

Combined Therapy with Chemotherapeutic Agents and Herpes Simplex Virus Type 1 ICP34.5 Mutant (HSV-1716) in Human Non-Small Cell Lung Cancer

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ABSTRACT

A replication-selective herpes simplex virus type 1 ICP34.5 mutant (HSV-1716) has shown efficacy both *in vitro* and *in vivo* against human non-small cell lung cancer (NSCLC) cell lines but complete eradication of tumor has not been accomplished with a single viral treatment in our murine xenograft models. Therefore, strategies to enhance the efficacy of this treatment were investigated. We determined the oncolytic activity of HSV-1716 in NCI-H460 cells in combination with each of four chemotherapeutic agents: mitomycin C (MMC), *cis*-platinum II (*cis*-DDP), methotrexate (MTX), or doxorubicin (ADR). Isobologram analysis was performed to evaluate the interaction between the viral and chemotherapeutic agents. The oncolytic effect of HSV-1716 in combination with MMC was synergistic in two of five NSCLC cell lines. In the other three cell lines, the combined effect appeared additive. No antagonism was observed. The *in vivo* effect of this combination was then examined in a murine xenograft model. NCI-H460 flank tumors were directly injected with HSV-1716 (4×10^6 PFU) followed by intravenous MMC administration (0.17 mg/kg) 24 hr later. After 3 weeks, the mean tumor weight in the combined treatment group was significantly less than either individual treatment in an additive manner. The synergistic dose of MMC neither augmented nor inhibited viral replication *in vitro* and HSV-1716 infection did not upregulate DT-diaphorase, which is the primary enzyme responsible for MMC activation. In summary, the combination of HSV-1716 with common chemotherapeutic agents may augment the effect of HSV-based therapy in the treatment of NSCLC.

OVERVIEW SUMMARY

We investigated the effects of a herpes simplex virus type 1 ICP34.5 mutant (HSV-1716) combined with several chemotherapeutic agents against human non-small cell lung cancer (NSCLC). Mitomycin C (MMC) with HSV-1716 was the most efficacious combination in killing cells and demonstrated a synergistic effect by isobologram analysis. The other combinations tested exhibited an additive effect. In a murine xenograft model, MMC also augmented an HSV-based oncolytic effect in an additive manner. Since the synergistic dose of MMC neither augmented nor inhibited viral replication *in vitro* and HSV-1716 infection did not upregulate the major enzyme of MMC activation, DT-di-

aphorase, the mechanism of synergy remains elusive. These data suggested that the combination of HSV-based oncolytic therapy with chemotherapy may improve efficacy over either individual modality alone in the treatment of NSCLC.

INTRODUCTION

LUNG CANCER is the leading cause of cancer death—the estimated incidence and mortality in the United States are 171,600 cases and 158,900 deaths in 1999, respectively (Landis *et al.*, 1999). Approximately 20 to 25% of patients have resectable lung cancer at the time of initial diagnosis (Jett, 1993; Nesbitt *et al.*, 1995). However, chemotherapy and/or radio-

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therapy have not significantly improved the survival time of patients with inoperable disease. The overall 5-year survival for patients with inoperable non-small cell lung cancer (NSCLC) is about 5 to 10% (Elias, 1993; Jett, 1993; Nesbitt *et al.*, 1995). Approximately 75% of patients will relapse with metastases and 50 to 75% will experience disease recurrence in the chest (Elias, 1993). The lack of successful therapeutic regimens led us to examine a novel therapeutic approach using genetically engineered viruses.

Advances in virology and molecular biology spurred the engineering of recombinant viruses with specific properties, creating new interest in virus-based therapy of solid tumors. One such group of viruses is the replication-selective herpes simplex virus type 1 (HSV-1) mutants (Whitley *et al.*, 1998; Coukos *et al.*, 1999b; Kirn, 1999). These mutants have been engineered by deleting or mutating genes that govern viral replication or neurovirulence. They can cause lytic infection with subsequent cell death in rapidly dividing cells but not in stationary cells. The mutants harboring deletions or mutations in the thymidine kinase (TK) gene (*UL23*) (Sanders *et al.*, 1982; Martuza *et al.*, 1991; Boviatsis *et al.*, 1994b; Jia *et al.*, 1994), the ICP6 gene (*UL39*) encoding the large subunit of ribonucleotide reductase (RR) (Boviatsis *et al.*, 1994b; Mineta *et al.*, 1995; Yazaki *et al.*, 1995; Kramm *et al.*, 1997), or both copies of the $\gamma 34.5$ gene (Chambers *et al.*, 1995; Kesari *et al.*, 1995; Randazzo *et al.*, 1995; Andreansky *et al.*, 1997) have demonstrated efficacy in tumors in the CNS, including malignant glioma, malignant meningioma, and metastatic melanoma. ICP34.5, encoded by the $\gamma 34.5$ gene, can prevent premature host cellular protein shutoff in the infected host through inhibition of the phosphorylation of the eukaryotic translation initiation factor eIF-2 α (Cassady *et al.*, 1998a), as well as viral exit from the cell (Brown *et al.*, 1994). ICP34.5 is also responsible for neurovirulence (Chou and Roizman, 1994; Valyi-Nagy *et al.*, 1994). Although the neurovirulence of the wild-type (wt) HSV-1 strain 17+ is approximately 500-fold greater in mice than wt HSV-1 strain F (Dix *et al.*, 1983; Valyi-Nagy *et al.*, 1994; Kucharczuk *et al.*, 1997), ICP34.5-deleted mutants of both strains have exhibited 10⁵- to 10⁶-fold attenuation in neurovirulence and can replicate more efficiently in rapidly dividing compared with quiescent cells (MacLean *et al.*, 1991; Chou and Roizman, 1994; Valyi-Nagy *et al.*, 1994). Because of their specificity for rapidly dividing cells, cytolytic effect, and lack of neurovirulence, ICP34.5 mutants were also investigated in non-CNS tumors (Coukos *et al.*, 1999c). They have shown efficacy in the treatment of intraperitoneal malignant mesothelioma (Kucharczuk *et al.*, 1997), epithelial ovarian cancer (Coukos *et al.*, 1999a), and subcutaneous malignant melanoma (Randazzo *et al.*, 1997), reducing tumor size and/or prolonging survival in a dose-dependent manner. HSV-G207, which is an ICP34.5 mutant of the F strain with an insertion of *Escherichia coli lacZ* gene in the ICP6 gene, also demonstrated efficacy in the treatment of breast and colon cancer both *in vitro* and *in vivo* (Toda *et al.*, 1998a,b) as well as CNS tumors (Mineta *et al.*, 1995; Yazaki *et al.*, 1995). Furthermore, the side effect profiles in the dose escalation phase I clinical trials using the ICP34.5 deletion mutant HSV-1716, or the ICP34.5- and RR-deleted HSV-1 mutant G207, for treatment of malignant glioma have been minimal (Brown *et al.*, 1998; Markert *et al.*, 1999) and thus,

both HSV-1716 and HSV-G207 are promising therapeutic agents for cancer therapy.

Since lung tissue expressed high mRNA concentrations of herpes virus entry mediator (HVEM) (Montgomery *et al.*, 1996), we investigated the efficacy of HSV-1716 *in vitro* and *in vivo* against human NSCLC cell lines (Abbas *et al.*, 1998). We have shown that HSV-1716 is efficacious, but complete destruction of the tumor mass has not been accomplished with viral treatment alone in our lung cancer xenograft models. Therefore, strategies to augment the efficacy of this treatment are being investigated. Several methods have been reported to enhance the HSV-based oncolytic effect. For example, the oncolytic effect of HSV-R3616, lacking both copies of the ICP34.5 gene, was augmented by ionizing radiation (Advani *et al.*, 1998) or by coexpression of interleukin 4 (IL-4) (Andreansky *et al.*, 1998) in the treatment of malignant glioma. In addition, administration of an HSV-based amplicon expressing IL-12 increased the oncolytic effect of HSV-G207 in an experimental colon cancer model (Toda *et al.*, 1998a). As preliminary data, an HSV amplicon vector carrying IL-2 showed therapeutic efficacy in treating intraperitoneal metastatic gastric carcinoma in nude mice and increased killing activity of splenocytes (Tsuburaya *et al.*, 1998). Coadministration of HSV amplicon vectors expressing the chemokine RANTES and the T cell-costimulatory ligand B7.1 also resulted in approximately 85% reduction of subcutaneous murine lymphoma nodules (Kutubuddin *et al.*, 1998). We have developed "carrier cells," which are *ex vivo*-infected cells to deliver HSV mutants, and demonstrated the increase in the uptake of virus into tumor and significant reduction in tumor burden and prolonged survival in a model of ovarian cancer (Coukos *et al.*, 1999a). Furthermore, the efficacy of replication-selective HSV ribonucleotide reductase-deleted mutants can be augmented by expression of a suicide gene such as the endogenous TK or heterogeneous CYP2B1 and subsequent administration of prodrug, ganciclovir or cyclophosphamide (Boviatsis *et al.*, 1994a; Chase *et al.*, 1998).

Another potential strategy would be to utilize chemotherapeutic agents to modulate the oncolytic effect of HSV mutants. A vast number of chemotherapeutic agents such as mitomycin C (MMC), *cis*-platinum II (*cis*-DDP), doxorubicin (ADR), and methotrexate (MTX) have been studied for the treatment of lung cancer. However, among more than 50 agents tested through the early 1980s, only 5 agents including MMC and *cis*-DDP demonstrated significant antitumor activity as a single agent in more than 15% of lung cancer patients (Joss *et al.*, 1984; Gralla and Kris, 1986). MMC is an alkylating agent and produces intrastrand cross-linking of DNA. It is known to be activated to a cytotoxic intermediate by bioreduction. *cis*-DDP is a platinum antitumor compound and forms strong bonds between proteins and nucleic acid. MTX or ADR showed only marginal effects as single agents but they are often included in combination chemotherapy regimens, most containing *cis*-DDP. MTX is an antifolate. It inhibits dihydrofolate reductase, which is necessary in nucleotide biosynthesis. ADR is an anthracycline and inhibits DNA synthesis by forming a complex between topoisomerase I and II and DNA (Holland *et al.*, 1997). These four compounds have been shown to induce apoptosis (Barry *et al.*, 1990; Ohmori *et al.*, 1993; Fukuda and Saijo, 1994; Castaneda and Kinne, 1999). Chemotherapy has been combined with one

or more different modalities (i.e., radiation) to increase the overall patient outcome.

The rationale for combining these chemotherapeutic agents with an HSV-based oncolytic therapy is as follows: (1) combining conventional chemotherapeutic agents with HSV-1 mutant may achieve a greater effect than use of either modality alone; (2) a combination of agents with different toxicological profiles may result in increased efficacy without increased overall side effects; (3) the effect of chemotherapy could impact on infected and uninfected cells in the tumor; (4) no overlapping resistance between HSV-1 ICP34.5 mutants and chemotherapeutic agents has been described previously; and (5) the combination may allow the reduction of the HSV-1 mutant dose, thus decreasing the cost of treatment. Here, we examined the efficacy of HSV-1716 in combination with various common chemotherapeutic agents against NSCLC *in vitro* and *in vivo*. The interactions between the viral and chemotherapeutic modalities *in vitro* were analyzed quantitatively by the isobologram method (Carter *et al.*, 1988). All chemotherapeutic agents tested augmented the oncolytic effect of HSV-1716 and no antagonism was observed. In addition, the combination of MMC with HSV-1716 produced a synergistic effect *in vitro* and an additive effect *in vivo*.

MATERIALS AND METHODS

HSV-1716

HSV-1716 was originally engineered in the laboratory of S.M. Brown (Glasgow, Scotland) from the wild-type (wt) HSV strain 17+, and passaged for use as described (Kucharczuk *et al.*, 1997). The genome of this virus contains a 759-bp deletion in each copy of the *RL1* gene, which encodes ICP34.5 (MacLean *et al.*, 1991). These deletions remove most of the sequences encoding ICP34.5, resulting in a mutant that fails to make this protein (McKie *et al.*, 1994). It should be noted that since wt HSV infection can cause encephalitis in humans (Whitley, 1996), we chose to test the antitumor efficacy only of a strain attenuated for neurovirulence.

Cell lines and culture

The human NSCLC cell lines A549, NCI-H460, Calu-1, Calu-3, and NCI-H322 were obtained from the American Type Culture Collection (Rockville, MD). All the cell lines were maintained at 37°C with 5% CO₂ in RPMI 1640 (A549, NCI-H460, and NCI-H322), McCoy's 5A (Calu-1), or Eagle's minimum essential medium (Calu-3), supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (100 µg/ml; Life Technologies, Gaithersburg, MD). Calu-3 medium also included nonessential amino acids (0.1 mM; Life Technologies) and sodium pyruvate (1 mM). Cells were harvested during log phase with 0.05% trypsin-EDTA.

Chemotherapeutic agents

Mitomycin C (MMC), doxorubicin (ADR), and methotrexate (MTX) were purchased from Calbiochem (La Jolla, LA). *cis*-platinum II (*cis*-DDP) was obtained from Sigma (St. Louis,

MO). All stocks of agents at 100 mM were made with phosphate-buffered saline (PBS) and stored at -80°C. These agents were diluted with medium without FBS just before use.

Combination of a common chemotherapeutic agent with HSV-1716 in human NSCLC cell lines in vitro

In preliminary experiments, we examined the chemosensitivity to MMC, *cis*-DDP, ADR, and MTX, as well as the oncolytic effect of HSV-1716 in the aforementioned human NSCLC cell lines (data not shown). In order to observe the interaction between the virus and drugs, we used suboptimal doses of each modality, resulting in less than 50% killing of tumor cells when given a single modality. Cells were plated in 96-well plates at a density of 3000 (A549, NCI-H460, and Calu-1) or 5000 (NCI-H322 and Calu-3) cells per well. Twenty-four hours later, the cells were infected with HSV-1716 at each appropriate multiplicity of infection (MOI) for 1 hr in serum-free medium (50 µl). Subsequently, medium (50 µl) containing 4% heat-inactivated FBS and various concentrations of chemotherapeutic agents was added to the appropriate wells. Cells were then incubated at 37°C in 5% CO₂. When the cells in the control wells were confluent on day 3 to 5, cell proliferation assays were performed with a chromogenic kit (CellTiter 96 aqueous non-radioactive cell proliferation assay; Promega, Madison, WI) that measures viable cell dehydrogenase activity by absorbance at 490 nm, using a microplate reader (EL-800; Bio-Tek Instruments, Winooski, VT). The percentage control was defined as the ratio of the mean absorbance of 18 treatment wells minus the blank to the mean absorbance of 6 untreated matched controls minus the blank. Blank is defined as the mean absorbance of six wells containing medium alone. Each experiment was performed at least twice.

Comparison of cells infected in vitro with HSV-1716 and treated with MMC pre- or postinfection

Three thousand NCI-H460 and A549 cells were plated in 96-well plates. After 24 hr MMC or medium was added for 3 hr in the pretreatment group (A) or posttreatment group (B), respectively. After washing the cells with PBS, HSV-1716 was added for 1 hr under serum-free conditions. Medium containing FBS with MMC (group B) or without MMC (group A) was then added and incubated for 3 hr. Medium on both groups was then replaced with complete medium without MMC. Cell viability was assessed by cell proliferation assay after 48 hr as described above.

One-step growth curves

NCI-H460 cells were plated in six-well plates at a density of 300,000 cells per well in medium (RPMI 1640) with 10% heat-inactivated FBS, incubated overnight, and subsequently infected with HSV-1716 at an MOI of 0.1 for 1 hr. In parallel experiments, MMC (0.1 µM) was added for 3 hr before or after infection, or MMC (0.1, 0.3, or 1.0 µM) was added for 48 hr after infection. A sample was harvested 1 hr after exposure to HSV-1716 (0 hr) as well as 6, 19, 24, and 48 hr later by mechanical scraping in their medium. The samples were stored at -80°C and titered by plaque assay on baby hamster kidney (BHK) cell monolayers as described previously (Kucharczuk *et al.*, 1997).

Flow cytometry cell cycle analysis

Cells were cultured in 25-cm² flasks and treated with HSV-1716 at an MOI of 10. After 24 hr, cells were harvested with a 0.05% trypsin-EDTA solution. Collected cells were fixed in 80% ethanol at -20°C for at least 24 hr, treated with RNase A (500 µg/ml; Sigma) for 30 min at room temperature, stained with propidium iodide (PI, 20 µg/ml), and analyzed with an EPICS XL flow cytometer (Coulter, Hialeah, FL). Data were analyzed by the Cellfit program. Cells displaying less than G₀ (hypodiploid) DNA content were labeled as being located in sub-G₀, and were considered to exhibit fragmented DNA, i.e., to be apoptotic (Darzynkiewicz *et al.*, 1997). Results were expressed as means ± SD.

Cytosol preparations of HSV-infected cells

Cells in exponential growth were infected with HSV-1716 at an MOI of 0.1 or 10. At 6, 19, and 42 hr after infection, cells were then washed twice with PBS and scraped into PBS, using a cell scraper. Cells were then collected by centrifugation at 150 × *g* for 10 min at 4°C. The pelleted cells were suspended in a buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1.5 mM MgCl₂, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated for 10 min at 4°C (Lee *et al.*, 1993). The cell pellet was disrupted by sonication on ice for 10 sec with a sonicator (VirTis, Gardiner, NY). The homogenate was centrifuged at 105,000 × *g* for 30 min at 4°C. Protein concentrations were measured with a protein assay kit (Bio-Rad, Hercules, CA).

Immunoblot analysis

The immunoblot analysis of DT-diaphorase (DTD) expression was performed as previously described (Lee *et al.*, 1993; Molnar-Kimber *et al.*, 1998) with the following modifications. The cytosolic proteins (1 or 3 µg) were first separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane at 0.8 mA/cm² for 2.5 hr. After transfer, the nitrocellulose was blocked with 4% bovine serum albumin (BSA) in PBS and the protein blots were incubated for 2 hr with a mouse anti-human DT-diaphorase monoclonal antibody (KM1015; 2 µg/ml) generously provided by N. Hanai (Kyowa Hakko, Tokyo, Japan). After rinsing, a goat anti-mouse IgG alkaline phosphatase-conjugated antibody (1:7500; Promega) was added for 1 hr. Visualization was performed with bromochloroindolyl phosphate (BCIP; Amersham, Piscataway, NJ) and nitroblue tetrazolium (NBT; Boehringer Mannheim, Indianapolis, IN).

In vivo xenograft flank model

Six- to 8-week-old CB17 severe combined immunodeficient (SCID) mice (Taconic, Germantown, NY) were housed in isolation units at the Wistar Institute (Philadelphia, PA). NCI-H460 cells were cultured under standard conditions and harvested in log phase with 0.05% trypsin-EDTA solution. After addition of serum-enriched medium, cells were centrifuged at 300 × *g* for 5 min at 4°C and resuspended in RPMI 1640. Each injection contained 2 × 10⁶ cells in 200 µl of 50% Matrigel (Becton Dickinson Labware, Bedford, MA) in RPMI 1640 medium. After tumors were established (average tumor volume, 163

mm³), mice were divided into 4 groups of 14. The tumors were directly injected with medium or HSV-1716 at a dose of 4 × 10⁶. Mice were given bolus MMC (0.17 mg/kg) or PBS intravenously 24 hr later. The tumor size was measured and volume was calculated at weekly intervals as 0.4 mm × length (mm) × width (mm) = tumor volume (mm³). Tumor growth curves showed mean estimated volume ± standard error of the mean. After a period of 3–4 weeks, mice were sacrificed and their tumors were weighed. All animal protocols were approved by the Animal Use Committees of the Wistar Institute and the University of Pennsylvania in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health #85-23, revised 1985).

Statistical analysis

Nonparametric response surface methods (Greco *et al.*, 1995) were employed to analyze the data from a series of drug-virus interaction studies performed *in vitro*. A three-dimensional (*x*, *y*, and *z* axes) response surface was defined by dose levels of MMC and HSV-1716 and the resulting outcome (percent control), respectively. As the dose levels of each modality spanned several logs in magnitude, a log base 10 transformation was applied to the data prior to application of nonparametric response surface methods. The nonparametric response surface was fit with bivariate splines without smoothing, using SAS PROC G3GRID, and plotted with SAS PROC G3D. Isobologram analysis was then performed on the basis of the generated response surface by SAS PROC GCONTOUR. An isobole is a contour plot of constant response (e.g., percent control) associated with a combination of two agents. Isobologram analysis requires the comparison of the observed isobole curve (usually convex or concave) at a particular level of response to the Loewe line of additivity, which is constructed by connecting the two single-agent concentrations that produce that particular level of response. In the concentration ranges employed in this series of experiments, the single-agent concentrations produced responses (percent control) in the range of 80–90%. The reason we demonstrated synergy in the 80–90% range, and not at lower values, was not because the combination of agents (synergy) was not observed at lower values, but instead was because earlier studies were performed to investigate the relevant concentrations at which interactions could be discerned. A selected isobole is then compared to the Loewe line of additivity (a diagonal northwest-southeast [NW-SE] line). If the isobole is upward concave (arcs beneath the additivity line) synergy is demonstrated. If the isobole is downward convex (arcs above the additivity line) antagonism is demonstrated. If the isobole closely follows the line, no interaction is demonstrated (Carter *et al.*, 1988). The interaction index, as proposed by Berenbaum (1981), was then estimated from the isobologram. Synergy, antagonism, or no interaction (additivity) are demonstrated if the interaction index is <1, >1, or 1, respectively. A mathematical model was then used to assess the statistical significance of the interaction. Piecewise linear models were fit using bivariate splines. The fit of the model without interaction terms was compared with the fit of a model with interaction terms, by the likelihood ratio test (Mood *et al.*, 1974), which allowed statistical significance of the interaction to be determined (*p* value). The model was constructed using Stata software with the xreg

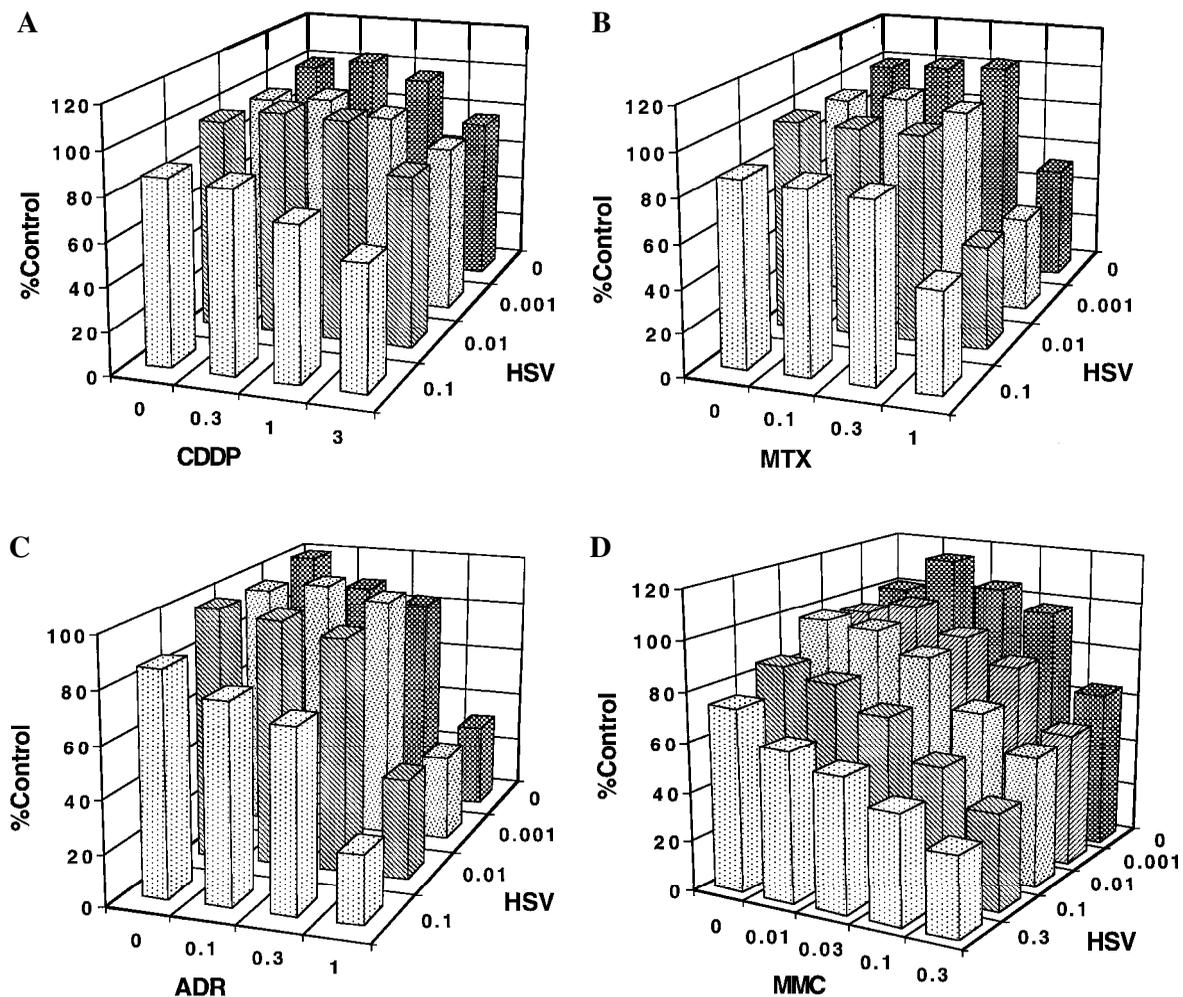


FIG. 1. Three-dimensional graphical representations of the dose–response results for the interactions between HSV-1716 and chemotherapeutic agents in NCI-H460 *in vitro*. (A) *cis*-DDP (CDDP); (B) MTX; (C) ADR; (D) MMC. *x* axis, HSV-1716 (MOI); *y* axis, chemotherapeutic agents (μM); *z* axis, percent control (i.e., the percentage of cell proliferation relative to nontreated control cells). Cell viability was measured by a cell proliferation assay (see Materials and Methods).

procedure, that accounted for the correlation among replicates at each MMC–HSV dose level combination. All *in vitro* interaction analyses were performed with SAS software (release 6; SAS Institute, Cary, NC) or Stata software (release 5; Stata, College Station, TX).

Tumor weights in *in vivo* experiments were expressed as means \pm SE. Differences in mean tumor weights in the animal experiments were determined by one-way analysis of variance (ANOVA). Post hoc comparisons among groups were done with the Fisher test (StatView; SAS Institute). Statistical significance was set at $p < 0.05$.

A linear model with terms for the effects of MMC and HSV-1716 and an interaction term was fit to the *in vivo* data. The test of synergy between MMC and HSV-1716 was conducted by a one-sided test on the coefficient of the interaction term. *In vivo* interaction analysis was performed in SPSS (SPSS, Chicago, IL).

RESULTS

Effect of four common chemotherapeutic agents on the oncolytic effect of HSV-1716 as assessed in NCI-H460 in vitro

The effectiveness of the combined treatment of several concentrations of four chemotherapeutic agents with several MOIs of HSV-1716 was evaluated in the NSCLC cell line NCI-H460 (a large cell carcinoma). Cells were treated with each agent just after infection. It should be noted that the highest dose of the agents used in these *in vitro* studies was within (*cis*-DDP, MTX, and ADR) or below (MMC) the peak serum concentrations measured after intravenous administration in human (Boobis *et al.*, 1991; McEvoy, 1999). Multiple dose–response graphical representations, indicating the cytotoxic effects of four different combinations of each chemotherapeutic agent and HSV-

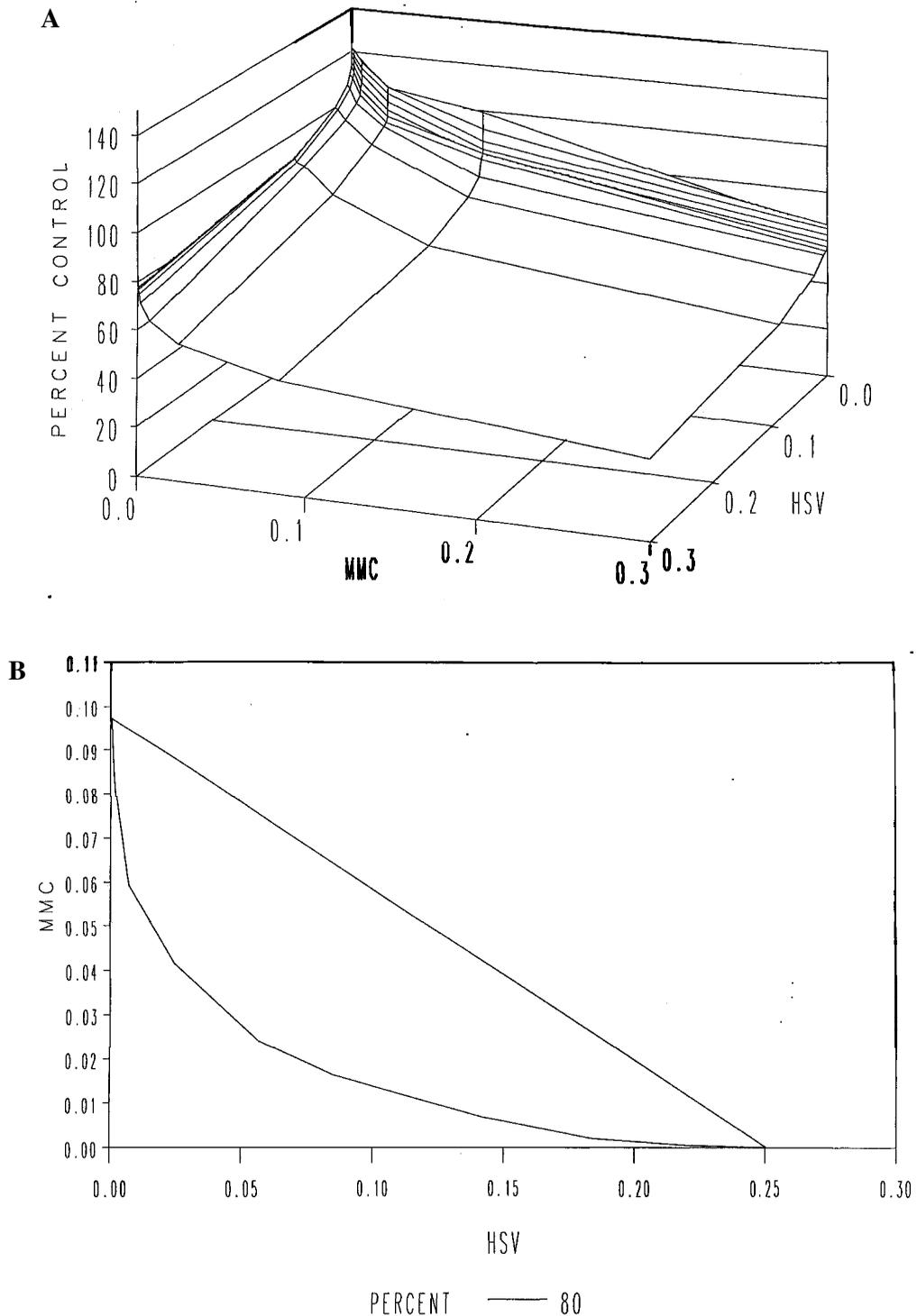


FIG. 2. (A) Dose-response surface for various concentrations of MMC and HSV-1716 in NCI-H460. The surface was constructed on a log base 10 transformation of the data because of the dose levels of each modality spanned several logs in magnitude. On retransformation to original units for plotting, it is apparent that data were concentrated in the lowest dose levels, as depicted by the grid lines. (B) Isobole contour plot showing combinations of MMC and HSV-1716 concentrations in NCI-H460, that product 80% control. Synergy is demonstrated by upward concave isobole in relation to the Loewe line of additivity (diagonal NW-SE line). *x* axis, HSV-1716 (MOI); *y* axis, MMC (μM); *z* axis, percent control.

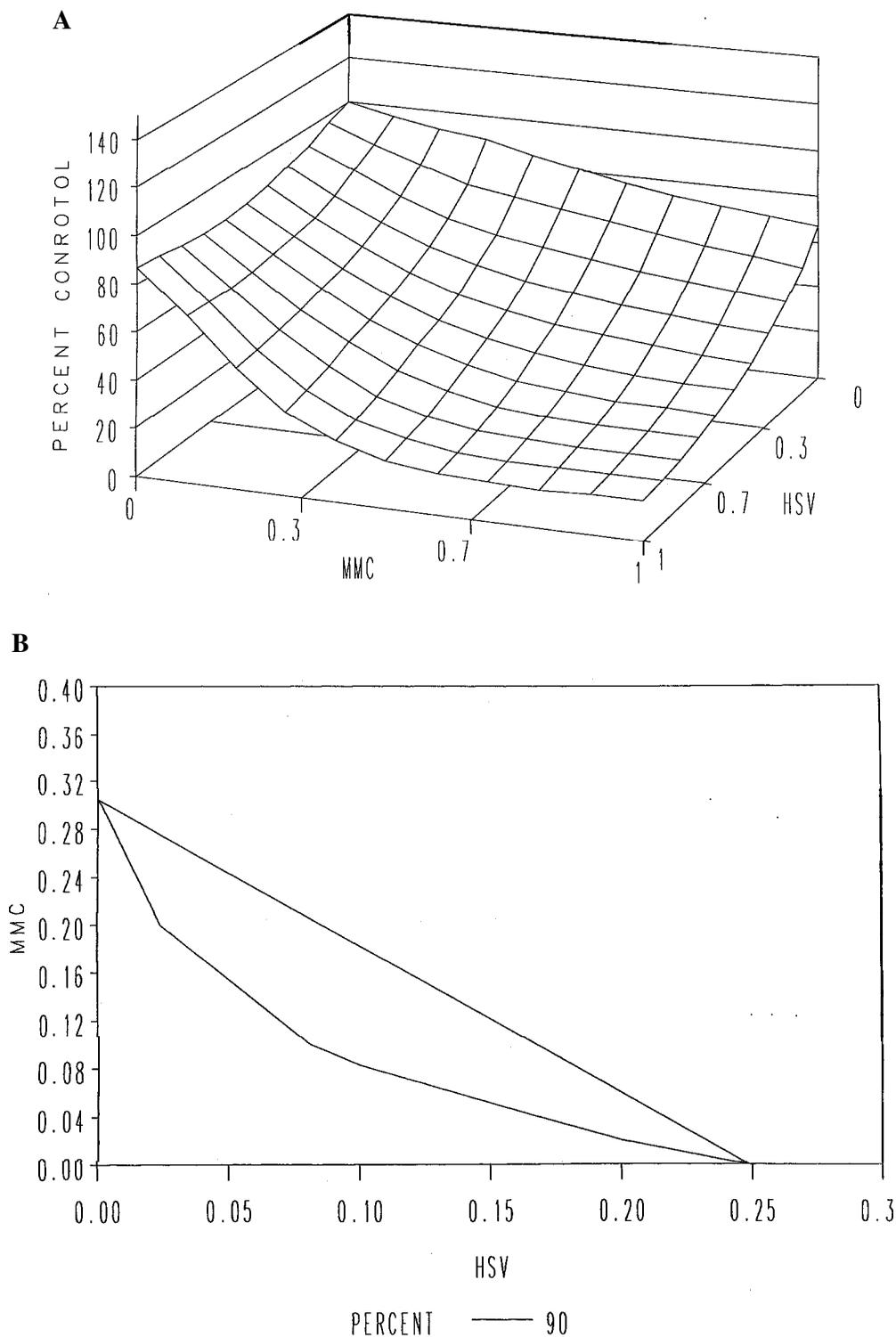


FIG. 3. (A) Dose-response surface for various concentrations of MMC and HSV-1716 in A549 cells. (B) Isobole contour plot showing combinations of MMC and HSV-1716 concentrations in A549 that produce 90% control. Synergy is demonstrated by upward concave isobole in relation to the Loewe line of additivity (diagonal NW-SE line). *x* axis, HSV-1716 (MOI); *y* axis, MMC (μM); *z* axis, percent control.

1716, were displayed in three-dimensional (3D) bar charts (Fig. 1A–D). These figures illustrate the data observed at specific dose combinations of the two agents. Figures 2A and 3A display fitted response surfaces, which represent continuous surfaces extrapolated across all possible dose combinations of the two agents, within the test range. Isobolograms were then generated to determine the relationship: synergism, additivity, or antagonism. The only combination in which the effect was significantly synergistic was MMC with HSV-1716 ($p = 0.00001$). For example, HSV-1716 at an MOI of 0.1 produced 85.9% cell survival and MMC at 0.1 μM showed 96.7% survival in NCI-H460. When HSV-1716 and MMC were combined at these concentrations, a 53.6% survival was obtained, demonstrating 29% greater effect than just an additive effect (Fig. 1D). The isobole based on ED_{80} (80% effective dose) values is below the Loewe line of additivity, indicating the presence of synergy in this combination (Fig. 2B). The interaction index based on the isobologram is 0.497, which indicates a synergistic effect. The other three agents (*cis*-DDP, MTX, or ADR) combined with HSV-1716 resulted in an additive effect. No antagonistic interactions were observed in these studies. UV-treated virus did not show synergism (data not shown).

Combination of MMC with HSV-1716 in five NSCLC cell lines

To determine whether the combination of MMC with HSV-1716 was synergistic in all histopathological types of NSCLC, we tested this combination against four additional NSCLC cell lines (Calu-1 [squamous cell carcinoma], Calu-3 [adenocarcinoma], NCI-H322 [bronchoalveolar carcinoma], and A549 [undifferentiated lung carcinoma]), representing each of the major histopathological types of non-small cell lung cancer. A summary of interactions resulting from statistical analyses in these five NSCLC lines is shown in Table 1. A synergistic effect was observed in A549 (Fig. 3; $p < 0.00001$, interaction index = 0.655) as well as NCI-H460 (Fig. 2). The combined therapeutic effect in the other three cell lines appeared additive. No antagonistic effect was observed with the combination of MMC with HSV-1716 in any cell line tested. We also observed synergism between additional ICP34.5 mutants and MMC in A549 (data not shown).

Comparison of cells infected in vitro with HSV-1716 and treated with MMC pre- or postinfection

To determine whether the timing of MMC administration affected the combined oncolytic effect, H460 and A549 were treated with MMC for 3 hr before or after infection with HSV-1716. Results are shown in Fig. 4. There were no significant differences in antitumor activity between cells treated with MMC before or after infection with HSV-1716 ($p = 0.11$ to 0.97 in NCI-H460, $p = 0.24$ to 0.90 in A549; data not shown).

One-step growth curves

One possible mechanism by which the combination of MMC with HSV-1716 could be synergistic is that MMC may augment the ability of HSV-1716 to replicate in NCI-H460 or A549 cells. The effect of MMC on viral replication was measured in one-step growth curve experiments. NCI-H460 cells were in-

fectured with HSV-1716 at an MOI of 0.1 for 1 hr and then MMC at 0.1, 1.0, or 3.0 μM was added. At several points, cells were harvested and titered as described previously (Kucharczuk *et al.*, 1997). As shown in Fig. 5, pretreatment with 0.1 μM MMC for 3 hr or posttreatment with 0.1 μM MMC for either 3 or 48 hr did not significantly change the burst size in comparison with control (42.1, 57.1, 53.3, and 60.3, respectively). However, the addition of high-dose MMC (1.0 or 3.0 μM) for 48 hr after infection clearly reduced burst size (13.7 and 0.2, respectively). Similar results were observed in A549 (data not shown). Although high-dose MMC may lessen burst size, the synergistic dose of MMC (0.1 μM) neither augmented nor inhibited HSV-1716 replication.

Cell cycle analysis

To examine whether HSV-1716 infection can induce apoptosis and/or affect cell cycle progression, cell cycle analyses by flow cytometry were performed. NCI-H460 and A549 cells were infected with HSV-1716 at an MOI of 10 and harvested after 24 hr of incubation. In NCI-H460, apoptosis was observed in $30.4 \pm 6.0\%$ of cells infected with HSV-1716 compared with $2.5 \pm 0.3\%$ in noninfected control (Table 2). HSV-1716 infection of A549 induced only a threefold increase in sub- G_0 fraction, which was significantly less than NCI-H460 (Table 2, $p < 0.0005$).

Since the cell cycle phase can influence the sensitivity to MMC (Crooke and Bradner, 1976), the effect of HSV-1716 infection on progression of cell cycle was determined by fluorescence-activated cell sorting (FACS). In both cell lines tested, HSV-1716 infection did not significantly alter the percentage of cells in the S phase (Table 2). Taken together, these data suggest that the synergism between HSV-1716 and MMC in NCI-H460 and A549 was due to neither HSV-1716-induced apoptosis nor accumulation in S phase.

DT-diaphorase expression in HSV-infected cells

Another possible mechanism of synergism between MMC and HSV-1716 is that HSV infection may upregulate NAD(P)H:quinone oxidoreductase, which is known as DT-diaphorase (DTD). Cytotoxic activity of MMC is dependent on its intracellular reductive activation to form cross-linked DNA. DTD, which catalyzes the two-electron reduction of quinones and other nitrogen oxides, is known to play a major role in activating MMC. Thus, the level of DTD expression was investigated by immunoblot analysis of protein lysates from four human NSCLC cell lines (NCI-H460, A549, Calu-1, and

TABLE 1. STATISTICAL ANALYSES OF THE INTERACTIONS BETWEEN MMC AND HSV-1716 IN NSCLC LINES

Cell line	Pathological type	Interaction	p Value
Calu-1	Squamous	Additive	NS ^a
Calu-3	Adeno	Additive	NS
NCI-H322	Bronchoalveolar	Additive	NS
NCI-H460	Large cell	Synergy	<0.00001
A549	Undifferentiated	Synergy	<0.00001

^aNS, Not significant.

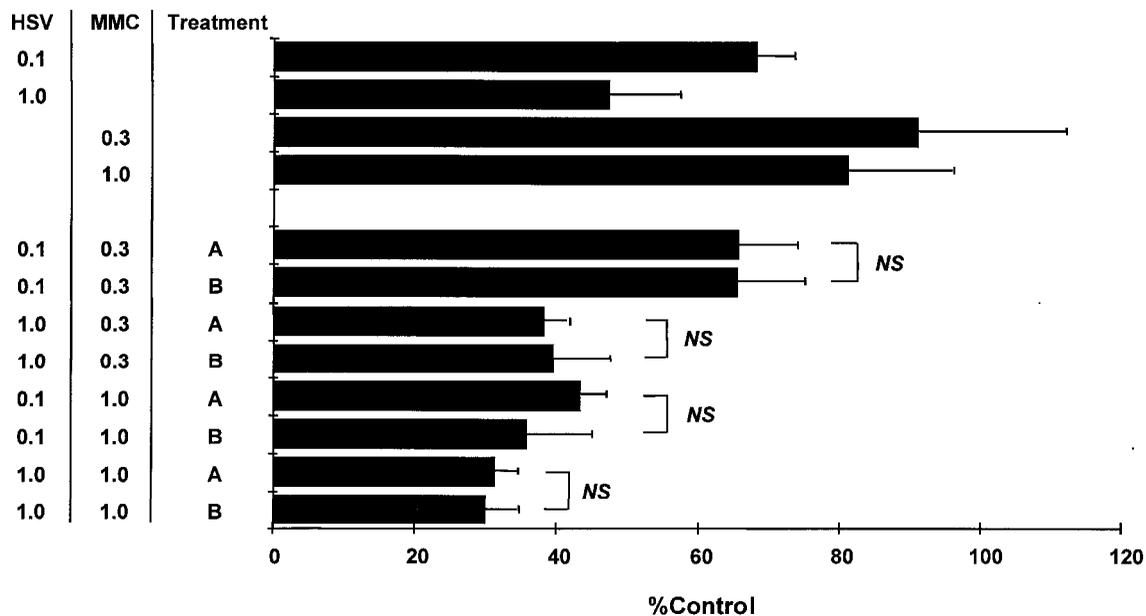
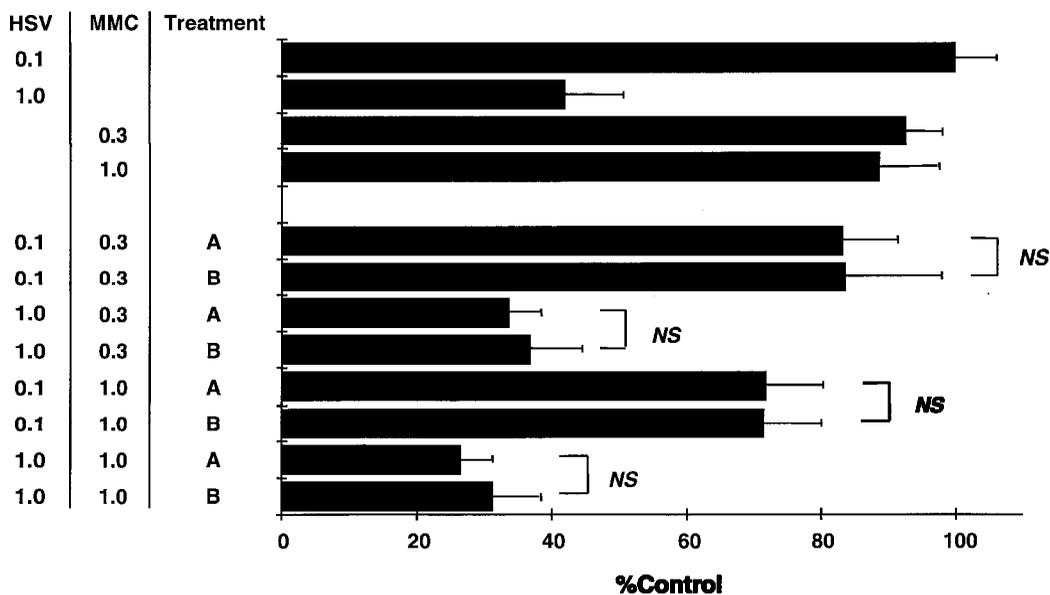
A: NCI-H460**B: A549**

FIG. 4. Comparison of cells infected *in vitro* with HSV-1716 and treated with MMC pre- or postinfection. (A) NCI-H460 or (B) A549 cells were infected with HSV-1716. MMC was administered 3 hr before or after the infection for 3 hr. Cell viability was measured 48 hr later by cell proliferation assays (MTS). *x* axis, % control. Error bars indicate means \pm SD. *y* axis, doses of each treatment (HSV [MOI] and MMC [μ M]). Treatment A or B means MMC administration for 3 hr before or after HSV-1716 infection, respectively. No significant difference was demonstrated between pre- and posttreatment of MMC in combination with HSV-1716 in NCI-H460 and A549. Error bars indicate means \pm SD.

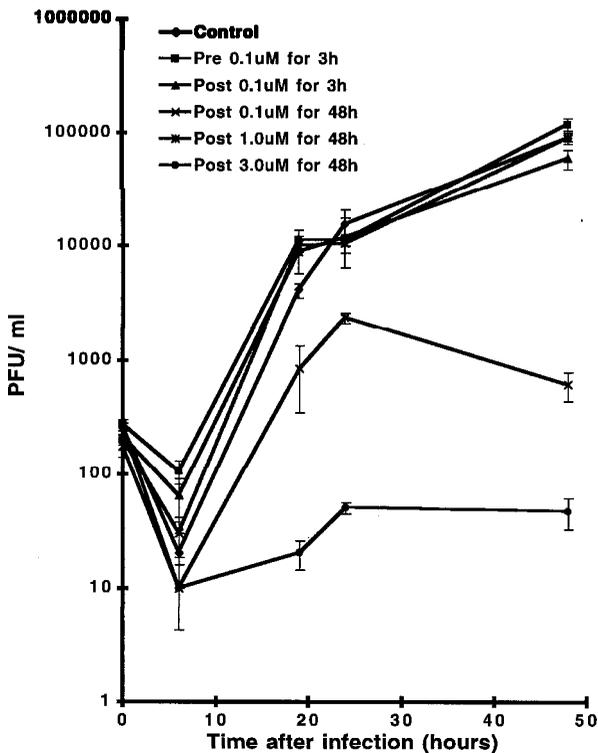


FIG. 5. The effect of MMC on HSV-1716 replication in NCI-H460. NCI-H460 was infected with HSV-1716 at an MOI of 0.1 and then 0.1, 1.0, or 3.0 μM MMC was added. At several time points, cells were harvested by scraping and virus titers were determined by immunoperoxidase plaque assay. Although addition of 1.0 or 3.0 μM MMC clearly reduced burst size, low-dose MMC (0.1 μM) neither augmented nor inhibited HSV-1716 replication. Error bars indicate means \pm SE. *x* axis, time (hr) after infection; *y* axis, plaque-forming units (PFU).

NCI-H322) that were mock or HSV infected. To maximize the detection of HSV-1716 impact on DTD expression, DTD expression was analyzed in protein lysates isolated from cells infected at an MOI of 10 at both 6 and 19 hr postinfection to observe modulation by early and late HSV genes, respectively. The level of DTD expression was also assessed in cells infected at an MOI of 0.1 for 42 hr to mimic the synergistic conditions *in vitro*. Each sample was analyzed at 1 and 3 μg , which were shown in odd- and even-numbered lanes, respectively, to optimize the ability to detect differences in expression levels. All cell lines expressed the 32-kDa DTD protein band, although at different levels (Fig. 6A and B, lanes 1 and 2). As shown in the immunoblot depicted in Fig. 6A, DTD expression was slightly decreased (up to twofold) in HSV-1716-infected NCI-H460 cells (lanes 5 and 7) and A549 cells (lanes 3, 5, and 7). The decrease in DTD was most evident in NCI-H460 cells infected at an MOI of 10 (NCI-H460; lanes 5 and 7). In NCI-H322 or Calu-1 (Fig. 6B), in which MMC did not demonstrate synergism with HSV-1716, DTD levels in infected cells did not change (NCI-H322; lane 5), modestly decreased (Calu-1; lanes 3 and 4 and lanes 7 and 8), or slightly increased (Calu-1, lanes 5 and 6; NCI-H322, lanes 3 and 4 and lanes 7 and 8) compared with uninfected control (Fig. 6B, lanes 1 and 2). Although HSV-1716 infection demonstrated slight effects on DTD expression, there was no distinct relationship between DTD expression level and synergism in MMC and HSV-1716 combined therapy.

Efficacy of combination of MMC with HSV-1716 in vivo

The *in vivo* oncolytic efficacy and the interaction of MMC in combination with HSV-1716 was assessed in a murine xenograft model. Preliminary experiments determined that MMC at 0.17 mg/kg or 4×10^6 PFU of HSV-1716 yielded tumor sizes of 70 to 80% of untreated control (data not shown). Although these doses may not yield maximum efficacy in this

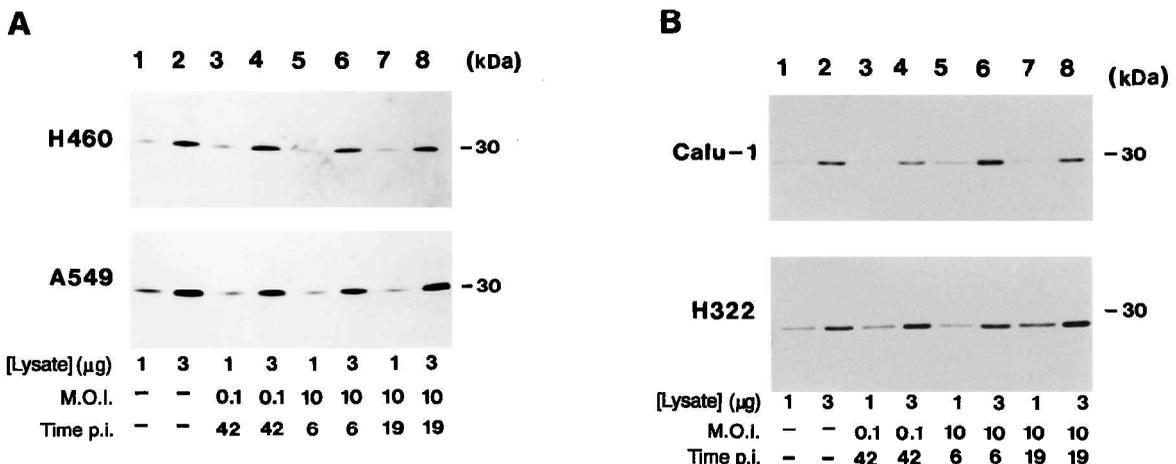


FIG. 6. Expression of DTD in the NSCLC lines NCI-H460 and A549 (A) and (B) Calu-1 and NCI-H322. Cells were uninfected (lanes 1 and 2) or infected with HSV-1716 at an MOI of 0.1 (lanes 3 and 4) or 10 (lanes 5–8), and harvested at 42 hr (lanes 3 and 4), 6 hr (lanes 5 and 6), and 19 hr (lanes 7 and 8) postinfection (p.i.). Cell lysates were prepared, measured, and 1 or 3 μg was separated by 12% SDS-PAGE as described in Materials and Methods.

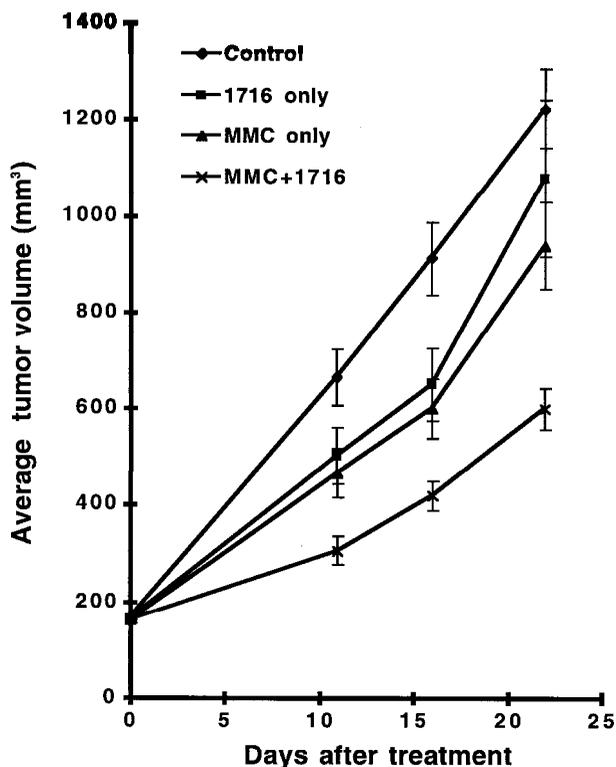


FIG. 7. HSV-1716 treatment of subcutaneous human lung cancer tumors (NCI-H460) with or without intravenous administration of MMC in SCID mice. HSV-1716 (4×10^6 PFU) was injected intratumorally. Mice were given bolus MMC (0.19 mg/kg) intravenously 24 hr later. Tumor size was measured and tumor volume was calculated at regular intervals. Error bars indicate means \pm SE. x axis, time after treatments (days); y axis, calculated mean tumor volume (mm^3).

model, these doses allowed us to investigate the interaction between the two agents. The efficacy of wt HSV combined with chemotherapy was not investigated because this dose of wt HSV would lead to fulminant infection and death of SCID mice (Kucharczuk *et al.*, 1997; Advani *et al.*, 1999). Established NCI-H460 tumors were treated with vehicle, MMC, HSV-1716, or both MMC and HSV-1716. Tumors were directly injected with either 4×10^6 PFU of HSV-1716 or medium. Twenty-four hours later, MMC (0.17 mg/kg) or PBS was administered intravenously. MMC was administered as a single bolus injection to simulate the common procedure of bolus MMC treatment to minimize severe myelosuppression (Verweij and Pinedo, 1990). To examine the effects over time, the tumor volume was measured and calculated at indicated intervals (Fig. 7). There was a significant decrease in tumor volume between control and all treatment groups (Fig. 7). The combined treatment group had significantly lower tumor volumes than did each single agent-treated group. Mice were sacrificed and tumors were weighed on day 22. As shown in Fig. 8, the final mean tumor burden in the vehicle-treated group was 1.406 ± 0.079 g. Treatment with HSV-1716 alone reduced tumor burden on average 19.8% to 1.127 ± 0.139 g ($p = 0.03$ versus control). Treatment with MMC alone reduced tumor burden on average 20.2% to 1.122 ± 0.070 g ($p = 0.03$ versus control). When both modalities were combined, the mean tumor weight was 0.793 ± 0.047 g ($p = 0.01$ versus HSV-1716 alone and $p = 0.01$ versus MMC alone), significantly less than either individual treatment, yielding only an additive effect. Combined treatment reduced tumor burden on average 43.6% compared with control and there was 29.3% reduction on average over MMC alone and 29.6% on average over HSV-1716 alone. Low-dose MMC, HSV-1716, or combined therapy did not adversely affect the weight gain of the experimental animals. Thus, the *in vivo* oncolytic effect of

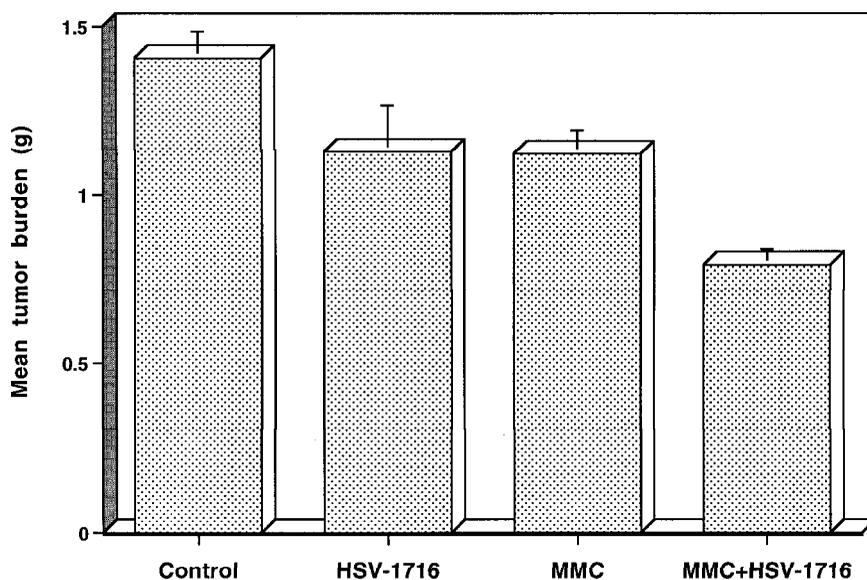


FIG. 8. Combined efficacy of MMC with HSV-1716 in NCI-H460 *in vivo*. SCID mice were sacrificed on day 22 and tumors were weighed. Mean tumor burden in the combined treatment group was significantly less than that produced by either individual treatment, yielding an additive effect, not a synergistic effect ($p = 0.39$). Combined treatment reduced tumor burden 43.6% compared with the control, which presented a further 29.3% reduction over MMC alone or 29.6% over HSV-1716 alone. Error bars indicate means \pm SE.

combined MMC and HSV-1716 therapy resulted in suppressed tumor growth in an additive manner.

DISCUSSION

The treatment of human NSCLC remains a formidable problem inasmuch as therapeutic advances have not significantly improved clinical outcome. The utilization of replication-selective HSV-1 (e.g., HSV-1716) as a therapeutic modality shows much promise. In our laboratory, HSV-1716 has shown efficacy against human NSCLC. However, total eradication of human NSCLC tumor nodules (NCI-H460 or A549) in our murine xenograft models has not been achieved by virus alone (Abbas *et al.*, 1998). Therefore, strategies to improve the efficacy of this form of treatment appear to be important. In efforts to augment the efficacy, we and others have investigated combination therapy with radiation (Advani *et al.*, 1998, 1999; Blank *et al.*, 1999; Bouvet *et al.*, 1999) or cytokines, including IL-4 (Andreansky *et al.*, 1998) and IL-12 (Toda *et al.*, 1998a). Administration of replication-selective HSV-1 mutants utilizing a cell-based delivery system also appears to enhance the uptake of virus into tumor (Coukos *et al.*, 1999a). Here, we described the combination studies of chemotherapeutic agents with HSV-1716 in models of NSCLC by an analytic statistical method. MMC showed a strong synergistic effect in two of five NSCLC cell lines (large cell or undifferentiated carcinoma). In the other three cell lines, the interactions appeared additive. The four chemotherapeutic agents tested augmented the oncolytic effect of HSV-1716 and no antagonism was observed.

The combination of MMC with HSV-1716 yielded an additive effect in our murine xenograft model. Since the half-life of MMC is 25–90 min (Verweij and Pinedo, 1990), one likely interpretation is that the duration of MMC exposure with HSV-1716 infection is important in inducing a synergistic response. In support of this hypothesis, a 3-hr MMC treatment combined with HSV infection yielded only an additive effect whereas a 48-hr MMC treatment combined with HSV-1716 infection resulted in synergistic oncolytic activity (Figs. 2 and 4). These results suggest that synergistic interactions may correlate with a longer duration of MMC contact with tumor cells. Although human clinical procedures commonly utilize an intermittent dosing schedule using bolus injections every 4–8 weeks to avoid severe myelosuppression (Verweij and Pinedo, 1990), the use of daily low-dose or continuous injection of MMC instead of a single bolus injection may yield greater efficacy in combination with HSV-1716. This strategy may minimize the potential MMC inhibition of HSV replication. Inhibition of a virus-based oncolytic effect by chemotherapeutic agents was a concern because these agents are known to block cell proliferation and may also inhibit viral DNA replication. However, our data suggested that the use of chemotherapeutic agents in combination with replication-selective HSV-1716 augmented the efficacy of HSV-based oncolytic therapy. Although we did not measure replication of HSV-1716 in H460 tumor nodules in these combination studies, it is feasible that replication of HSV-1716 may peak within several days. Alternatively, since the ability of H460 to support replication of HSV-1716 was comparable to that of SKOV3 and A2780 *in vitro*, it is also feasible that replication of HSV-1716 *in vivo* in H460 cells may be evident as

late as 3 weeks postinfection, analogous to our findings of the presence of HSV-1716 by immunohistochemistry in HSV-1716-treated SKOV3 or A2780 tumors in murine xenograft models (Coukos *et al.*, 1999a). HSV-1716-positive cells were also observed in melanoma flank tumors up to 10 days after HSV-1716 administration (Randazzo *et al.*, 1997). Regardless of the duration of HSV-1716 replication, it is likely that multiple administrations of HSV-1 ICP34.5 mutants would have improved efficacy in comparison with a single administration, as demonstrated in murine xenograft models of lung, cervical, and CNS cancer (Advani *et al.*, 1999; A.E. Abbas *et al.*, 1998).

A combination of adenovirus (Ad)-mediated gene therapy with anti-cancer drugs has also been shown to exhibit a greater effect than either modality alone. Nielsen *et al.* demonstrated that paclitaxel had a synergistic or an additive effect when combined with Ad-mediated *p53* gene therapy in several cancer models (Nielsen *et al.*, 1997). Cheon *et al.* combined an Ad vector, containing the herpes simplex virus thymidine kinase (HSV-TK) gene driven by the osteocalcin promoter, with a low dose of MTX and demonstrated a significant increase in efficacy and prolonged survival in a murine osteosarcoma model (Cheon *et al.*, 1997). A replication-selective adenovirus, ONYX-015, in combination with 5-fluorouracil (5-FU) or *cis*-DDP showed a greater effect than either individual modality and prolonged survival (Heise *et al.*, 1997). Furthermore, Tong *et al.* demonstrated that a topoisomerase I inhibitor, topotecan, in combination with Ad-TK gene therapy yielded a dose-dependent synergistic interaction and that Ad-TK gene therapy enhanced the effect of subsequent chemotherapy in an ovarian cancer model (Tong *et al.*, 1996). Thus, the combination of virus-based therapy with chemotherapy for the cancer treatment appears to be promising.

The safety profile of the replication-selective HSV mutants has been investigated in rodents, *Aotus* monkeys, and currently in phase I human clinical trials (Randazzo *et al.*, 1996; Brown *et al.*, 1998; Hunter *et al.*, 1999; Markert *et al.*, 1999). In several rodent models, the replication of HSV-1716 was restricted to mesothelioma or melanoma tumor nodules (Randazzo *et al.*, 1996; Kucharczuk *et al.*, 1997). Anti-HSV staining or HSV DNA was not detected by immunohistochemistry or polymerase chain reaction (PCR), respectively, in surrounding normal tissues, or in distant organs including lung, liver, kidney, brain, and spleen (Kucharczuk *et al.*, 1997). HSV-1716 replication, in contrast to replication of wt HSV 17+, was also severely restricted in normal human skin xenografts on the flanks of SCID mice (Randazzo *et al.*, 1996). Intracerebral inoculation of G207 into the HSV-1-sensitive *Aotus* monkeys resulted in a rise in serum anti-HSV antibody levels but no observable HSV pathology was detected by histopathological examination or magnetic resonance imaging (Hunter *et al.*, 1999). There was also no evidence of HSV dissemination in the G207-injected *Aotus* monkeys (Hunter *et al.*, 1999). No limiting toxicities have been observed in the dose escalation phase I clinical trials investigating HSV-1716 or HSV-G207 for the treatment of localized glioblastoma multiforma (Brown *et al.*, 1998; Markert *et al.*, 1999).

The mechanism(s) of synergistic activity in the combination of MMC with HSV-1716 was postulated to be one or more of the following: (1) MMC augmenting the oncolytic activity of HSV-1716, (2) HSV-1716 enhancing the anti-tumor effect of

MMC, or (3) the antitumor effects of the two agents occurring at two distinct points on the same pathway. To investigate potential mechanisms by which MMC could synergize with the oncolytic effect of HSV-1716, it was noted that MMC but not three other chemotherapeutic agents synergized with HSV-1716. Since these four agents have all been shown to induce apoptosis (Barry *et al.*, 1990; Ohmori *et al.*, 1993; Fukuda and Saijo, 1994; Castaneda and Kinne, 1999), the induction of apoptosis alone was not sufficient to yield a synergistic effect. The distinct responses most likely reflected the different mechanisms of each drug. The second hypothesis was that MMC at the synergistic dose may augment viral replication, analogous to a previous report showing that ionizing radiation of cells infected with an ICP34.5 mutant enhanced its replication (Advani *et al.*, 1998). However, our data show that although high-dose MMC reduced the replication efficiency of HSV-1716, the synergistic dose of MMC (0.1 μ M) neither augmented nor inhibited viral replication (Fig. 5). Whereas varying the administration schedule of topotecan with Ad-TK gene therapy can modulate the synergistic activity between these two agents in ovarian cancer (Tong *et al.*, 1998), no significant differences in the effectiveness of the combined therapy of MMC and HSV-1716 infection were observed by varying the time of MMC administration. These data do not support hypothesis 1; that is, the mechanism of the synergism between MMC and HSV-1716 is not caused by MMC augmenting the oncolytic activity of HSV-1716.

A third hypothesis is that HSV-1716 may augment the anti-tumor activity of MMC. Whereas MMC is a bioreductive alkylating agent (Kennedy *et al.*, 1985) and is known to induce apoptosis, the mechanisms utilized by wt HSV-1 and mutants to kill infected cells and the mechanism by which cell death proceeds are still a matter of investigation. Galvan and Roizman showed that some wt HSV-infected cells undergo cell death via apoptosis while other cells die a nonapoptotic death, depending on the cell type (Galvan and Roizman, 1998). It has been proposed that ICP34.5-deleted mutants will induce apoptosis (Chou and Roizman, 1992; Chou *et al.*, 1994) because ICP34.5 protein inhibits premature shutoff of host protein synthesis (Cassady *et al.*, 1998a,b), and we demonstrated that HSV-1 mutants lacking ICP34.5 can induce cell death via apoptosis in the NSCLC cell line NCI-H460 (Table 2). However, in the NSCLC line A549 (Table 2), cell death via nonapoptotic mechanisms appeared dominant, similar to wt HSV (Galvan and Roizman, 1998). Thus, it is likely that ICP34.5 mutants may induce cell death by either apoptotic or nonapoptotic mechanisms, similar to wt HSV-1 (Galvan and Roizman, 1998).

The observation that MMC can more effectively kill cells in the S phase of the cell cycle (Crooke and Bradner, 1976) raised the possibility that HSV-1716 infection may influence the rate of cell cycle progression in NSCLC lines. For example, if HSV infection blocked cells from progressing through the S phase of the cell cycle, thereby increasing the percentage of cells in S phase, then the cells may exhibit greater sensitivity to chemotherapeutic agents inhibiting DNA synthesis, such as MMC. However, HSV-1716 infection did not result in the accumulation of NCI-H460 and A549 cells in S phase (Table 2) and the mechanism of the MMC and HSV-1716 synergism does not appear to involve HSV-1716 regulation of cell cycle progression in NSCLC cells.

Since the cytotoxic activity of MMC is dependent on its intracellular reductive activation, another possible mechanism of

TABLE 2. FLOW CYTOMETRY CELL CYCLE ANALYSES OF HSV-1716-INFECTED NSCLC CELL LINES

Cell line	HSV-1716 ^a	S phase (%) ^b	Apoptosis (%) ^c
NCI-H460	—	23.1 \pm 1.7	2.5 \pm 0.3
	+	26.7 \pm 4.4	30.4 \pm 6.0
A549	—	22.4 \pm 1.4	3.3 \pm 1.4
	+	24.8 \pm 2.5	11.4 \pm 5.5

^aHSV-1716: +, cells were infected with HSV-1716 at an MOI of 10 for 24 hr; —, noninfected cells.

^bS phase (%): percentage of cells in S phase.

^cApoptosis (%): percentage of cells in sub-G₀.

the synergy is the upregulation of the enzymes activating MMC by HSV infection. DT-diaphorase (DTD) catalyzes the two-electron reduction of MMC to form MMC hydroquinone, which has DNA-alkylating activity (Tomasz *et al.*, 1987; Siegel *et al.*, 1990). The level of cellular DTD correlates with MMC-induced cytotoxicity in NSCLC cell lines (Malkinson *et al.*, 1992) and the expression level of DTD in immunoblot was directly proportional to its activity (Marin *et al.*, 1997; Mikami *et al.*, 1998). Furthermore, MMC-resistant cell lines showed no or only marginal DTD activity (Begleiter *et al.*, 1989; Siegel *et al.*, 1990; Lee *et al.*, 1993; Pan *et al.*, 19985; Mikami *et al.*, 1996) and introduction of *NQO1* gene encoding DTD into DTD-negative St-4 cells rendered them several times more sensitive to MMC (Mikami *et al.*, 1996). Therefore, we investigated DTD expression in HSV-infected cells by immunoblot assays (Fig. 6). HSV infection demonstrated slight effects on DTD expression; however, we did not observe a distinct association with DTD expression and synergism between MMC and HSV-1716. Although it is possible that HSV may affect other enzymes that play a role in activation of MMC, such as NADPH-cytochrome P-450 reductase (Tomasz and Lipman, 1981; Pan *et al.*, 1984), xanthine oxidase (Pan *et al.*, 1984) or cytochrome *b*₅ reductase (Hodnick and Sartorelli, 1993), which catalyze the one-electron reduction of MMC to its semiquinone radical, these other enzymes did not appear to play a major role in sensitivity of lung cancer cells to MMC (Mikami *et al.*, 1996; Sakamoto *et al.*, 1997; Cummings *et al.*, 1998). The interactions between HSV-1 infection and these enzymes have not been described thus far. Since murine cytomegalovirus suppressed NADPH:cytochrome P450 through virus-induced interferon (Selgrade *et al.*, 1984), it is feasible that HSV-1 may not increase expression of NADPH:cytochrome P450. In addition, glutathione S-transferase (GST) has been considered to be important in inactivation of MMC (Nishiyama *et al.*, 1997). Although bacterial GST has been used in fusion proteins to elucidate function of various HSV genes (Mitchell *et al.*, 1997; Monahan *et al.*, 1998), the effect of HSV infection on activity of mammalian GST has not been described. If HSV infection inhibits on GST function in NCI-H460 and A549 but not Calu-1, Calu-3, and NCI-H322, it may explain the synergy between HV-1716 and MMC.

As an alternative mechanism, synergism has been shown to occur when two drugs such as cyclosporine A and rapamycin inhibit two distinct points in a given signal transcription pathway, such as T cell activation (Molnar-Kimber, 1996). Taken together, these data are consistent with the possibility that MMC

and HSV-1716 may synergize by affecting distinct points in the cell death pathway used in A549 and NCI-H460. This hypothesis would suggest that A549 and NCI-H460 undergo cell death according to pathways almost identical to those of the other NSCLC lines, but that utilize one or more distinct steps.

In summary, we have developed a novel therapeutic strategy for the treatment of NSCLC with an HSV-1 ICP34.5 mutant (HSV-1716) in combination with conventional chemotherapeutic agents. This combination therapy produced an additional therapeutic benefit over either individual modality. In particular, low-dose MMC produced a synergistic effect with HSV-1716 *in vitro* and an additive effect *in vivo*. The combination of HSV-based oncolytic therapy and chemotherapy could be used to treat NSCLC, especially to reduce localized tumor burden and tumor-associated symptoms. Since HSV-1 ICP34.5 mutants have been shown to be efficacious against many non-CNS malignancies such as breast, colon, ovarian, lung cancer, and malignant mesothelioma, combined therapy may augment the effectiveness of HSV-based oncolytic therapy in these as well as additional malignancies.

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