measured approximately two times each week after injection of MeP-dR, and an estimate of the weight (milligrams) was calculated as described under Materials and Methods. MeP-dR at these doses does not alter growth of nontransduced D54 (parental) tumors (data not shown). ● control, vehicle-treated; ○, 134 mg/kg/dose MeP-dR. This experiment has been repeated with very similar results.
within a few days of treatment) suggest that both dividing and nondividing tumor cells are being eliminated. Although a strategy that kills both dividing and nondividing tumor cells may serve an important role in cancer therapy, additional studies will be necessary to fully understand the mechanisms underlying these observations and their relationship to in vitro cell killing. For example, in vivo properties related to “pockets” of high-level intratumoral PNP expression, radius of diffusion for 6-methylpurine, and interstitial fluid clearance (prodrug and toxin), influence bystander killing in a fashion that does not apply to the in vitro situation. Because there are significant differences between in vitro and in vivo half-life of both prodrug and toxin, the experiments described here apply primarily to the process of in vivo tumor cell killing by E. coli PNP.

These studies also provide one test of the barriers to suicide gene therapy by demonstrating regressions of refractory cancers after only a single dose of prodrug. Much longer courses (e.g., several days to weeks of prodrug therapy for HSV-tk or cytosine deaminase (Freeman et al., 1993; Ram et al., 1993; Huber et al., 1994; Tapscott et al., 1994; Beck et al., 1995; Fick et al., 1995; Elshami et al., 1996; Sacco et al., 1996; Dilber et al., 1997; Imaizumi et al., 1998)) may eliminate the cells capable of prodrug activation, diminish bystander killing, and tend to disable the overall approach.

Important risks are engendered by an anticancer strategy designed to kill both dividing and nondividing cells. Bystander killing in this setting could damage normal tissues (e.g., surrounding the tumor or elsewhere in the host) and result in a loss of selectivity. On the other hand, the ability to kill nondividing tumor cells may ultimately be crucial to the treatment of many common human cancers, particularly those that are refractory to conventional therapies because of a low growth fraction. In this regard, antitumor effects noted in the present studies (and those of others) have been achieved without significant weight loss, animal death, or other evidence of unmanageable (collateral) damage to surrounding tissue (Parker et al., 1997; Martiniello-Wilks et al., 1998; Puhlmann et al., 1999). Although toxins generated and concentrated within a tumor mass are significantly diluted when they escape to the rest of the body, the success of any anticancer therapy (including suicide gene approaches) requires a measure of selective tumor targeting. Therefore, progress and efforts toward targeting therapeutic genes specifically to tumors (e.g., modifying vector tropism and tumor specific promoters) are of particular relevance to emerging suicide gene strategies such as those described here.

The unusually strong antitumor effects of a single dose of MeP-dR and the long retention time of 6-methylpurine and
its metabolites suggested that 6-methylpurine itself might be useful for intratumoral injection. Because the maximally tolerated (total) dose of 6-methylpurine is similar whether administered intratumorally or intraperitoneally, the results in Figs. 3 and 4 suggest that a substantial fraction of 6-methylpurine escapes into the systemic circulation after i.t. administration. Nevertheless, significant antitumor effects in the gliomas and complete regressions and cures in pancreatic cancers were noted after only three doses of 6-methylpurine.

Taken together, these experiments suggest the importance of direct intratumoral injection as a preclinical endpoint in the development of suicide gene therapies for cancer. In tumors such as the human glioma model tested here, intracellular generation of toxin by a suicide gene can help establish high local concentrations, a prolonged tumor half-life, and stronger tumor regressions with less systemic toxicity than can be achieved using intratumoral toxin inoculation. The experiments help clarify the mechanism of action of a drug (6-methylpurine) with features well suited for use in experimental approaches to human cancer therapy.

Acknowledgments

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References


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