GS-9191 Is a Novel Topical Prodrug of the Nucleotide Analog 9-(2-Phosphonylmethoxyethyl)Guanine with Antiproliferative Activity and Possible Utility in the Treatment of Human Papillomavirus Lesions[▽]

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GS-9191 is a novel double prodrug of the nucleotide analog 9-(2-phosphonylmethoxyethyl)guanine (PMEG) designed as a topical agent to permeate skin and be metabolized to the active nucleoside triphosphate analog in the epithelial layer. The prodrug was shown to be metabolized intracellularly to 9-(2-phosphonylmethoxyethyl)-N⁶-cyclopropyl-2,6-diaminopurine (cPrPMEDAP) and subsequently deaminated to PMEG. The active form, PMEG diphosphate, was shown to be a potent inhibitor of DNA polymerase α and β while showing weaker activity against mitochondrial DNA polymerase γ (50% enzyme inhibition observed at 2.5, 1.6, and 59.4 μ M, respectively). GS-9191 was markedly more potent than PMEG or cPrPMEDAP in a series of human papillomavirus (HPV)-positive cell lines, with effective concentrations to inhibit 50% cell growth (EC₅₀) as low as 0.03, 207, and 284 nM, respectively. In contrast, GS-9191 was generally less potent in non-HPV-infected cells and primary cells (EC₅₀s between 1 and 15 nM). DNA synthesis was inhibited by GS-9191 within 24 h of treatment; cells were observed to be arrested in S phase by 48 h and to subsequently undergo apoptosis (between 3 and 7 days). In an animal model (cottontail rabbit papillomavirus), topical GS-9191 was shown to decrease the size of papillomas in a dose-related manner. At the highest dose (0.1%), cures were evident at the end of 5 weeks, and lesions did not recur in a 30-day follow-up period. These data suggest that GS-9191 may have utility in the treatment of HPV-induced lesions.

Human papillomaviruses (HPV) cause proliferative lesions in the skin and squamous mucosa, predominantly in the form of hyperplasias, papillomas, and condylomas. Currently recommended treatments for HPV-mediated diseases include patient-applied treatments (podofilox gel or imiquimod cream), provider-applied treatments (cryotherapy, podophyllin resin, and trichloroacetic acid), or surgical removal of the lesion (infrared coagulation, laser surgery, and a loop electrical excision procedure) (5). The primary pharmacological therapy for external anogenital warts is topically applied imiquimod cream (Aldara), a nucleoside-like compound that acts as an immune response modifier. This therapy has limited efficacy in males relative to females (33% versus 72%, respectively), a high incidence of recurrence, local tolerability issues, and a relatively long duration of treatment (up to 16 weeks) (26). Podofilox gel (Condylox package insert; Watson Pharmaceuticals, Inc., Corona, CA), which is also applied externally, is an antiproliferative agent (antimitotic agent) with similar efficacy as imiquimod (12, 27, 28). Cidofovir, a nucleotide analog with antiproliferative and antiviral activity (1), has been tested as a potential treatment for anogenital warts and has shown positive effects (25; also J. Douglas, T. Corey, S. Tyring, J. Kreisel,

B. Bowden, D. Crosby, T. Berger, M. Conant, B. McGuire, H. S. Jaffe, presented at the 4th Conference on Retroviruses and Opportunistic Infections, Washington DC, 22 to 26 January, 1997). While the existing antiproliferative agents have proven therapeutic utility, there is a need for new agents that are better tolerated and have an improved response rate and/or a shorter duration of treatment.

The acyclic nucleotide, 9-(2-phosphonylmethoxyethyl)guanine (PMEG) forms an active phosphorylated metabolite, PMEG diphosphate (PMEG-DP), in cells, which inhibits the growth of various transformed cell lines due to potent inhibition of the nuclear DNA polymerases α , δ and ϵ , resulting in inhibition of DNA synthesis and/or DNA repair (15, 16, 21). In animal models, PMEG has antiproliferative effects; however, the utility of PMEG as an antiproliferative agent is limited by its poor cellular permeability and toxicity (18, 23). An N⁶-substituted prodrug of PMEG, 9-(2-phosphonylmethoxyethyl)-N⁶-cyclopropyl-2,6-diaminopurine (cPrPMEDAP) has similar antiproliferative effects in vitro (9, 13) and reduced toxicity in vivo but, like PMEG, is negatively charged at physiologic pH and has poor permeability into the skin. As HPV infects the skin, topical administration of an active agent can be directly applied to the lesion, which should increase efficacy while decreasing the potential for systemic toxicity. GS-9191 (1-phenylalanine, N,N'-[[[2-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]ethoxy]methyl]phosphinylidene]bis-, bis(2-methylpropyl) ester) is a lipophilic (logD of 3.4) prodrug that was designed to increase

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the permeability and accumulation of metabolites (specifically PMEG-DP) into cells.

The antiproliferative effects of GS-9191 were evaluated in a series of HPV-transformed cell lines, the metabolism and mechanism of action were confirmed *in vitro*, and preliminary efficacy was obtained in the cottontail rabbit papillomavirus (CRPV) model.

MATERIALS AND METHODS

Compounds. GS-9191, cPrPMEDAP, and PMEG were synthesized by the Department of Medicinal Chemistry, Gilead Sciences, Inc. Solutions of GS-9191 in 100% dimethyl sulfoxide and solutions of cPrPMEDAP and PMEG in pH 7.0 phosphate buffer were utilized for *in vitro* assays. PMEG monophosphate (PMEG-MP), PMEG-DP, cPrPMEDAP-MP, and cPrPMEDAP-DP were synthesized by TriLink BioTechnologies (San Diego, CA). [Purine-8-¹⁴C]GS-9191 (55 mCi/mmol) and [purine-8-³H]cPrPMEDAP (16.6 mCi/mmol) were synthesized by Moravsek Biochemicals (Brea, CA), dissolved in dimethyl sulfoxide and 50% ethanol, respectively. Podofilox was purchased from Acme Biosciences (Belmont, CA); cidofovir was synthesized by Gilead Sciences, Inc.

Cells. Six cervical carcinoma cell lines carrying integrated copies of HPV subtypes (HPV-16 in SiHa and CaSki cells; HPV-18 in HeLa, MS-751, and C-41 cells; and HPV-39 in ME-180 cells), three HPV-negative carcinoma cell lines (cervical carcinoma HT-3; tongue squamous carcinoma SCC-4 and SCC-9), and human embryonic lung fibroblasts (HEL) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (Gibco/Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine (Gibco/Invitrogen), and antibiotics (100 units/ml penicillin, and 100 µg/ml streptomycin [Gibco/Invitrogen]). Primary cells from healthy human donors, including adult foreskin keratinocytes (PHK), cervical keratinocytes (CK), bronchial epithelial cells (NBE), and dermal fibroblasts (PHF), were purchased from Cambrex (Walkersville, MD) and cultured in special medium supplied by Cambrex (serum-free keratinocyte growth medium for PHK and CK; serum-free small airway epithelial cell growth medium for NBE; 2% serum-containing fibroblast growth medium for PHF).

Metabolism of GS-9191 *in vitro*. The intracellular metabolic profile of GS-9191 was determined in SiHa cells incubated with [¹⁴C]GS-9191 for 24 h. Cells were washed with culture medium and phosphate-buffered saline and detached from the flask using trypsin. Cells were then resuspended in 80% methanol, incubated overnight at -20°C to allow for cell lysis, and centrifuged at 14,000 rpm for 15 min to remove cellular debris. The methanol extract was lyophilized, and the dry pellet was dissolved in the 25 mM K₂HPO₄ (pH 6.0) and 5 mM tetrabutylammonium bromide in water for liquid chromatography coupled to in-line radioactivity detection. Analytes were separated using reverse phase liquid chromatography on a Prodigy ODS(3) (150 by 4.6 mm; 5-µm particle size) column (Phenomenex, Torrance, CA) using a buffer containing 25 mM K₂HPO₄ and 5 mM tetrabutylammonium bromide (pH 6.0), a flow rate of 1.2 ml/min, and a multistage linear gradient from 0 to 70% acetonitrile over 65 min. Metabolite peaks were identified from authentic standards for PMEG, PMEG-MP, PMEG-DP, cPrPMEDAP, cPrPMEDAP-MP and cPrPMEDAP-DP, separated using the same analytical methodology and detected by UV detection.

In vitro cellular metabolism experiments were performed with GS-9191 using SiHa, CaSki, PHK, CK, and HEL cells. Cells were incubated with a 1 µM concentration of compound for 32 h. Cells were collected (as described above) and resuspended in 0.2% formic acid. The concentrations of GS-9191 and its metabolites (cPrPMEDAP and PMEG) were determined by liquid chromatography coupled to tandem mass spectrometry using a Sciex API-4000 triple quadrupole mass spectrometer (MDS Sciex/Applied Biosystems, Foster City, CA) operating in positive electrospray ionization mode. Analytes were separated using a Aqua C₁₈ column (50 by 2.0 mm; 5-µm particle size; Phenomenex), a buffer of 0.2% formate, and a multistage linear gradient from 0 to 90% acetonitrile over 12 min. The amount of analyte in each sample was determined using seven-point standard curves prepared in cell extract from untreated cells. Low- and high-quality control samples were analyzed at the beginning and end of each analytical run to ensure accuracy and precision within 20%. Intracellular concentrations of compounds were calculated based on the number of cells the intracellular extract was derived from and the measured cell volume for each cell type (SiHa, 1.33 pl/cell; CaSki, 1.3 pl/cell; PHK, 4.44 pl/cell; CK, 9.02 pl/cell; HEL, 4.17 pl/cell). The area under the intracellular concentration curve over 32 h

(AUC₀₋₃₂), metabolism efficiency, and intracellular concentrations required to inhibit cell proliferation were then calculated.

Hydrolase activity was measured using a modification of a previously described method (3). Metabolites were quantified by high-performance liquid chromatography, and activity was expressed as pmoles of metabolites produced per minute per microgram of enzyme sample. *In vitro* deamination assays were performed as previously described (22). To measure deaminase activity, 2 nM [³H]cPrPMEDAP was incubated at 37°C for 16 h with cell extracts; extracts were analyzed by high-performance liquid chromatography.

Antiproliferation assay. Cells were detached from culture flasks using trypsin, counted, and plated in 96-well culture plates (250 to 1,000 cells per well, optimized for each cell type). The following day, fivefold serial dilutions of compounds were added in duplicate. Either no compound was added to control wells or 10 µM colchicine (a mitosis inhibitor) was added to represent 100% proliferation or 0% proliferation, respectively. Seven days after the addition of compounds, culture plates were treated with 10% trichloroacetic acid at 4°C for 1 h to allow cell-derived proteins to bind to the bottom surfaces of the plates. The plates were washed with water, stained with 0.4% sulforhodamine B in 1% acetic acid for 10 min, and extensively washed with 1% acetic acid. The remaining dye was solubilized in 10 mM Trizma base to generate purple coloring that was quantified by measuring the absorbance at a wavelength of 490 nm. From the experimental data, sigmoidal dose-response curves were generated, and 50% effective concentration (EC₅₀) values were calculated using GraphPad Prism software, version 4.0 for Windows (GraphPad Software, San Diego, CA).

Inhibition of DNA polymerases. Human DNA polymerases α and β were purchased from CHIMERx (Milwaukee, WI). DNA polymerase γ expressed in baculovirus was provided by Bill Copeland (National Institute of Environmental Health Sciences, Research Triangle Park, NC). For the DNA polymerase α and β assays, serial dilutions of compounds were incubated in the reaction mixture (1 µM [³H]dGTP, 1 µM [³H]dUTP, 10 µM dATP, 10 µM dCTP, 9 µM dUTP, 9 µM dGTP, 0.4 µM oligo-DNA template, 0.4 µM oligo-primer [a mixture of four different sequences], 0.2 mg/ml bovine serum albumin, 1 mM dithiothreitol, 10% glycerol, 40 mM HEPES [pH 7.5], 7.5 mM MgCl₂, and 0.0033 units of DNA polymerase) at 37°C for 30 min. The reaction mixture was applied to a DEAE filter plate. The plate was washed with phosphate buffer, and the remaining radioactivity was measured with a Topcount microplate reader. For the DNA polymerase γ assay, fourfold serial dilutions of compounds were incubated with the reaction mixture (0.5 µM [³H]dGTP, 50 µM dATP, 50 µM dCTP, 50 µM TTP, 100 µg/ml activated DNA, 1 mg/ml bovine serum albumin, 3 mM dithiothreitol, 5% glycerol, 100 mM KCl, 5 mM MgCl₂, and 50 ng/ml DNA polymerase) at 30°C for 10 min. The reaction mixture was spotted onto Whatman 3MM paper disks (Whatman, Inc., Piscataway, NJ). The disks were washed with 5% trichloroacetic acid-1% Na-pyrophosphate, followed by a wash in 100% ethanol. Dry disks were counted for radioactivity. The 50% inhibitory concentrations (IC₅₀s) were calculated using Sigmaplot software (Systat Software, Inc., Point Richmond, CA) or GraphPad Prism for DNA polymerase α/β and γ, respectively.

Inhibition of DNA synthesis, cell cycle analysis, and apoptosis. Labeling with 5-bromo-2'-deoxyuridine (BrdU) was performed by adding 20 µl of 100 µM BrdU without removing compounds, followed by 3 or 8 h of incubation. At the end of incubation, cells were fixed, and the amount of BrdU incorporated into DNA was quantified by an enzyme-linked immunosorbent assay using a Cell Proliferation ELISA and BrdU (colorimetric) Kit (Roche Diagnostics, Penzberg, Germany). From the experimental data, sigmoidal dose-response curves were generated and the EC₅₀ values were calculated using GraphPad Prism software. For cell cycle analyses, cells were plated in six-well plates at about 70% confluence and cultured for 48 h before the addition of GS-9191 at 10 times the antiproliferative EC₅₀ (0.3 nM for SiHa; 30 nM for PHK) or of podofilox at 10 times the EC₅₀ (370 nM for SiHa; 55 nM for PHK). Nuclear DNA was stained with propidium iodide (PI) using a CycleTEST PLUS kit (Becton Dickinson, Franklin Lakes, NJ) and analyzed by flow cytometry. The percentage of cells at each cell cycle stage was calculated using ModFit LT software (Verity Software House, Topsham, ME). To assess apoptosis, after 3- and 7-day incubations with GS-9191 in designated cell types, cells were detached from plates by trypsin, washed, double stained with PI and annexin V, and analyzed by flow cytometry.

Antiviral testing in CRPV model. All animal studies were approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University, College of Medicine. Male and female New Zealand White rabbits (5 per group) were infected with a CRPV stock at dilutions of 10⁻¹ and 10⁻² (two sites each on each rabbit) as described by Christensen et al. (7). Treatments began on day 14 after CRPV infection and consisted of once-daily topical application of 100 µl of compound to papillomas for 5 days/week for 5 weeks. Placebo ointment containing propylene glycol, petrolatum, sorbitan sesquioleate, and microcryst-

FIG. 1. Intracellular metabolites formed following a 24-h incubation of SiHa cells with 1 μ M [14 C]GS-9191. Metabolites were separated using liquid chromatography coupled to an in-line radioactivity detector. The metabolites cPrPMEDAP, PMEG, PMEG-MP, and PMEG-DP were identified based on retention of authentic standards. No phosphorylated cPrPMEDAP species were detected. dpm, disintegrations per minute.

talline wax served as the negative control, and 0.5% cidofovir in solution was used as the positive control. GS-9191 was formulated as an ointment and administered at three concentrations: 0.01%, 0.03%, and 0.1%. The sizes of papillomas were measured weekly in three dimensions, and a geometric mean diameter was calculated for each papilloma. Statistical analysis of geometric mean diameter over time was compared with mean size of placebo-treated papillomas at the same time point using a Student's *t* test. *P* values of <0.05 were considered to be statistically significant. When sites with adverse effects (redness/erosions) were identified, treatments were temporarily stopped until sites healed. A portion of the animals were held after completion of dosing to monitor whether recurrence of lesions occurred.

RESULTS

Intracellular metabolism of GS-9191. In order to understand the metabolic pathway of GS-9191 activation in cells, [14 C]GS-9191 was incubated with SiHa cells, and the intracellular metabolites were characterized. As shown in Fig. 1, the predominant intracellular metabolites observed following a 24-h incubation were cPrPMEDAP, PMEG, PMEG-MP, and

PMEG-DP. Based on the intracellular metabolite profile and results obtained with the related PMEG prodrug used as an anticancer agent, GS-9219 (22), the presumed intracellular activation pathway of GS-9191 to PMEG-DP is shown in Fig. 2.

Intracellular samples taken at an earlier time point (8 h) showed the predominant metabolite to be cPrPMEDAP (data not shown), suggesting that the first step in the intracellular activation of GS-9191 is release of cPrPMEDAP. The lysosomal carboxypeptidase, cathepsin A, has been shown to play an important role in the initial hydrolysis of other nucleotide phosphonoamidate prodrugs (3). We tested the ability of purified cathepsin A to hydrolyze GS-9191 and found that it catalyzed the reaction at a rate of 25,000 pmol/min $\cdot$ μ g. Moreover, GS-9191 metabolites accumulated 7 to 15 times less efficiently in cathepsin A-negative cells (GM5076 and GM2438) than in cathepsin A-positive cells (HEL, SiHa, and GM5400) (data not shown). Combined, these results suggest a role of this protease in the initial activation step of GS-9191. Following the cleavage of GS-9191, cPrPMEDAP requires deamination to result in formation of PMEG and, ultimately, formation of the putative active metabolite PMEG-DP. Consistent with the importance of deamination in the activation of cPrPMEDAP, the deaminase inhibitor (deoxycytosine) significantly reduced the antiproliferative activity of cPrPMEDAP but not that of PMEG (data not shown).

Antiproliferative activity of GS-9191 in cell culture. The antiproliferative activity of GS-9191 and its metabolic products cPrPMEDAP and PMEG (Fig. 2) were examined in six HPV-transformed carcinoma cell lines, three HPV-negative carcinoma cell lines, and five primary cell cultures. GS-9191 exhibited EC₅₀s ranging from 0.03 nM to 15 nM (Table 1) and was generally more active against HPV-transformed cells than against non-HPV-transformed cell lines and primary cells. GS-9191 was substantially more potent than cPrPMEDAP and PMEG, suggesting that the prodrug more effectively delivers

FIG. 2. Putative pathway for the intracellular activation of GS-9191. The lipophilic prodrug GS-9191 efficiently enters the cell by passive diffusion (1). Enzymatic ester hydrolyzes (2), and spontaneous chemical conversion (3) results in formation of a monophenylalanine intermediate metabolite. Either a pH- or enzyme-dependent process (4) results in formation of free phosphonate N⁶ prodrug cPrPMEDAP, which is then deaminated by a cytosolic N⁶-methyl-AMP aminohydrolase to form PMEG (5). The presence of two negative charges results in poor membrane permeability for cPrPMEDAP and PMEG (6) although more efficient carrier-mediated processes have been identified (7) (24). PMEG can then be phosphorylated by a two-step enzymatic process to the active metabolite PMEG-DP (8 and 9).

TABLE 1. Antiproliferative activity of GS-9191 and related compounds

Cell type	EC ₅₀ (nM) ^a			
	GS-9191	cPrPMEDAP	PMEG	Cidofovir
HPV-transformed cervical carcinoma cell lines ^b				
SiHa	0.03 ± 0.01	290 ± 110	210 ± 90	>100,000
CaSki	2.03 ± 0.97	1,410 ± 620	5,530 ± 1,1610	>100,000
HeLa	0.71 ± 0.46	6,930 ± 1,120	2,160 ± 1,010	>100,000
MS-751	0.04 ± 0.02	3,310 ± 550	2,800 ± 560	>100,000
C-4I	0.44 ± 0.07	1,330 ± 1,150	470 ± 170	3,100 ± 2,030
ME-180	1.83 ± 1.52	8,320 ± 690	2,570 ± 380	64,700 ± 17,400
HPV-negative carcinoma cell lines ^c				
HT-2	5.42 ± 4.75	7,040 ± 1,030	1,930 ± 1,300	>100,000
SCC-4	5.03 ± 6.76	1,430 ± 550	160 ± 130	>100,000
SCC-9	15.3 ± 11.1	10,100 ± 2,020	3,150 ± 1,600	>100,000
Primary cells ^d				
PHK	2.91 ± 3.33	3,700 ± 3,540	550 ± 770	14,400 ± 21,600
CK	2.10 ± 0.02	1,380 ± 970	>100,000	51,700 ± 67,000
NBE	0.98 ± 0.30	2,300 ± 2,150	670 ± 390	31,900 ± 18,100
HEL	7.44 ± 8.62	4,820 ± 22,000	290 ± 220	23,500 ± 32,700
PHF	4.75 ± 2.98	12,700 ± 8,950	1,580 ± 870	>100,000

^a Results represent the mean ± standard deviation of 3 to 6 experiments, except for cidofovir in HPV-negative cell lines ($n = 1$). Podofilox showed nonspecific activity of between 4.6 and 26 nM across all cell types.

^b Cell lines transformed with HPV16 (SiHa and CaSki), HPV18 (HeLa, MS-751 and C-4I), and HPV39 (ME-180).

^c HPV-negative carcinoma cell lines derived from cervix (HT-3) and tongue (SCC-4 and SCC-9).

^d Primary cells are skin keratinocytes (PHK), cervical keratinocytes (CK), bronchial epithelia (NBE), lung fibroblasts (HEL), and skin fibroblasts (PHF).

PMEG-DP into cells. Cidofovir, which exhibited efficacy against anogenital warts in a phase I/II study (25), was much less potent than GS-9191, with EC₅₀s ranging from 3 μ M to greater than 100 μ M. GS-9191 was also found to be more potent than podofilox in HPV-transformed cells.

Correlation of cellular metabolism with EC₅₀. To better understand the relationship of antiproliferative activity to the intracellular metabolism of GS-9191 in HPV-transformed cell lines and primary cells, cells were incubated with 1 μ M GS-9191 for 32 h, followed by analysis of intracellular metabolites by liquid chromatography-tandem mass spectrometry. As summarized in Table 2, a good correlation between the EC₅₀ of GS-9191 and intracellular levels of PMEG was observed. For example, the relatively resistant CaSki cell line showed a large deficiency in both hydrolysis and deamination relative to the more sensitive SiHa cell line. Primary cells accumulated 3.2- to 40-fold lower total levels of GS-9191 and its metabolites. Primary cells also showed poor deamination relative to the SiHa

cell line. The higher prodrug hydrolysis rates in SiHa and HEL cells were confirmed by experiments in cell lysates (data not shown).

Activity of PMEG-DP in DNA polymerase assays. Previous studies have shown that PMEG-DP is a potent chain-terminating inhibitor of cellular DNA polymerases (14). The metabolic products of GS-9191 (PMEG-MP, PMEG-DP, cPrPMEDAP-MP, and cPrPMEDAP-DP) were tested in in vitro DNA polymerase α , β , and γ assays (Table 3). Consistent with the hypothesis that PMEG-DP is the active metabolite, only PMEG-DP was able to inhibit DNA polymerase α with an IC₅₀ of 2.5 μ M. While all compounds showed some inhibition of DNA polymerase β , PMEG-DP was at least eightfold more potent than the other metabolites. PMEG-DP only weakly inhibited the mitochondrial DNA polymerase γ .

Inhibition of cellular DNA synthesis and S-phase arrest. The time course of DNA synthesis inhibition in SiHa cells is shown in Table 4. Inhibition occurred as early as the first 3 h,

TABLE 2. Correlation of the efficiency of cellular metabolism of GS-9191 to antiproliferative activity in different cell types

Cell type	EC ₅₀ (nM)	Intracellular AUC ₀₋₃₂ (μ M · h) ^a				Metabolism efficiency (%) ^b	
		A	B	C	Total (A+B+C)	Hydrolysis [(B+C)/(A+B+C)]	Deamination [C/(B+C)]
		GS-9191	cPrPMEDAP	PMEG			
SiHa	0.03	28,000	6,810	1,960	36,800	24	22
CaSki	2.0	89,800	3,750	170	93,700	4.2	4.3
CK	2.1	10,400	991	33.4	11,400	9.0	3.3
PHK	2.9	1,510	814	47.9	2,380	36	5.6
HEL	7.4	1,620	835	26.0	2,480	35	3.0

^a AUC₀₋₃₂ was calculated based on intracellular concentrations measured at 1, 2, 4, 6, 8, 24, and 32 h for each metabolite in each cell type during an incubation with 1 μ M GS-9191.

^b Metabolism efficiency, expressed as the percentage of intracellular metabolites, represents the ability of each cell type to hydrolyze the bis-amidate promoieties and deaminate cPrPMEDAP.

TABLE 3. Inhibition of cellular DNA polymerases by the phosphorylated metabolites of GS-9191

Metabolite	Inhibition of DNA polymerase (IC ₅₀ [μM]) ^a		
	α (n = 4)	β (n = 3)	γ (n = 2)
PMEG-MP	>100	13.5 ± 2.12	ND
PMEG-DP	2.5 ± 0.97	1.6 ± 0.53	59.4 ± 17.6
cPrPMEDAP-MP	>100	33 ± 2.83	ND
cPrPMEDAP-DP	>100	23 ± 1.41	>150

^a Values are the means ± standard deviations of 2 to 4 experiments (n) done in duplicate. ND, not determined.

with maximal inhibition occurring during the first 9 h (EC₅₀ during 6 to 9 h, 1.0 nM) and potency gradually decreasing thereafter. Consistent with their activities measured in the 7-day antiproliferation assays, both cPrPMEDAP and PMEG showed lower potency than GS-9191 (>100-fold less potent).

The inhibition of DNA synthesis was further examined in SiHa, CaSki, HEL, and PHK cells by measuring the amount of incorporation of BrdU into cellular DNA following exposure to serial dilutions of GS-9191 (Fig. 3). Dose-dependent inhibition of BrdU incorporation was observed in all cells. GS-9191 inhibited DNA synthesis with the highest potency in SiHa cells (EC₅₀ of 0.89 nM) and was least potent in HEL cells (EC₅₀ of 275 nM). These results suggest that the inhibition of cellular DNA synthesis is the common mechanism responsible for the antiproliferative activity of GS-9191.

Cell cycle profiles of SiHa cells and PHK cells treated with GS-9191 and the mitosis inhibitor podofilox were examined by nuclear DNA staining followed by flow cytometry (Fig. 4). In untreated SiHa cells, 23% of cells were found in the S phase. After a 48-h treatment with a 10-fold EC₅₀ concentration of GS-9191 (0.3 nM, calculated from the EC₅₀ obtained in the antiproliferation assay), the percentage of cells in the S phase increased to 65.6%, suggesting that S-phase progression was inhibited. In contrast, treatment of SiHa cells with podofilox resulted in an increase in the percentage of cells in the G₂/M phase (7.8% to 45%). PHK cells treated with 10-fold EC₅₀ concentrations of GS-9191 and podofilox also exhibited increased percentages of cells in the S phase (19.5% to 36.1%) and in the G₂/M phase (8.9% to 16.5%), respectively. S-phase arrest was also observed in CaSki and A431 skin carcinoma cells exposed to GS-9191 (data not shown).

Induction of apoptosis as a consequence of DNA chain termination. Studies were done to further characterize the mechanism of the antiproliferative activity of GS-9191. HPV-transformed carcinoma cell lines are reported to be relatively resistant to apoptosis induced by DNA damage, due to the downregulation of p53 by the viral E6 protein (11, 20). When SiHa cells were treated with GS-9191 at concentrations ranging from 2.7 to 270 nM, dose- and time-dependent apoptosis induction was observed (Table 5). Only 3.4% of cells treated with 2.7 nM GS-9191 became apoptotic by day 3, while 0.9 nM was sufficient to reduce DNA synthesis by 50% within 24 h (Fig. 3), and 0.3 nM was sufficient to arrest more than 65% of cells in the S phase within 48 h (Fig. 4). By day 7, 33.1% of cells treated with 2.7 nM GS-9191 became apoptotic. These data suggest that significant accumulation of DNA damage is required for SiHa cells to initiate apoptosis. Higher concentra-

TABLE 4. Time dependence of inhibition of cellular DNA synthesis as measured by BrdU labeling in HPV-16-transformed SiHa cervical carcinoma cells treated with PMEG, cPrPMEDAP, and GS-9191

BrdU labeling time (h)	EC ₅₀ (nM) ^a		
	PMEG	cPrPMEDAP	GS-9191
0–3	8,970	8,840	74.0
3–6	3,040	1,860	7.8
6–9	2,580	1,500	1.0
24–27	3,630	2,300	1.8
48–51	6,020	2,650	7.9
72–75	10,100	>20,000	11.5

^a EC₅₀, concentration required to inhibit 50% of DNA synthesis. Values are the average of two experiments.

tions of GS-9191 induced apoptosis in a proportion of cells (15 to 20%) at day 3 and in the majority of cells (>80%) by day 7. Similarly delayed apoptosis was observed in CaSki, HeLa, and CK-1 cells following treatment with GS-9191 (data not shown). Cidofovir also caused apoptosis, but the concentration required to induce apoptosis was orders of magnitude higher than that for GS-9191.

Testing in CRPV model. To see if the in vitro findings could translate into in vivo efficacy, studies were done in the CRPV rabbit model. In the CRPV model, topical treatment of the papillomas with 0.5% cidofovir had a weak suppressive effect on papilloma size (34% decrease on day 48) and/or growth rates. Topical GS-9191 showed significant dose-related suppression of papilloma growth (Fig. 5). At the mid and high doses of GS-9191 (0.3% and 0.1%), there was a greater than 70% reduction in papilloma size by the end of the dosing period compared to the controls. The lowest dose (0.01%) had more modest effects (53% decrease) but still appeared to have a greater effect than cidofovir. Table 6 shows that cures (lack of visible papilloma) were achieved in some animals at the mid dose (two of five) and most animals at the high dose (four of five). After a period of more than 1 month without treatment, recurrence of papillomas occurred in only one animal in the mid dose group. At the highest dose (0.1%) there were indications of local irritation at the dosing site, which required that dosing be interrupted for a few days for some animals. No systemic toxicities were observed with treatment as assessed by body weight change and clinical chemistry (data not shown).

DISCUSSION

This article describes the characterization of a novel topical prodrug of PMEG which may have utility for the treatment of HPV-induced disease. The strategy employed was to take a compound with good intrinsic potency but poor permeability and a toxic liability and design a prodrug (and formulation) that could effectively deliver the potent active molecule to the disease site while limiting the exposure to nonaffected areas.

Intracellularly, the prodrug moiety of GS-9191 was hydrolyzed to produce cPrPMEDAP. Subsequently, the N⁶-cyclopropyl prodrug moiety of cPrPMEDAP was deaminated to produce PMEG, which was then rapidly phosphorylated to generate the pharmacologically active species, PMEG-DP. We have shown in our previous work that N⁶-methyl-AMP amino-

FIG. 3. Inhibition of cellular DNA synthesis by GS-9191. Cells were incubated with serial dilutions of GS-9191 for 32 h, and inhibition of cellular DNA synthesis was determined by BrdU incorporation. Data are expressed as means $\pm$ standard deviations of two independent experiments. EC₅₀s are indicated by arrows. OD, optical density.

hydrolase is able to convert cPrPMEDAP to PMEG in vitro (22). Cellular uptake of GS-9191 was sufficient to result in consistently greater activity than podofilox, a marketed agent for HPV-induced genital warts, in in vitro antiproliferative assays. GS-9191 also showed greater activity relative to cidofovir, a nucleotide analog with a similar mechanism of action that has shown efficacy in early clinical trials (25).

The relative potency of GS-9191 in different cell lines was related to their ability to cleave GS-9191 to its active form. This is clearly shown in CaSki cells, where, despite good cellular loading with total material, only a small percentage (~4%) of prodrug was converted to PMEG. Based on these results, deamination of cPrPMEDAP may be an important determinant in the sensitivity of transformed cells to GS-9191. The

implications of this result will have to be assessed further in vivo.

Consistent with prior reports, PMEG-DP was found to be incorporated efficiently into DNA by DNA polymerases α and δ while incorporation by DNA polymerase β , γ , and ϵ appeared less efficient (8, 14, 16, 17). Of note, PMEG-DP is a more potent inhibitor for DNA polymerase α/δ than cidofovir DP (2). DNA polymerases α (primase) and δ are required for synthesis of the lagging strand and the leading strand of chromosomal DNA, respectively. We have confirmed that, of the GS-9191 metabolites, only PMEG-DP inhibits DNA polymerase α (Table 3). Once PMEG-DP is incorporated, the DNA chain can no longer elongate because PMEG-DP lacks the 3'-like hydroxyl moiety, essential for the formation of the phos-

FIG. 4. GS-9191 induces cell cycle arrest at the S phase. SiHa and PHK cells were incubated for 48 h with no compound, 10× EC₅₀ concentration of GS-9191 (0.3 nM for SiHa and 30 nM for PHK), or podofilox (370 nM for SiHa and 55 nM for PHK). The amount of DNA in each nucleus was quantified by PI staining, followed by flow cytometric analysis.

TABLE 5. Induction of apoptosis following 3- and 7-day incubations with cidofovir or GS-9191 in an HPV-16-transformed cervical carcinoma cell line (SiHa)

Compound	Concentration (nM)	Cell survival at the indicated time point ^a			
		Day 3 (% of cells)		Day 7 (% of cells)	
		Live	Apoptotic	Live	Apoptotic
GS-9191	0	97.8 ± 0.4	1.9 ± 0.6	97.4 ± 0.7	1.3 ± 1.0
	2.7	95.2 ± 2.6	3.4 ± 1.5	65.5 ± 25.9	33.1 ± 26.1
	27	83.7 ± 17.7	14.6 ± 16.2	17.4 ± 6.8	82.2 ± 7.1
	270	79.6 ± 23.7	19.6 ± 23.3	15.5 ± 7.5	84.1 ± 7.9
Cidofovir	180,000	98.1 ± 0.8	1.6 ± 0.9	62.2 ± 18.0	37.2 ± 18.0

^a Results represent the mean ± standard deviation of two to three experiments.

phodiester bond with the next incoming nucleotide (8). Thus, PMEG-DP works as an obligate chain terminator of DNA synthesis.

Our results suggest that inhibition of DNA polymerases by PMEG-DP is likely the prevailing mechanism of action, and this activity alone may explain the *in vitro* antiproliferative activity of GS-9191. Following application of GS-9191 to cells, inhibition of DNA synthesis appears to be maximal between 6 and 24 h, but activity still remained for 75 h. Apoptosis appears to be a downstream consequence of the inhibition of DNA synthesis and S-phase arrest. SiHa cells are transformed by the high-risk genotype HPV-16, whose E6 protein is extremely efficient in downregulating p53 and is overexpressed due to the integration of the viral genome into the host chromosome (11, 20). Even in a cell line thought to be resistant to apoptosis, GS-9191 was found to be a relatively potent proapoptotic agent. *In vivo*, GS-9191 is expected to show selectivity for rapidly dividing papilloma cells (compared to normal keratinocytes, the majority of which are quiescent) because the inhibition of chromosomal DNA replication affects only cells in the S phase of the active cell cycle.

The CRPV model is well characterized and has been used previously to evaluate the antipapillomavirus activity of various treatments (4, 6, 7, 19). The model involves the induction of discrete papillomas on the back of a rabbit within 28 days by infecting scarified skin with CRPV virus. Papilloma formation

TABLE 6. Treatment of CRPV with cidofovir or different doses of GS-9191

Day or event	Presence of visible papillomas (no. of papillomas/no. of sites) after the indicated treatment ^a				
	Placebo	Cidofovir	0.01% GS-9191	0.03% GS-9191	0.1% GS-9191
48	5/5	5/5	5/5	3/5	3/5
62	5/5	4/5	5/5	3/5	1/5
71	ND	ND	ND	3/4	0/4
84	ND	ND	ND	3/4	0/4
Cure ^b	0/5	1/5	0/5	2/5	4/5
Recurrence ^c		ND		1/2	0/4

^a Papillomas were induced with a 10⁻² dilution of CRPV (five sites per treatment group). ND, not determined.

^b Cure is defined as a lack of visible papilloma.

^c Recurrence in cured animals during a 32-day follow-up period.

occurs in a predictable manner, and the life cycle and virological components of CRPV infections parallel cutaneous and genital HPV infections in humans (6). The effects of cidofovir, PMEG, and cPrPMEDAP have been characterized in the CRPV model. Cidofovir has been extensively studied, including testing of various durations and frequencies of topical, subcutaneous, or intralesional treatments (7, 10). Cidofovir showed strong antipapilloma effects leading to cures when delivered topically at 1% to lesions of small to moderate size; large papillomas could be cured by intralesional treatment. Systemic administration was least effective. When PMEG was given subcutaneously to CRPV-infected rabbits at doses of 0.01, 0.1, or 1 mg/kg twice a day, the highest dose showed some therapeutic effect, but treatment was terminated after 10 days due to severe toxicity (18). The intermediate metabolite of GS-9191, cPrPMEDAP, showed a ~50% reduction in papilloma growth and was tolerated when dosed topically at 1%, while a similar topical administration of 0.1% PMEG for 8 weeks was not tolerated (6). In our study, topical GS-9191 demonstrated reduction in papilloma growth, cures, and a low rate of recurrences at doses where topical GS-9191 was generally well tolerated. This experiment illustrated improvements in efficacy and tolerability relative to those previously reported with other agents.

Combined, these results show that GS-9191 acts as an effective prodrug, delivering high levels of PMEG-DP to HPV-transformed cells. Due to the lack of an HPV viral polymerase, the active metabolite PMEG-DP has to exert its antiviral effect by inhibiting host cell polymerases, and partial selectivity for virally infected cells is likely derived based on the increased replication index of infected cells and by local administration. The results found in these studies suggest that GS-9191 may have utility in the clinical treatment of genital warts caused by HPV infection, especially in patient populations where current therapies lack efficacy.

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FIG. 5. Effect of topical GS-9191 on CRPV-induced papilloma growth. Papillomas were induced with a 10⁻² dilution of CRPV. Treatment with 100 μ l of topical cidofovir or GS-9191 began on day 14. Data are expressed as means ± standard errors of the mean (SEM) of five papilloma measurements. The asterisk indicates statistical significance ($P < 0.05$). GMD, geometric mean diameter.

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