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Inhibition of HSV-1 replication and reactivation by the mutation-insensitive transcription inhibitor tetra-*O*-glycyl-nordihydroguaiaretic acid

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Abstract

Methylated derivatives of nordihydroguaiaretic acid (NDGA)were previously shown to be potent mutation-resistant inhibitors of herpes simplex virus type 1 (HSV-1) which target Sp1 protein binding to critical viral promoters. The hydrophobic nature of these agents, however, renders them relatively water-insoluble and, therefore, limits their applicability. We report here on the anti-HSV-1 properties of a related but water-soluble glycylated derivative of NDGA, tetra-*O*-glycyl-NDGA (G_4N). In yield reduction assays, G_4N inhibited replication of laboratory and clinical strains of wild type HSV-1 and ACV-resistant ($HSV\text{-}1^R$) strains of HSV-1 in a dose-dependent manner, with average IC_{50} values of 4.7 and 3.2 μM against wild-type and $HSV\text{-}1^R$ strains, respectively. An MTT-based cytotoxicity assay revealed a TC_{50} value of 73.2 μM for G_4N on Vero cells, with no reduction in viability detected at concentrations below 30 μM. Similar to its methylated counterparts, G_4N was found to inhibit transcription of the HSV-1 ICP4 gene, a major immediate early viral regulator, and gel mobility shift assays showed it can block Sp1 protein binding to cognate sites on the ICP4 promoter. In anticipation of its potential use as a systemic anti-HSV-1 agent, we tested G_4N in a murine trigeminal ganglia (TG) explant model system, and found G_4N was able to prevent HSV-1 reactivation from explanted and cultured latently infected TG.

Keywords: Herpes simplex virus type 1; Tetra-*O*-glycyl-nordihydroguaiaretic acid; G_4N M_4N; Sp1; Latency

1. Introduction

The herpes simplex virus type 1 (HSV-1) is an enveloped DNA virus afflicting greater than 70% of the human population. In humans, an HSV-1 infection normally results in primary and recurrent mucoepithelial lesions (herpes labialis) and in some instances leads to disseminated disease, encephalitis and blindness. Following a primary infection, HSV-1 establishes a permanent latent infection within the sensory ganglia of the host. This latent reservoir of virus may then reactivate following appropriate stimuli and serve as a site for future recurrent infection (Whitley, 1996; Roizman and Sears, 1996). At particular risk of recurrence, due to the increased severity of infection and the generation of drug-resistant strains of HSV-1, are those with compromised immune systems such as AIDS patients, cancer patients, and patients undergoing organ transplantation (De Logu et al., 2000).

Clinically, the most widely used and successful chemotherapeutic agents in the systemic treatment of HSV-1 infection are nucleoside analogue agents, in particular acyclovir (ACV) which targets viral DNA synthesis via HSV thymidine kinase activity (Hirsch et al., 1996). Studies have shown nucleoside analogue agents to also be successful topical treatments for herpes labialis (Straten et al., 2001) and systemic agents for minimizing the establishment and reactivation from latency (Efstathiou et al., 1999). Despite the effectiveness and high selectivity of nucleoside analogue agents, however, the development of ACV-resistant and nucleoside analogue-resistant strains of mutant HSV have been reported in immunocompromised individuals (Crumpacker et al., 1982; Crumpacker, 2001). The ability of HSV strains to readily mutate in response to conventional chemical agents underscores a need to continually develop novel anti-HSV agents that will substitute for or complement ACV and nucleoside analogues.

During an HSV-1 lytic infection, viral genes are expressed in a tightly regulated cascade. In this cascade, several of the HSV-1 genes are transcriptionally regulated by Sp1 protein

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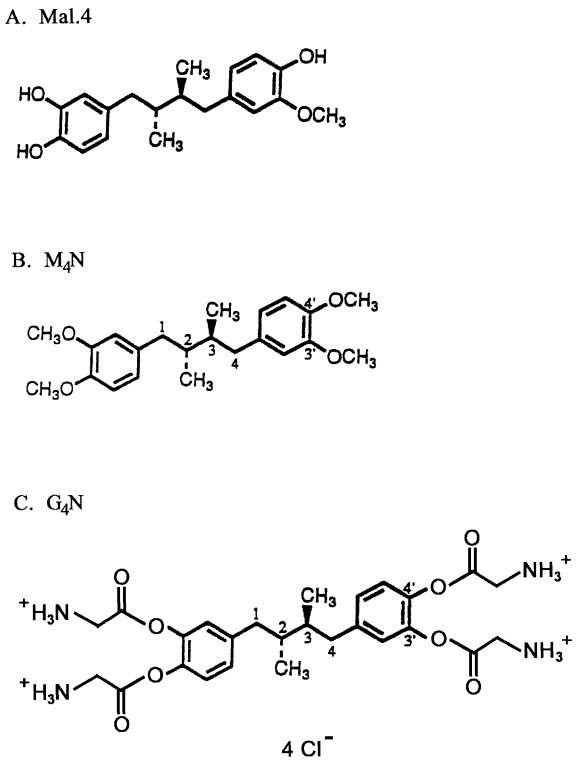

Fig. 1. Molecular structures of Mal.4, M_4N and G_4N. (A) 1-(3,4-Dihydroxyphenyl)-4-(3-methoxy-4-hydroxyphenyl)-2,3-dimethylbutane (3-*O*-methyl-NDGA; Mal.4). (B) Meso-1,4-bis(3,4-dimethoxyphenyl)-(*2R*,*3S*)-dimethylbutane tetra-*O*-methyl-nordihydroguairetic acid (tetra-*O*-methyl-NDGA, M_4N). (C) Meso-1,4-bis(3,4-dimethoxyphenyl)-(*2R*,*3S*)-dimethylbutane tetra-*O*-glycyl-nordihydroguairetic acid (tetra-*O*-glycyl-NDGA, G_4N).

binding, and may serve as targets for viral inhibition. The immediate early ICP4 gene is an attractive target as it is among the first genes to be expressed in the lytic cascade, and its expression is absolutely essential for HSV-1 replication and for the expression of subsequent viral genes.

We previously reported that the naturally occurring plant lignan, 3′-*O*-methyl-NDGA (Mal.4; Fig. 1A), and a synthetic derivative, tetra-*O*-methyl-NDGA (M_4N; Fig. 1B), both inhibit HIV replication in a dose-dependent manner, and prevent the binding of the host transcription factor, Sp1, to cognate binding sites on the HIV LTR promoter. Subsequently, we reported that M_4N prevents replication of HSV-1, also an Sp1-regulated virus, in a dose-dependent manner. Inhibition by M_4N of transcription from the HSV-1 ICP4 gene promoter and interference by M_4N of Sp1 protein binding to cognate sites on the HSV-1 ICP4 gene promoter was also demonstrated. Importantly, by utilizing an inhibitory mode of action distinct from that of ACV, we demonstrated that M_4N is completely effective against mutant ACV-resistant HSV-1. Moreover, by targeting a host cellular factor, M_4N exhibited complete mutation-insensitivity following 10 viral passages, whereas, ACV, tested concurrently, rapidly led to the generation of ACV-resistant forms of HSV-1 (Chen et al., 1998).

Mal.4 and M_4N, however, are highly hydrophobic molecules that are insoluble in physiologic solutions and are impractical as systemic therapeutic agents. Therefore, a series of chemically related hydrophilic compounds were synthesized and screened for antiviral activity against HSV-1. One of these compounds, tetra-*O*-glycyl-NDGA (G_4N; Fig. 1C), which has four terminal glycyl groups instead of the four methyl groups of M_4N, is water soluble and demonstrated strong antiviral activity in our preliminary studies (Huang et al., 2002). In this report, we demonstrate that G_4N inhibits HSV-1 replication in Vero cells by a mechanism similar to Mal.4 and M_4N and can be effectively used to prevent the reactivation of HSV-1 in explanted, latently-infected mouse trigeminal ganglia (TG). These results indicate that G_4N has the potential to be an effective therapeutic agent for the systemic treatment of recurrent herpes simplex virus.

2. Materials and methods

2.1. Cells and viruses

Vero cells (African green monkey kidney cells) and CV-1 cells (African green monkey kidney cells) were purchased from the American Type Culture Collection, Rockville, MD and were maintained in Minimal Essential Media (Gibco) supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. KOS and McKrae strains of HSV-1 were obtained from Dr. N. Fraser (University of Pennsylvania). ACV-resistant HSV-1 (HSV-1^R) strains, F59878 and T28387, were obtained from Dr. K. Thompson (University of Chicago). All HSV-1 strains and stocks were propagated in Vero cells. Reactivation of KOS HSV-1 from latently infected murine TGs were assayed on CV-1 cell monolayers.

2.2. Viral yield reduction assay

Vero cells grown to confluency in 24-well plates were infected for 1 h with HSV-1 at a multiplicity of infection (m.o.i.) of 0.01 in the absence of drug. At 1 h post-infection (p.i.), the viral inoculum was removed, the cells were washed thoroughly with phosphate buffered saline (PBS), and medium containing G_4N or ACV (Sigma) at different concentrations was added. Fresh G_4N stock must be prepared for each experimental testing. There were duplicate wells for each concentration of drug tested. After 30 h of incubation in a humidified 37 °C, 5% CO_2 incubator, the cells were sonicated and the lysate, cleared by brief centrifugation, was collected and frozen at −80 °C. Frozen samples were then thawed, diluted, and virus yield was determined by plaque formation assay on Vero cells. Cells grown to confluency in 24-well plates were exposed to dilutions of lysate (210 μl per well) for 1 h, after which time the viral inoculum was removed and replaced with media containing

0.5% methyl cellulose (1.2 ml per well). After 3 days of incubation at 37 °C, the methyl cellulose media was removed and the cells were fixed and stained for 20 min with 0.06% formalin, 1% crystal violet and 0.64% NaCl. The stained cells were rinsed gently with tap water and air dried. Titers of virus yield were then determined by the enumeration of plaques. The IC_{50} was determined as the drug concentration required to reduce plaque formation by 50%.

2.3. MTT cytotoxicity assay

Vero cells grown to confluency or to 50% confluency in 24-well plates were exposed to media containing different concentrations of G_4N in a volume of 1.5 ml per well. The G_4N concentrations were the same ones used in the viral yield reduction experiments and duplicate wells of each concentration were tested. After a 30 h incubation, the media was replaced with 0.5 ml per well MTT solution PBS containing 5% fetal bovine serum, penicillin, streptomycin and 0.5 mg/ml MTT (Sigma). After a 2-h incubation, the MTT solution was replaced with 1 ml of DMSO. The cells were rocked gently for 20 min to allow solubilization of the incorporated formazan crystals by the DMSO. The OD_{570} value of each DMSO sample was measured and the relative number of viable cells was determined after correction of the values for absorbance due to turbidity from cell debris (OD_{690}). The TC_{50} was calculated as the drug concentration which reduced cell viability by 50%. The viability of murine TG was assayed in a similar manner.

2.4. RT-PCR

Total RNA was isolated from HSV-1 infected Vero cells (MOI of 1) by the method of Chomczynski and Sacchi (1987). HSV-1 ICP4 mRNA levels were measured semi-quantitatively by RT-PCR analysis using oligonucleotide primers specific for a portion of the ICP4 coding region (nucleotides +1113 to +1411 from the start site of transcription). The upstream primer was 5′-GCAGTACGCCCTGATCAC-3′ and the downstream primer was 5′-CAGGCTGGTCAGCAGGAA-3′. In brief, RNA samples were treated with RNAsin (Promega) and RNAse-free DNAse to remove any contaminating genomic DNA. First strand synthesis was primed with the ICP4-specific downstream primer and performed using MMLV RT enzyme and PCR_X Enhancer (Gibco) at 42 °C for 45 min. Amplification of the first strand synthesis products was then carried out by PCR using Taq DNA polymerase and PCR_X Enhancer under the following thermocycler conditions: 94 °C for 3 min, 30 cycles of 94 °C for 55 s, 60 °C for 55 s, 72 °C for 1 min and a final 10 min 72 °C extension. The PCR products were visualized by electrophoresis on a 1.8% agarose gel and ethidium bromide staining. The bands were photographed, scanned and band intensities were quantified using IMAGEQUANT software. RT-minus negative controls which received Milli-Q H_2O in place of MMLV RT enzyme were performed for all samples and showed there was no DNA contamination (Park, JHU, Ph.D. Thesis, 2002). RNA was isolated at the time before much viral DNA replication had occurred and, thus, the viral genome copy number was still relatively low (approximately MOI of 1), therefore, standard DNAse treatment (2 U per 38 μl reaction) was sufficient to avoid contamination with viral DNA.

2.5. Gel mobility shift assay

The DNA template used for the gel mobility shift assay was a 36 bp DNA fragment spanning nucleotides −101 to −66 from the start site of transcription of the HSV-1 ICP4 promoter, which contains the Sp1 protein binding sites most important for ICP4 gene transcription. The template was formed by annealing the single stranded complementary oligonucleotides: 5′-GTTCGGGGCGGGCCCGCCTGGGGGGCGGGGGGCCGG-3′ and 5′-CCGGCCCCCCGCCCCCCAGG and labeling with ^{32}P-dATP using the Klenow fragment of DNA Polymerase I (Promega). The labeled DNA (5 ng) was incubated for 30 min at room temperature with various concentrations of G_4N in reaction buffer containing 10 mM Tris (pH 7.5), 0.7 mM HEPES (pH 7.7), 30 mM KCl, 1 mM EDTA, 0.8 mM $MgCl_2$, 0.6 μM $ZnSO_4$, 10% glycerol, 5 mM DTT and 0.5 mg/ml BSA. Recombinant Sp1 protein (Sjottem et al., 1997) was then added and the reaction mixture was incubated at room temperature for an additional 30 min. Reactions were electrophoresed on a 5% polyacrylamide gel in 0.25× TBE buffer containing 2.5% glycerol. The dried gel was then exposed to X-ray film for autoradiography and ^{32}P emissions were quantified by phosphorimager analysis using a Packard Instant Imager.

2.6. Mouse trigeminal ganglia system of HSV latency and reactivation

Mice were anesthetized by an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and both corneas were lightly scarified using a 26 gauge needle. A 5 μl aliquot of KOS HSV-1 containing 1.0×10^6 pfu was then placed on each eye and the eye was gently massaged. After >28 days p.i., mice were euthanized and the TG were rapidly and aseptically explanted into growth media and incubated in a humidified 37 °C, 5% CO_2 incubator. Various concentrations of freshly made G_4N in PBS (or a PBS control) were administered to the TGs every 48 h for a period of 8 h up to either 5 or 14 days post-explantation. Prior to each 8-h G_4N treatment, a sample of culture media was transferred to CV-1 cell monolayers to assay for the presence of reactivated infectious HSV-1 virions. At the end of G_4N treatment, an MTT cytotoxicity assay was performed to ensure that all TGs remained viable. After MTT analysis, PCR using HSV-1 specific primers was performed to ensure all TGs had been latently infected with HSV-1.

3. Results

3.1. G_4N inhibits replication of wild type and ACV-resistant HSV-1 in Vero cells

Results from our previous studies showed that M_4N inhibits the growth of HSV-1 (Sm44 strain) in Vero cell cultures in a dose-dependent fashion with an IC_{50} of 11.7 μM. This was shown in comparison with ACV which had an IC_{50} of 7.54 μM. It was also demonstrated that because of its novel mode of inhibition, M_4N exhibits no viral cross-resistance against ACV-resistant strains of HSV-1 (HSV-1^R; Chen et al., 1998). In this study, G_4N was evaluated in comparison with ACV for its inhibitory activity against four strains of HSV-1: two wild-type strains (KOS, a laboratory strain and McKrae, a virulent clinical isolate), and two HSV-1^R mutant strains. The viral yield reduction assay results are shown in Fig. 2. G_4N inhibited wild-type HSV-1 production in a dose-dependent manner with IC_{50} values of 3.6 and 4.5 μM, and IC_{90} values of 15.7 and 34.3 μM for KOS and McKrae strains of HSV-1, respectively. ACV inhibited both wild type strains in a similar dose-dependent manner with IC_{50} values of 5.4 and 3.9 μM. Thus, G_4N exhibits very similar antiviral potency against wild-type HSV-1 strains as ACV, and it appears to be slightly more effective than M_4N, although this may be due to a difference in viral strain.

Cross-resistance is observed among different drugs targeting like viral mechanisms. Since G_4N is hypothesized to function as M_4N and target a mechanism distinct from ACV, it was expected that there would be no cross-resistance of HSV-1^R strains against G_4N. Cross-resistance against G_4N was tested using the two HSV-1^R strains, F59878 and T28387, under assay conditions and drug concentrations identical to those used for the wild-type viruses. The results (Fig. 3) show that the IC_{50} values for ACV were 20–40-fold higher against the HSV-1^R strains than against wild-type strains. G_4N, however, retained complete effectiveness against both HSV-1^R strains with IC_{50} values of 2.1 and 4.3 μM. Thus, there is no cross-resistance of HSV-1^R strains against G_4N.

3.2. Cytotoxicity of G_4N towards Vero cells

The cytotoxicity of a prospective antiviral drug must be investigated in order to gauge its safety, and to investigate whether the observed antiviral effect is an indirect result of a deleterious effect upon the host cell. This is of particular concern for G_4N which targets a host cellular factor.

The cytotoxicity of G_4N toward Vero cells was determined by MTT analysis using the same drug concentrations and incubations times as the viral yield reduction experiments. Since G_4N may exhibit different toxicities against dividing and non-dividing cells, its toxicity was tested for both 100% confluent non-dividing cells and 50% confluent actively dividing cells. After 30 h of incubation, no toxicity was seen up to 30 μM (Fig. 4). At 50 μM of G_4N, 70.1% of non-dividing Vero cells, and 40.8% of actively dividing Vero cells remained viable. At 100 μM, 27.0% of non-dividing cells, and 7.3% of dividing Vero cells remained viable. This gives a TC_{50} value for G_4N towards Vero cells of 73.2 μM for non-dividing cells, and 46.9 μM for dividing cells. The IC_{50} value for G_4N of 3.6–4.5 μM strongly indicates that the G_4N-induced inhibition of HSV-1 does not result from

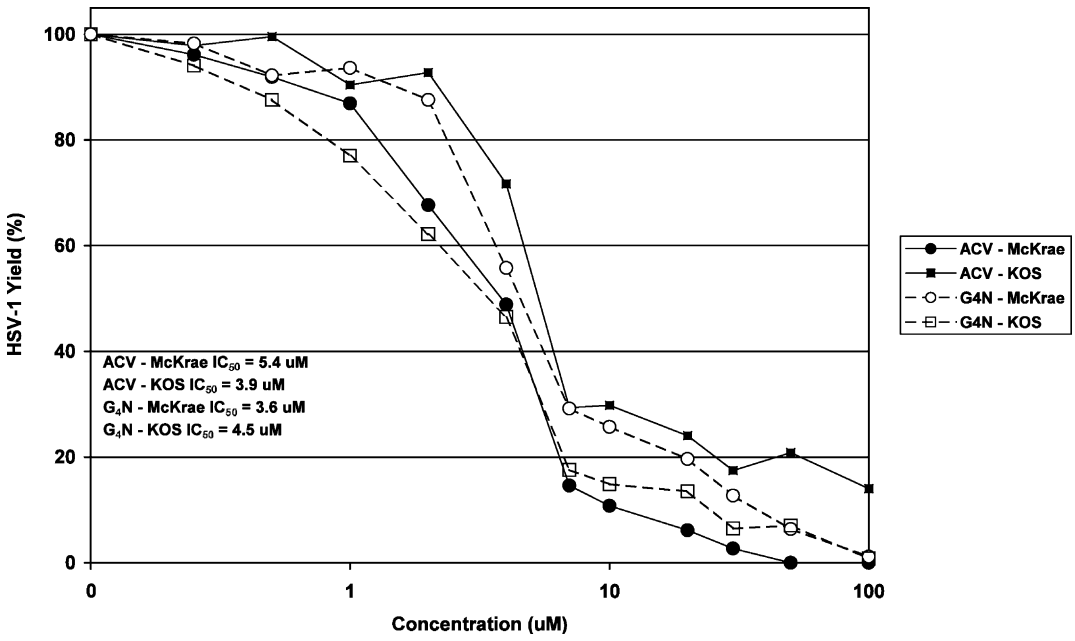

Fig. 2. Inhibition of wild-type HSV-1 by G_4N and ACV. The effect of G_4N and ACV on the replication of two wild-type strains of HSV-1 in Vero cells was determined by viral yield reduction assay. KOS, a laboratory HSV-1 strain; McKrae, a virulent clinical isolate of HSV-1. Each concentration was done in duplicate. The IC_{50} was determined as the drug concentration required to reduce plaque formation by 50%.

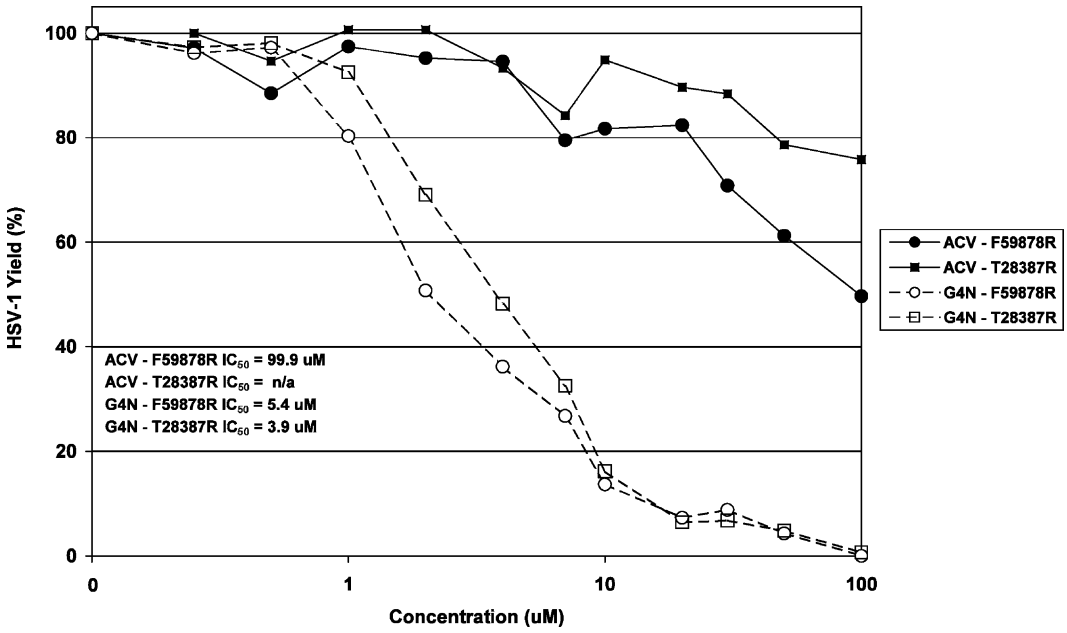

Fig. 3. Inhibition of ACV-resistant strains of HSV-1 by G_4N and ACV. Using the same conditions as used in Fig. 2, the reduction of viral yield by G_4N and ACV was tested using the two HSV-1^R strains, F59878 and T28387.

host cell injury, since the cells remain completely viable at this concentration.

3.3. Inhibition of HSV-1 ICP4 gene transcription by G_4N during lytic infection

Earlier reporter gene experiments showed that M_4N inhibits transcription from the HSV-1 ICP4 gene promoter, presumably by interfering with required host Sp1 transcription factor binding to the ICP4 promoter region.

To examine whether G_4N prevents HSV-1 ICP4 gene transcription during a lytic infection, ICP4 mRNA levels in HSV-1 infected, G_4N-treated Vero cells were measured by RT-PCR. Confluent Vero cells were infected with HSV-1 at an m.o.i. of 1 and treated with varying concentrations of G_4N. At 3 h p.i., when ICP4 gene transcription is at a maximum, RNA was isolated and analyzed by RT-PCR using HSV-1 ICP4-specific primers. The PCR product of 298 bp was resolved on an agarose gel, stained with ethidium bromide and photographed (Fig. 5A). RT-minus negative

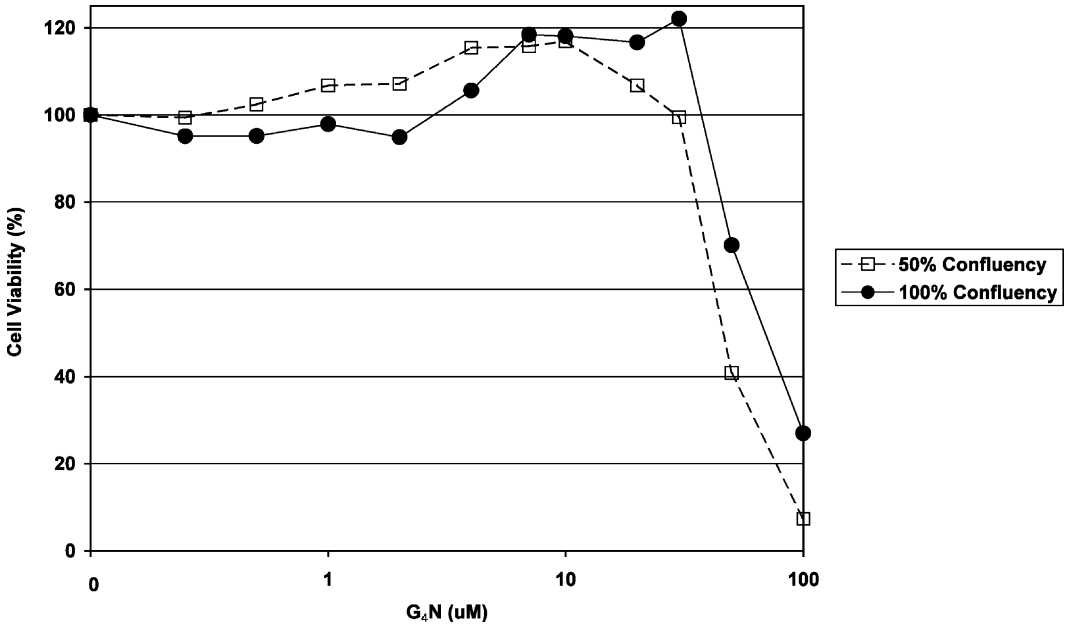

Fig. 4. Cytotoxicity of G_4N towards Vero cells. Cell viability of actively dividing (50% confluent) and stationary (100% confluent) Vero cells incubated in G_4N-containing media was measured using MTT analysis. Viability was expressed relative to the no drug treatment control (100%).

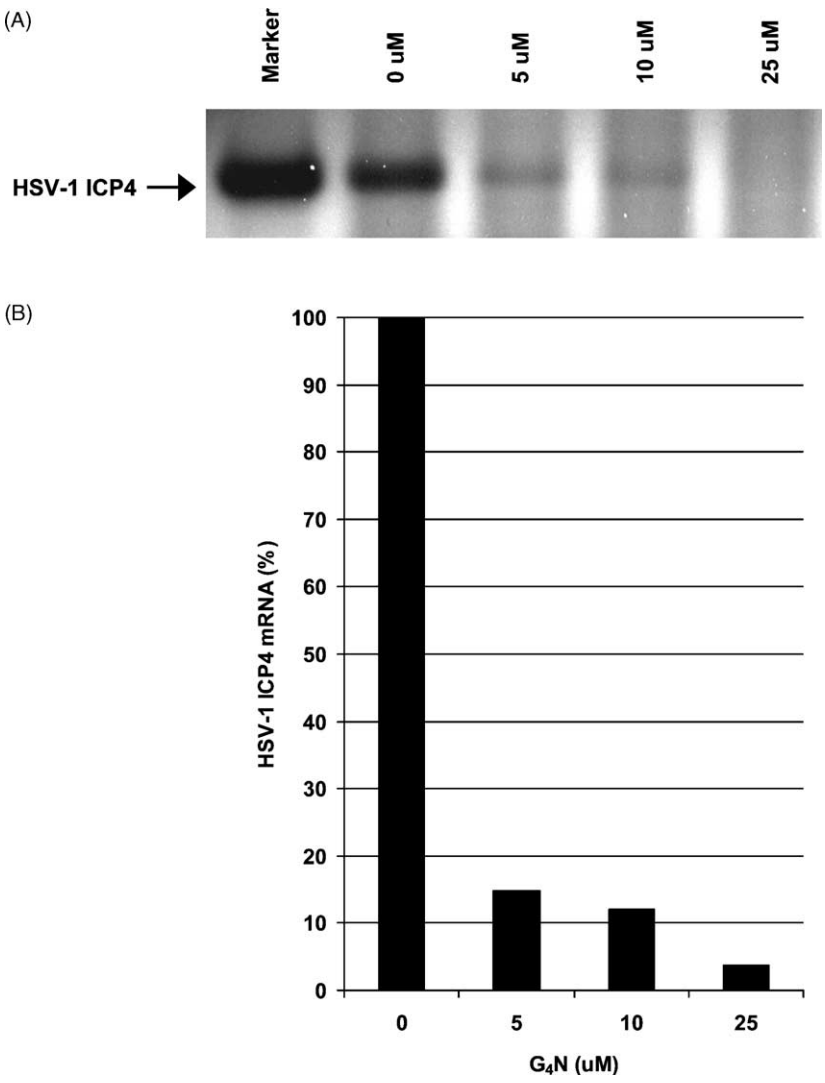

Fig. 5. RT-PCR analysis of G_4N inhibition of HSV-1 ICP4 gene transcription in vivo. Confluent Vero cells were infected with HSV-1 (MOI of 1) and treated with varying concentrations of G_4N. At 3 h p.i., RNA was isolated and ICP4 mRNA levels was detected by RT-PCR using ICP4 specific primers. DNA bands were resolved by agarose gel electrophoresis, stained with ethidium bromide, and quantified by phosphorimager analysis with background subtraction. (A) Reduction by G_4N of HSV-1 ICP4 mRNA expression, resolved on a 1.8% agarose minigel. (B) HSV-1 ICP4 bands from (A) were quantitated using IMAGEQUANT software and represented graphically. The 0 μM band represents 100% expression.

controls showed there was no detectable DNA contamination (Park, 2002). Quantitation of the 298 bp band by densitometry followed by background subtraction (Fig. 5B) showed ICP4 mRNA levels were reduced to 15% of untreated controls at 5 μM G_4N. At 10 and 25 μM G_4N, ICP4 mRNA levels were further reduced to 12 and 4%, respectively, showing that G_4N causes a dose-dependent decrease in HSV-1 ICP4 gene transcription during a lytic infection.

3.4. G_4N inhibits binding of Sp1 protein to the HSV-1 ICP4 gene promoter in vitro

Previous experiments showed that the mode of viral inhibition by Mal.4 and M_4N was due to interference with the binding of the Sp1 transcription factor to its cognate binding sites on critical HIV and HSV viral gene promoters (Gnabre et al., 1995; Chen et al., 1998). We hypothesized that structurally similar G_4N inhibits HSV-1 ICP4 gene transcription via the same mechanism. The ability of G_4N to prevent Sp1 protein binding to the HSV-1 ICP4 promoter was examined here in vitro by electrophoretic mobility shift analysis (EMSA). A portion of the HSV-1 ICP4 promoter region containing the most proximal Sp1 protein binding sites, reported to be the most important for gene transcription (Jones and Tjian, 1985), was labeled and pre-incubated for 30 min with varying concentrations of G_4N. Recombinant Sp1 protein was then added to the reaction for an additional 30 min and the mixture was electrophoresed on a non-denaturing polyacrylamide gel (Fig. 6A). The relative intensities of the bands corresponding to Sp1 protein–DNA complexes were quantified by phosphorimager analysis, and are shown in Fig. 6B. The data shows that G_4N interferes

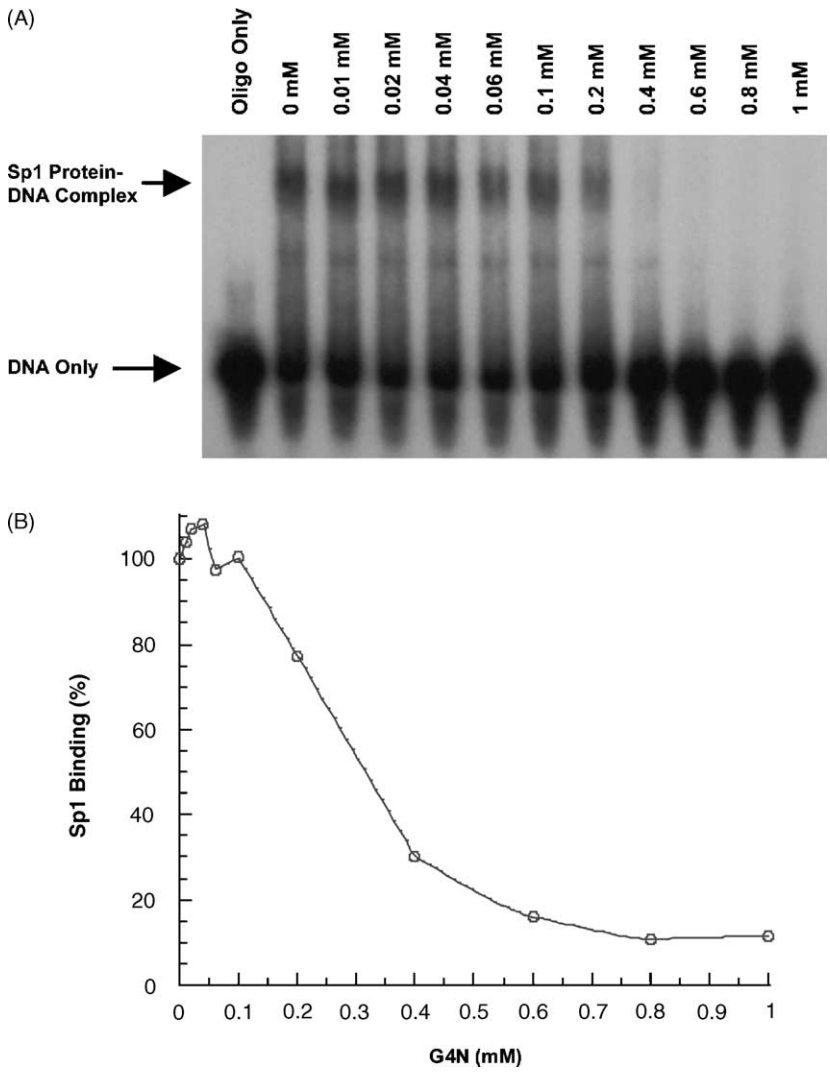

Fig. 6. EMSA of G_4N inhibition of Sp1 protein binding in vitro. (A) Autoradiogram of EMSA using an HSV-1 ICP4 template. The DNA template used spanned nucleotides −101 to −66 from the start site of transcription of the HSV-1 ICP4 promoter region and contains the Sp1 protein binding sites most important for ICP4 gene transcription. Arrows indicate positions of free and Sp1-bound DNA. (B) Graphical representation of phosphorimager data from HSV-1 ICP4 EMSA showing the percent Sp1 protein binding relative to the no drug treatment control (100%).

with Sp1 protein binding to DNA in a dose-dependent fashion. As G_4N concentration increases from 0 to 1 mM, the amount of Sp1 protein–DNA complex decreases, while the quantity of unbound DNA increases. Additional experiments in which the Sp1 protein was pre-incubated with G_4N followed by addition of the labeled oligo, or pre-incubated with the oligo followed by G_4N addition, seem to indicate that G_4N blocks Sp1 protein binding to DNA by associating with the DNA and not the protein (Huang et al., 2002).

3.5. G_4N suppresses HSV-1 reactivation from latency

The capacity for HSV-1 to establish permanent latency within the sensory neurons of the host and to periodically reactivate is a unique and clinically important aspect of HSV biology. The need for effective systemic treatment of recurrent herpes virus infection in at-risk patients prompted us to examine whether G_4N could be used to block the reactivation of HSV from latency. The mouse TG latency and explantation-induced reactivation model is a popular animal model for studying molecular and cellular aspects of HSV latency and reactivation, and was used in this study to assess the ability of G_4N to inhibit reactivation. In an initial experiment which proceeded for 5 days, TGs from 20 latently infected mice were induced to reactivate by explantion to media. Ten explanted TGs were treated with 40 μM G_4N, and ten were treated with PBS in an identical fashion. As shown in Table 1, three of the ten untreated control TGs had reactivated by Day 5 p.e., whereas, none of the ten G_4N-treated TGs reactivated. In a second experiment which was continued for 14 days p.e., eight explanted TGs received 40 μM G_4N, and eight received PBS. After 14 days, six of the eight untreated TGs had reactivated, whereas, only three of the eight G_4N-treated TGs had reactivated (Table 1).

Table 1
Effect of G_4N treatment upon reactivation of latent HSV-1

Experiment	0 μM		40 μM	
	+/Total	%	+/Total	%
1	3/10	30	0/10	0
2	6/8	75	3/8	37.5
Total	9/18	50	3/18	16.7

HSV-1 was induced to reactivate by explanting TG's from latently infected mice (<28 days p.i.) to media. Viral reactivation was detected on CV-1 cell monolayers. Drug treatment was every other day for 8 h. The *P*-value calculated by Fisher exact test is 0.07504 for the relationship between total treated vs. untreated ganglia. At the conclusion of the experiment, cell viability of all TG's was confirmed by MTT analysis, latent infection of all TG's was confirmed by PCR analysis using HSV-1 specific primers.

In both experiments combined, a total of nine out of 18, or 50%, of the explanted TGs showed HSV-1 reactivation from latency in the absence of G_4N treatment. However, with 40 μM G_4N treatment, only three out of 18, or 16.7% of the TGs showed HSV-1 reactivation. Treatment with 40 μM G_4N, thus, led to a 67% decrease in HSV-1 reactivation. The *P*-value of 0.07504 as calculated by Fisher exact test for the relationship between total treated versus untreated ganglia, indicates the data is promising yet is short of significance. As the results of the Fisher test varies considerably with sample size, and the sample size tested here is relatively small, further testing will be conducted to verify the *P*-value. At the conclusion of the TG explantation experiments, the relative viability of the TGs was tested using the MTT-based cytotoxicity assay to ensure that the observed inhibitory effect of G_4N was not the result of cell injury. All of the TGs remained completely viable and there was no difference in viability between the G_4N-treated and untreated groups (data not shown). In addition, PCR analysis using HSV-1-specific primers confirmed that all of the TGs had been successfully infected with HSV-1 and contained latent HSV-1 (data not shown).

4. Discussion

In previous studies we introduced and demonstrated the potent antiviral activity and mutation-insensitivity of Mal.4 and M_4N (Gnabre et al., 1995; Chen et al., 1998). With this report we have furthered our studies of the antiviral effects of NDGA derivatives to include a water soluble compound, G_4N, that effectively inhibits replication of wild-type and ACV-resistant HSV-1, and suppresses HSV-1 reactivation from latently infected TG.

The molecular mode of action underlying the antiviral activity of Mal.4 and M_4N is the prevention of host Sp1 protein binding to cognate sites on critical viral promoters, thus, leading to transcriptional inhibition. Several lines of evidence strongly indicate that G_4N functions via the same mechanism. G_4N inhibited HSV-1 replication with an IC_{50} value similar to Mal.4 and M_4N. In gel mobility shift experiments G_4N inhibited Sp1 protein binding to the HSV-1 ICP4 promoter in vitro in a dose-dependent fashion. RT-PCR analysis demonstrated dose-dependent G_4N inhibition of ICP4 gene transcription in virally infected cells. In addition to these experimental results, similar selective indices, a similar chemical structure, and the absence of cross-resistance in ACV-resistant ($HSV\text{-}1^R$) strains among all three compounds are further evidence of a shared mechanism.

In our current model, transcriptional inhibition of viral genes occurs as molecules of drug associate with particular sequences of DNA, thereby, interfering with the binding of Sp1 protein. Using reporter constructs transfected into mammalian cells, this sequence-specific interference has been demonstrated as Mal.4 prevented transcription from gene promoters containing the consensus Sp1 binding sites, GGGCGG, whereas, promoters devoid of Sp1 sites were relatively unaffected (Gnabre et al., 1995). Gel mobility shift experiments in which M_4N was pre-incubated with either Sp1 protein or with labeled DNA indicate that M_4N associates with the DNA rather than the protein (Huang et al., 2002). Specificity of G_4N towards Sp1 binding is demonstrated in vitro in the gel mobility shift experiments, and correlates with the reduction by G_4N of ICP4 gene expression seen in RT-PCR analysis. However, maximal HSV-1 ICP4 expression also requires binding of the VP16–Oct1–HCF complex (Cleary and Herr, 1995) to the TAATGARAT (R = purine) sequences located within the ICP4 promoter. Whether G_4N will also associate with AT-rich sequences, and whether such a G_4N–DNA complex will prevent VP16 or Oct1 activity is not yet known. As we have shown that 3-*O*-methyl-NDGA (Mal.4) does not affect transcription factor binding to NF-κB binding sites, nor does it appreciably affect transcription factor binding to USF binding sites (Gnabre et al., 1995), if glycylated NDGA functions similarly to methylated NDGA, it should also not indiscriminately prevent transcription factor binding. In addition, it is equally uncertain whether prevention of VP16 binding would contribute significantly to transcriptional inhibition, since deletion mutants have shown that VP16 binding is not essential to viral gene expression (Spector et al., 1991).

The average IC_{50} of 4.1 μM obtained from the viral yield reduction experiments correlates well with the RT-PCR analyses of G_4N inhibition of ICP4 transcription in infected Vero cells. However, the concentrations of G_4N required to prevent Sp1 binding to the ICP4 promoter in the gel mobility shift experiments appears inconsistent with the viral yield reduction experiments. This may be because the in vitro conditions of the gel mobility shift reactions almost certainly differs greatly from the environment within the nuclei of infected Vero cells. It is very unlikely that G_4N would function equivalently in the two environments. A second reason may be that the viral yield reduction data reflects G_4N's sum effects on all the viral genes. Sp1 binding sites are found in the promoters of many immediate-early and early HSV

genes, any or all of which may be affected by G_4N to varying degrees, and lead to viral inhibition. The gel mobility shift data shows G_4N's effects on only two of the eight ICP4 Sp1 binding sites. It is, therefore, likely that lower concentrations of G_4N would suffice to inhibit viral replication, than what is required to inhibit Sp1 binding at particular ICP4 Sp1 binding sites.

Since the G_4N inhibitory mechanism is completely distinct from the antiviral mechanism of nucleoside analogue agents, there is little possibility of cross-resistance between these two classes of drugs. We have shown in previous studies that mutant HSV-1^R strains have no resistance against M_4N or Mal.4, and the studies presented here demonstrate G_4N is as equally potent against HSV-1^R as it is against wild-type HSV-1. In addition to being effective as a sole treatment against mutant HSV-1^R, G_4N's independent mode of action also raises the possibility of combining it with nucleoside analogue treatments in an antiviral cocktail which targets multiple points in HSV-1 replication.

Another corollary to the mode of action of Mal.4, M_4N and G_4N is mutation-insensitivity. The capacity of harmful viral and bacterial strains to mutate in response to chemotherapy presents a rapidly increasing problem in new drug design and the implementation of therapeutic strategies. Viral inhibitors which target viral factors have the advantages of high specificity and low cytoxicity. However, in the clinical setting, prolonged use of these drugs places selective pressure upon the viral population to generate harmful resistant strains containing mutant proteins against which the drug is ineffective. Mutant ACV-resistant strains of HSV-1 have been commonly reported to contain altered TK and DNA polymerase enzymes (Hirsch et al., 1996), and repeated use of AZT and even the most effective reverse transcriptase (RT) and protease inhibitors leads to resistant HIV strains containing mutations found within HIV RT and protease enzymes (Richman, 2001). In contrast, host factors, unlike viral factors, are under no selective pressure to mutate and are in general structurally invariable. Thus, inhibitors like G_4N which target host factors have the advantages of being relatively mutation-resistant, are potentially effective against a spectrum of related or unrelated viruses, and most significantly will be less prone to generate drug-resistant viral strains. This should be qualified, however, by the unlikelihood of any single drug, including G_4N, displaying complete mutation-resistance over prolonged clinical treatment. It is conceivable that resistance to G_4N may be conferred by mutations to the Sp1 binding site which prevent G_4N association but retain Sp1 binding, or by the use of alternative transcription factors for viral gene transcription.

The Sp1 protein is an abundant and ubiquitous transcription factor that regulates the basal expression of many cellular genes in addition to playing critical roles in cell growth and development. Therefore, inhibition of its binding to DNA by G_4N might be expected to have a profound deleterious effect on the viability of host cells. However, the data presented here for G_4N and our previous studies with Mal.4 and M_4N show, quite to the contrary, that these compounds are relatively non-toxic to cells in culture. Experiments also indicate that G_4N and M_4N are likely to be less cytotoxic to primary, non-dividing cells than to proliferating cells in culture. In fact, we have previously shown that M_4N can selectively eliminate C3 cell-induced tumors in mice while sparing the surrounding normal stationary cells (Heller et al., 2001), and 8 h treatment of explanted mouse TG with G_4N showed no cytotoxicity up to 120 μM. We hypothesize that stationary cells whose Sp1-regulated cell cycle genes are inactive and require less transcription factor activity would be less sensitive to the inhibitory activities of M_4N and G_4N, and would, thus, suffer less toxic effects than proliferating cells. Of particular note with regard to toxicity, are the findings that subdermal injection of M_4N showed no toxicity in mice (Heller et al., 2001), and topical application of G_4N to dorsal regions of guinea pigs twice daily at concentrations up to 150 mM also showed no toxicity (Park, 2002). The low absorption of M_4N by cells may contribute to the low toxicity seen in animals including mice, rats, rabbits and dogs (Heller, 2002). Similar experiments have been done for G_4N in guinea pigs, and examinations of animal tissues of following local administration of G_4N in vivo showed no histological differences from non-treated tissues (Park, 2002) and will be reported separately.

The requirement of Sp1 in endogenous gene expression in eukaryotic cells has not been extensively studied. Sp1 has been associated with the cell cycle, chromatin remodeling, and the maintenance of methylation-free CpG islands. It has been shown that many chromosomal genes previously shown to be Sp1-dependent in gene transfection experiments, including several cell cycle genes, remain active in the absence of Sp1. While Sp1 protein is necessary for early embryonic development, it is not required for cell growth and differentiation (Marin et al., 1997). Although a high rate of proviral transcription is essential for viral replication in differentiated cells, there may be no such great demand for such large output of cellular transcripts in quiescent host cells. In contrast, rapidly dividing cells do require elevated levels of cell cycle protein in order to carry out cell division in a time-dependent manner. We have found treatment of C3 cells with 40 μM M_4N for 72 h caused a marked decrease in CDC2 protein and mRNA levels, which requires Sp1 binding. The same treatment, however, did not affect levels of cyclin B protein, whose promoter does not require Sp1 protein binding, nor did it affect GAPDH transcription (Heller, 2002).

Even in proliferating Vero cells, G_4N exhibits no cytotoxicity within the therapeutic range of 0–30 μM, with cytotoxic effects being evident only at concentrations above this range. A TC_{50} value of 73.2 μM combined with an IC_{50} value of 4.5 μM yields a selective index (TC_{50}/IC_{50}) for G_4N in Vero cells of 16.3, which is similar to the selective index of 13.7 reported previously for M_4N. Contingent upon activity seen in infected laboratory animals, it may be speculated that the relatively low selective index for G_4N indicates that when possible it should be used therapeutically at

its lowest effective concentration to avoid toxicity. Due to this, G_4N may be most useful as a routine complement to ACV in controlling and precluding the exponential growth of ACV-resistant (HSV-1^R) strains of HSV. Since HSV-1^R strains remain sensitive to G_4N, the small initial population of HSV-1^R mutants generated during early ACV treatment may be kept under control by supplementing with low concentrations of G_4N. If so, supplementing ACV with small amounts of G_4N may result in a continually low IC_{50} value for ACV, thus, reducing the dosage, the toxicity, and the cost of treatment.

Whereas, the insolubility of Mal.4 and M_4N restricts their antiviral use to very localized applications such as the topical treatment of herpes labialis, G_4N's high water-solubility extends the advantages of this class of compounds to the systemic treatment of recurrent HSV-1 infection. The precise molecular mechanisms of HSV reactivation from latency are not well understood. Perhaps the best inhibitors of HSV reactivation would act upon the signal transduction pathway leading to HSV reactivation. Given the little which is known about signaling mechanisms involved in HSV-1 reactivation, however, it may be difficult at this point to demonstrate that G_4N inhibition of a cellular promoter associated with HSV-1 reactivation contributes to the inhibition we observed. However, since G_4N can block the initiation of transcription of even the earliest viral genes, it may be particularly effective at preventing HSV reactivation, since it would preclude the initiation of the HSV lytic gene expression cascade. In contrast, nucleoside analogue agents are active only at the point of viral DNA replication when HSV has already reactivated and the lytic gene transcription cascade has progressed beyond immediate early gene and early gene expression.

Indeed, animal studies of the impact of nucleoside analogue agents upon HSV latency and reactivation have focused upon reducing the number of latently infected neurons or reducing the viral copy number per cell during the establishment of latency, with the implication that these reductions would in turn reduce the frequency and severity of future HSV reactivation (Efstathiou et al., 1999). Such studies have focused mainly on valaciclovir and famciclovir (oral pro-drugs of ACV and penciclovir, respectively) and have shown some success in reducing the establishment of HSV latency in mice when administered early and at high dosage. Several clinical trials have shown oral ACV to be highly effective in the suppression of HSV recurrence even during prolonged treatment over several years (Kaplowitz et al., 1991). ACV effectively interrupts the further progress of viral DNA replication, thereby, suppressing a recurrence as well as an acute infection; however, it does not act upon the reactivation mechanism. The mechanisms causing HSV reactivation are yet unclear, however, are likely to directly impinge upon *a* gene expression, which G_4N inhibits. The results above show that G_4N directly and significantly reduces the frequency of HSV reactivation from explanted latently infected mouse TG. Therefore, as a systemic agent G_4N may be useful in more urgent clinical cases, such as those in which the patients are severely immunocompromised, where it is necessary to prevent the initiation of any HSV reactivation rather than reduce its severity. Experiments demonstrating G_4N's ability to prevent in vivo HSV reactivation in latently infected animals are now being prepared.

As with the issue of ACV-resistance, G_4N has the potential to be most useful therapeutically as a complement to nucleoside analogues in the systemic treatment of HSV reactivation. It is a common practice to administer systemic and prophylactic anti-HSV treatment to HSV-seropositive patients in order to minimize the frequent HSV-1 reactivation resulting from tissue damaging procedures such as excimer laser keratectomy for the correction of corneal refractive errors (Gilbert, 2001), cytoreductive chemotherapy in malignant cancer treatment (Khan and Wingard, 2001), or facial restructuring of the face and neck (Asbell, 2000). Guinea pig experiments have been performed which show M_4N and G_4N prevent HSV-1 induced cutaneous lesions and viral shedding when applied as a topical agent in vivo (Park, 2002). Supplementing current systemic nucleoside analogue regimens with G_4N may improve their efficacy by providing a two-pronged treatment of HSV reactivation, with G_4N reducing the frequency of reactivation, and both nucleoside analogue reagents and G_4N reducing the severity of reactivation.

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References

Asbell, P.A., 2000. Valacyclovir for the prevention of recurrent herpes simplex virus eye disease after excimer laser photokeratectomy. Trans. Am. Ophthalmol. Soc. 98, 285–303.

Chen, H., Teng, L., Li, J., Park, R., Mold, D.E., Gnabre, J., Hwu, J.R., Tseng, W.N., Huang, R.C., 1998. Antiviral activities of methylated noridhydroguaiaretic acids. Part 2. Targeting herpes simplex virus replication by the mutation insensitive transcription inhibitor tetra-*O*-methyl-NDGA. J. Med. Chem. 41, 3001–3007.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guianidium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

Cleary, M.A., Herr, W., 1995. Mechanisms and flexibility in DNA sequence recognition and VP16-induced complex formation by the Oct-1 POU domain. Mol. Cell. Biol. 14, 2090–2100.

Crumpacker, C., 2001. Antiviral agents. In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B. et al. (Eds.),

Fields Virology, 4th edition. Lippincott Williams and Wilkins, Philadelphia, pp. 393–433.

Crumpacker, C.S., Schnipper, L.E., Marlow, S.I., Kowalsky, P.N., Hershey, B.J., Levin, M.J., 1982. Resistance to antiviral drugs of herpes simplex virus isolated from a patient treated with acyclovir. N. Engl. J. Med. 306, 343–346.

De Logu, A., Loy, G., Pellerano, M.L., Bonsignore, L., Schivo, M.L., 2000. Inactivation of HSV-1 and HSV-2 and prevention of cell-to-cell virus spread by *Santolina insularis* essential oil. Antiviral Res. 48, 177–185.

Efstathiou, S., Field, H.J., Griffiths, P.D., Kern, E.R., Sacks, S.L., Sawtell, N.M., Stanberry, L.R., 1999. Herpes simplex virus latency and nucleoside analogues. Antiviral Res. 41, 85–100.

Gilbert, S., 2001. Improving the outcome of facial resurfacing—prevention of herpes simplex virus type 1 reactivation. J. Antimicrob. Chemother. 47, 29–34.

Gnabre, J.N., Brady, J.N., Clanton, D.J., Ito, Y., Dittmer, J., Bates, R.B., Huang, R.C., 1995. Inhibition of human immuno deficiency virus type 1 transcription and replication by DNA sequence-selective plant lignans. Proc. Natl. Acad. Sci. 92, 11239–11243.

Heller, J.D., 2002. Discovery and mechanism of the anti-cancer chemotherapeutic tetra-*O*-methyl nordihydroguaiaretic acid. Ph.D. thesis, Johns Hopkins University, UMI Dissertations Publishing.

Heller, J.D., Kuo, J., Wu, T.C., Kast, M.W., Huang, R.C., 2001. Tetra-*O*-methyl nordihydroguiaretic acid induces G_2 arrest in mammalian cells and exhibits tumoricidal activity in vivo. Cancer Res. 61, 5499–5504.

Hirsch, M.S., Kaplan, J.C., D'Aquila, R.T., 1996. Antiviral agents. In: Fields, B.N., Knipe, D.M., Howley, P.M., Chanock, R.M., Melnick, J.C., Monath, T.P. et al. (Eds.), Fields Virology, 3rd edition. Raven Press, Philadelphia, pp. 431–466.

Huang, R.C., Li, Y., Giza, P.E., Gnabre, J.N., Abd-Elazem, I.S., King, K.Y., Hwu, J.R., 2002. Novel anti-viral agent tetraglycylated nordihydroguaiaretic acid hydrochloride as a host dependent viral inhibitor. Antiviral Res, in press.

Jones, K.A., Tjian, R., 1985. Sp1 binds to promoter sequences and activates herpes simplex virus 'immediate-early' gene transcription in vitro. Nature 317, 179–182.

Kaplowitz, L.G., Baker, D., Gelb, L., Blythe, J., et al., 1991. Prolonged continuous acyclovir treatment of normal adults with frequently recurring genital herpes simplex virus infection. J. Am. Med. Assoc. 265, 747–751.

Khan, S.A., Wingard, J.R., 2001. Infection and mucosal injury in cancer treatment. J. Natl. Cancer Inst. Monogr. 29, 31–36.

Marin, M., Karis, A., Visser, P., Grosveld, F., Philipsen, S., 1997. Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation. Cell 89, 619–628.

Park, R., 2002. Inhibition of the Herpes simplex virus type I by three NDGA derivatives: Mal.4, M_4N, and G_4N. Ph.D. thesis, Johns Hopkins University, UMI Dissertations Publishing.

Richman, D.D., 2001. HIV chemotherapy. Nature 410, 995–1001.

Roizman, B., Sears, A.E., 1996. Herpes simplex viruses and their replication. In: Fields, B.N., Knipe, D.M., Howley, P.M., Chanock, R.M., Melnick, J.C., Monath, T.P. et al. (Eds.), Fields Virology, 3rd edition. Raven Press, Philadelphia, pp. 2231–2295.

Sjottem, E., Andersen, C., Johansen, T., 1997. Structural and functional analyses of DNA bending induced by Sp1 family transcription factors. J. Mol. Biol. 267, 490–504.

Spector, D., Purves, F., Roizman, B., 1991. Role of alpha-transinducing factor (VP16) in the induction of alpha genes within the context of viral genomes. J. Virol. 65, 3504–3513.

Straten, M.V., Carrasco, D., Lee, P., Tyring, S.K., 2001. A review of antiviral therapy for herpes labialis. Arch. Dermatol. 137, 1232–1235.

Whitley, R.J., 1996. Herpes simplex viruses. In: Fields, B.N., Knipe, D.M., Howley, P.M., Chanock, R.M., Melnick, J.C., Monath, T.P. et al. (Eds.), Fields Virology, 3rd edition. Raven Press, Philadelphia.