The therapeutic combination of the herpesvirus simplex virus type 1 (HSV-1) thymidine kinase (TK) gene and the prodrug, ganciclovir (GCV), has found great utility for the treatment of many types of cancer. After initial phosphorylation of GCV by HSV-1 TK, cellular kinases generate the toxic GCV-triphosphate metabolite that is incorporated into DNA and eventually leads to tumor cell death. The cellular and pharmacological mechanisms by which metabolites of GCV lead to cell death are still poorly defined. To begin to address these mechanisms, different mutated forms of HSV-1 TK at residue Gln-125 that have distinct substrate properties were expressed in mammalian cell lines. It was found that expression of the Asn-125 HSV-1 TK mutant in two cell lines, NIH3T3 and HCT-116, was equally effective as wild-type HSV-1 TK for metabolism and sensitivity to GCV, bystander effect killing and induction of apoptosis. The major difference between the two enzymes was the lack of deoxypyrimidine metabolism in the Asn-125 TK-expressing cells. In HCT-116 cells expressing the Glu-125 TK mutant, GCV metabolism was greatly attenuated, yet at higher GCV concentrations, cell sensitivity to the drug and bystander effect killing were diminished but still effective. Cell cycle analysis, 4′,6′-diamidine-2′-phenylindole dihydrochloride staining, and caspase 3 activation assays indicated different cell death responses in the Glu-125 TK-expressing cells as compared with the wild-type HSV-1 TK or Asn-125 TK-expressing cells. A mechanistic hypothesis to explain these results based on the differences in GCV-triphosphate metabolite levels is presented.

Delivery and expression of herpesvirus thymidine kinase (HSV-1 TK) in combination with ganciclovir (GCV) has shown great clinical promise as a gene therapy of different cancers (1–4). GCV is a prodrug that must be initially phosphorylated by HSV-1 TK and then cellular kinases to the toxic triphosphate form, GCVTP, that incorporates into cellular DNA and may act as an inhibitor of DNA polymerase 6 (5–7). The basis for the original clinical trials was the ability of 10% or less HSV-1 TK-expressing cells to mediate a bystander effect whereby non-TK-expressing cells also became sensitive to GCV killing (1, 8, 9). In vitro, the primary mechanism of the bystander effect has been determined to be the gap junction mediated transfer of GCV metabolites to neighboring non-HSV-1 TK-expressing cells (10–13). In response to GCV phosphorylation and/or metabolite transfer, most cell types have been reported to undergo apoptosis, which appears to be the cellular mechanism by which both the HSV-1 TK-expressing cells and bystander cells ultimately die (5, 11, 14). Recently, GCV has been reported to induce S- and G2/M phase cell cycle arrest in HSV-1 TK-expressing cells (5, 15–17), and these changes were associated with modulation of Cdc2/cyclin B activities (16) and increased levels of cyclin B1 (15). In vivo, it is clear that initial HSV-1 TK/GCV tumor cell killing results in a complex inflammatory stimulation of the immune system that affects all tumor cells (1, 18–21). Regression of tumors distant from the primary HSV-1 TK-expressing site and establishment of anti-tumor immunity has also been demonstrated, and this has been termed the distant bystander effect (22–24).

All of the effects of GCV metabolites must somehow be linked to their incorporation into DNA and the disruption of the cell cycle, which in many cell systems results in induction of apoptosis, yet these links are still not clear. It is apparent, however, that phosphorylation of GCV by HSV-1 TK leads to a broad range of diverse pharmacological, cellular, and physiological effects in vitro and in vivo. Optimizing these effects and understanding the biochemical mechanism by which GCV acts could lead to improved therapeutic and clinical outcomes for genetic therapies of cancer utilizing HSV-1 TK. A separate study has described the characterization of the enzymatic properties and cell killing properties of three site-specific mutations of Gln-125 in HSV-1 TK to Asp, Asn, or Glu.2 It was observed that when expressed in the human colon tumor cell line HCT-116 and treated with GCV, the Glu-125 mutant was equally effective at cell killing as the wild-type (Gln-125) or Asn-125 HSV-1 TKs. This was despite the diminished enzymatic properties of Glu-125 HSV-1 TK compared with wild-type Gln-125 TK, which included a 7-fold higher \( k_{m} \) for GCV and a 83-fold decrease in \( k_{m}/K_{m} \). In this current report, further characterization of cell lines expressing these mutant HSV-1 TKs and analysis of the differences in cellular responses to GCV are evaluated. Besides determining that the Asn-125 TK enzyme acts just as efficiently as wild-type enzyme in these cell lines, we report differences in the cell cycle progression, apoptosis induction, bystander killing, and GCV dose effects of the Glu-125 TK enzyme. These studies demonstrate how cellular expression of different HSV-1 TK mutants with distinct enzymatic properties can be used to evaluate the unique pharmacological properties of GCV.

* This work was supported in part by a grant from the Arkansas Science and Technology Authority (to R. R. D.) and by National Institutes of Health Grant CA77938-01 (to R. R. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biochemistry, Slot 516, University of Arkansas for Medical Sciences, 4301 W. Markham, Little Rock, AR 72205. Tel.: 501-686-5419; Fax: 501-686-8169; E-mail: drakerickr@exchange.uams.edu.

§ The abbreviations used are: HSV-1, herpes simplex virus type 1; TK, thymidine kinase; GCV, ganciclovir; GCVTP, toxic triphosphate form of GCV; DAPI, 4′,6′-diamidine-2′-phenylindole dihydrochloride.

** This paper is available on line at http://www.jbc.org
Expression and Characterization of HSV-1 TKs in Cell Lines—A Moloney murine leukemia virus derived plasmid for the expression of HSV-1 TK, termed pLENTK, has been previously constructed (12). A unique BspEI-MluI restriction fragment within the HSV-1 TK sequence of pLENTK contains the Gin-125 mutation site. This fragment was removed from wild-type plasmid and replaced with the analogous fragments encoding each mutant. The new pLEN-mutant TK constructs were introduced into the NIH-3T3 cells by transfection. Each cell line expressing wild-type HSV-1 TK plasmid, each mutant TK plasmid was transfected individually into the murine fibroblast cell line, NIH-3T3, and the human colon tumor cell line, HCT-116, using Lipofectin reagent (Life Technologies, Inc.) (2 μg of plasmid, 14 μl of lipid/1 × 10^6 cells). Cells were maintained in RPMI 1640 medium and selected with G418 (200 μg/ml for 2 weeks) as described previously (12). At least eight individual G418-resistant cell clones were picked and grown up for further characterization. Each clone was screened initially for growth inhibition by 25 μM GCV. Those clones that were sensitive were further analyzed for HSV-1 TK protein expression by Western blot analysis with a polyclonal, rabbit anti-HSV-1 TK antibody (a gift from Dr. Margaret Black, Washington State University). For each clone, cell numbers were normalized to 1 × 10^5, and equal protein loading was confirmed for each sample by gel staining. Blotted HSV-1 TK protein bands were visualized on film using ECL chromophore reagents (Amersham Pharmacia Biotech). Each HSV-1 TK cell line set used was characterized for comparative metabolite expression levels of HSV-1 TK to one another as determined by the Western blot analyses.

Metabolic Labeling with [3H]Nucleosides—For metabolic labeling, cells (1–2 × 10^5) were labeled in triplicate in 2 μCi of [3H]GCV (8 μM) for 18 h, and then nucleotides were extracted from pelleted cells in 0.2 ml of 70% methanol at 4 °C for 15 min as described previously (12, 25). An aliquot of each methanol-soluble supernatant was analyzed for radioactivity by scintillation counting. The methanol insoluble pellets, representative of a crude DNA fraction, were resuspended in 0.15 ml of water and also counted for radioactivity. For deoxypyrimidines, cells were grown to confluency in 60-cm^2 plates, and either 2 representative of a crude DNA fraction, were resuspended in 0.15 ml of 0.15 M LiCl for GCV or 0.35 M LiCl for thymidine/dC as determined by the Western blot analyses.

RESULTS

Metabolic labeling with [3H]GCV, [3H]Thymidine, and [3H]dT—In a separate study, three site-specific mutations of Asp, Asn, and Gin were introduced on a plasmid DNA by site-directed mutagenesis (25). The mutant increasing (473 M GCV using an Apo-Alert CPP32/Caspase 3 kit with the peptide substrate, DEVD-pNA, as per manufacturer's instructions (CLONTECH). GCV-treated and untreated cells were grown in 25-cm^2 flasks, and cell numbers were determined using a hemocytometer prior to analysis. Assays were done in triplicate with protein extracts derived from 2 × 10^6 cells. The amount of Caspase 3-like activities were quantitated using a Shimadzu UV/Vis spectrophotometer at 405 nm.

Cell Cycle Analysis—Parental HCT-116 cells and each HSV-1 TK-expressing cell line were grown to 80% confluence in 25-cm^2 flasks and treated for 24 h plus or minus 25 μM GCV. Following drug incubation, cells were removed by trypsin, and total cell numbers were determined. The cells were then washed twice in phosphate-buffered saline and fixed in 1 ml of 70% ethanol at 4 °C for at least 1 day. Just prior to cell cycle analysis, the ethanol was removed and cell pellets were resuspended in 0.1% bovine serum albumin plus RNase (0.1%) and propidium iodide (50 μg/ml) for 30 min at room temperature to stain DNA. DNA content was measured using a FACS Calibur flow cytometer (Becton Dickinson), and data were analyzed using MODFIT LT (Verity Software House) computer software.

Differential GCV-mediated Cell Killing by Mutant HSV-1 TKs

MATERIALS AND METHODS

Cell Cycle Analysis—Parental HCT-116 cells and each HSV-1 TK-expressing cell line were grown to 80% confluence in 25-cm^2 flasks and treated for 24 h plus or minus 25 μM GCV. Following drug incubation, cells were removed by trypsin, and total cell numbers were determined. The cells were then washed twice in phosphate-buffered saline and fixed in 1 ml of 70% ethanol at 4 °C for at least 1 day. Just prior to cell cycle analysis, the ethanol was removed and cell pellets were resuspended in 0.1% bovine serum albumin plus RNase (0.1%) and propidium iodide (50 μg/ml) for 30 min at room temperature to stain DNA. DNA content was measured using a FACS Calibur flow cytometer (Becton Dickinson), and data were analyzed using MODFIT LT (Verity Software House) computer software.

Differential GCV-mediated Cell Killing by Mutant HSV-1 TKs

HCT-116 cells were treated for 2 days at a range of 0%:100%, 25%:75%, 50%:50%, and 75%:25%, 100%:0%. After 2 days, 25 μM GCV was added to each cell line in triplicate (3 × 10^5 cells/well) in 8-well plate (3 × 10^5 cells/well) in 1 ml of fresh medium on a separate plate. After 2 days, the medium was removed, and cells from each well were diluted from 1:10 to 1:10,000 in 1 ml of fresh medium on a separate plate. After 7 days, surviving cell colonies were fixed and stained for counting.

DAPI Staining of Apoptotic Cells—Parental HCT-116 cells and each HSV-1 TK-expressing cell line were plated in triplicate (5 × 10^5 cells/well) in 8-well plastic chamber slides (Lab-Tek) and left untreated or treated with 25 μM GCV for 36 or 34 h. At either time point, cells were washed with phosphate-buffered saline followed by staining in 1 μg/ml DAPI in 100% methanol for 10 min (26). After rinsing, the stained cells were visualized with a DAPI-specific filter on a Zeiss fluorescent microscope at 40× magnification.

Caspase 3 Assay—Caspase 3-like activity was determined in parental HCT-116, and each HSV-1 TK-expressing cell line was treated for 50 h plus or minus 25 μM GCV using an Apo-Alert CFP32/Caspase 3 Colorimetric Assay kit with the peptide substrate, DEVD-pNA, as per manufacturer's instructions (CLONTECH). GCV-treated and untreated cells were grown in 25-cm^2 flasks, and cell numbers were determined using a hemocytometer prior to analysis. Assays were done in triplicate
Differential GCV-mediated Cell Killing by Mutant HSV-1 TKs

Table I
Total methanol soluble and insoluble ³H-labeled metabolites

| Cell Line     | NIH3T3   | HCT-116   |
|---------------|----------|-----------|
|               | [³H]GCV  | [³H]GvDNA | [³H]GCV  | [³H]GvDNA | [³H]T    | [³H]dC   |
| Parent        | 2.8      | 2.8       | 1.1      | 1.2       | 2.4     | 1.1      |
| Gln-125 TK    | 498      | 48        | 1216     | 141       | 52.0    | 4.5      |
| Asn-125 TK    | 585      | 42        | 1085     | 133       | 8.0     | 1.2      |
| Glu-125 TK    | 3.6      | 2.6       | 33       | 27        | 3.3     | 0.9      |
| Asp-125 TK    | ND       | ND        | 1.0      | 1.6       | ND      | ND       |

Table II
Phosphorylated metabolites of [³H]GCV and [³H]thymidine from HCT-116 and NIH3T3 cell clones

| Cell Line     | NIH3T3   | HCT-116   |
|---------------|----------|-----------|
|               | GCVMP    | GCVDP     | GCVT    |
| Parent        | 0.2      | 0         | 0       |
| Gln-125 TK WT | 12       | 22        | 232 (48)|
| Asn-125 TK    | 11       | 21        | 272 (71)|
| Glu-125 TK    | 0        | 0         | 1.6 (0.4)|

| Cell Line     | NIH3T3   | HCT-116   |
|---------------|----------|-----------|
|               | GCVMP    | GCVDP     | GCVT    | TMP     | TTP     |
| Parent        | 0.4      | 0         | 0       | 0.1     | 0.2     |
| Gln-125 TK    | 96       | 101       | 415 (30)| 1.2     | 9.5     |
| Asn-125 TK    | 95       | 104       | 533 (44)| 0.2     | 0.9     |
| Glu-125 TK    | 3.0      | 2.5       | 18 (5)  | 0.2     | 0.4     |

FIG. 1. Clonal dilution assays for GCV sensitivity. Parental HCT-116 (diamond), wild-type HSV-1 TK cells (triangle), Asn-125 TK cells (square), and Glu-125 TK cells (circle) were treated with 0, 0.1, 1, or 10 μM GCV in triplicate for 24 h. After this time, each well of cells was sequentially diluted from 1:10 to 1:10,000 in 1 ml of fresh medium on a separate 24-well plate as described under "Materials and Methods." After 7 days, surviving cell colonies were fixed in 100% methanol, stained with 0.1% methylene blue, and counted.
results in induction of apoptosis (5, 11, 14, 15). Therefore, two late stage apoptosis assays, nuclear DAPI staining and caspase-3 activation, were done for GCV treatments of the three HSV-1 TK-expressing HCT-116 cell lines. As shown in Fig. 4, DAPI-stained nuclei of wild-type and Asn-125 TK-expressing cells treated with GCV for 36 or 84 h indicated progressive increases in condensed and fragmented nuclei characteristic of apoptosis. Also, the DAPI staining of these cell lines indicates a GCV-specific nuclear swelling of preapoptotic cells and enhanced staining of nucleoli. This nuclear swelling in response to GCV has been observed within 12 h of GCV administration in wild-type HSV-1 TK HCT-116 cells (data not shown) and has also been reported for other GCV treated HSV-1 TK cell lines (16, 17). For the Glu-125 HSV-1 TK-expressing cells, 36 h of GCV treatment led to fewer swelled nuclei and little evidence of apoptotic nuclei, although distinct staining of condensed nucleoli was observed. Even after 84 h of GCV treatment of these cells, there were still comparatively fewer changes in nuclear morphologies of the Glu-125 TK cells compared with the wild-type or Asn-125 HSV-1 TK-expressing cells, although there was more apparent nuclear swelling. Under identical treatment conditions, GCV treatments of parental, non-HSV-1 TK-expressing HCT-116 cells indicated none of the nuclear swelling or apoptotic fragmentations seen in the three HSV-1 TK-expressing cell lines (data not shown).

A more direct analysis of apoptotic activity was done by assaying the activation of the executioner protease, caspase 3. Activation of the zymogen form of caspase 3 has been implicated as a component of the late execution phase of apoptosis, and the substrate proteins cleaved by activated caspase 3 and related enzymes are responsible for the end stage morphological and intracellular changes associated with apoptotic cell death (27, 28). Caspase 3 activity was determined in different cell extracts derived from GCV-treated and control cells using a colorimetric assay with the peptide substrate DVED as described under “Materials and Methods.” As shown in Fig. 5, the DVEDase activity of GCV-treated Glu-125 HSV-1 TK cells was three times lower than that observed for GCV-treated wild-type or Asn-125 HSV-1 TK-expressing cells. Co-incubation of GCV-treated wild-type HSV-1 TK-expressing cells with the competing peptide DVED resulted in caspase 3 activities near untreated control cell values (data not shown). Thus, the results of the DAPI staining and caspase 3 assays are consistent with an altered apoptotic response and cell death pathway in GCV-treated Glu-125 HSV-1 TK-expressing cells. The cumulative results of this study are consistent with two distinct cell death responses induced by GCV treatment in the same HCT-116 cell line background that is dependent on the distinct enzymatic properties of HSV-1 TK.

DISCUSSION

The killing of tumor cells with HSV-1 TK and GCV is a complex interactive sequence of biochemical and cellular events involving incorporation and accumulation of GCVMP into DNA, disruption and inhibition of the cell cycle, gap junction metabolite transfer, and apoptosis. Because of all these interactions, defining the exact sequential processes involved and the primary cellular targets of GCV metabolites has proven difficult. In our report, we describe a new approach to characterizing the action of GCV by using HSV-1 TKs with altered and distinct enzymatic properties. By using the HCT-116 cells that have been previously characterized for bystander effect sensitivity (12) and apoptosis events, and by normalizing expression levels of the HSV-1 TKs in these cell lines, we sought to establish a cell system in which the major variable for GCV sensitivity would be the inherent activity of the expressed HSV-1 TK. Comparing the effects of the expressed Glu-125 TK
enzymes with wild-type HSV-1 TK in the HCT-116 cells led to the unexpected observations of differential intracellular responses and cell death mechanisms in response to GCV as indicated by the DAPI staining, cell cycle analysis, and caspase 3 assays.

The expressed Asn-125 TK mutant appears to act identically to wild-type HSV-1 TK when expressed in HCT-116 and NIH3T3 cells, the only major difference being the lower metabolism of deoxyxpyrimidine substrates by the Asn-125 TK. The catalytic efficiency ($k_{\text{cat}}/K_m$) of the purified Asn-125 TK and Glu-125 TK enzymes for GCV have been determined to be four times and 82 times lower, respectively, than wild-type HSV-1 TK, yet both enzymes were still effective at mediating GCV killing of HCT-116 tumor cells. This lower efficiency did not seem to affect the amount of GCV metabolites generated by the expressed Asn-125 TK, because they were equivalent to wild-type HSV-1 TK (Tables I and II). The Glu-125 TK proved ineffective when expressed in NIH3T3 cells, which was also reflected by the GCV metabolite levels. These very low GCV metabolite levels and minimal DNA incorporation are likely the reasons for this inactivity in the NIH3T3 cells, but why this occurs is not clear. It is possible that the NIH3T3 cells have comparatively more active nucleotide phosphatase or efflux activities than the HCT-116 cell lines. These activities could be saturated in the wild-type or Asn-125 TK-expressing cells but not in the lower metabolizing Glu-125 TK-expressing cells. In contrast, the low amount of GCVTP generated in the Glu-125 TK-expressing HCT-116 cells was sufficient to cause a 2-log reduction in cell colonies (Fig. 1) and generate effective bystander killing (Fig. 2).

In several studies that analyzed the effects of GCV on cell cycle inhibition and DNA synthesis in HSV-1 TK-expressing tumor cells, the general conclusion was that the primary mechanistic action of GCV was related to its accumulation and incorporation into DNA (5, 15, 16). The resulting DNA damage and instability activates cell cycle regulatory proteins that lead to cell cycle arrest in S and early G2 phases (15–17). Eventually this cell cycle inhibition leads to cell death via apoptosis (5, 15) or by a nonapoptotic process (16, 17). Interestingly, two different cell death mechanisms were described for the same murine melanoma B16F10 cell lines in response to GCV. One study demonstrated induction of apoptosis in response to GCV (15), whereas the other concluded that cell cycle arrest caused cell death and did not require induction of apoptosis (16). This is intriguing because our data suggest that distinct cell death responses to GCV are occurring in the same HCT-116 cell line. It was also demonstrated that GCV metabolites had no direct effects on the synthesis of DNA (5), this despite in vitro studies showing that GCVTP is a selective inhibitor of DNA polymerase $\delta$ ($K_i = 2 \mu M$) (7). Another critical pharmacological feature of GCV related to its incorporation into DNA is its ability to cause multi-log cell killing at short exposure times and very low drug doses compared with other nucleoside drugs like 1-$\beta$-arabinofuranosylcytosine or acyclovir (5, 25). As can also be seen in our results in Figs. 1 and 2, low doses of GCV can cause significant cell killing (5). It is likely this low dose aspect of GCV that is the primary reason for its effectiveness in mediating the bystander effect (13). Using the same GCV metabolite analysis procedure as described herein, we have detected a range of GCVTP metabolite levels that vary widely between different tumor cell lines (ranging from 0.5 to 533 pmol/10⁶ cells) (12, 29). Even the lowest range of GCVTP levels were effective for the particular cell line analyzed. In regards to our mutant Glu-125 TK, it is likely that expression of this gene in other cell lines will result in similar variable killing and dose effects (as seen with the NIH3T3 and HCT-116 cells) depending on the inherent properties of each cell line.

How do the results from the Asn-125 TK- and Glu-125 TK-expressing HCT-116 cells relate to the previously reported mechanistic studies of GCV? Clearly the metabolic labeling and cell killing data for the poor GCV metabolizing Glu-125 TK highlights the low dose effectiveness of the drug in killing tumor cells. Especially noteworthy is the ability of 10% Glu-125 TK-expressing cells to cause a 1-log reduction in cell colonies in the bystander effect assays (Fig. 2). Whatever the primary cellular target of GCVTP is in the HCT-116 cells, it is ex-
Differential GCV-mediated Cell Killing by Mutant HSV-1 TKs

Parental HCT-116 and the three HSV-1 TK-expressing cell lines were treated with 25 μM GCV for 24 h. The distribution of cells in G0/G1, S, and G2/M phases of the cell cycle were quantified by flow cytometric analysis as described under “Materials and Methods.” The corresponding DNA histograms are presented in Fig. 3.

| Cell line | Cell cycle phase |
|-----------|-----------------|
|           | G0/G1 | S  | G2/M |
| HCT-116 (−GCV) | 43.3 | 35.0 | 21.7 |
| HCT-116 (+GCV) | 39.0 | 44.0 | 17.1 |
| WT Gln-125TK (−GCV) | 65.8 | 18.4 | 15.8 |
| WT Gln-125TK (+GCV) | 60.1 | 39.9 | 0 |
| Asn-125TK (−GCV) | 63.4 | 18.0 | 18.6 |
| Asn-125TK (+GCV) | 70.0 | 30.0 | 0 |
| Glu-125TK (−GCV) | 62.4 | 8.5 | 29.1 |
| Glu-125TK (+GCV) | 39.0 | 61.0 | 0 |

GCV as compared with the wild-type and Asn-125 TK cell lines. The cumulative data indicate a comprehensively different response of the Glu-125 TK-expressing HCT-116 cells to GCV than for HCT-116 cells expressing wild-type HSV-1 TK or Asn-125 TK. We hypothesize that the Glu-125 TK-expressing HCT-116 cells are undergoing necrosis in response to GCV rather than apoptosis; however, it remains to be proven. A necrotic response to GCV has been demonstrated in vivo with the B16 melanoma model, and it was determined that a necrotic cell death in response to GCV was more immunostimulatory than apoptotic cell death (30).

Based on the fact that the Glu-125 TK-expressing cells only require minimal levels of GCVTP to ultimately lead to some form of cell death, it is apparent that the levels of GCVTP generated in the wild-type and Asn-125 TK-expressing cells are in large excess of those required to kill the cell. We propose that in the wild-type and Asn-125 TK-expressing cells, the excess GCVTP and possibly other GCV metabolites saturate the primary cellular target and with time accumulate and interact with other secondary or tertiary cellular targets. As an acyclic analog of both dGTP and GTP, GCVTP could act as a mimic of GTP and inhibit GTPases or other GTP-binding proteins involved in the regulation of the cell cycle, signal transduction cascades, and/or cytoskeletal organization. This secondary or tertiary inhibition of cellular targets by GCVTP could be the cause of, or at least potentiate, the induction of apoptosis. This excess in GCVTP would not accumulate to the levels observed for the wild-type or Asn-125 TK cell lines in the Glu-125 TK cells, and thus the result is less of an apoptotic response. We have observed that GCV treatment of wild-type HSV-1 TK-expressing HCT-116 cells leads to modulation of protein levels of the Bcl-2 family members, Bak and Bcl-XL. Also, co-incubation of GCV with the protein kinase inhibitor, UCN-01 (7-hydroxystaurosporine), in these same cells can increase the amount and rate of the apoptotic response.3 Although these latter observations are consistent with the proposed secondary and tertiary effects of GCVTP on apoptosis, our hypothesis remains to be tested. Utilizing the Glu-125 TK mutant for comparative analysis with wild-type expressing HSV-1 TK cells should allow us to test this hypothesis in the HCT-116 cells and other cell lines. More extensive cell cycle studies and analysis of changes in cell cycle regulatory proteins in response to GCV should prove most informative in this regard. Because of the in vivo stimulation of the immune system in response to

3 McMasters, R., Wilbert, T. N., Jones, K. E., Pitliky, K., Saylor, R. L., Moyer, M. P., Chambers, T. C., and Drake, R. R. (2000) Cancer Gene Ther., in press.
GCV treatment in many animal studies (18–24, 30), it will also be interesting to determine whether the altered cell death response in tumors expressing the Glu-125 TK translates to any differences in the in vivo immune response in animal tumor models.

REFERENCES

1. Freeman, S. M., Whartenby, K. A., Freeman, J. L., Abboud, C. N., and Marrogi, A. J. (1996) Semin. Oncol. 23, 31–45
2. Klatzmann, D., Valery, C. A., Bensimon, G., Marro, B., Boyer, O., Molnar-Kimber, K., Diquet, B., Salzmann, J. L., and Philipp, J. (1998) Hum. Gene Ther. 9, 2595–2604.
3. Sterman, D. H., Treat, J., Litzy, L. A., Amin, K. M., Conrod, L., Molnar-Kimber, K., Recio, A., Knox, L., Wilson, J. M., Albelda, S. M., and Kaiser, L. R. (1998) Hum. Gene Ther. 9, 1083–1092.
4. Bonini, C., Ferrari, G., Verzeletti, S., Servida, P., Zappone, E., Ruggieri, L., Ponsoni, M., Rossini, S, Mavilio, F., Traversari, C., and Bordignon, C. (1997) Science 276, 1719–1724.
5. Rubsam, L. Z., Davidson, B. L., and Shewach, D. S. (1998) Cancer Res. 58, 3873–3882.
6. Smee, D. F., Boehme, R., Chenow, M., Binko, B. P., and Matthews, T. R. (1985) Biochem. Pharmacol. 34, 1049–1056.
7. Eisley, D. D., Lee, S. H., Miller, W. H., and Kuchta, R. D. (1995) Biochemistry 34, 2504–2510.
8. Culver, K. W., Ram, Z., Wallbridge, S., Ishii, H., Oldfield, E. H., and Blaesa, E. M. (1992) Science 256, 1550–1552.
9. Freeman, S. M., Abboud, C. N., Whartenby, K. A., Packman, C. H., Koeplin, D. S., Moolten, P. L., and Abraham, G. N. (1993) Cancer Res. 53, 5274–5283.
10. Bi, W. L., Parysek, L. M., Warnick, R., and Stambrook, P. J. (1995) Hum. Gene Ther. 4, 725–731.
11. Denning, C., and Pitts, J. D. (1997) Hum. Gene Ther. 8, 1825–1835.
12. McMasters, R., Jones, K. E., Saylors, R. L., Hendrix, E. M., Moyer, M. P., and Drake, R. R. (1998) Hum. Gene Ther. 9, 2253–2261.
13. Rubsam, L. Z., Boucher, P. D., Murphy, P. J., KuKuruga, M and Shewach, D. S. (1999) Cancer Res. 59, 669–675.
14. Hamel, W., Magnelli, L., Chiarugi, V. P., and Israel, M. A. (1996) Cancer Res. 56, 2697–2702.
15. Wei, S., Chao, Y., Hung, Y., Lin, W., Yang, D., Shib, Y., Ch'ang, L., Whang-Peng, J., and Yang, W. K. (1998) Exp. Cell Res. 241, 66–75.
16. Halloran, P. J., and Fenton, B. G. (1998) Cancer Res. 58, 3855–3865.
17. Kaneko, Y., and Tsukamoto, A. (1995) Cancer Lett. 96, 105–110.
18. Freeman, S. M., Ramesh, R., and Marrogi, A. J. (1997) Lancet 349, 2–3.
19. Vile, R. G., Castleden, S., Marshall, J., Camplejohn, R., Upton, C., and Chong, H. (1997) Int. J. Cancer 71, 267–274.
20. Hall, S. J., Sanford, M. A., Atkinson, G., and Chen S.-H. (1998) Cancer Res. 58, 3221–3225.
21. Mullen, C. A., Anderson, L., Woods, K., Nishino, M., and Petropoulos, D. (1998) Hum. Gene Ther. 9, 2019–2030.
22. Bi, W., Kim, Y.-G., Feliciano, E. S., Pavelic, L., Wilson, K. M., Pavelic, Z. P., and Stambrook, P. J. (1997) Cancer Gene Ther. 4, 246–252.
23. Kianmanesh, A. R., Perrin, H., Paniz, Y., Fahr, M. Nagy, H. J., Houssin, D., and Klatzmann, D. (1997) Hum. Gene Ther. 8, 1807–1814.
24. Wei, M. X., Bougnoux, P., Sacre-Salem, B., Peyrat, M., Lhuillery, C., Salzmann, J., and Klatzmann, D. (1998) Cancer Res. 58, 3221–3225.
25. Drake, R. R., McMasters, R., Kriss, S., Hume, S. D., Rechtin, T. M., Saylors, R. L., Chiang, Y., Govindarajan, R., and Munshi, N. C. (1997) Antiviral Res. 35, 177–185.
26. Adam, L., Crepin, M., Savin, C., and Israel, L. (1995) Cancer Res. 55, 5156–5160.
27. Rudel, T., and Bokoch, G. M. (1997) Science 276, 1571–1574.
28. Thornberry, N. A., and Lazebnik, Y. (1998) Science 281, 1312–1316.
29. Kim, Y-G, Bi, W., Feliciano, E. S., Drake, R. R., and Stambrook, P. J. (1999) Cancer Gene Ther., in press.
30. Melcher, A., Todryk, S., Hardwick, N., Ford, M., Jacobson, M., and Vile, R. G. (1998) Nat. Med. 4, 581–587.