Expression of *Escherichia coli* purine nucleoside phosphorylase (PNP) activates prodrugs and kills entire populations of mammalian cells, even when as few as 1% of the cells express this gene. This phenomenon of bystander killing has been previously investigated for herpes simplex virus-thymidine kinase (HSV-TK) and has been shown to require cell to cell contact. Using silicon rings to separate *E. coli* PNP expressing cells from non-expressing cells sharing the same medium, we demonstrate that bystander cell killing by *E. coli* PNP does not require cell-cell contact. Initially, cells expressing *E. coli* PNP convert the non-toxic prodrug, 6-methylpurine-2'-deoxyriboside (MeP-dR) to the highly toxic membrane permeable toxin, 6-methylpurine (MeP). As the expressing cells die, *E. coli* PNP is released into the culture medium, retains activity, and continues precursor conversion extracellularly (as determined by reverse phase high performance liquid chromatography of both prodrug and toxin). Bystander killing can also be observed in the absence of extracellular *E. coli* PNP by removing the MeP-dR prior to death of the expressing cells. In this case, 100% of cultured cells die when as few as 3% of the cells of a population express *E. coli* PNP. Blocking nucleoside transport with nitrobenzylthioinosine reduces MeP-dR mediated cell killing but not MeP cell killing. These mechanisms differ fundamentally from those previously reported for the HSV-TK gene.

Pro-drug activation by the HSV-TK\(^1\) gene has been suggested as a potentially useful therapy for abnormal proliferation of cells in the setting of coronary artery restenosis (1), AIDS (2), graft versus host disease (3), rheumatoid arthritis,\(^2\) and a variety of human tumors (4–6). We have previously described an approach in which extremely toxic purine bases, capable of inhibiting protein synthesis, RNA synthesis, and DNA synthesis can be generated inside human cells by *E. coli* purine nucleoside phosphorylase (PNP) (7–10). The systemic toxicity of the strategy was minimized by careful prodrug dosing, leading to tumor remissions and cures in animals carrying tumors that stably expressed *E. coli* PNP (9). The usefulness of the approach has been confirmed by several other laboratories, using a variety of viral and non-viral methods to deliver the PNP gene *in vitro* and *in vivo*, and has been suggested as an important alternative to the HSV-TK gene (11–15).

The ability of enzyme/prodrug systems to kill nearby cells that are not expressing an exogenous enzyme might allow eradication of these cells even when only a small fraction of the cells express the foreign gene (11, 16). A few enzyme/prodrug systems have exhibited this important property of "bystander killing," including HSV-TK/ganciclovir, *E. coli* cytosine deaminase/5-fluorocytosine, and *E. coli* PNP/6-methylpurine-2'-deoxyriboside (MeP-dR) (8, 16, 17). Of the three, only PNP/MeP-dR has been shown to kill an entire culture of cells when approximately 1 in 100 cells stably express the transgene (7–9). In transient transfection, expression of *E. coli* PNP in as few as 1 in 1000 cells kills all of the bystander cells (7, 8).\(^3\) The mechanism of killing involves the cleavage of MeP-dR by *E. coli* PNP to release the cell toxin 6-methylpurine (MeP), which can freely diffuse in and out of cells (Fig. 1). Measured levels of MeP production correlate with the degree of cell killing (8, 9). The importance of the development of methods that optimize bystander killing is evident, since it is not currently possible to deliver a reporter or therapeutic gene to greater than 10 to 20% of cells in most abnormal tissue with any of the available *in vivo* gene delivery protocols.

We have hypothesized that potent bystander killing by *E. coli* PNP is mediated by intracellular generation of the MeP toxin that is released into the local environment, and that cell contact is not necessary for killing to occur. In this study, we show that the *E. coli* PNP/MeP-dR system can kill bystander cells physically separated from PNP expressing cells. We demonstrate a role for *E. coli* PNP released by dying cells in the extracellular conversion of prodrug to toxin and define the kinetics of *E. coli* PNP activity with respect to prodrug exposure time and concentration. Furthermore, we show that cell proliferation is abolished in a fashion that exhibits both time and dose dependence upon either the amount of prodrug added or the level of *E. coli* PNP expressed.

### Experimental Procedures

**Cell Lines—**D54 were human glioma cells obtained from the American Tissue Type Collection. MT539, mouse glioma cells, were a generous gift from Yancy Gillespie, University of Alabama, Birmingham. All cells were cultured in minimal essential medium with Earle's salts and 10% fetal bovine serum. D54-PNP and MT539-PNP cells were generated using an amphotropic retrovirus and the shuttle vector pLNSX to express *E. coli* PNP under SV40 promoter control (as described elsewhere (7, 8)).

**Cloning Ring Assay—**To create a cloning ring barrier, the upper
D54 cells in 96-well plates were treated for 1 h with either 100 μM MeP-dR or vehicle was placed into each well. To fix the cells, the medium was aspirated and replaced by 2.5% glutaraldehyde in phosphate-buffered saline with Mg²⁺ and Ca²⁺, and the cells were incubated at room temperature for 10 min. The wells were then washed once with phosphate-buffered saline and covered with 2 ml of phosphate-buffered saline until the entire plate was ready to stain. Following fixation of all of the wells in a plate, the cells were stained by incubating 1 ml of staining solution (1 mg/ml crystal violet in 70% ethanol) in each well for 10 min at room temperature and then immersing the plate in a beaker under running water until no more dye was released.

Measurement of PNP Activity in D54 Cell Pellets and Medium—D54-PNP cells were seeded at 30% confluence and grown for 5 days in a T-75 flask with or without MeP-dR. Cells and culture medium were removed from the flasks at the beginning of the experiment and after 5 days of culture. The samples were prepared and the activity of E. coli PNP was determined using MeP-dR as a substrate as described (8). The amount of MeP produced was determined by reverse phase high performance liquid chromatography (8).

Cell Proliferation Assays—MT539 mouse glioma cells were grown in 96-well plates and 100 μM MeP-dR was added. Proliferation was measured every other day using a cell proliferation assay (Celltiter 96 AQ, Promega) which detects the accumulation of tetrazonium salts by living cells (below). D54 human glioma cells were seeded at 3 × 10⁴ cells/cm² in 96-well plates. For the time course assay, 100 μM drug in 100 μl of medium was added to each well. At 2, 4, 8, 24, 48, 72, and 96 h, the drug was removed, the cells were washed with 250 μl of medium, and fresh medium was added. For studies of MeP-dR and MeP dose dependence, increasing concentrations of drug were incubated for 4 days. The cells were then washed with 250 μl of medium and fresh medium was added. Proliferation was measured every day by tetrazolium dye assay (18). The MTT was then removed and 100 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (5 mg/ml MTT in phosphate-buffered saline) was added and incubated at 37 °C for 2 h. The MTT was then removed and 100 μl of dimethyl sulfate was added. After pipetting, the chromom containing dimethyl sulfate solution was transferred to another 96-well tray and absorbance at 490 nm was read and compared with a standard curve for cell number calculation. Each point represents the average of three wells.

Nucleoside Transport Assay—S-(p-Nitrobenzyl)-6-thio-inosine (NBMPR) (Sigma) is an inhibitor of nucleoside transport and is prepared as a 10 mM stock solution in 50% dimethyl sulfate/sterile water. D54 cells in 96-well plates were treated for 1 h with either 1 μM NBMPR in medium or with vehicle (medium with an equivalent amount of dimethyl sulfate). The cells were then washed and treated for 6 h with vehicle, 1 μM NBMPR, 1 μM NBMPR plus 100 μM MeP-dR or MeP, or 100 μM MeP-dR or MeP. Following treatment, cells were washed and treated with MTT assay described above.

RESULTS

PNP/MeP-dR Bystander Killing Occurs after the Death of E. coli PNP Expressing Cells and Without Cell Contact—To investigate whether or not bystander killing of non-E. coli PNP cells requires cell contact, cloning rings were attached with vacuum grease to the center of cell culture wells. For each pair of wells, the outside of the ring was seeded with D54 parental (non-E. coli PNP expressing) “bystander” cells, while the inside of each ring was seeded with either D54-PNP cells (which constitutively express E. coli PNP) or D54 parental cells. In columns A and C, cells inside the ring are D54-PNP cells, and in columns B and D, the inside cells are D54 parental cells. Columns A and B were treated with 100 μM MeP-dR for 6 days. On each day, a row of cells was fixed and stained with crystal violet.

FIG. 1. Conversion of MeP-dR to MeP by E. coli PNP.

FIG. 2. Bystander killing occurs in the absence of cell contact. D54 cells seeded inside or outside a cloning ring (removed) are separated by a thin ring of vacuum grease. All surrounding cells (outside the ring) are D54 parental cells. In columns A and C, cells inside the ring are D54-PNP cells, and in columns B and D, the inside cells are D54 parental cells. Columns A and B were treated with 100 μM MeP-dR for 6 days. On each day, a row of cells was fixed and stained with crystal violet.
tative decrease in growth. Over the next 4 days, D54-PNP center cells treated with MeP-dR declined to near zero. D54-parental bystander cells surrounding the D54-PNP cells treated with MeP-dR had stopped growing by day 3 (Fig. 2, column A). These bystander cells then declined rapidly to less than 1% of control cells (based on microscopic visual examination). No decrease in growth was seen in MeP-dR treated D54-parental center wells (Fig. 2, column B) or in untreated wells (Fig. 2, columns C and D). The lack of cell contact did not limit the ability of PNP transduced cells to kill bystander cells in the presence of MeP-dR. The decrease in the number of parental cells on day 6 lagged behind the death of D54-PNP cells by about 2 days (Fig. 2, column A), suggesting that the parental cells were exposed to a lethal event about 2 days after the D54-PNP cells were exposed.

_E. coli PNP Released into the Cell Medium Is Active and Stable Over Time_—To test whether _E. coli_ PNP was released by dying _E. coli_ PNP expressing cells, and whether the extracellular enzyme could contribute to MeP-dR conversion, we determined the amount of _E. coli_ PNP activity present in either the medium or a cell pellet from a culture of D54-PNP cells (Fig. 3). Day 0 represents the baseline amount of _E. coli_ PNP activity in cell pellet and culture medium of D54-PNP cells 24 h after plating. The amount of _E. coli_ PNP activity in the cell pellets and medium from D54-PNP cells grown for 5 days in the presence or absence of 100 μM MeP-dR was determined. All D54-PNP cells treated with MeP-dR died by day 5 of drug treatment. The amount of _E. coli_ PNP activity found in the medium of cultures killed with MeP-dR was 10-fold greater than the amount of PNP activity in medium of untreated cells. The _E. coli_ PNP activity in the culture medium of D54-PNP cells treated with MeP-dR represents the total PNP activity released by the dying cells over several days. The total amount of _E. coli_ PNP activity in the untreated D54-PNP cell pellet at day 5 was approximately 50% lower than the activity in the culture medium of cells after treatment with MeP-dR, which could suggest that the rate of PNP degradation inside of cells is higher than in the medium, or that the freeze/thaw protocol for cell lysis is a less efficient way to recover _E. coli_ PNP than the release of the enzyme from dying cells. These results suggest that extracellular _E. coli_ PNP released from dying cells remains active and stable over time.

**The Rate of MeP-dR Conversion Correlates with an Increased Rate of Cell Death**—To confirm the release and stability of extracellular _E. coli_ PNP, a second glioma cell line transduced with _E. coli_ PNP (MT-539-PNP) was mixed in varying proportions with non-expressing cells (MT-539 parental) (9). Cell proliferation and the concentrations of MeP-dR and MeP in the medium were measured over time. Even at 1% MT-539-PNP cells, all of the cells in the mixture died after MeP-dR treatment (Fig. 4). However, both the rate of cell death and the rate of MeP-dR conversion directly correlated with the increase in the proportion of cells expressing the PNP gene. MeP-dR conversion continued to rise at a steady rate even after all the cells in the culture were dead, confirming the stability of PNP enzyme in the medium. Because the rate of conversion was constant over time and dependent upon the percentage of cells initially expressing PNP, there appears to be little loss of _E. coli_ PNP activity due to degradation inside or outside of the cell. The smallest amount of MeP-dR converted to MeP that was sufficient to kill 100% of parental cells was approximately 4% (4 μM MeP). Equivalent amounts of MeP added directly to parental cells have been shown to mediate 100% cell killing in numerous cell lines (8) (Fig. 5D).

**A Brief in Vitro Exposure to Prodrug or Toxin Leads to Cell Death**—In D54-PNP cells, an exposure to 100 μM MeP-dR for as little as 1 h led to a 50% decrease in growth of the cells by day 4 (Fig. 4A). Exposure for 4 h or longer led to complete cell population death. A similar time course is seen when D54 parental cells are treated with MeP (Fig. 5B). When D54-PNP cells were treated with various concentrations of MeP-dR for 4 days, as little as 0.78 μM MeP-dR caused a 25% reduction in growth (Fig. 5C). When grown in concentrations of 3.1 μM MeP-dR or greater, all of the D54-PNP cells eventually died (by day 8, data not shown). The dose-response of D54 parental cells...
to MeP mirrors the D54-PNP response to MeP-dR (Fig. 5D). In contrast, D54 parental cells grown in 100 μM MeP-dR for 4 days showed growth reduction of less than 20% and no change in growth at concentrations of MeP-dR at 50 μM and below (data not shown). These results show that a brief exposure of PNP expressing cells to high concentrations of MeP-dR (100 μM for 4–8 h) or a prolonged exposure to 30-fold less MeP-dR (equal to or greater than 3.1 μM for 4 days) killed all cells. In addition to providing evidence of prodrug dose dependence, these experiments suggest the possibility that a variety of dosing schedules of MeP-dR might be therapeutically useful in the development of in vivo models of E. coli PNP cell killing.

Prior to the Death of PNP Expressing Cells, Substantial Intracellular Conversion of Prodrug Leads to High Bystander Killing—To determine the relative contributions to prodrug conversion of intracellular E. coli PNP and extracellular (free) enzyme, a cloning ring assay was performed with D54-PNP cells inside and D54 parental cells outside of the rings. The experiments were designed to briefly expose cells to MeP-dR, and remove the prodrug at a time before substantial cell lysis had occurred. In Fig. 6A, the treated wells were exposed to 100 μM MeP-dR for 24 or 48 h, washed, and given fresh medium, as shown. In wells treated with MeP-dR for 24 h, substantial bystander killing was evident on day 8 of the experiment, while in wells exposed to MeP-dR for 48 h, nearly all bystander cells were dead by day 5 (Fig. 6, A and C). The cell density of MeP-dR-treated D54-PNP cells (inside the ring) did not change between day 1 and day 2, suggesting that cell death was not pronounced during this period. However, profound bystander killing was observed by day 8, even when the prodrug, generated toxin, and any extracellular E. coli PNP enzyme were removed at the end of day 1. These results suggested that a strong bystander killing effect does not depend on the death of E. coli PNP expressing cells and consequent release of active enzyme into the extracellular environment.

To further examine the effect of intracellular prodrug conversion on bystander killing, 1 and 3% mixtures of D54-PNP cells and D54 parental cells were treated with 100 μM MeP-dR for 48 h (a time period prior to a decrease in cell number). Afterward, the medium was exchanged (to remove any prodrug, toxin, or extracellular enzyme), and cell growth was measured using a MTT assay (Fig. 6D). Four days after exchanging medium, the 3% mixture was completely killed. The 1% mixture grew briefly and then declined. Although PNP mediated cell killing can be augmented by extracellular conversion of prodrug by free enzyme (Fig. 4), the result in Fig. 6D suggests that even without extracellular enzyme involvement, as few as 3% of cells need to express E. coli PNP for MeP-dR treatment to cause complete bystander killing.

MeP-dR Utilizes a Nucleoside Transporter to Enter Cells—The human equilibrative nucleoside transporter (hENT1) has recently been cloned and characterized (19). Because hENT1 facilitates the transport of nucleosides but not bases, hENT1 might be involved in MeP-dR transport, but not MeP transport. We used the drug NBMPR, an inhibitor of hENT1 transport, to block MeP-dR transport into D54-PNP cells (Fig. 7A). D54-PNP cells treated with 1 μM NBMPR and 100 μM MeP-dR for 6 h grew modestly throughout the experiment. In the absence of NBMPR treatment, all of the MeP-dR-treated cells died. This result indicated that nucleoside transporter inhibition blocks cellular entry and cell killing by MeP-dR. The NBMPR had no effect on D54 parental cells treated with 100 μM MeP (Fig. 7B) showing that uptake mechanisms for MeP differ from that of MeP-dR.

**DISCUSSION**

Sensitization to MeP-dR has a high bystander effect in vitro. When 1% of a population of cells express the E. coli PNP gene, substantial toxicity to non-expressing cells occurs after addition of MeP-dR (Figs. 4 and 6). The mediator of the bystander killing is the toxin MeP (8) (Figs. 1, 4, and 5) which can diffuse between cells without the need for cell-cell contact (Fig. 2). A short time (1 h) is sufficient for a toxic effect of MeP-dR to occur, and a large therapeutic index between the lethal toxin dose in transduced cells (3–6 μM) and the dose of prodrug known to be tolerated in parental cells (100 μM, data not
FIG. 6. Effect of exposure to MeP-dR for 24 and 48 h on bystander killing. A, D54-PNP cells (inside rings) and D54 parental cells (outside rings) were exposed to 100 μM MeP-dR for 24 or 48 h, and then washed and given fresh medium (on days 1 or 2). Wells were fixed and stained on days 1, 2, 5, and 8. B, ×100 magnification of D54-PNP cells inside of the rings. C, ×100 magnification of D54 parental cells outside of the rings.

D, 1 and 3% mixtures of D54-PNP cells and parental cells, or parental cells by themselves in a 96-well plate were treated with 100 μM MeP-dR for 48 h, and then washed and given fresh medium. Growth was measured every day using a MTT assay.
shown) was observed in vitro. The early death of expressing cells and subsequent death of non-expressing cells was observed in mixing experiments (Figs. 2, 4, and 6). Moreover, bystander cells continue to die even after the death of the PNP expressing cells (Figs. 2 and 6). The PNP enzyme is very stable and can participate in extracellular prodrug conversion even after producing cells have died. However, substantial bystander killing with the HSV-TK gene has been a basis for multicenter clinical trials of central nervous system diseases, rheumatologic conditions, and other disease states. However, HSV-TK and cytosine deaminase cannot generate toxins that kill resting cells. Moreover, both of these alternatives lead to variable bystander killing when fewer than 10% of cells express the sensitization gene. Finally, at least for HSV-TK, extracellular enzyme does not participate in cell killing.

One can argue that the generation of very potent agents within a tumor or other tissue could lead to uncontrolled systemic toxicity, or that the surprising stability of the E. coli PNP enzyme after cell death might be limiting in vivo, due to the generation of toxin in undesirable sites within an animal host. However, the E. coli PNP approach has recently been supported by in vivo studies which show complete tumor regressions and cures of tumors stably expressing the E. coli PNP gene, without apparent host toxicity (9). The careful administration of the prodrug, therefore, appears to allow substantial anti-tumor effects despite both the highly toxic agents generated, and the stability of the E. coli PNP.

In summary, we have shown that during the course of bystander killing, the prodrug, MeP-dR, kills cells expressing E. coli PNP first. Killing of bystander cells by expressing cells is efficiently mediated by a membrane permeable toxin produced by both intracellular PNP and by extracellular enzyme released by dying cells. Bystander cell killing is dose and time dependent. The toxin is approximately 30-fold more potent than the prodrug in the cells studied here, and the levels of toxin that are generated are sufficient to explain the bystander killing that is observed. These features provide a way to study cell sensitization in a number of cell types and human disease states, that take advantage of a simple and efficient mechanism for bystander killing.

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Fig. 7. Blockage of the nucleoside transporter in D54-PNP cells inhibits MeP-dR action, but not MeP. Proliferation of cells in the presence of a combination of 1 μM NBMPR, 100 μM MeP-dR, or 100 μM MeP compared with vehicle control for: A, D54-PNP; or B, D54 parental cells.
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