Antiviral Activities of Methylated Nordihydroguaiaretic Acids. 2. Targeting Herpes Simplex Virus Replication by the Mutation Insensitive Transcription Inhibitor Tetra-*O*-methyl-NDGA

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We had previously reported that tetramethyl-*O*-NGDA (M_4N), a synthetic derivative of the naturally occurring nordihydroguaiaretic acid (NDGA), is able to inhibit HIV Tat transactivation by blocking host Sp1 protein at the Sp1 cognate binding site on the HIV LTR promoter. The present studies were undertaken to examine whether M_4N is able to inhibit the replication of herpes simplex virus (HSV), another Sp1-regulated virus. The results showed that in Vero cells, M_4N inhibits at micromolar levels ($IC_{50} = 43.5\ \mu M$) the expression of the herpes immediate early gene (α-ICP4), which is essential for HSV replication. An electrophoretic mobility shift assay, examining Sp1 binding to the α-ICP4 promoter, showed a significant inhibition of the control bands: 88% inhibition of the fast moving band (FMB) and 45% of the slow moving band (SMB), at 100 μM of drug concentration. Comparative studies between M_4N and acycloguanosine (acyclovir, ACV) in cultured Vero cells revealed an interesting pattern in the drug sensitivity (IC_{50}) and cytotoxicity (TC_{50}) parameters. For M_4N, the IC_{50} varied between 11.7 and 4 μM in 10 passages of HSV-1 and 4 passages of HSV-2 with no indication for a requirement of higher drug concentration. In contrast, for acyclovir, the IC_{50} increased from 7 μM in the first passage to 444 μM in the tenth passage of HSV-1, and $>88\ \mu$M for the fourth passage of HSV-2, indicating a rapid build-up of drug resistance against acyclovir. While the selective index (SI), defined as the ratio: TC_{50}/IC_{50}, remained relatively constant for M_4N; it dropped 60-fold for acyclovir in the endpoints of viral passages. Drug sensitivity for M_4N toward the acyclovir-sensitive strain (sm44) and the acyclovir-resistant strain (ACV-10) of HSV-1 was similar, indicating no cross-resistance between M_4N and acyclovir in their anti-HSV effects. These results may have an important clinical relevance since HSV has been shown to be a factor for spreading of HIV.

Introduction

High rates of mutation and rapid turnover of viral population are typical traits of fast growing viruses. These traits have somewhat hindered the effectiveness of using inhibitors of viral proteins as antiviral drugs. The anti-HIV compounds that are currently being used to treat AIDS patients include six reverse transcriptase inhibitors (zidovudine, didanosine, zalcitabine, stavadine, lamivudine and nevirpapine) and three protease inhibitors (saquinavir, ritonavir, and indinavir). A commonly used drug for herpes simplex viruses (HSV-1 and HSV-2) is acyclovir (ACV), a guanosine analogue. It has recently been observed that these drugs, while very potent suppressers of wild-type viruses, gradually lose their effectiveness with the appearance of a group of populated viral mutants.[1−3] Increasingly high drug dosages and long-term treatments are required to circumvent this problem in the clinical setting. Prolonged excessive treatment with these drugs, however, is detrimental to the patients, and treatments are generally very costly.

Most, if not all, viruses, including those replicatively active mutants, are host dependent. They require the participation of certain cellular factors for supporting viral growth. Host cellular factors, unlike viral proteins, are not under mutational pressure and are, in general, structurally invariable. Thus, compounds that block the usage of these cellular factors at different stages of the viral life cycle are likely to be good candidates as mutation insensitive antiviral drugs. Several studies using cellular factors as alternative targets for inhibition of HIV-1 have been explored.[4]

A plant lignan, 3-*O*-methylnordihydroguaiaretic acid (Mal.4), has been shown to suppress HIV replication in a dose dependent manner, in part, by inhibiting transcription from the HIV long terminal repeat (LTR) promoter. Electrophoretic mobility shift analysis has shown that Mal.4 prevents the binding of the eukaryotic transcription factor, Sp1, to its cognate binding sites on the HIV LTR promoter, thus indicating a likely mechanism for transcriptional inhibition.[5] In anticipation of the possible clinical use of lignans in the treatment of viral diseases, methods for the synthesis of preparative

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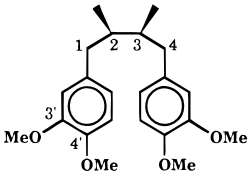

Figure 1. Molecular structure of M_4N, *meso*-1,4-bis(3,4-dimethoxyphenyl)-(2*R*,3*S*)-dimethylbutane tetra-*O*-methylnordihydroguaiaretic acid, tetra-*O*-methyl-NDGA [FW 358.2].

amounts of nine different methylated NDGA were established. The anti-HIV transactivation activities of these methylated NDGA isomers were compared with Mal.4 isolated from *Larrea tridentata*.[5−8] It was found that chemically synthesized 3-O-methyl-NDGA showed identical anti-HIV promoter activity as Mal.4 isolated from the plant. In addition, the fully methylated tetra-*O*-methyl-NDGA (M_4N) (Figure 1) is 3 times more active than the synthetic 3-*O*-methyl-NDGA in anti-HIV assays and can be synthesized with a high yield and low cost from the parent compound, NDGA.[8]

The present study was undertaken to examine whether the chemically synthesized M_4N is able to inhibit the replication of herpes simplex virus (HSV), another Sp1-regulated virus. The HSV ICP4 gene is among the first genes to be expressed in an HSV lytic infection, and its expression is essential for HSV replication and for the proper regulation of subsequent HSV gene transcription.[9] Its promoter region possesses eight Sp1 consensus binding sites, five of which are required for ICP4 gene expression,[10] thus making it a good candidate for M_4N inhibition. The ability of M_4N to prevent transcription from the HSV ICP4 gene promoter was tested in vivo by transfecting Vero cells with a plasmid containing the ICP4 gene promoter linked to a secreted reporter gene and then measuring the inhibitory effect of M_4N on transcription from the HSV ICP4 promoter. A potential mechanism by which M_4N prevents transcription from the ICP4 gene promoter was studied by electrophoretic mobility shift which involved the binding of Sp1 protein to the ICP4 gene promoter in the presence and absence of the drug. We report that M_4N prevents in vivo transcription from the ICP4 gene promoter in a dose-dependent fashion and also inhibits the binding of Sp1 to the two proximal-most Sp1 binding sites on the ICP4 promoter, indicating a likely mechanism for the transcriptional inhibition. We further show that M_4N is a mutation insensitive inhibitor of HSV replication in Vero cell cultures. While HSV builds up resistance against acyclovir quickly in Vero cells, M_4N is able to suppress the growth of HSV-1 and HSV-2 with a consistently low drug concentration following many viral passages without showing drug resistance.

Results

Inhibition of HSV ICP4 Gene Promoter Activity by Tetra-*O*-methyl-NDGA. The activity of the HSV ICP4 gene promoter was examined in the SEAP assay by measuring levels of secreted alkaline phosphatase (SEAP) produced from Vero cells transfected with pHSV1a$_4$ SEAP and equimolar ratios of the plasmids pGH290,[11] and pET.GST.1.POU.oir$^+$, encoding for the VP16 protein and the POU domain of Oct-1 protein,[12] respectively, to maximize the in vivo HSV ICP4 transcriptional activity. The inhibitor, M_4N, showed a

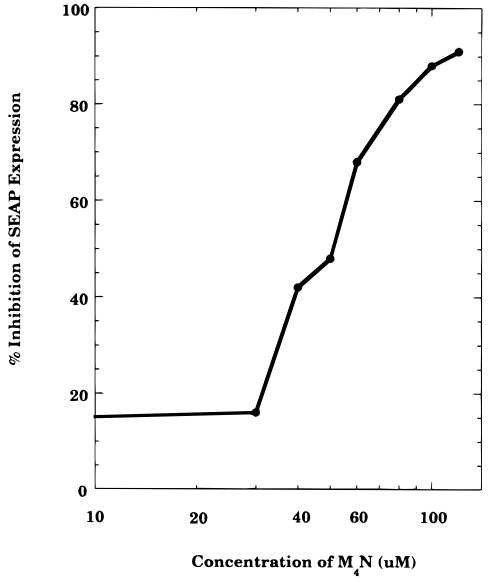

Figure 2. (A) Inhibition of HSV-1 ICP4 promoter activity by M_4N in tissue culture. Vero cells were cultured and transfected with pHSV1a$_4$SEAP expression plasmids as described in the Experimental Section. SEAP activity was analyzed as previously described.[6] Data points represent the percent inhibition of secreted alkaline phosphatase (SEAP) activity by M_4N from proportional numbers of Vero cells transfected with the pHSV1a$_4$SEAP expression plasmid. The IC_{50} of M_4N was 43.5 μM. Vero cells were cultured, transfected, and incubated with different concentrations of M_4N as described in the Experimental Section. Proportional cell numbers were determiend by SRB analysis.

dose−response inhibition of this HSV ICP4 promoter activity. The effect of the compound started at 30 μM with 16% inhibition and reached a maximum activity (91% inhibition) at 120 μM (Figure 2). The IC_{50} of M_4N was 43.5 μM. The naturally occurring 3-*O*-methyl-NDGA (Mal.4) had a similar dose−response activity (data not shown). The intrinsic cytotoxic effect of M_4N was assessed on the same Vero cell cultures (after the SEAP assay) by the sulforhodamine B assay of cell viability.[13] Any reduction in SEAP expression due to cell death was corrected by calculating the SEAP activity per transfected cell. Within the effective concentration range of the compound ($IC_{50} = 43.5\ \mu$M), the percent cell viability was >90% (data not shown).

Inhibition of Sp1 Binding by Tetra-*O*-methyl-NDGA. The mechanism of the inhibition observed in the tissue culture experiments was further investigated by examining the effects of M_4N on Sp1 protein binding to the HSV ICP4 promoter using an electrophoretic mobility shift assay (EMSA).[14] The oligonucleotide used in the EMSA was a portion of the HSV ICP4 gene promoter containing the two most proximal Sp1 binding sites, reported to be the most important regions for transcription.[15] Specific Sp1 protein binding was confirmed by a reduction in Sp1 protein−DNA complexes upon addition of a 160-fold excess of unlabeled HSV ICP4 promoter fragment (data not shown). The alternative occupation of a single or dual sites resulted in the production of two bands: the faster migrating band (FMB) corresponds to the occupation of a single site (presumably either of the two sites), and the slower migrating band (SMB) corresponds to the occupation of both sites. The relative intensities of the bands corre-

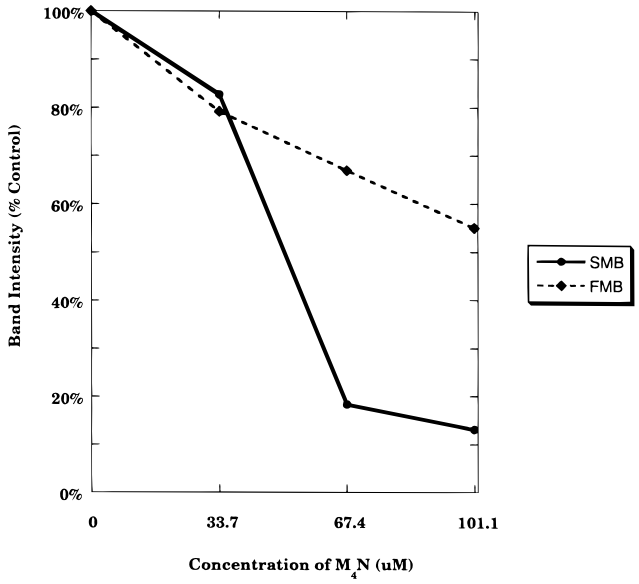

Figure 3. Electrophoretic mobility shift analysis (ESMA) of Sp1 protein binding to the HSV-1 ICP4 promoter. Phosphorimager analysis of EMSA; slower migrating band (SMB) and faster migrating band (FMB) intensities vs M_4N concentration. The upstream regulatory region of the HSV-1 ICP4 promoter containing the two proximal-most Sp1 binding sites (see Materials and Methods) was radiolabeled and preincubated with and without a 160-fold excess of unlabeled oligonucleotides and different concentrations of M_4N. Sp1 protein was added and each sample was electrophoresed on a 7% nondenaturing polyacrylamide gel. M_4N concentrations used were within its solubility range. The faster migrating band (FMB) corresponds to Sp1 protein binding to only one Sp1 binding site; the slower migrating band (SMB) corresponds to Sp1 binding to both Sp1 binding sites.

sponding to Sp1 protein−DNA complexes were quantitated by phosphorimager analysis. The data showed that M_4N interferes with Sp1 binding to the ICP4 promoter fragment in vitro. With increasing concentrations, the slower migrating band diminished more rapidly than the faster migrating band. This suggests that M_4N prevents Sp1 binding in a preferential manner (Figure 3).

Comparison of the Effectiveness of Tetra-*O*-methyl-NDGA and Acyclovir Following Passages of HSV in Vero Cells. Drug resistance against acyclovir has been universally observed for HSV in cell cultures. Since NDGA derivatives target a step of the viral life cycle that involves no viral proteins, the effect of M_4N is expected to be insensitive to viral protein mutations. To test this hypothesis we examined the effect of M_4N on HSV-1 (HIV Sm44 strain) and HSV-2 (HSV-2 SAV strain) replication and compared this effect with that of acyclovir at different viral passages. A series of concentrations of M_4N and acyclovir were used to inhibit either HSV-1 or HSV-2 in Vero cells. The drug-treated viruses, harvested at their IC_{50} at the first passage, were used to infect a second batch of Vero cells. The IC_{50} for M_4N or acyclovir was determined again for these second viral passages. A series of separate experiments were conducted in this sequential manner for 3, 5 and 10 HSV-1 passages and 4 HSV-2 passages with triplicates for each concentration of both drugs. The intrinsic cytotoxicity of the two drugs on the Vero cells used in this study was measured using a tetrazoliuar-based calorimetric assay for cell viability.[16] It was

Table 1. Comparative Potency (IC_{50}s) and Relative Safety (Selective Indexes,[a] SI) between M_4N and ACV at Different HSV Passages of Drug Treatment

	IC_{50} (μM)		SI	
HSV-1/2 passage	M_4N	ACV	M_4N	ACV
1	11.7	7.54	13.7	589
2	4.4	37.8	36.4	117
3	8.24	>88.8	19.4	50
4	5.97	138.4	26.8	32
5	6.48	111.0	24.7	40
6	6.98	130.7	22.9	34
7	6.98	189.8	22.9	23
8	9.91	444	16.1	23
9	8.82	378.8	18.1	11
10	4.77	444	33.5	10
1[b]	4.18	6.66	38.3	667
2[b]	11.08	>44.4	14.4	100
3[b]	11.08	62.8	14.4	70
4[b]	11.08	>88.8	14.4	50

[a] The selective index was defined as the ratio: TC_{50}/IC_{50}. The average TC_{50} for M_4N was 160 μM; the TC_{50} for acyclovir (ACV) was 444 μM. [b] HSV-2 passage.

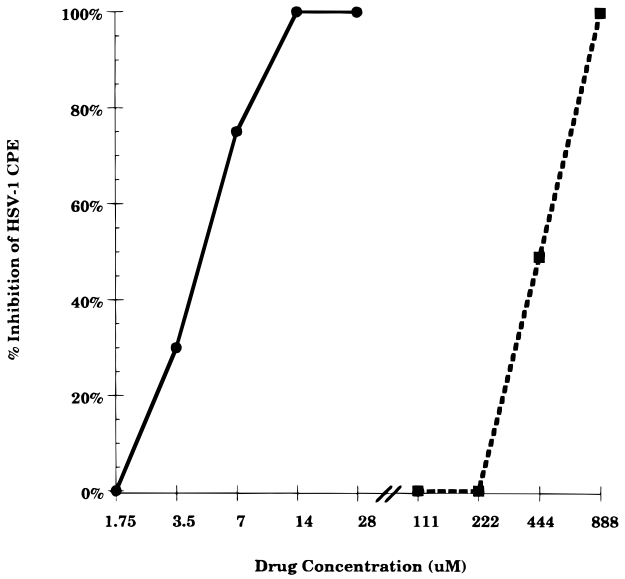

Figure 4. Dose−response inhibition of HSV-1 cytopathic effects by M_4N and ACV. Different concentrations of M_4N and acyclovir in an equal volume of 100% DMSO were added to Vero cells 2 h after viral infection. Growth inhibition (% CPE) was monitored and the IC_{50} calculated. Virus recovered from cultures at the IC_{50} was used as a stock to infect Vero cells for obtaining second passage of virus at its IC_{50}. The experiments were conducted serially for 10 viral passages: (●) dose-dependent effects of M_4N on long-term M_4N-treated HSV-1 (M_4N-10); (■) dose-dependent effects of acyclovir on long-term ACV-treated HSV-1 (ACV-10) following 10 viral passages.

determined three times again with triplicates for each drug concentration to obtain the average TC_{50} values (TC_{50} 160 mM for M_4N and 444 mm for ACV). We have found that the IC_{50} for ACV developed reproducibly and quickly for both HSV-1 and HSV-2 while IC_{50} for M_4N remained reproducibly constant following viral passages.

The SI (selective index, the ratio between TC_{50} and IC_{50}) has generally been used to assess drug safety. As shown in Table 1 and Figure 4, the IC_{50} of HSV-1 for acyclovir increased quickly from 7.5 to 444 μM by the 10th passage, an approximately 60-fold increase. The IC_{50} for M_4N, however, fluctuated between 4.7 and 11.7 μM within the 10 passages, but showed no obvious rising

trend. A 100-fold difference in required drug concentrations between M_4N and acyclovir was observed at the 10th HSV-1 passage. The same trend was also observed for HSV-2. For example, the IC_{50} for acyclovir rose quickly from 6.6 to >88 μM by the fourth passage while the IC_{50} for M_4N remained within the 4−11 μM range in four viral passages. The SI value of acyclovir for the sensitive strain of HSV at first viral passage was initially found to be as high as 589 (Table 1). However, viral drug resistance toward acyclovir developed quickly in Vero cells (Table 1). By the tenth passage of the original acyclovir-sensitive strain, a large population of acyclovir resistant viruses had developed. The SI for acyclovir for this resistant virus strain decreased to 10. For HSV-2, the results showed similar trends. The SI value for acyclovir was 667 at the first passage. By the fourth passage, it dropped quickly to less than 50. In contrast, M_4N had a low SI value at the first passage (SI = 13.7), and it remained fairly constant during 10 viral passages tested for HSV-1 and 4 viral passages tested HSV-2. In fact for the 10th passage viruses, M_4N showed a higher selective index (33.5) than acyclovir (10) (Table 1).

Assessment of Cross Resistance between Acyclovir and M_4N-Treated HSV-1 Strains. Cross resistance is fairly common among different drugs targeting the same viral protein.[17] Since the mode of action of acyclovir is entirely different from that of M_4N, cross resistance between the two seems unlikely. To check this hypothesis, and to repeat the drug sensitivity test as reported in Table 1 and Figure 4, we examined the effect of acyclovir and M_4N on three different HSV-1 strains (the originally M_4N- and acyclovir-sensitive Sm44 strain, a strain collected from the 10th passage of the M_4N-treated Sm44 virus (M_4N-10) and another one derived from the 10th passage of the acyclovir-treated SM44 virus (ACV-10)). The results of this experiment are in agreement with our predictions. M_4N was found to inhibit both acyclovir-sensitive (ACV-S) and acyclovir-resistant (ACV-10) strains equally as measured by both the CPE-CVS and PFU-CVS methods. In contrast, a low concentration of acyclovir was sufficient to inhibit both the ACV-S and M_4N-10 strains (IC_{50} <4 μM), while an exceedingly high concentration of acyclovir (IC_{50} of 444 μM) was required to inhibit the ACV-10 strain (Table 1). No viral cross resistance against M_4N and acyclovir was observed (Figure 5).

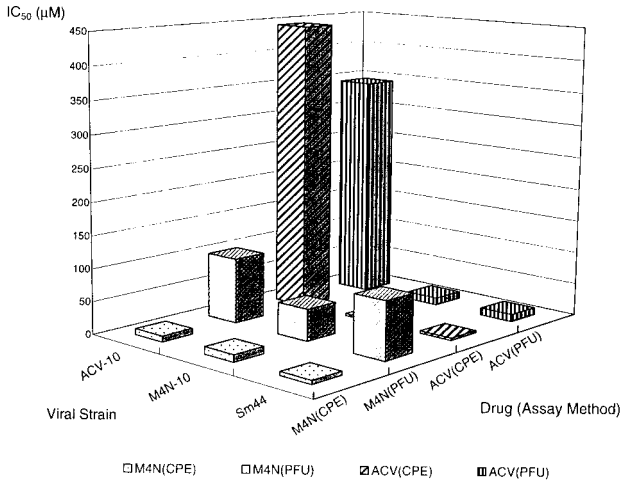

Figure 5. Inhibition of three different strains of HSV-1 in Vero cells. Three different strains of HSV-1 were tested for their drug sensitivities toward M_4N and ACV: original (HSV-1 Sm44, both ACV and M_4N sensitive), HSV-1 following 10 passages in M_4N (M_4N-10), and HSV-1 following 10 passages in ACV (ACV-10) The IC_{50} of M_4N for Sm44, M_4N-10 and ACV-10 were 5.64, 10.9, and 8.93 μM, respectively, as measured by the CPE assay, and 87.9, 48.3, and 99.9 μM, respectively, as measured by the PFU-CVS assay. In contrast, the IC_{50} of ACV for Sm44, M_4N-10, and ACV-10 were 3.59, 3.37, and 444 μM, respectively, as measured by the CPE assay. The IC_{50} for both Sm44 and M_4N-10 are <11.4 μM but reaches 344.1 μM for ACV-10, as measured by the PFU-CVS assay. Profile of drug sensitivities showed no cross-resistance between M_4N and ACV in inhibition of HSV-1.

Discussion

The ability of harmful viral and bacterial strains to mutate in response to conventional chemical agents poses a rapidly increasing problem in new drug design and the development of therapeutic strategies. In these studies we showed that M_4N inhibits in vivo transcription from the HSV ICP4 promoter, a gene essential for HSV replication and one of the earliest genes expressed in the HSV lytic transcriptional program. This may define the probable gene target of the anti-HSV activity of the compound. Furthermore, electrophoretic mobility shift analyses revealed that, at low concentrations, M_4N inhibits the binding of Sp1 protein to Sp1 binding sites on the HSV ICP4 promoter, indicating a likely mechanism for the observed transcriptional inhibition.

We further showed that M_4N is a mutation-insensitive drug. The mutation-insensitive effect of the compound may be attributed to its direct interference with the binding of host-encoded Sp1 protein to its cognate binding site, GGGCGG. Indeed, any mutation of this site by the virus in itself prevents Sp1 protein binding and thereby also precludes transcriptional activity. Such an antiviral strategy should highly restrict the ability of either virus to mutate against the drug activity. This is in contrast to the antiviral mechanism of such nucleoside analogue inhibitors as 3′-azido-3-deoxythymidine, AZT,[18] or acyclovir,[19] against which the respective viruses may readily mutate without necessarily compromising any of their replicative abilities. Also, the Sp1 protein has been shown to regulate the gene activity of several other pathological viruses, thus suggesting a possible use for this general antiviral agent in supplementing some of the more specialized drugs in treating multiple infections such as HIV and HSV.

Due to the fact that Sp1 is an important cellular transcription factor,[20] the possible inhibitory effect of this class of compounds on the expression of Sp1-regulated cellular genes[21] must be discussed. We mentioned earlier that Mal.4 cannot displace Sp1 once it is stably bound to its enhancer sites.[5] It is therefore likely that methylated NDGA will have a greater effect on Sp1-regulated genes in proliferating cells than on the expression of Sp1-regulated housekeeping genes in stationary cells. In the former case, the drug will be able to compete with Sp1 protein for the Sp1 sites in gene promoters during DNA synthesis, while in the latter case, the drug may have little effect on the transcribing chromatin of housekeeping genes with Sp1 protein stably bound at their promoters.

The relatively low selective index of M_4N dictates its use at the lowest effective concentration to avoid cellular

Table 2. Toxicity Studies of M_4N in Mice[a]

A. group	no. of mice	route	treatment: no. of injections per day	days of injection	mortality
100 mg/kg	5	ip	1	7	0/5
50 mg/kg	5	ip	1	7	0/5
50 mg/kg	5	ip	2	5	1/5
25 mg/kg	5	ip	1	7	0/5

B. group	no. of mice	route	treatment: no. of injections per day	days of injection	body weight changes
300 mg/kg	5	ip	1	1	−2.66%
50 mg/kg	5	ip	1	7	−2.80%
control	5	ip	1	7	−10.10%

[a] In A, tetramethyl-NDGA was dissolved in 100% DMSO and injected intraperitoneally using the amount and times as defined per group of mice. In B, the control group received DMSO alone. There were no mortality observed in any mice group during the experimental period. Mice were weighed every day to monitor the body weight changes for a period of 14 days following drug injection. The percent body weight changes were calculated and compared to the control group.

toxicity. This is substantiated by the higher cell viability (>90%) observed with M_4N at its effective concentration (IC_{50} = 43.5 μM) against HSV. M_4N, unique in its insensitivity to mutations in HSV, may best be used in combination with acyclovir to specifically control the acyclovir-resistant strains of HSV. We further postulate that since acyclovir-resistant mutants (ACV-10, Figure 5) are exceedingly sensitive to M_4N, the small pool of acyclovir-resistant mutants generated during the early viral passages in medium containing acyclovir might be brought under control by low concentrations of M_4N. If this is true, one would expect a lower IC_{50} for acyclovir during viral passages when the treatment is supplemented with a small concentration of M_4N. Using M_4N and acyclovir together may thus give a synergistic effect in abolishing both the wild-type and the mutant viruses. By suppressing the acyclovir-resistant strains of HSV frequently found in HIV and HSV doubly infected patients,[22] the low concentration of M_4N should have no significant long-term effects on the host while lowering the dosage of acyclovir and therefore the cost. Because NDGA derivatives target HIV and HSV by the same mechanism (as transcriptional inhibitors) they may also be effective in suppressing HIV strains that carry viral protein mutations similar to those described for HSV in this communication. Large scale synthesis of several hydrophilic NDGA derivatives is currently in progress. Results from some of the initial animal studies support the nontoxicity nature of this class of compounds at a concentration of 300 mg/kg (Table 2). This indicates that these agents may well be used as specific chemotherapeutic drugs with a reasonable safety margin to inhibit a variety of Sp1-regulated viral replication and to supplement AZT or protease inhibitors for the treatment of HIV positive patients as well.

Experimental Section

Materials and Methods. Chemicals. Tetra-*O*-methyl-NDGA (M_4N) (Figure 1) was synthesized and purified as previously described.[8] Acyclovir (ACV) was purchased from Ke-Yi Pharmaceutical Company. Both M_4N and acyclovir stocks were made in filter-sterilized 100% DMSO (Sigma D2650) at a concentration of 0.2−1.0 mg/mL and stored at −80 °C. Crystal violet was purchased from Hua-Bei Pharmaceutical Co., and methyl cellulose M450 was purchased from Shanghai Chemical Co.

Cells and Viruses. Vero cells (ATCC) and herpes viruses (HSV-1 Sm44 and HSV-2 Sav) were used in all experiments. The virus strains were propagated in Vero cells. The titers of virus stocks were determined and kept at −80 °C until used.

Plasmids. Three plasmids, pHSV1α_4SEAP, pGH290, and pET.GST.1.POU.ori$^+$, were used for the present study. pHSV1α_4SEAP was constructed by ligating the ICP4 promoter-containing *Xmn*I/*Bam*HI fragment of plasmid, pBR322 HSV-1 ICP4 (a pBR322 vector containing the −380/+30 nucleotides of the HSV-1 ICP4 promoter, from p175CAT[23]) across the *Xmn*I and *Bgl*II sites of pSEAP-basic (CLONTECH), which contains the SEAP reporter gene. Plasmid pGH290, which expresses VP16 protein from a CMV promoter, and plasmid p175CAT were obtained from Dr. Gary Hayward (Johns Hopkins Medical Institution). Plasmid pET.GST.1.POU.ori$^+$ which expresses the POU domain of Oct-1 was obtained from Dr. Winship Herr (Cold Spring Harbor Laboratory, NY).

Transfection. Vero cell cultures were seeded at a density of 1.50×10^5 cells/well in 24-well tissue culture plates (Falcon 3047) and grown for 24 h. Cells were then transfected by Lipofectamine (Gibco) according to the manufacturer's directions at optimal concentrations and incubation times. Briefly, cells were washed once with 400 μL/well of Optimem I culture medium (Gibco) and 170 μL of the same medium was added to each well. Six hundred microliters of fresh Optimem I medium containing approximately 1 μg of DNA was mixed with 600 μL of the same medium containing 40 μL of Lipofectamine reagent and let stand at room temperature for 30 min. Forty microliters per well of this transfection cocktail was then added to the cells and cultures were incubated for 8 h in a humidified 5% CO_2, 95% air, 37 °C incubator. A stock solution (33.4 mM in 100% DMSO) of M_4N was diluted in DMSO to the appropriate concentrations. Seventeen microliters of the inhibitor was added to 1.7 mL of complete Medium 199 media in 2 mL cryovials and vortexed for 30 s. The transfection cocktail was aspirated from each well and replaced with 550 mL of the inhibitor−DMSO−media mixture. The cultures were incubated for an additional 36−48 h in a humidified 5% CO_2, 95% air, 37 °C incubator. All samples were run in triplicate. The cultures were then analyzed by the SEAP assay.[6]

Secreted Alkaline Phosphatase (SEAP) Assay. Four hundred twenty microliters of media was removed from each well of the transfected cultures and heated at 65 °C for 5 min to inactivate endogenous phosphatases. (The SRB assay is to be performed on the remaining 120 μL culture.) One hundred microliters of 2× SEAP buffer (2 M diethanolamine, 1 mM $MgCl_2$, 20 mM L-homoarginine) was combined with 100 μL of each sample in a 96-well microtiter plate. Twenty-two microliters of substrate solution (120 mM 4-nitrophenyl phosphate disodium salt hexahydrate (Fluka) in 1× SEAP buffer) was then added to each 200 μL sample, and OD_{405} values were read every 20 min on an ELISA microplate reader (set at 37 °C with a 5 s shake before each reading). SEAP activity corresponds to the linear rate of change in OD_{405} values. The SEAP assay results were corrected for cytotoxic effects for M_4N by measuring the number of cells in each well using a sulforhodamine B (SRB) assay.[13]

One-quarter volume (40 μL) of ice cold 50% TCA was added to the remaining 120 μL well cultures and incubated for 1 h at 4 °C to fix the cells. Cells were then gently rinsed five times under tap water, and the plates were sharply flicked to remove the excess moisture and dried at room temperature. The fixed cells were stained for 30 min by adding 360 μL/well of 0.4% (w/v) sulforhodamine B (Sigma; SRB) in 1% acetic acid. The plates were then washed four times with 1% acetic acid, sharply flicked to remove excess moisture, and dried at room temperature. Five hundred microliters per well of unbuffered Tris-base (pH 10.7) was added to solubilize the stain, and 200 μL of each sample was read at OD_{564} on an ELISA microplate

reader in 96-well plates. The concentration of solubilized dye corresponds to the number of cells per culture.

Electrophoretic Mobility Shift Assay (EMSA). The following complementary oligonucleotides containing the two proximal-most HSV1 ICP4 SP1 binding sites: 5-GTTC-GGGGCGGGCCCGCCTGGGGGGCGGGGGG CCGG-3′, and 3′-CCGCCCGGGCGGACCCCCCGCCCCCCGGCC-5′ (Research Genetics, Inc.) were annealed at a final concentration of 100 μg/mL. The double-stranded oligos were then end-labeled by incubating 1 μg of annealed oligo, 1.2 μM α-^{32}P dCTP (3000 μCi/mmol) (Amersham), 40 μM dATP, 40 μM dGTP, and 5 units of the Klenow fragment of DNA polymerase in 20 μL of buffer (50 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM DTT, 5 mM NaCl), at room temperature for 45 min. The reaction was terminated by the addition of 1 μL of 0.5 M EDTA. An aliquot of 1.1 μL of the radiolabeled DNA solution was removed and added to 14.9 μL of H_2O to make 16 μL of a 3 ng/μL solution.

One microliter of the radiolabeled oligonucleotide (3 ng) was preincubated with 8 μL of 2× binding buffer (50 mM HEPES pH 7.9, 2 mM EDTA, 10 mM DTT, 300 mM KCl, 0.10% NP40, 20% glycerol, 5 mM $MgCl_2$) and the required concentrations of competitor oligos, inhibitors, and DMSO in a final volume of 16 μL (6.25% DMSO) at 30 °C for 30 min followed by 2.5 h incubation at room temperature, a critical step to achieve efficient binding of inhibitors to the oligonucleotides. The samples were cooled on ice, 1 μL (0.5 fpu/μL) of Sp1 protein (Promega) was added to each tube, and the samples were incubated at 18 °C for 15 min. The samples were then electrophoresed on a 7% nondenaturing polyacrylamide gel.[5] After electrophoresis, the gel was dried and relative band intensities were quantitated by phosphorimager analysis with proportional background signal subtraction.

Antiviral Assays. Vero cells were infected with 100 TCID 50 of HSV-1 or HSV-2. At 2 h postinfection (pi), different concentrations of M_4N and acyclovir in equal volumes of 100% DMSO were diluted with either liquid medium or 0.5% methyl cellulose MEM medium and added to triplicate plates of the cells. Control samples were concomitantly run with the test compounds with uninfected cells to assess the intrinsic cytotoxicity of the drug. The concentration of drug required for 50% viral inhibition (IC_{50}) and 50% cell cytotoxicity (TC_{50}) was calculated by three methods. (1) The CPE method: The cytopathic effect (CPE) was routinely monitored by inverted microscope and scored. (2) The CPE-CVS method: Cells were stained with crystal violet (CVS) and the OD_{540} of each well was determined using an Elisa microplate reader.[24] (3) The PFU-CVS method: Vero cells were seeded in a 24-well culture plates and infected with HSV-1 as above. Test compounds dissolved in DMSO were appropriately diluted with 0.5% methyl cellulose MEM medium and added to the infected monolayer instead of liquid culture medium. After incubation, the cell culture plates were stained with crystal violet and the plaque-forming units (PFU) were counted. The final DMSO concentration in the medium never exceeded 10%.

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