Suicide gene/prodrug therapy using salmonella-mediated delivery of *Escherichia coli* purine nucleoside phosphorylase gene and 6-methoxypurine 2′-deoxyriboside in murine mammary carcinoma 4T1 model

Wei Fu, Hekui Lan, Shenghua Liang, Tong Gao and Daming Ren[1]

The State Key Laboratory of Genetic Engineering, Institute of Genetics, Fudan University, 220 Han Dan Road, Shanghai 200433, China

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Attenuated salmonella have been reported to be capable of both selectively growing in tumors and expressing exogenous genes for tumor-targeted therapy. As 6-methoxypurine 2′-deoxyriboside (MoPdR) is similar to 6-methylpurine 2′-deoxyriboside in structure, we aimed to evaluate the antitumoral effect of the *Escherichia coli* purine nucleoside phosphorylase (ePNP) gene, using an attenuated salmonella-mediated delivery system, in combination with MoPdR. A novel mutant serovar Typhimurium (SC36) was used to carry the pEGFP-C1-ePNP vector that contains an enhanced green fluorescent protein and an ePNP gene under the control of the cytomegalovirus promoter. The function of the ePNP expression vector was confirmed *in vitro* using the enzymic conversion of MoPdR into methoxypurine. We also observed a high bystander effect induced by the ePNP/MoPdR system with a very low proportion (1%) of ePNP-positive cells and 5 μg/mL MoPdR, although the growth of parental cells was affected appreciably by MoPdR. The killing effect and increased apoptosis induced by SC36 carrying the ePNP expression vector (SC/ePNP) were detected by cytotoxicity assay and propidium iodide staining flow cytometry analysis, in combination with MoPdR. SC/ePNP was given orally to mice bearing mammary carcinomas, and its antitumor effect was evaluated. SC/ePNP plus MoPdR significantly inhibited tumor growth by approximately 86.6–88.7% and prolonged the survival of tumor-hosting mice. Our data support the view that MoPdR combined with the ePNP gene could be used in gene-directed enzyme prodrug therapy. Attenuated salmonella could be a promising strategy to improve ePNP/MoPdR bystander killing due to its preferential accumulation and anticancer activity in tumors. (*Cancer Sci* 2008; 99: 1172–1179)

A hypoxic microenvironment is characteristic of many solid tumors. Current limitations of gene therapies for malignant tumors include lack of cancer-specific targeting strategy and insufficient tumor delivery.[1] To resolve these problems, *Salmonella enterica* serovar typhimurium (*S. typhimurium*) strains, which are facultative anaerobes, have been considered intriguing candidates because of their selective growth in tumors and essential ability to deliver exogenous genes encoding therapeutic proteins.[2,3] To increase their applicability for treatments, the *S. typhimurium* strains were first attenuated by purine and other auxotrophic mutations to improve tumor-specific targeting as well as to reduce toxicity.[2–7] Then genetic modification of the salmonella lipid A was carried out to reduce septic shock and the lipid A-attenuated *S. typhimurium* has been evaluated in a phase I clinical trial.[4,8] Recently, Zhao *et al.*[9–11] developed *S. typhimurium* strains A1 and A1-R and detected real-time imaging of *S. typhimurium* A1-R-induced exogenous gene expression *in vitro* using fluorescence microscopy. Experience from clinical trials of cancer gene therapy indicates that no single therapeutic strategy can effectively eradicate a fairly large tumor, whereas combined gene therapy represents a more reliable approach to combat tumor growth.[12,13] The idea of combining salmonella-mediated gene therapy with stimulation of the host antitumor immune response drove studies of cancer gene therapy. Therefore these tumor-targeting bacteria have been used to deliver genes encoding angiogenic inhibitors,[14–16] prodrug-converting enzymes,[17] or cytokines[18,19] aiming to enhance their oncolytic effects.

Of several gene-directed enzyme prodrug therapy systems, we were interested in the system described by Sorscher *et al.*[20] It has been reported that the *Escherichia coli* purine nucleoside phosphorylase (ePNP) gene could convert 6-methylpurine 2′-deoxyriboside (MePdR) into a toxic substance named 6-methylpurine. As prokaryotic PNP enzymes differ fundamentally in sequence, structure, and function from their eukaryotic counterparts, MePdR should be a poor substrate for mammalian PNP.[21–25] Therefore, expression of ePNP is able to kill a number of cancer cells *in vitro* when a small fraction of cells (e.g. 0.1–3%) express this suicide gene, in combination with MePdR treatment.[20,26–30] Expression of suicide genes will not be achieved in all the tumor cells, so a bystander effect is necessary to produce toxic metabolites to kill not only the positive cells but also the bystander cells.[31–33] The ePNP/MePdR system differs from other gene-directed enzyme prodrug therapy systems because the toxic metabolites of this system will readily cross the cell membrane and not require direct cell-to-cell contact or the presence of a gap junction.[34] In previous papers, the ePNP/MePdR system has been reported to be an efficient suicide gene/prodrug system with significant antitumor activities on ovarian cancers,[29] gliomas,[35] prostate cancers,[36] melanomas,[37] pancreatic cancers,[38,39] hepatomas,[40,41] and bladder tumors.[42]

Love and Remy[43] reported that metabolism of various methylated purines, such as 6-methoxypurine, used identical metabolic pathways to 6-methylpurine. When some cells, such as mammalian cells or purine-requiring mutants of bacteria, could not use methylated purines, they come to a low growth rate, reduced cell yield, and derepression of purine synthesis. As 6-methoxypurine 2′-deoxyriboside (MoPdR) is similar to MePdR in structure, we wondered whether MoPdR could substitute

[1]To whom correspondence should be addressed. E-mail: dmren@fudan.edu.cn

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MePdR and use the identical principle of the ePNP/MePdR strategy to inhibit tumor growth. To fully explore the potentially antitumoral effect of the salmonella-mediated ePNP gene combined with MoPdR, we constructed a recombinant plasmid expressing the ePNP gene (pEGFP-C1-ePNP), which contained an enhanced green fluorescent protein (EGFP) under the control of the cytomegalovirus promoter, and transformed it into a live attenuated purine–auxotrophic strain of *S. typhimurium* (SC36). Then the function of the ePNP expression vector was confirmed *in vitro* using the enzymic conversion of MoPdR into methoxypurine. Furthermore, the bystander effect and the apoptosis efficiency of the ePNP/MoPdR system were assessed *in vitro* by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and flow cytometry analysis. The *in vivo* antitumor activities were also addressed in terms of tumor growth and survival rate in mice with mammary carcinoma.

Materials and Methods

Plasmids, bacterial strains, and media. Full-length cDNA of purine nucleoside phosphorylase (EC2.4.2.1, PNPase) gene (*DeoD*) was amplified using polymerase chain reaction (PCR) from *E. coli* DH5α genomic DNA. Genomic DNA preparation was carried out as described.[44] Specific primers for PCR were designed according to the published sequences: sense primer 5′-GG*GAATTC*GATGGCTACCCCACACATTAA-3′; and antisense primer 5′-AT*GTCGAC*TTACTCTTTATCGCCCAGCAG-3′. The *Eco*RI and *Sal*I restriction sites (underlined and italicized) were added to facilitate the PCR product (approximately 720 bp) and inserted into the eukaryotic expression vector pEGFP-C1 (Clontech) by standard homologous recombination techniques. pEGFP-C1 encodes a red-shifted variant of wild-type GFP that has been optimized for brighter fluorescence and higher expression in mammalian cells. The plasmid constructions were confirmed by DNA sequencing. The auxotrophic *S. typhimurium* LB5000 (LT2Trp Met Erpsl flaA R–M+) and SL3261 (WARY hisG46 aroA del 407 Fusaricres, R+M+) were provided by Professor Bruce A.D. Stocker (Stanford University School of Medicine, Stanford, CA). A novel purine–auxotrophic mutant SC36 (his G aro A cys pur I) with a low residual virulence was gained by diethyl sulfate mutagenesis of SL3261, as previously described.[45] Bacterial strains were routinely grown at 37°C in Luria broth (LB) or agar supplemented with kanamycin (50 μg/mL). pEGFP-C1 and pEGFP-C1-ePNP were first transformed into LB5000 using electrotransformation in cuvettes (0.2 cm electrode gap; Eppendorf) with a single pulse of 12.5 kV/cm (2.5 kV, 200 Ω, 25 μF). After amplification of the colony, the plasmids were extracted from the transferred LB5000 (3S Spin Plasmid Miniprep Kit V3.1; Shanghai Shenergy Biocolors Bioscience & Technology Co. Ltd.) then introduced into SC36 (as carried out for LB5000) to generate SC/pEGFP and SC/ePNP.

Cells, cell culture, and bacterial infection. Murine mammary carcinoma 4T1 cell lines were obtained from Shanghai No.1 People's Branch Hospital (China). The cells (10^5 cells/well) were cultured in 12-well plates overnight in antibiotic-free RPMI-1640 media (Gibco BRL) in a 5% CO_2 atmosphere at 37°C. Bacteria (SC/pEGFP or SC/ePNP) were shaken in LB broth with kanamycin (50 μg/mL) overnight at 37°C to reach the late logarithmic phase of growth (OD_{600} of approximately 4) and suspended in antibiotic-free media. Then 2.5×10^7 c.f.u. of bacteria (at a multiplicity of infection of 1:250) were directly added into 12-well plates and co-incubated with tumor cells for 3 h. Subsequently, the cells were washed three times with phosphate-buffered saline (PBS) and replenished with complete media (containing 100 U/mL penicillin and 100 μg/mL streptomycin) and further cultured for 1 or 2 days. The infected cells were collected and some of them were used to monitor the gene expression by reverse transcription (RT)-PCR (AccessQuick; Promega). Some of the infected cells (10^4 cells/well) in 96-well plates, cultured with complete media (containing 100 U/mL penicillin and 100 μg/mL streptomycin) and MoPdR (previously synthesized using the same principle)[46] were used for cytotoxicity assay[47] and propidium iodide staining flow cytometry analysis.[48]

Western blot analysis for caspase-3. Total proteins of the infected cells were extracted (TRIzol reagent; Invitrogen) and their concentration was determined by Bio-Rad protein assay buffer. Total proteins were separated on 8–12% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Amersham, Piscataway, NJ). The membranes were blocked for 1 h in 5% non-fat dry milk (Shanghai Bright Dairy and Food) in PBS then incubated with primary antibodies, detected by the appropriate secondary antibodies, and revealed with an enhanced chemiluminescence system (Amersham Life Sciences). The primary antibodies were anti-caspase-3 (Santa Cruz Biotechnology) and anti-actin (Sigma).

Stable positive cell lines and functional tests. The cell lines were transfected with pEGFP-C1 or pEGFP-C1-ePNP (Lipofectamine 2000; Invitrogen) and selected with G418 at a concentration of 400 μg/mL. Fresh media with G418 were replaced every 3 days. Fifteen days after the cell culture, the cell clones with G418 resistance (named 4T1/ePNP and 4T1/pEGFP) were obtained. Resistant clones were selected and their ePNP expression was monitored by RT-PCR. The positive clones highly expressing ePNP were selected for observing the bystander effect by MTT assay[49] and analyzing enzymic conversion by a high-performance liquid chromatography (HPLC)-based assay.[20]

Bacterial distribution *in vivo* after oral inoculation. Four-week-old female BALB/c mice were obtained from BK Company (Shanghai, China). To eliminate enteric flora, all mice were fed with sterilizing water containing neomycin (5 g/L), streptomycin (5 g/L), and penicillin (5 U/μL). After the first 2 weeks of antibiotic therapy, tumor xenografts were established by subcutaneous inoculation of tumor cells (5×10^6 cells) into the right flanks of mice. When the tumors had reached a mean volume of approximately 80 mm^3, mice were fed with 1×10^9 c.f.u. of SC/ePNP. At different time points, three mice in each group were killed and their tumors, livers, spleens, kidneys, and gastrointestinal tracts were excised, weighed, and homogenized in 2 mL of ice-cold, sterile PBS. The bacteria were quantified by plating serial dilutions of the homogenates onto LB agar plates containing kanamycin (50 μg/mL). The plates were incubated overnight at 37°C and the bacterial colonies were counted.

Assessment of ePNP expression *in vivo*. The ePNP mRNAs in the tissues of SC/ePNP-treated mice were determined by RT-PCR. At different time points, total cellular RNA was isolated from the tissues (TRIzol reagent; Invitrogen) and reverse-transcribed into cDNA (AccessQuick; Promega). The cDNA was amplified by PCR to determine β-actin and ePNP mRNA expression. The primer design was based on the published sequences: sense primer 5′-ACCCACACTGTGGCCCATCTA-3′ and antisense primer 5′-CGGAACCGCTCATTGCC-3′ for β-actin, and sense primer 5′-GGGAATTCGATGGCTACCCCACACATTAA-3′ and antisense primer 5′-ATGTCGACTTACTCTTTATCGCCCAGCAG-3′ for ePNP for PCR amplification of 289-bp and 720-bp fragments, respectively. To assess the functionality of ePNP/MoPdR in the tissues, an HPLC-based assay was developed measuring the catabolism of the prodrug MoPdR to methoxypurine. Seven days after oral inoculation, homogenates of tissues (Liquid N2) were generated and lysis was completed by three cycles of freezing and thawing. Cell debris was removed by centrifugation (15 000*g* × 10 min) followed by determination of the protein content of the supernatants (Bradford protein assay kit; Sangon). Then 100 μL (0.1 mg/sample) of the supernatant was incubated with 900 μL of 5 μg/mL MoPdR at 37°C for 24 h and the enzymic conversion analyzed by HPLC.[20]

Analysis of anti-tumor effect *in vivo*. To eliminate enteric flora, all mice were fed with sterilizing water containing neomycin (5 g/L), streptomycin (5 g/L), and penicillin (5 U/μL). After the first 2 weeks of antibiotic therapy, the animals were inoculated subcutaneously with 5×10^6 murine tumor cells. When the tumors reached a mean volume of approximately 80 mm^3, mice were randomly grouped ($n = 5$ animals per group) and fed with sterilizing water without antibiotics. All SC/ePNP-treated mice received 1×10^9 c.f.u. of SC/ePNP and control mice received SC/pEGFP or buffer only. For bacteria colonization, all salmonella-treated mice were fed with sterilizing water containing kanamycin (800 mg/L) the day before or during the study. Subsequently, intraperitoneal injections of MoPdR (10 mg/kg body weight) were started 7 days after bacterial inoculation, when the mean volume of tumors was over 200 mm^3. The MoPdR treatments were used daily, four times. All blank mice received 0.9% normal saline solution. Tumor diameters were measured at regular intervals with calipers, and the tumor volume in mm^3 was calculated by the formula: volume = $1/2 \times \text{length} \times \text{width}^2$. Survival rates were monitored after bacterial infection. Mice bearing mammary carcinoma 4T1 were killed when tumors reached 4000 mm^3 or beforehand if they showed signs of distress. These time points were defined as survival time.

Statistical analysis. Statistical analysis of the data was carried out using the Student–Newman–Keuls' test and Primer statistical software (SPSS Base 10.0 for Windows; SPSS, Chicago, IL). Results were considered statistically significant at $P < 0.05$.

Results

Vector functional tests *in vitro*. The function of the ePNP expression vector (pEGFP-C1-ePNP) was confirmed by HPLC after SC/ePNP infection *in vitro*, and the ratio of MoPdR conversion in the supernatants of SC/ePNP-infected cells was 95% after 96 h (Fig. 1). Moreover, SC/ePNP-infected cells showed a strong sensitivity to MoPdR (Fig. 2), indicating that plasmid pEGFP-C1-ePNP construction was functional.

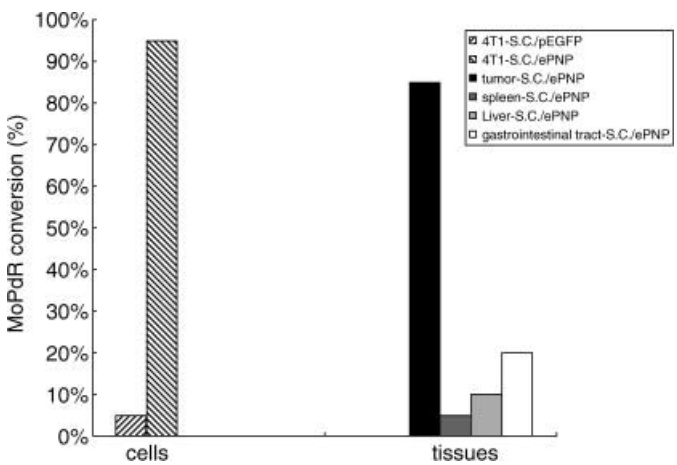

Fig. 1. Enzymic conversion. Murine mammary carcinoma 4T1 cells (10^4 cells/well) were cultured 96-well plates in media with 5 μg/mL 6-methoxypurine 2′-deoxyriboside (MoPdR) for 96 h. The supernatants (0.1 mg/100 μL) of the tissues were incubated with 900 μL of 5 μg/mL MoPdR at 37°C for 24 h. Subsequently, lysates from the cells and tissues were analyzed for the concentrations of MoPdR in the supernatants by high-performance liquid chromatography at a wavelength of 254 nm. 4T1-SC/ePNP, right diagonal; 4T1-SC/pEGFP, left diagonal; gastrointestinal tract-SC/ePNP, white; liver-SC/ePNP, hoar; spleen-SC/ePNP, dark grey; tumor-SC/ePNP, black.

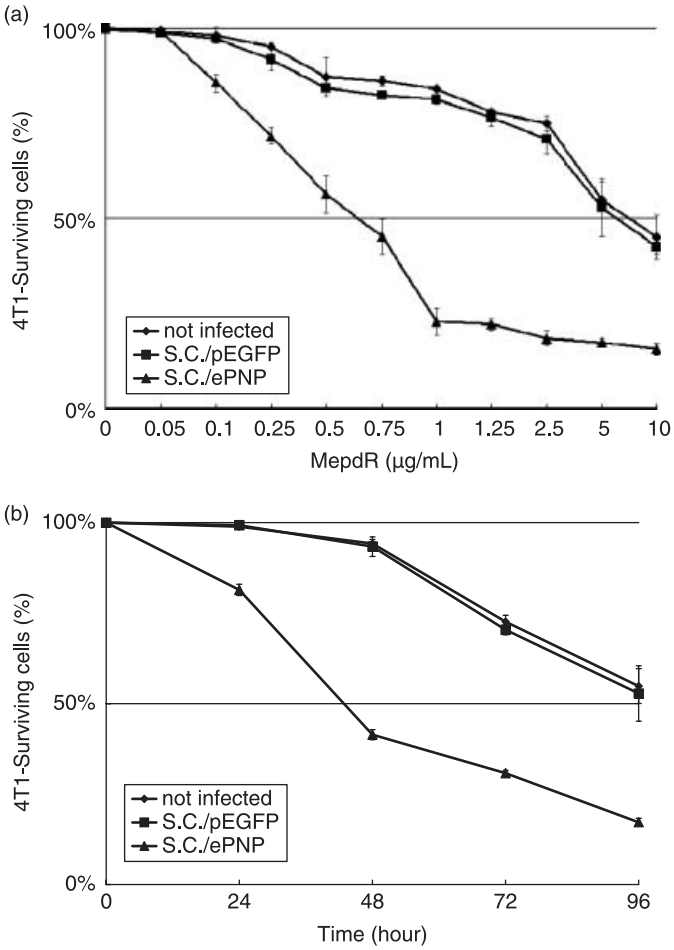

Fig. 2. Sensitivity of tumor cells to treatment with the *Escherichia coli* purine nucleoside phosphorylase gene and 6-methoxypurine 2′-deoxyriboside (ePNP/MoPdR). (a) The infected murine mammary carcinoma 4T1 cells were treated with MoPdR concentrations ranging from 0.05 to 10 μg/mL. The absorbance of each well at a wavelength of 570 nm was measured using an enzyme-linked immunosorbent assay reader (Bio-Rad) 96 h later. (b) At the concentration of 5 μg/mL MoPdR, cell survival was quantified at indicated time points by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Data are representative of at least three separate experiments; each point represents the mean ± SE and is expressed as a percentage relative to the same cells non-treated. SC/ePNP, triangle; SC/pEGFP, square.

Effects of SC/ePNP increased spontaneous apoptosis of cells. Incubated with different concentrations of MoPdR (ranging from 0 to 10 μg/mL), parental or SC/pEGFP-infected cells were resistant to MePdR, with an IC_{50} (kill 50%) higher than 5 μg/mL. In contrast, SC/ePNP-infected cells were susceptible to MoPdR, with an IC_{50} lower than 0.75 μg/mL (Fig. 2a). Moreover, the sensitivity of the infected cells to MoPdR was time-dependent at the concentration of 5 μg/mL MoPdR. The prodrug treatments led to approximately 19% of cell death after 24 h and 83% of cell death after 96 h (Fig. 2b). The apoptosis results showed that SC/ePNP treatment increased the spontaneous apoptosis of the infected cells compared to the control cells at different concentrations of MoPdR (ranging from 0 to 0.75 μg/mL). The greatest increase in the number of apoptosis cells was at the final concentration of 0.75 μg/mL MoPdR, and the ratio was 19.45% (Fig. 3a). As indicated in Fig. 3b, treatment with SC/ePNP and 5 μg/mL MoPdR led significant expression of caspase-3 to be time-dependent in 4T1 tumor cell lines,

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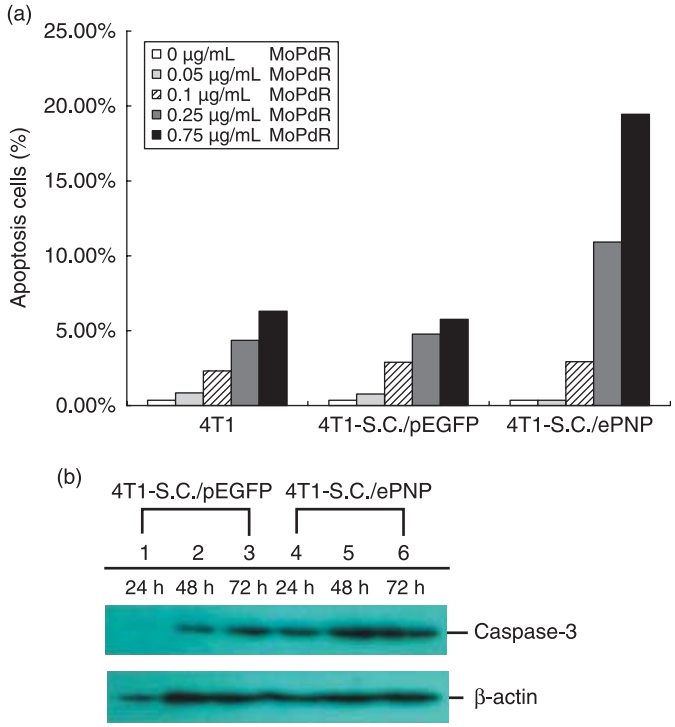

Fig. 3. Assessment of apoptosis in 4T1 murine mammary tumor cells. (a) Enhancement of apoptosis. After incubation with 6-methoxypurine 2′-deoxyriboside (MoPdR) at different concentrations (0–0.75 μg/mL) for 96 h, harvested cells were fixed overnight with precooled ethanol and treated with 50 μg/mL propidium iodide (Sigma Chemical) and 10 μg/mL RNaseA for 30 min at 37°C. A minimum of 1.5×10^4 events were analyzed on a fluorescence-activated cell sorting flow cytometer with an argon laser tuned at 488 nm and use of CellQuest software (Becton Dickinson, Rutherford, NJ). Results are expressed as the percentage of apoptotic cells after *S. typhimurium* carrying pEGFP-c1 (SC/pEGFP) and *S. typhimurium* carrying pEGFP-c1-ePNP (SC/ePNP) treatment with respect to the same cells non-treated, in combination with MoPdR. (b) Western blot analysis. The infected cells were treated with 5 μg/mL MoPdR and total cellular proteins were isolated at indicated time points and subjected to Western blot analysis using anti-caspase and anti-β-actin antibody.

beginning at 24 h and peaking at 48 h after infection, compared with that of SC/pEGFP-infected cells.

Bystander effect. In the absence of ePNP-positive cells, the growth of parental cells was affected slightly even when the MoPdR concentration reached 5 μg/mL. At the concentration of 5 μg/mL MoPdR, the significant bystander effect could be detected even with a very low proportion (1%) of ePNP-positive cells. Furthermore, in the conditions of 1 μg/mL MoPdR and 5% ePNP-positive cells, more than 50% of cells were killed. These results indicated that a high bystander effect was achieved (Fig. 4).

Bacterial distribution and gene expression in tissues. The kinetics of bacterial distribution after oral inoculation was detected to be in a time-dependent pattern. Significant accumulation of SC/ePNP in the tumors could be retained for at least 12 days, with a peak level observed at day 7, and the tumor-to-normal tissue ratios were as high as 10 000:1. SC/ePNP entirely disappeared from liver and spleen 16 days after oral treatment (Fig. 5a). As shown in Fig. 5b, expression of the ePNP gene was detected in the tumors at day 7 and day 12. In contrast, low expression of the ePNP gene in other tissues could be detected at day 7, but not at day 12. Futhermore, an HPLC-based assay evaluated the functionality of ePNP/MoPdR in the tissues and showed that higher conversion of MoPdR was detected in tumors than in other tissues (Fig. 1). Taken together, these results showed that SC/ePNP, when given to mice bearing established tumors, was preferentially accumulated and retained in large amounts in tumors for at least 12 days.

Antitumoral effect of the ePNP gene combined with MoPdR. We found the intraperitoneal injections of MoPdR were no more than 10 mg/kg body weight/day, otherwise an excessive dose of MoPdR led to significant weight loss of mice. Therefore, this dose was used for all MoPdR treatment experiments *in vivo*. As shown in Fig. 6a, tumor growth of mice bearing 4T1 mammary carcinoma was significantly retarded by the salmonella-mediated ePNP/MoPdR system. There was no statistical difference between the four groups (PBS-treated, MoPdR-treated, SC/pEGFP-treated, and SC/ePNP-treated groups) at any time point during the experiments. The mean tumor volume of mice treated with SC/ePNP plus MoPdR was lowered by 86.6%, 87.2%, 87.6%, and 88.7%, compared with that of mice treated with SC/ePNP ($P < 0.001$), SC/pEGFP ($P < 0.001$), MoPdR ($P < 0.001$), and PBS ($P < 0.001$), respectively. As shown in Fig. 6b. SC/ePNP plus MoPdR also prolonged the survival of mice with mammary carcinoma as compared with SC/ePNP-treated ($P = 0.0034$), SC/pEGFP-treated ($P = 0.0026$), MoPdR-treated ($P = 0.0017$), and PBS-treated ($P = 0.0015$) counterparts. In conclusion, the treatment of the ePNP gene and appropriate dose of MoPdR could significantly suppress tumor growth with the initial mean tumor volume >200 mm^3 when the intraperitoneal injections of MoPdR were no more than 10 mg/kg body weight/day.

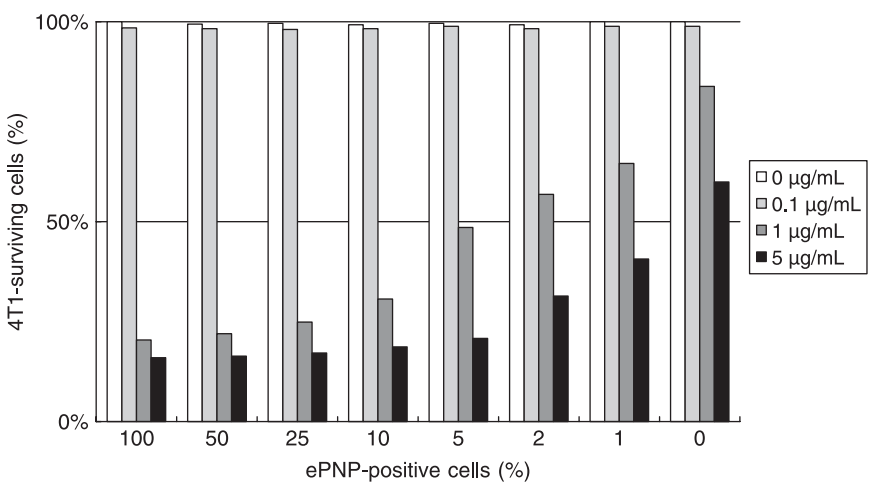

Fig. 4. Bystander effect. To investigate the bystander effect *in vitro*, *Escherichia coli* purine nucleoside phosphorylase (ePNP)-positive cells and parental cells were mixed in different proportions (0:100; 1:99; 2:98; 5:95; 10:90; 25:75; 50:50; and 100:0). Twenty-four hours later, all cells were cultured in RPMI-1640 media containing 6-methoxypurine 2′-deoxyriboside at different concentrations (0–5 μg/mL) for 3 days. The percentage of surviving cells was calculated to evaluate the survival ratios of 4T1 mammary carcinoma cells by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay. Data are representative of at least three separate experiments. Survival ratios are expressed as percentages relative to the corresponding mixture of untreated control cells.

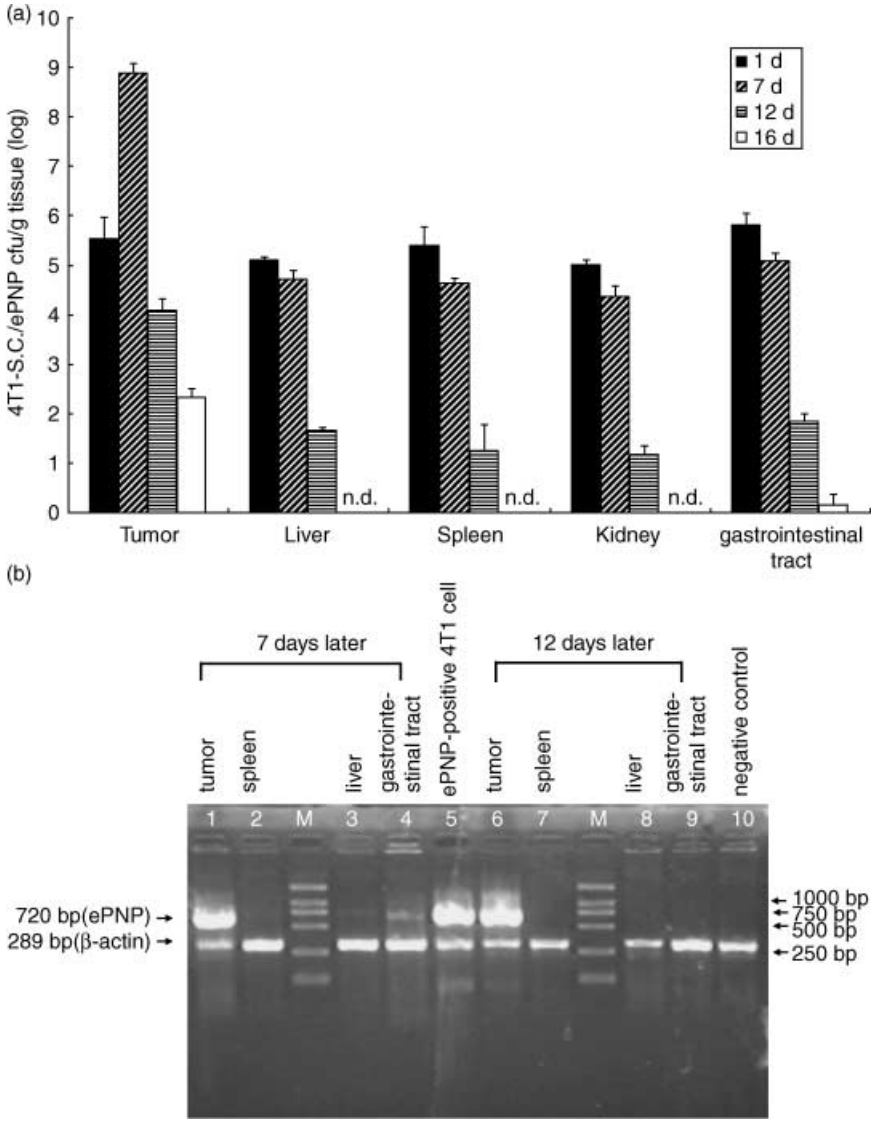

Fig. 5. Bacterial distribution in murine tissues. (a) Preferential accumulation of salmonella in the tumors of mice given *S. typhimurium* carrying pEGFP-c1-ePNP (SC/ePNP). Mice bearing mammary carcinoma 4T1 cells were given 1×10^9 c.f.u. of SC/ePNP, orally. The amounts of SC/ePNP accumulated in tumors, livers, spleens, kidneys, and gastrointestinal tracts were determined at 1, 7, 12, and 16 days post-infection. Each value represents the mean ± SD from three mice. n.d., not detected. (b) Reverse transcription–polymerase chain reaction (RT-PCR) analysis for gene expression. At different time points after bacterial inoculation (7 and 12 days), the tissues of mice bearing mammary carcinoma 4T1 tumors were tested for ePNP expression by RT-PCR. M, molecular marker profile.

Discussion

Herein, for the first time, we showed a eukaryotic expression vector encoding the ePNP gene through *S. typhimurium*-mediated gene delivery in cancer therapy, in combination with MoPdR. Previous studies reported that various methylated purines, such as 6-methoxypurine and 6-methylpurine, used identical metabolic pathways and the limited use of them by mammalian cells led to a low growth rate and reduced cell yield.[43] As MoPdR is similar to MePdR in structure, we wondered whether an exploitation of MoPdR could work as functionally as MePdR to retard or stop tumor growth in mice.

The infection of bacteria in human tumor was recognized as early as 1868. Subsequently, tumor-targeted attenuated bacterial strains, such as *S. typhimurium, Clostridium* and *Bifidobacterium*, have been developed as antitumor agents capable of preferentially amplifying within tumors and inhibiting tumors growth.[2,16,50] Salmonella strains have been reported to have innate antitumor activity towards both primary and metastatic tumors and the ability to deliver proteins capable of metabolizing chemotherapeutic drugs directly within tumors.[2–7] In the work described here, we used a new mutant *S. typhimurium* named SC36 (his G aro A cys pur I), with low residual virulence gained by diethyl sulfate mutagenesis of SL3261. We observed the SC36-mediated heterogeneous gene expression *in vitro* and *in vivo* and preferential accumulation of SC36 within implanted tumors (Figs 1,5). Our data, combined with that reported previously,[51] suggest that attenuated *S. typhimurium* appears to only survive in tissues that become hypoxic and provide nutrients for it to grow. This evidence prompted us to explore whether SC36 carrying a eukaryotic expression vector encoding the ePNP gene was capable of both targeting tumor cells and suppressing tumor growth, in combination with MoPdR.

Although the mechanisms contributing to bacterial infection to mammalian cells are not completely understood, Schoen *et al*.[52] summarized that invasion of bacterial carrier strains into host cells has been shown to be important in cellular mechanisms of bacteria-mediated delivery. Previous studies done by Critchley *et al*.[53] reported that bacterial invasion could sensitize mammalian cells to the action of MePdR. In the MTT assay described here, SC/ePNP-infected cells showed a strong sensitivity to MoPdR (Fig. 2). We also observed that parental or infected cells were susceptible to MoPdR at the final concentration of 10 μg/mL. This is probably responsible for the toxicity of MoPdR to cells, and an excessive dose of MoPdR led to significant cell death. Furthermore, propidium iodide staining showed that the ePNP/

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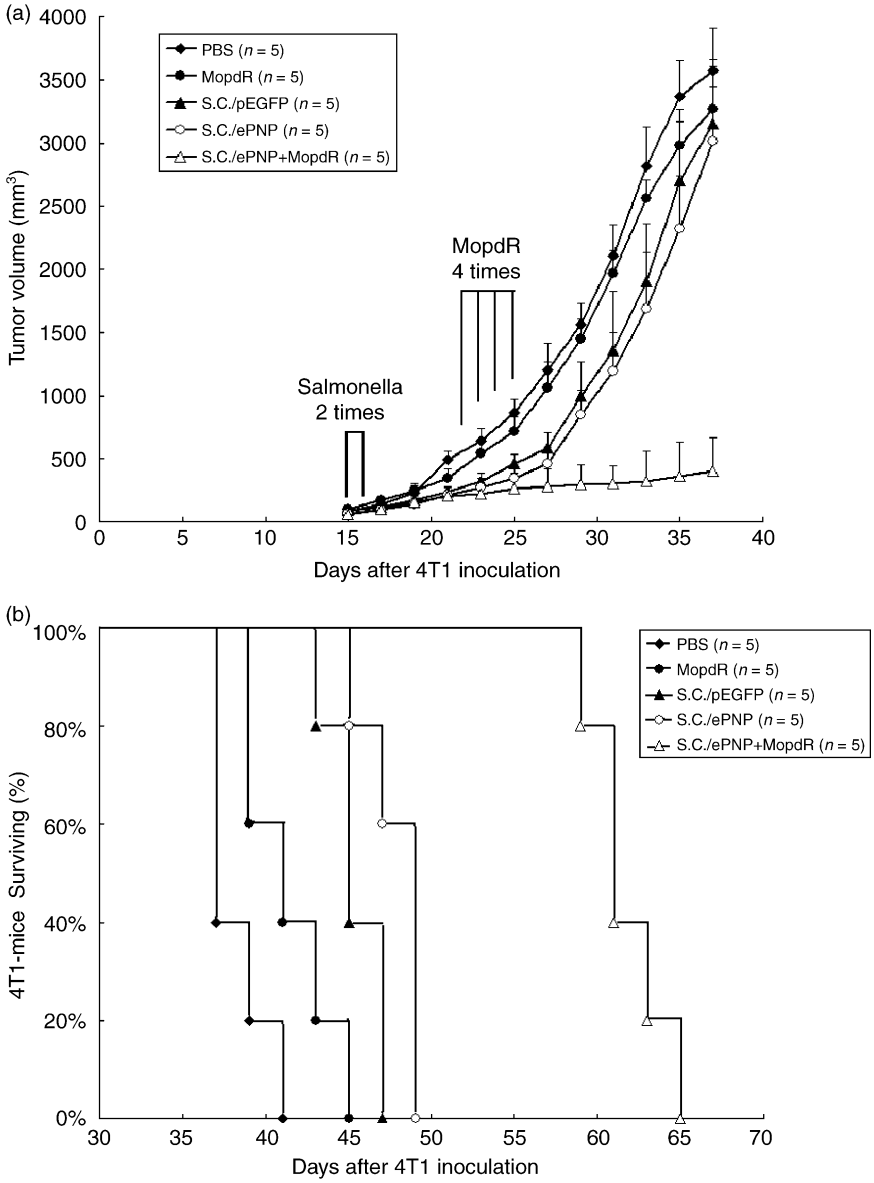

Fig. 6. Antitumor effects of the *Escherichia coli* purine nucleoside phosphorylase (ePNP) gene combined with 6-methoxypurine 2′-deoxyriboside MoPdR in mice bearing murine mammary carcinoma 4T1 tumors. (a) When tumors had reached the mean volume of approximately 80 mm^3, 4T1-bearing mice were divided into five groups. There was no statistical difference between the five groups at the start of treatment (phosphate-buffered saline [PBS], 78.63 ± 8.91; MoPdR, 80.11 ± 10.75; *S. typhimurium* carrying pEGFP-c1 [SC/pEGFP], 77.93 ± 10.59; *S. typhimurium* carrying pEGFP-c1-ePNP [SC/ePNP], 81.25 ± 8.47; the combination of SC/ePNP and MoPdR [SC/ePNP+MoPdR], 80.19 ± 12.14). Tumor volumes were compared by two-tailed Student's *t*-test. Data are presented as the mean ± SEM. (b) Survival curves of mice bearing 4T1 tumors among different groups are shown. Data were analyzed by the log–rank test, $P < 0.05$.

MoPdR system increased spontaneous apoptosis (Fig. 3), consistent with previous reports.[30] It is possible that the concomitant presence of apoptotic cells, bacterial products such as lipopolysaccharides,[54] and bacterial DNA[55] could act as 'danger signals'[56,57] for these infiltrating cells. The local abundance of these danger signals associated with the phagocytosis of dead cancer cells by activated macrophages is likely to initiate an immune response against specific cancer antigens and increase spontaneous apoptosis.[53] We also observed a high bystander killing effect induced by the ePNP/MoPdR system with 1% ePNP-positive cells and 5 μg/mL MoPdR, although the growth of parental cells was affected appreciably by MoPdR (Fig. 4). These data suggest that *S. typhimurium* could act as an antitumor agent to deliver genes, and apoptosis induced by the ePNP/MoPdR system was observed to have a high bystander effect.

In this study, with the help of MoPdR, we showed that attenuated *S. typhimurium* carrying the ePNP gene expression vector, given orally, significantly slowed tumor growth and prolonged survival periods in mice with established tumors (Fig. 6). Furthermore, *in vivo* studies show that the combination of MoPdR and the ePNP gene has positive synergistic antitumoral properties against experimental murine tumors, compared with treatments using MoPdR or the ePNP gene alone. To achieve complete tumor regression in further experiments, multiple inoculations of the bacteria might be important to prolong gene production and to increase antitumor activity.[58] Zhao *et al.*[9–11] showed that weekly treatment of bacteria was more effective than a two-dose treatment. Moreover, this combined approach could be improved by using cytokines (e.g. interleukin [IL]-4, IL-12, or IL-18) that have the capacity to induce the production of γ-interferon to achieve the inhibition of cancer growth and reduce the dose of prodrugs.[19] Alternatively, combining this approach with other therapeutic strategies, such as radiotherapy, might be more effective for tumor regression and prodrug abasement.[59] Although attenuated *S. typhimurium* is effective to deliver plasmids carrying exogenous genes, the continuous loss of recombinant plasmids during cell division was observed and the mice required antibiotic treatment to reduce competing microflora. To make SC36 safer for clinical trials, it should be important to develop tumor-specific expression vectors. Mengesha *et al.*[60]

developed a hypoxia inducible promoter (HIP-1) system and found that HIP-1 could confine gene expression strictly to the tumor. By this token, the use of tumor-specific gene promoters is an attractive option for salmonella-mediated gene delivery of the ePNP/MoPdR system.

In conclusion, MoPdR could be used in antitumor therapy associated with the ePNP gene. Because of some characteristics of attenuated salmonella, such as preferential accumulation, selective expression, and positive synergistic antitumor activity, our data support the idea that tumor-targeting *S. typhimurium* could improve antitumor efficacy of the ePNP/MoPdR system in murine models. As substantial weight loss, limiting lethality, or other toxicities were not observed in these experiments with appropriate MoPdR doses, we expect that the simple method detailed in this study will be applicable for more tumor models in further research.

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