Expression of MRP4 Confers Resistance to Ganciclovir and Compromises Bystander Cell Killing*

Received for publication, April 5, 2002, and in revised form, June 24, 2002
Published, JBC Papers in Press, June 24, 2002, DOI 10.1074/jbc.M203262200

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The multidrug resistance protein MRP4, a member of the ATP-binding cassette superfamily, confers resistance to purine-based antiretroviral agents. However, the antiviral agent ganciclovir (GCV) has not been shown to be a substrate of MRP4. GCV is important not only in antiviral therapy, but also in the selective killing of tumor cells modified to express herpes simplex virus thymidine kinase (HSV-TK). We therefore tested the effect of MRP4 on the cytotoxicity of GCV, on the ability of GCV to kill cells genetically modified to express HSV-TK, and on the bystander effect in which unmodified target cells are killed by GCV. Cells overexpressing MRP4 had markedly increased resistance to the cytotoxicity of GCV. Although, expression of recombinant HSV-TK increased the intracellular concentration of GCV nucleotide, cells were rescued by the cytoprotective effect of MRP4. In cells that overexpressed MRP4, intracellular accumulation of GCV metabolites was reduced, efflux of these metabolites was increased, and resistance to bystander killing was increased. Therefore, MRP4 can strongly reduce the susceptibility of HSV-TK-expressing cells to GCV, and its overexpression in adjacent cells protects them from bystander cell death. These findings indicate that a nucleotide transporter, such as MRP4, modulates the cellular response to GCV and thus may influence not only the efficacy of antiviral therapy, but also prodrug-based gene therapy, which is critically dependent upon bystander cell killing.

The multidrug resistance proteins (MRPs)¹ are a family of ATP-binding cassette transporters (ABC transporters; for an overview, see //nutrigene.4t.com/humanabc.html) that mediates drug efflux and multidrug resistance (1). We previously demonstrated that MRP4 (also known as ABC4) severely reduces the antiviral efficacy of several nucleoside reverse transcriptase inhibitors, such as zidovudine (3’-azido-3’-deoxythymidine) (AZT) and 9-(2-(phosphonomethoxy)ethyl)-adenine (PMEA), in mammalian cells and that this effect corresponds with increased ATP-dependent efflux of their nucleotide derivatives (2). These findings were, in part, replicated by Lee et al. (3). A better understanding of the role of MRP4 as a nucleotide efflux transporter may lead to improved nucleoside-based therapies for HIV, herpes viruses, and cancer.

Ganciclovir (9-(1,3-dihydroxy-2-propoxymethyl)guanine (GCV)) is widely used against cytomegalovirus infection in patients with AIDS (4–6) but is poorly tolerated in combination with AZT (7). Although the molecular basis of this interaction is unknown, in vitro studies indicate that the combination is extremely cytotoxic (8, 9). We therefore postulated that MRP4 might be involved in the cytotoxicity induced by the AZT-GCV combination. If so, such an interaction could have important therapeutic potential because of the widespread use of GCV as a prodruk in gene therapies for cancer (10, 11). Transduction of tumor cells with the herpes simplex virus thymidine kinase (HSV-TK) gene and treatment with GCV is a common anticancer gene therapy (10–12). HSV-TK specifically converts GCV to its monophosphate nucleotide form, GCV-monophosphate, which is then further anabolized by cellular kinases, and accumulation of these metabolites causes cell death (5, 12). However, the cytotoxicity of this therapy varies dramatically. The mechanism of this variability is unknown, but it does not appear to reflect the level of HSV-TK expression (13, 14).

The variable toxicity of GCV to HSV-TK gene-modified tumor cells also markedly affects the efficacy with which bystander (adjacent, non-transduced) tumor cells are killed (15). In vitro studies show that bystander cell death is associated with the accumulation of GCV metabolites (16); however, this effect is variable and idiosyncratic, and the mechanism accounting for variable accumulation of these metabolites is unclear (17, 25). Some investigators believe that direct intercellular communication by gap junctions is a prerequisite for GCV metabolism transfer (18), while others do not (19, 20). Other
studies suggest that diffusion of GCV metabolites and the proximity of bystander cells are important variables (21–23). Although a combination of these mechanisms is likely, it is unknown if nucleotide efflux plays a role. We reasoned that because the efflux transporter MRP4 transports some natural and modified nucleotides (2, 24) its expression could impact HSV-TK based gene therapy by hastening the removal of cytotoxic GCV metabolites. However, it is unknown if MRP4 plays a role in GCV metabolism and cellular egress.

We investigated whether MRP4 affects the cellular accumulation and cytotoxicity of GCV in cells specifically overexpressing MRP4 and in cells we engineered to overexpress MRP4. The cDNA encoding human MRP4 was isolated and stably introduced into MCF-7 cells that expressed negligible immunoreactive MRP4. These cells had enhanced resistance to GCV cytotoxicity and to bystander killing. We also investigated the effect of MRP4 on the cytotoxicity of GCV in cells engineered to express HSV-TK. Taken together, our results show that MRP4 plays a key role in the intracellular accumulation and cytotoxicity of GCV metabolites. These findings reveal ganciclovir egress by MRP4 as a novel mechanism contributing to the variable response in gene therapy based on HSV-TK expression.

MATERIALS AND METHODS

Materials—[8–3H]Ganciclovir, [adenine-8-3H]bis(pivaloyloxymethyl)-9-[2-phosphonomethoxy)ethyl]-adenine (bis-POM-[3H]PMEA), [3H]vinblastine, [3’-5’,7’-3H]methotrexate (MTX), [5-3H]gemcitabine, [8–14C]6-[8-11032H1]TH1032 ME).

Tunometer (BD Biosciences) using laser excitation at 488 nm. The percent-

were performed as described previously (2).

with a recombinant murine retrovirus (pLENTK) encoding HSV-TK

role in GCV metabolite accumulation and cellular egress.

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portion, yielded a 5.8-kb

5’-untranslated regions of this cDNA fragment were removed, and the complete 4.2-kb cDNA encoding the 1325 amino acids of MRP4 was used to construct the expression vector MSCV-MRP4-ires. Myristic acid contains the murine stem cell virus (MSCV) long terminal repeat as well as a ribosomal entry site (IRES) to permit expression of green fluorescent protein (GFP) and was kindly provided by Dr. Robert Hawley, Holland Laboratory, American Red Cross, Rockville, MD). The complete MRP4 cDNA was also subcloned into the EcoRI site of pcDNA3 (Invitrogen) to generate pcDNA3-MRP4.

MRP4 Expression in Cell Lines—HEK293T cells were transfected with pEQ-PAM2-E and pvSVG expression vectors containing MSCV-IRES-GFP or MRP4-IRES-GFP constructs by calcium phosphate precipitation (29), and supernatants containing retroviral particles were used to transfect the MCF7 cell line. GFP+ cells were identified by FACS. SAOS-2 cells transfected by calcium phosphate precipitation with either pcDNA3 or pcDNA3-MRP4 were selected in G418 (1000 µg/ml).

Metabolic Labeling with [3H]GCV—CEMs HSV-TK and CEMr1- HSV-TK cells were treated in triplicate with 1 µC of [3H]GCV (plus 1 µC unlabeled GCV), washed, and resuspended in fresh medium. Nucleotides were extracted from pelleted cells in 0.2 ml of 7% methanol at 4°C for 15 min (22). Radioactivity was measured in an aliquot of each methanol supernatant by scintillation counting. The proportion of phosphorylated GCV was measured in an aliquot of medium by anion exchange chromatography (22).

Clonal Dilution Assays of Bystander Effect—The HSV-TK expressing SW620-TK cells were plated with acceptor cells that did not express HSV-TK (total, 2 × 10^5 cells/dish) and incubated for 24 h at 37°C. Medium was removed, drugs were added, and cells were incubated at 37°C in the indicated intervals. Viability and inhibition of cell growth were determined by direct counting of trypan blue-dyed cells on a hemocytometer. A 3-4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Sigma) was used according to the manufacturer’s instructions to determine cellular toxicity. Each assay included duplicate samples for each drug concentration, and all experiments were performed at least twice.

Flow Cytometry—After exposure to GCV, cells were harvested, washed, and resuspended at ~1 × 10^5 cells/ml in propidium iodide staining solution (0.05 mg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100). Immediately before analysis, cells were treated for 30 min at room temperature with RNase (Calbiochem, San Diego, CA) at a final concentration of 14 µg/ml and filtered through nylon mesh. Fluorescence was measured on a Becton Dickinson FACScan flow cytometer (BD Biosciences) using laser excitation at 488 nm. The percent-

ages of cells in different phases of the cell cycle were determined by using the Modfit computer program (Verify Software House, Topsham, ME).

Sub-G1 Determination—Analysis of the DNA content less than G0 was used as an additional measurement of apoptosis as previously reported (31). Briefly, after drug treatment both floating and attached cells were harvested in a propidium iodide solution (50 µg/ml propidium iodide in 0.1% sodium citrate and 0.1% Triton X-100), treated with 5 µg/ml RNase (Calbiochem) for 30 min at room temperature, and then analyzed by flow cytometry on a Becton Dickinson FACScan (BD Biosciences) using laser excitation at 488 nm. The percent-

of sub-G1 cells was determined.

DAPI Staining of Apoptotic Cells—CEMsss and CEMr1 cell lines expressing TK were plated (5 × 10^4 cells/well) in 8-well plastic chamber slides (Lab-Tek) and treated with the indicated concentrations of GCV. The cells were then washed with phosphate-buffered saline, stained in 1 µg/ml DAPI, and examined on a Zeiss fluorescent microscope at 40× magnification (28).

Assays of Drug Accumulation—Cells (2 × 10^6 cells/ml growth me-

were seeded in 6-cm dishes 24 h prior to the assay. They were then washed with Hanks’ balanced salt solution and incubated for the desired interval with the radiolabeled drug dissolved in warmed medium. Aliquots of cells were removed, washed with chilled phosphate- buffered saline, solubilized in 1 ml of 0.5 N NaOH, and assayed for radioactivity.

Cloning of MRP4 cDNA—The human MRP4 cDNA (expressed se-

sequence tag 38,091, described in Ref. 2) was used to screen a human lung λ ZAPII cDNA library (Stratagene) by plaque hybridization. A 4.5-kb cDNA that lacked the initiator methionine was identified, and a 5’ portion of this cDNA was used to screen a human lung 5’-STRETCH PLUS cDNA library (Clontech). Ten positive clones that contained only the 5’ portion of MRP4 were isolated. The 5’ portion of MRP4, together with the overlapping 4.5-kb clone of the 3’ portion, yielded a 5.8-kb MRP4 cDNA (GenBank™ accession number: AY081219). The 5’- and 3’-untranslated regions of this cDNA fragment were removed, and the complete 4.2-kb cDNA encoding the 1325 amino acids of MRP4 was used to construct the expression vector MSCV-MRP4-ires. Myristic acid contains the murine stem cell virus (MSCV) long terminal repeat as well as a ribosomal entry site (IRES) to permit expression of green fluorescent protein (GFP) and was kindly provided by Dr. Robert Hawley, Holland Laboratory, American Red Cross, Rockville, MD). The complete MRP4 cDNA was also subcloned into the EcoRI site of pcDNA3 (Invitrogen) to generate pcDNA3-MRP4.

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Clonal Dilution Assays of Bystander Effect—The HSV-TK expressing SW620-TK cells were plated with acceptor cells that did not express HSV-TK (total, 2 × 10^5 cells/dish) and incubated for 24 h at 37°C. Medium was removed, cells were trypsinized, and each well was diluted to 1:10 to 1:10,000 with fresh medium. After incubation for 24 h, medium was removed, cells were trypsinized, and each well was diluted 1:10 to 1:10,000 with fresh medium. After 7 days, surviving cell colonies were fixed in methanol, stained, and counted (27).

RESULTS

Effect of MRP4 Overexpression on the Cell Cycle, Sensitivity to GCV, and Apoptosis—Immunoblot analysis showed the PMEA-resistant cell line, CEMr1 (4), expressed 6- to 8-fold more MRP4 and slightly less MRPl than did the CEMss parent cell line (Fig 1A). Treatment with GCV caused an indistinguishable cell cycle arrest in both CEMr1 and CEMss cells, i.e. the proportion of S-phase cells increased and the G1-phase decreased (Fig. 1B). However, the viability of GCV-treated CEMss cells decreased in a dose-dependent manner, whereas the viability of CEMr1 cells was only modestly affected by GCV (Fig. 1C); this finding was confirmed by measuring the population of cells with DNA content less than G1 in cultures of apoptotic cells (31) (Fig. 1D). CEMr1 and CEMss cells were equally sensitive to vinblastine and, as expected, retained their respective resistance and sensitivity to PMEA (Fig. 1E) (4). These results indicate that cells overexpressing MRP4 are sensitive to cell cycle arrest induced by GCV but are substantially more resistant to its cytotoxic effects.
Efflux of GCV Is Enhanced in HSV-TK-transduced Cells That Overexpress MRP4—Sensitivity to GCV can be enhanced by modifying targeted cells to express HSV-TK, but identically modified cells vary markedly in their sensitivity to GCV (13). To evaluate whether MRP4 modulates the GCV sensitivity, we modified CEMss and CEMr1 cells to stably express HSV-TK, isolated cells that expressed similar levels of HSV-TK (Fig 2 B), and treated them with GCV. Although expression of HSV-TK increased the sensitivity of both sets of cells to GCV, their relative sensitivity was preserved (Fig 2 A). The IC50 of GCV was 60 μM in the CEMr1-TK cells, a value five times that in the CEMss-TK cells (12 μM). Thus, MRP4 overexpression strongly influences sensitivity to GCV in cells that are and are not modified to express HSV-TK.

We compared efflux of GCV and its metabolites from CEMss-HSV-TK and CEMr1-HSV-TK cells by pretreating the cells with GCV, suspending them in drug-free medium for 4 h, and quantifying GCV and phosphorylated GCV in the medium by anion exchange chromatography (27). Sixty-one percent of GCV metabolites in the CEMr1-HSV-TK cells and 44% in the CEMss-HSV-TK cells were released (Fig. 2 C). Thirty percent more of the GCV metabolites exported by CEMr1-HSV-TK cells than by the CEMss-HSV-TK cells were phosphorylated. These findings indicate that cells that overexpress MRP4 readily remove GCV metabolites.

Accumulation of GCV Is Reduced in Cells That Overexpress MRP4—The initial uptake of GCV (0–5 min after exposure) was very rapid and was essentially identical in CEMss and CEMr1 cells (not shown). However, marked differences in GCV

![Fig. 1](image_url) Overexpression of MRP4 decreases the cytotoxicity but not the cell cycle arrest induced by GCV. A, a representative immunoblot analysis of MRP4 and MRP1 in CEMss and CEMr1 cells (100 μg of total cell lysate). B, the proportions of CEMss and CEMr1 cells in G, S, or S phase were determined after 48 h of exposure to various concentrations of GCV. C, viability (by trypan blue dye exclusion) of CEMss and CEMr1 cells after 48 h of exposure to GCV. D, the proportion of cells with sub-G1 DNA content (an indicator of apoptosis) was quantified by FACS after staining with propidium iodide. E, CEMss and CEMr1 cells were counted after exposure for 48 h to 80 μM PMEA or 5 nM vinblastine. The error bars represent the standard deviation from the mean value of two to three independent experiments with triplicate determinations.

![Fig. 2](image_url) Cells expressing MRP4 show increased efflux of GCV metabolites and remain GCV-resistant after transduction with HSV-TK. A, CEMss and CEMr1 were transduced with a plasmid encoding recombinant HSV-TK. Cells expressing HSV-TK were treated with GCV and assayed for viability. B, Western blot analysis of HSV-TK in CEMss and CEMr1 cells transduced with HSV-TK. C, efflux of GCV metabolites from CEMss-HSV-TK and CEMr1-HSVTK cells. D, cells were incubated with various concentrations of radiolabeled GCV, and intracellular accumulation of GCV was measured. Some cells were preincubated with indomethacin. The error bars represent one standard deviation from the mean, and their absence indicates the error was smaller than the symbol.
accumulation were observed after 60 min or more. CEMr1 cells accumulated substantially less drug than CEMss cells at every GCV concentration tested (Fig. 2D). Treatment with indomethacin, an inhibitor of MRP4 transport (see below), dramatically increased GCV accumulation in the CEMr1 cells (and to a lesser extent in the CEMss cells; Fig. 2D) and decreased their efflux of radiolabeled GCV nucleotides into the media to the level observed in CEMss cells (not shown).

**Isolation and Expression of Human MRP4**—A cDNA encoding human MRP4 was isolated from two independent human lung cDNA libraries (see “Materials and Methods”). Its coding sequence was identical to the reported sequence (GenBankTM/EBI accession number AF071202) with the exception of seven single-base substitutions. Of these, only four caused amino acid changes. These differences may reflect polymorphisms or may be related to derivation of the reported MRP4 cDNA from a drug-selected cell line; drug selection has been noted to cause such alterations in other mammalian ABC transporters (e.g. MDR1 and BCRP). These differences clearly do not affect the transport of PMEA (see Fig. 3B).

**Uptake of bis-POM-PMEA, Vinblastine, and Methotrexate by Cells Modified to Express MRP4**—The human breast carcinoma cell line, MCF-7, was shown by Western blotting to express very low levels of MRP4. We transduced MCF-7 cells with either the MSCV-MRP4-IRES-GFP or the control MSCV-IRES-GFP. B, uptake of bis-POM[^H]PMEA was measured in cells transduced with either MRP4 or empty vector. C, accumulation of[^H]vinblastine was measured in cells transduced with either MRP4 or empty vector. D, steady-state[^H]MTX concentration was measured as previously described (33). E, the osteosarcoma cell line Saos-2, which expresses minimal MRP4, was transfected with either pcDNA3 or pcDNA3-MRP4, and cells were selected in G418. Asterisks indicate the clones that were expanded for functional analysis. F, uptake of bis-POM[^H]PMEA was inversely proportional to the expression of MRP4. G, indomethacin inhibited MRP4 function, but probenecid did not. Uptake of bis-POM[^H]PMEA was measured in cells preincubated with no drug, with indomethacin, or with probenecid.

![Image](http://www.jbc.org/Downloaded from http://www.jbc.org)
was less than one-third of the uptake by control cells at every concentration tested. In contrast, vinblastine uptake was identical in test and control cells (Fig. 3C). Interestingly, whereas earlier studies suggested that MRP4 plays a role in MTX uptake and efflux (3), we found that the steady-state intracellular concentration of methotrexate was the same in test and control cells (Fig. 3D).

We next compared MRP4 function with the quantity of MRP4 protein expressed. Saos-2 human osteosarcoma cells, which express very little MRP4, were transfected with pcDNA3-MRP4 and selected in G418. Immunoblot analysis revealed a wide range of MRP4 expression (Fig. 3E). Although there appeared to be an inverse relationship between expression of MRP4 and expression of MRP1 in CEMss cells (Fig. 1A), MRP1 and MRP4 expression were completely unrelated in Saos-2 transfectants (Fig. 3E). Intracellular accumulation of PMEA was inversely proportional to the quantity of MRP4 expressed: accumulation of PMEA was decreased (~41%) at low levels of MRP4 expression and substantially (more than 95%) reduced at very high levels of MRP4 expression (Fig. 3F). Accumulation of PMEA in the Saos-2 MRP4 transfectants was increased over 600% by treatment with 100 μM indomethacin.

**Fig. 4.** Ectopic expression of MRP4 in MCF-7 cells reduces GCV uptake and cytotoxicity. A, MCF-7 and MCF-7-MRP4 cells were incubated for various intervals with radiolabeled GCV, and intracellular radioactivity was measured. B, MCF-7 and MCF-7-MRP4 cells were incubated with various concentrations of radiolabeled GCV, and intracellular radioactivity was measured. C, MCF-7 and MCF-7-MRP4 cells were pretreated with radiolabeled GCV, washed in ice-cold buffer, and resuspended in warmed drug-free medium. Intracellular radioactivity was measured at the indicated intervals. D, MCF-7 and MCF-7-MRP4 cells were exposed for 72 h to GCV at the indicated concentrations, and an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to determine viability. E, MCF-7 and MCF-7-MRP4 cells were exposed for 4 h to various concentrations of MTX. No difference in MTX sensitivity was seen at either 4 h or after 72 h of exposure (not shown).

**Fig. 5.** Clonal dilution bystander assay reveals MRP4 overexpressing MCF-7 cells are more resistant to cell killing by HSV-TK expressing cells. SW620-TK cells mixed in various proportions with MCF7 or MCF7-MRP4 cells were treated with 10 μM GCV for 24 h (7, 16). Surviving cell colonies were quantified (27). The values are obtained from triplicate determinations in a single experiment that was repeated twice with similar results.
Modified to Express MRP4 showed a modest increase (56%) in PMEA accumulation, pos-
effect (Fig. 3G). In contrast, 100 µM probenecid treatment had no
effect (Fig. 3G). Indomethacin-treated Saos-2 control cells also showed
a modest increase (56%) in PMEA accumulation, possibly reflecting inhibition of an endogenous transporter.

Accumulation, Efflux, and Cytotoxicity of GCV in MCF7 Cells Modified to Express MRP4—We observed no difference be-
tween control and MRP4-transfected MCF-7 cells in the initial rates of uptake (<5 min) of GCV, gemcitabine (a cytidine anal-
og). This finding is consistent with the patterns of mRNA expression we observed for the major nucleoside transporters
(hCNT1a, ENT2, and hENT1) (not shown). However, there were striking differences between test and control cells in GCV
accumulation: the steady-state concentration of GCV in the MRP4-transfected cells was only ~50% that in the vector cells
(Fig. 4A). This disparity was observed over a wide range of concentrations of GCV (Fig. 4B).

We investigated whether efflux was altered in the cells ex-
pressing MRP4. First, we treated these cells with ~1.5 times the amount radiolabeled GCV used to treat control cells to
obtain a comparable intracellular GCV concentration. We then washed the cells, placed them in drug-free medium, and mea-
sured intracellular radioactive GCV (Fig. 4C). The decline of intracellular GCV was more rapid in the MRP4-transfected
cells compared with vector cells (t1/2 s of ~16 and 32 min, respectively). This increased efflux was accompanied by a strik-
ing decrease in GCV-induced cytotoxicity (Fig. 4D) but not in
MTX-induced cytotoxicity (Fig. 4E). Similar results were ob-
tained in parallel experiments with Saos-2 cells modified to
express MRP4 (not shown).

Expression of MRP4 Affects Bystander Cell Killing—Because
MRP4 transports nucleotide derivatives (1–3, 24, 26, 32) in-
cluding GCV anabolites from cells, we reasoned that the killing of bystander cells (those that do not express TK) by GCV
could be significantly influenced by MRP4 expression. To explore this possibility, we used an SW620-HSV-TK cell line that activates
GCV and readily effluxes GCV nucleotides (27). We performed a
clonal dilution assay (27) in which either MCF-7 or MCF-7-
MRP4 cells were co-cultured with increasing percentages of the
SW620 HSV-TK cells (see “Materials and Methods”) (Fig. 5). As
the proportion of HSV-TK-expressing cells increases the deliv-
ery of GCV metabolite increases (30) leading to greater cell
death of bystander cells. The MCF-7-MRP4 cells are more
resistant to bystander killing. However, with very high propor-
tions of HSV-TK cells we see that MRP4 overexpression offers
less protection. These studies indicate that MRP4 plays a
strong role in bystander cell survival, but suggest its protection
may be overcome by increasing the proportion of HSV-TK ex-
pressing cells.

DISCUSSION

We have shown that expression of MRP4 affects the sensi-
tivity of cells to the therapeutically important antiviral gua-
nine analog GCV and that it modulates the direct and by-
stander cytotoxicity in which cells modified to express HSV-TK
are treated with GCV. Cells that overexpressed MRP4 accu-
ulated smaller quantities of GCV metabolites than did con-
trols. Although the sensitivity to GCV-induced cell cycle arrest
was not reduced in these cells, they were protected from GCV
cytotoxicity. This finding is consistent with a relationship be-
tween GCV cytotoxicity and the intracellular concentration of
the drug (7, 14). However, the effect did not extend to all cytoxins: sensitivity to methotrexate and vinblastine was not
affected.

Because we and others have demonstrated that GCV metab-
olites are readily transported from cells expressing HSV-TK
(17, 27, 30), we evaluated the effect of MRP4 expression on GCV cytotoxicity of cells gene-modified to express HSV-TK.

HSV-TK cells that overexpressed MRP4 had significantly en-
hanced resistance to GCV (Fig. 2, A and B). This finding has
strong implications for gene therapy with HSV-TK because
varying MRP4 expression among cells gene-modified with
HSV-TK would lead to decreased cytotoxicity.

The success of gene therapy requires not only that the HSV-
TK-modified cells export GCV nucleotides but also that the
bystander cells accumulate and retain sufficient nu-
cleotides to induce apoptosis. An increase in the extracellular
concentration of GCV can induce the killing of otherwise in-
tractable bystander cells (13), suggesting the existence of a
mechanism that excludes GCV nucleotides or decreases their
intracellular concentration in these cells. Bystander killing by
GCV can also be enhanced by prolonged exposure to the drug
(13). Our results strongly suggest that expression of MRP4 by
bystander cells protects them from GCV cytotoxicity when they
are co-cultured with initiator cells expressing HSV-TK (Fig. 5).
However, this protection decreases as the proportion of HSV-
TK-expressing cells increases, implying that a threshold level
of GCV nucleotide is exceeded and overrides the protective
effect of MRP4. Nevertheless, this result suggests that cells
overexpressing MRP4 would be less susceptible to the by-
stander effect because of their reduced accumulation of GCV
nucleotides.

Our results demonstrate that cellular retention and accumu-
lation of the guanine analog ganciclovir is strongly influenced
by MRP4. Expression of this nucleotide transporter may reduce
the antiviral efficacy of GCV therapy, which is often used to
treat or prevent cytomegalovirus disease in transplant recipi-
ents and patients with AIDS. It can also attenuate both direct
GCV cytotoxicity and bystander cell killing in cells modified to
express HSV-TK to induce selective cell killing. We have also
shown that cells that express MRP4 are much less susceptible
to GCV despite modification with an HSV-TK expression vec-
tor. These studies provide the mechanistic basis for future
studies evaluating the role of MRP4 expression in antiviral
response to GCV and in HSV-TK and GCV gene therapy with
solid malignancies (e.g. brain tumors) Finally, a challenge for
the future will be to determine the degree to which MRP4
modulates the levels of toxic metabolites near bystander cells.

Acknowledgments—We thank Dr. Richard Ashmun and Sam Lucas
for FACScan analysis, Dr. Dan Hua Pan for excellent technical assistance,
and Vicki Gray for help in preparing this manuscript. We also
thank Drs. Phil Potter and Brian Sorrentino for their critical comments
and Sharon Naron for excellent editorial advice.

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Expression of MRP4 Confers Resistance to Ganciclovir and Compromises Bystander Cell Killing

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J. Biol. Chem. 2002, 277:38998-39004.
doi: 10.1074/jbc.M203262200 originally published online June 24, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203262200

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