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A LONG ACTING SUICIDE GENE TOXIN, 6-METHYL PURINE, INHIBITS SLOW GROWING TUMORS AFTER A SINGLE ADMINISTRATION

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METHYL PURINE CANCER THERAPY

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Nonstandard abbreviations: PNP = purine nucleoside phosphorylase; 6-MeP-dR = 9-β-D-[2-deoxyribofuranosyl]-6-methylpurine; HSV-tk = herpes simplex virus thymidine kinase

Section Assignment: Chemotherapy, Antibiotics, & Gene Therapy

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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 intraperitoneal (IP) 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 *E. 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 CF PAC 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 non-dividing 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* ($T_{1/2}$ >24 hours, compared to a serum half life of < 1 hour). By HPLC, metabolites of [^{3}H]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.

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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 non-metastatic, locally invasive cancers account for over 100,000 cases per year, in the United States alone, and the majority lead to death (SEER, 1999 and DeVita, *et.al.*, 1997). Treatment of these types of cancer remains a significant therapeutic challenge. Non-surgical modalities (chemotherapy, 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) (Dionne, *et.al.*, 1998, Sadi and Barrack, 1991, Giangaspero, *et.al.*, 1987, Vescio, *et.al.*, 1990, and 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 *E. coli* cytosine deaminase (CD) have been tested previously for releasing concentrated chemotherapy within tumors, they seem less likely to be useful in exploring the above question (Ram, *et.al.*, 1993, Dilber, *et.al.*, 1997, Sacco, *et.al.*, 1996, Beck, *et.al.*, 1995, Elshami, *et.al.*, 1996, Fick, *et.al.*, 1995, Imaizumi, *et.al.*, 1998, Freeman, *et.al.*, 1993, and Huber, *et.al.*, 1994). Ganciclovir monophosphate (the toxin generated from ganciclovir by HSV-tk) cannot be given by direct intratumoral injection, because the plasma membrane is impermeant to

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phosphorylated nucleosides. Direct injection of even very high levels of 5-flurouracil, (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, since 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 anti-tumor effects *in vivo* (e.g. when 1 in 1000 cells express PNP) have been observed previously (Hughes, *et.al.*, 1995, Hughes, *et.al.* 1998, Parker, *et.al.*, 1997, and 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.*, 1999, and 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 non-dividing cells *in vitro* (Secrist, *et.al.*,1999 and Parker, *et.al.*, 1998). Whether the same is true *in vivo* is not known.

In the present study, we therefore examined *in vivo* anti-tumor activity and mechanism of

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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, 6-MeP-dR (9-β-D-[deoxyribofuranosyl]-6-methylpurine), 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 anti-tumor 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 hours) of toxic metabolites within tumor tissue. Based on the above results, we also examined anti-tumor effects after direct injection of 6-methylpurine into growing tumors *in vivo*.

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Methods

Establishment of human glioma tumors

pLNSX, a gift of Dr. D. Miller (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 x 10^7) were injected SC 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 (mg) 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-^{3}H], Moravek Biochemicals, Brea, California) 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 3 tumors. MeP-dR and its metabolites were eluted from a Spherisorb ODSI (5 μm) column (Keystone Scientific Inc., College, PA, USA) 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

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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 (unpublished observation), the phosphorylated metabolites should be only phosphates of 6-methylpurine-riboside. The fact that these metabolites were retained in the tumors for more than 24 hours 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 Chemical Company (St. Louis, MO, USA). A modification 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 preestablished tumors

The susceptibility of CFPAC-1 cells (Bradbury, *et.al.*, 1992) to 6-methylpurine was determined in SCID mice implanted subcutaneously in the flanks with 20 million CFPAC-1 cells. Tumors greater than 75 mm^3 tumor volume were injected intra-tumorally (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 the approximate amount of 6-methylpurine animals could tolerate when given i.p. In glioma tumors, 6-methylpurine was given by intratumoral injection in 100μl of normal saline every day for a total of 3 days (days 18, 19,

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and 20 post-implantation) 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 mm^3) was determined as described in (Dykes, *et.al.*, 1992), or for CFPAC tumors by measuring with calipers two dimensions for hemi-sperical-shaped tumors (length (l) and width (w); mass = $0.4lw^2$) or three dimensions for oval-shaped tumors (length (l), and width (w), and the distance between the closest edges (d); mass = $0.52lwd$). Mice died from the natural progression of their disease process or were euthanized by carbon dioxide inhalation when the tumor mass was greater than 1,500 mm^3, the tumor was ulcerated, or the animal displayed premorbid behavior (imminent death from lethargy, respiratory depression, and/or severe weight loss).

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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, intraperitoneally) caused tumors to regress to 30% of the original volume. 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.

In order to examine the *in vivo* metabolism of MeP-dR, the identity of the radioactivity associated with tumors after ip injection of [^{3}H]MeP-dR was determined. Table 1 demonstrates that there was a similar amount of radioactivity in both the D54 and D54-PNP tumors thirty minutes after injection of [^{3}H]MeP-dR. However, most of the radioactivity in the D54-PNP tumors 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 hours after injection of [^{3}H]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 hours after injection of [^{3}H]MeP-dR. These results indicated that the $T_{1/2}$ of 6-methylpurine metabolites in tumor tissue was over 24 hours. 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 (Figure 2).

The maximally tolerated dose of 6-methylpurine was found to be approximately 1 mg/kg body weight given daily (intraperitoneally) for 9 days. Figure 3 demonstrates anti-tumor 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

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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 Figure 4. At the highest dose tested (10 mg/kg 6-methylpurine/kg body weight given i.t. for 3 days), animals had no tumor growth but were dead by 18 days. Five mg/kg 6-methylpurine elicited significant anti-tumor effects and cures with no deaths (4% wt loss, 3 of 15 animals in this group and 1 of 10 mice given 1.7 mg/kg were long term survivors, (160 days)).

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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 non-proliferating 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, Gadi, *et.al.*, 2000, Martiniello-Wilkes, *et.al*, 1998, and Puhlmann, *et.al.*, 1999) and define threshold levels of prodrug conversion *in vivo* that may be useful predicting tumor regression in the future.

Because the plasma half-life of MeP-dR in mice is on the order of 20 minutes and the D54 tumor doubling time is approximately 10-15 days, the data shown in Figure 1 supports strong activity of the systemically administered drug against both the dividing and non-dividing tumor compartments. The regressions observed with this slow growing tumor (i.e. within a few days of treatment) suggest that both dividing and non-dividing tumor cells are being eliminated. While a strategy that kills both dividing and non-dividing tumor cells may serve an important role in cancer therapy, additional studies will be necessary in order 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

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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 (Ram, *et.al.*, 1993, Dilber, *et.al.*, 1997, Sacco, *et.al.*, 1996, Beck, *et.al.*, 1995, Elshami, *et.al.*, 1996, Fick, *et.al.*, 1995, Imaizumi, *et.al.*, 1998, Freeman, *et.al.*, 1993, Tapscott, *et.al.* , 1994, and Huber, *et.al.*, 1994)) 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 non-dividing 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 non-dividing 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, anti-tumor 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.*, 1999, and 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

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measure of selective tumor targeting. Therefore, progress and efforts towards targeting therapeutic genes specifically to tumors (e.g. modifying vector tropism, tumor specific promoters) are of particular relevance to emerging suicide gene strategies such as those described here.

The unusually strong anti-tumor 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 Figures 4 and 5 suggest that a substantial fraction of 6-methylpurine escapes into the systemic circulation after *i.t.* administration. Nevertheless, significant anti-tumor 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 pre-clinical 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 innoculation. 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.

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Footnotes

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Figure 1: Response of SC 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 2 times each week after injection of MeP-dR and an estimate of the weight (mg) was calculated as described in the Methods section. MeP-dR at these doses does not alter growth of non-transduced 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).

Figure 2: 6-MeP-dR cleavage *in vivo*. Non-tumor bearing animals were sacrificed various times after injection of (67 mg/kg IP) MeP-dR, and the amount of MeP-dR and 6-methylpurine in the plasma was determined using reverse phase HPLC. Within several minutes, low levels of 6-methylpurine were detected in the blood. Each data point represents the average (±SD) of 3 mice. A repeat of this experiment led to similar results.

Figure 3: Response of D54 CNS tumors to treatment with 6-methylpurine. Glioma tumors were established as above, and 6-methylpurine was given by intratumoral injection in 100μl of normal saline every day for a total of 3 days (days 18, 19, and 20 post-implantation). Six mice

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were studied per group. ●, median weight, control (vehicle) treated; ▾, 3 mg/kg/dose 6-methylpurine; ○, 4.5 mg/kg/ dose 6-methylpurine. Treatment with 6-methylpurine at a dosage of 4.5 mg/kg effected a reduction in tumor size that was statistically significant (for example, at day 49, p=0.0047, one-way analysis of variants).

Figure 4: Response of human pancreatic (CFPAC) tumors to direct intratumoral injection with 6-methylpurine. Tumors were established by inoculating 2 x 10^7 cells into the flanks of scid mice. When the tumors reached the size of approximately 75 mm^3, 6-methylpurine was given by intratumoral injection in 50 μl of normal saline every day for a total of 3 days. At least five animals were studied per group. Treatment with 6-methylpurine at a dosage of 5 mg/kg effected a reduction in tumor size in comparison to the vehicle treated control group that was statistically significant (for example, at day 21, $p<0.0001$, one-way analysis of variants).

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Table 1: Radioactivity in tumor tissue of animals treated with [^{3}H]MeP-dR. Tumors, established as in Figure 1 were removed 0.5, 4, and 24 hours after ip injection of [^{3}H]MeP-dR (67 mg/kg, 1.5 Ci/mole). Methanol extracts of each tumor were analyzed by reverse phase HPLC for MeP-dR or 6-methylpurine (MeP) and its metabolites. Each value is the average of 3 tumors ±SEM. This experiment was repeated with similar results. Compared to D54: *p< 0.02; **p<0.0005 (Student's t-test)

tumor	*E. coli* PNP activity	Time after injection	MeP-dR	MeP-metabolites	total
	(nmoles/mg/hr)	(hours)	(pCi)	(pCi)	(pCi)
D54-PNP	200	0.5	500 ± 260*	14,000 ± 4,200	14,500
		4	0	8,100 ± 550**	8,100
		24	0	7,200 ± 1,300*	7,200
D54	0	0.5	13,000 ± 3,100	1,400 ± 870	14,400
		4	0	1,800 ± 250	1,800
		24	0	1,300 ± 240	1,300

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Figure 1

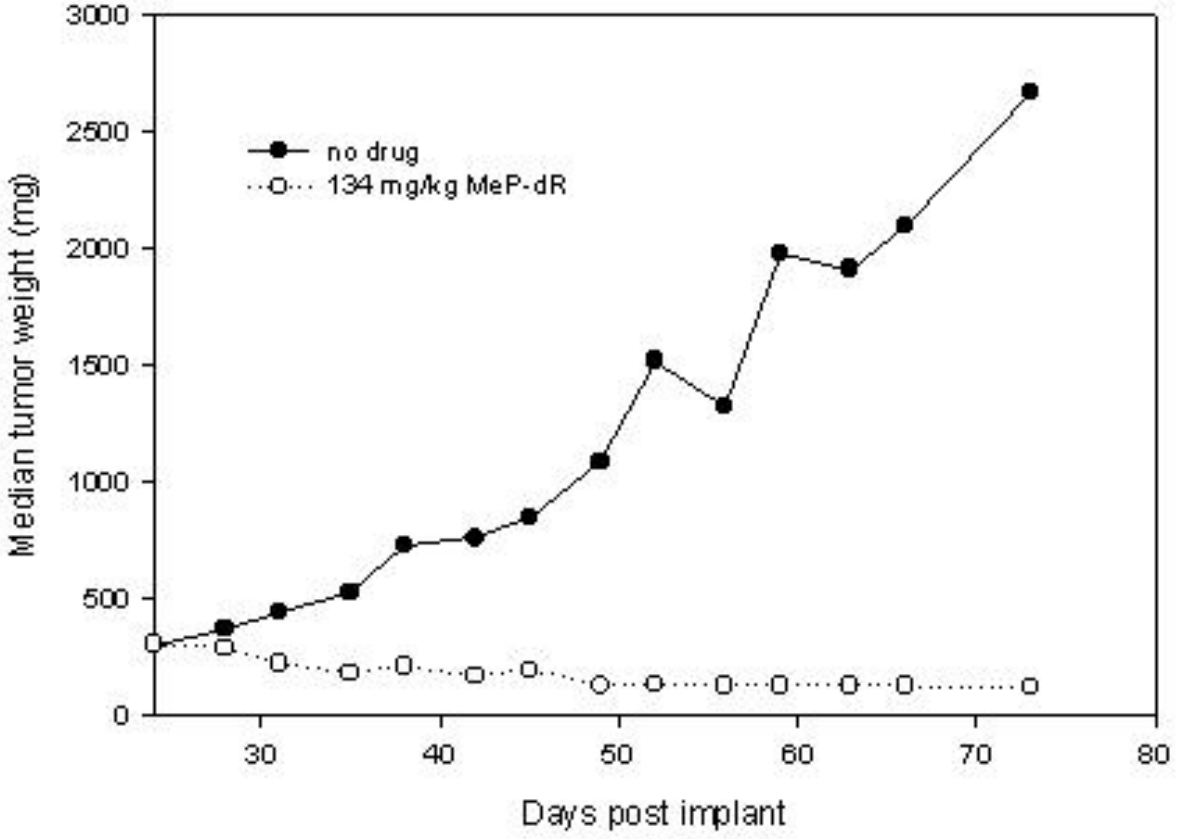

3000
2500
2000
1500
1000
500
0
Median tumor weight (mg)
no drug
134 mg/kg MeP-dR
30
40
50
60
70
80
Days post implant

Figure 2

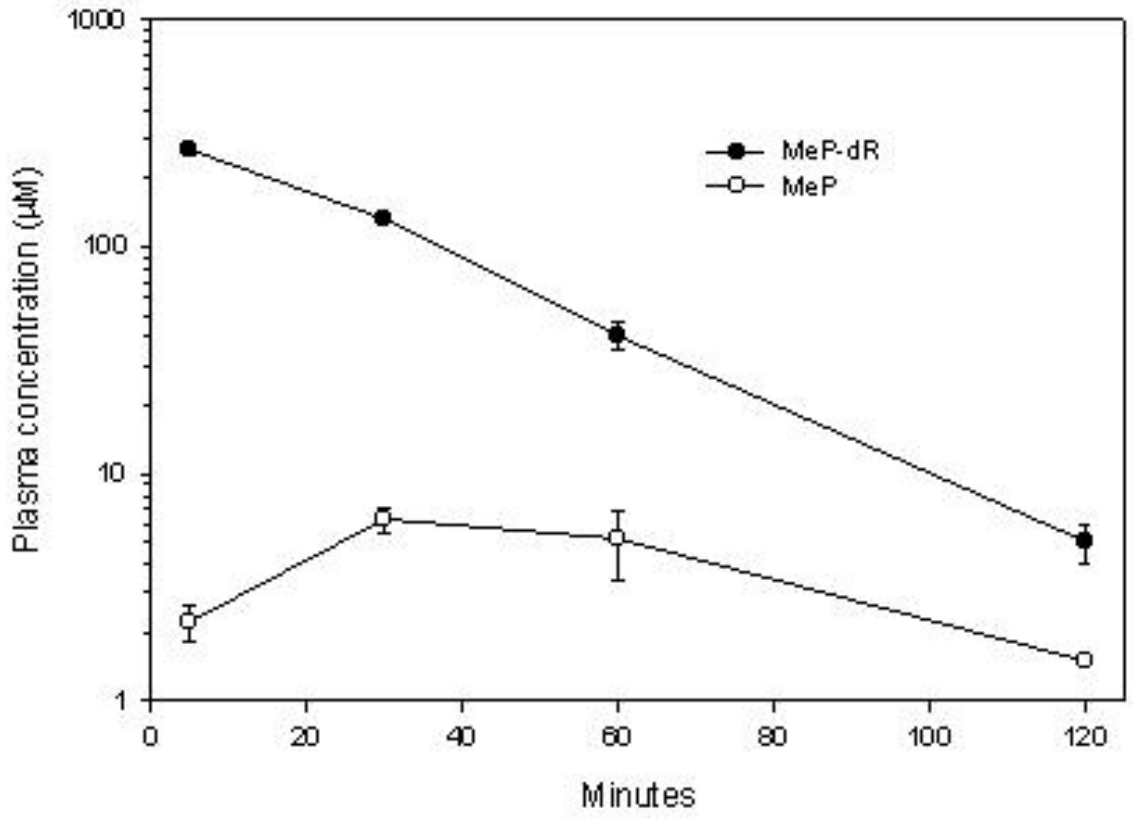

1000
100
10
1
Plasma concentration (µM)
0
20
40
60
80
100
120
Minutes
MeP-dR
MeP

Figure 3

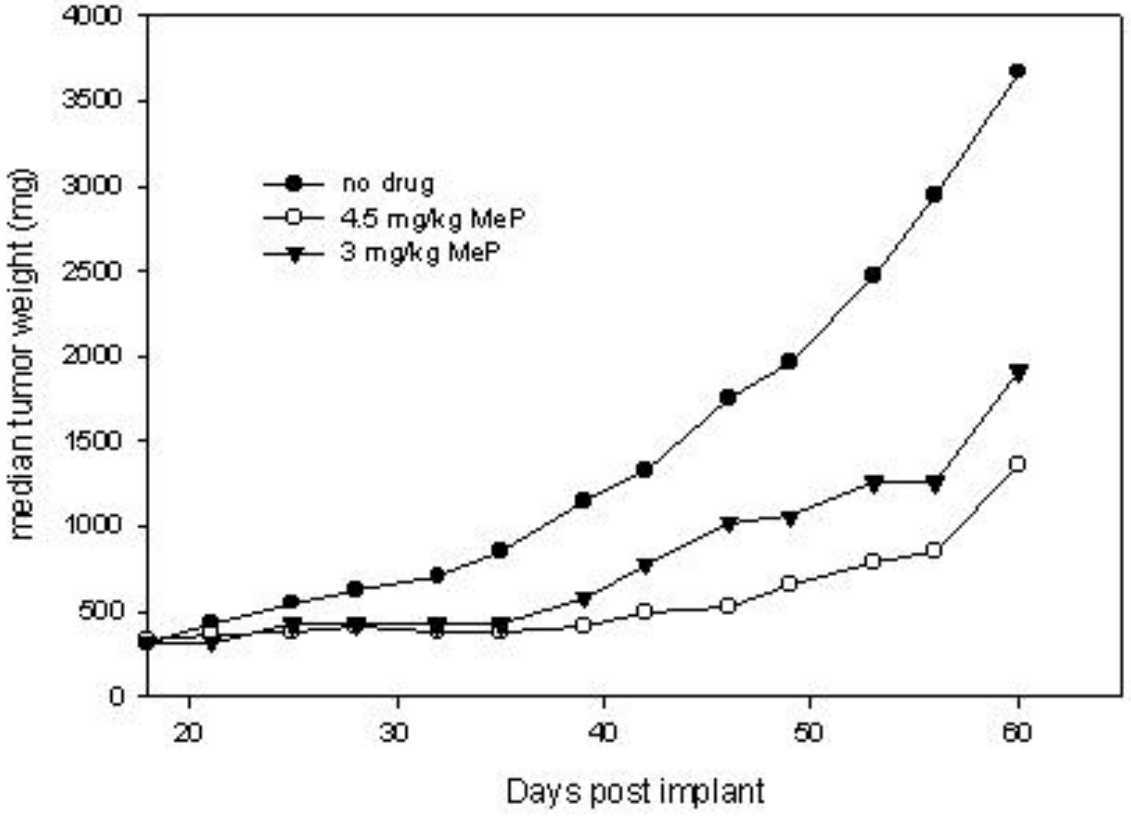
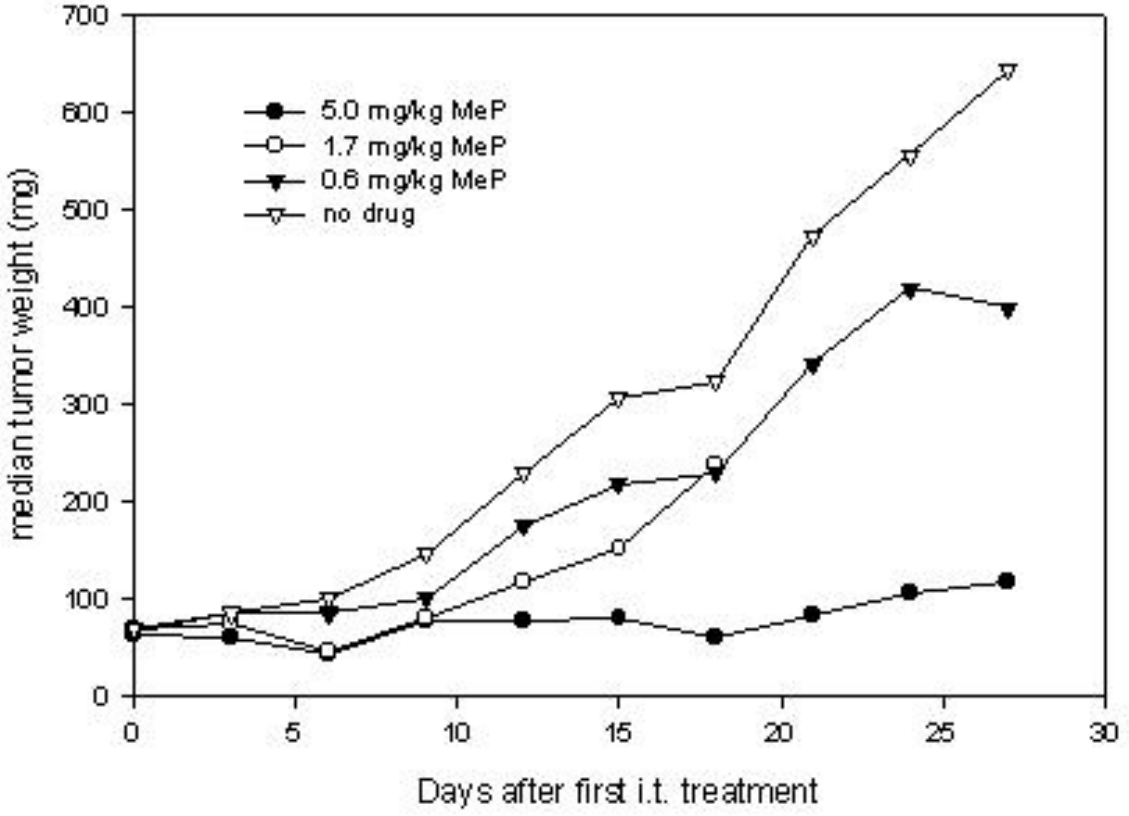

4000
3500
3000
2500
2000
1500
1000
500
0
median tumor weight (mg)
no drug
4.5 mg/kg MeP
3 mg/kg MeP
20
30
40
50
60
Days post implant

Figure 4


5.0 mg/kg MeP
1.7 mg/kg MeP
0.6 mg/kg MeP
no drug
median tumor weight (mg)
Days after first i.t. treatment
0
100
200
300
400
500
600
700
0
5
10
15
20
25
30