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Novel antiviral agent tetraglycylated nordihydroguaiaretic acid hydrochloride as a host-dependent viral inhibitor

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Abstract

A water soluble derivative of nordihydroguaiaretic acid (NDGA), G_4N (**2**), synthesized by reaction of NDGA (**1**) with *N*,*N*-dimethylglycine in the presence of dicyclohexylcarbodiimide and dimethylaminopyridine and then with HCl(g) (Scheme 1), competes effectively with the DNA binding domain of recombinant Sp1 protein for binding to the human immunodeficiency virus (HIV) LTR as demonstrated by an electrophoretic mobility-shift assay (EMSA). By blocking Sp1 binding to the HIV LTR, G_4N suppresses Sp1-regulated HIV Tat transactivation and replication in cultured cells with an IC_{50} of 12 μM similar to that of 3′-*O*-methyl-NDGA as we have previously reported. In addition simian immunodeficiency virus (SIV) replication was completely inhibited by G_4N at 5.0 μM. G_4N showed no toxic effect to 174 × CEM cells and H9 cells at 100 μM.

Keywords: Tetra-*O*-glycyl-nordihydroguaiaretic acid; G_4N; Human immunodeficiency virus; Sp1; Simian immunodeficiency virus

1. Introduction

We have previously reported that the natural product 3′-*O*-methyl-NDGA (Mal.4) and a synthetic derivative of NDGA, tetra-*O*-methyl-NDGA (M_4N) are able to inhibit human immunodeficiency virus (HIV) Tat transactivation by blocking host Sp1 protein at the Sp1 cognate binding site on the LTR promoter (Gnabre et al., 1995a,b, 1996; Hwu et al., 1998) and suppress HIV replication in culture cells (Gnabre et al., 1995a). In a related study, we have further shown that M_4N is able to inhibit the replication of herpes simplex virus type 1 (HSV-1) by suppression of another Sp1-regulated gene, immediate early gene *ICP4*, that is essential for HSV-1 replication (Chen et al., 1998). Moreover, NDGA derivatives can inhibit human papilloma virus (HPV) E6/E7 promoter activity by the same mechanism (Craigo et al., 2000).

Host proteins, such as the transcription factor Sp1, are not synthesized 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 potentially good candidates for mutation-insensitive antiviral drugs. M_4N is such a mutation-insensitive antiviral drug that was previously reported in our recent anti-HSV studies (Chen et al., 1998). It was found that while HSV-1 and -2 build up resistance against acyclovir (ACV), a guanosine analogue, there was no sign of drug resistance following long HSV passages in cultures containing M_4N (Chen et al., 1998). M_4N and other methylated NDGA derivatives however all have poor water solubility. A water soluble derivative, tetraglycylated NDGA·4HCl (G_4N), was therefore synthesized (Huang et al., 2002; King et al., in press). G_4N is readily soluble in water up to a maximum concentration of 150 uM while NDGA and methylated NDGA is not soluble in water even at 1 uM. G_4N was found to inhibit Sp1-regulated *ICP4*, an immediate early gene of HSV-1, transcription but had no effect on HSV entry (Park, 2002). It can suppress wild type as well as ACV—resistant HSV-1 ($HSV\text{-}1^R$) replication in Vero cells and prevent HSV-1 reactivation in explanted, mouse trigeminal ganglia (Park et al., in press). In this communication, we report that G_4N, by inhibition of Sp1-regulated proviral transcription, possesses antiviral activities against HIV-1 and SIV.

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2. Materials and methods

2.1. Synthesis and chemical structure of tetraglycylated NDGA, G_4N

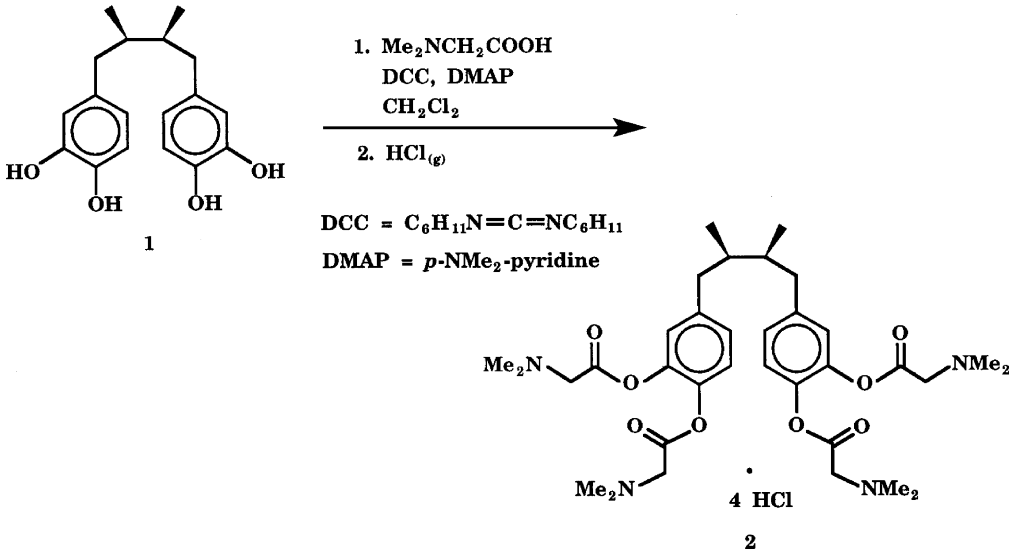
Tetraglycylated NDGA, G_4N, was synthesized by treating nordihydroguaiaretic acid with *N,N*-dimethylglycine in the presence of dicyclohexylcarbodiimide (DCC) and a catalytic amount of dimethylaminopyridine (DMAP) in CH_2Cl_2 (Huang et al., 2002; King et al., in press; King, 2000). The resultant derivatized NDGA was allowed to react with HCl(g) to successfully give the desired tetraglycylated NDGA (G_4N Mol. Wt. 788.1, Scheme 1) in 87% yield. Anhydrous G_4N is completely stable.

2.2. Production and purification of Sp1 protein

The Sp1 protein was produced from a bacterial expression plasmid, pGST-Sp1-167C, that carries a fusion of the glutathione-*S*-transferase gene to the region encoding the C-terminal 167 amino acids that contains three zinc-finger DNA binding domains of the protein (Sjottem et al., 1997). This protein is referred to as Sp1-167C in this paper. Cells (*Escherichia coli* strain BL21) harboring the plasmid were grown in LB medium containing 100 mg/ml ampicillin and induced by the addition of IPTG (0.10 mM). After an overnight incubation at 37 °C, the majority of the fusion protein accumulated as insoluble inclusion bodies. The cells were pelleted by centrifugation, washed once with 20 mM Tris–HCl, pH 7.8 and then suspended in 50 mM Tris–HCl, pH 7.8, 200 mM NaCl, 1.0 mM EDTA, 5% glycerol, 0.10 mM PMSF at a concentration of 1×10^{10} cells/ml. A 1/10 volume of 50 mM EDTA, 10% Triton X-100 was added followed by the addition of lysozyme (150 μg/ml). The cells were incubated on ice for 1.0 h and then frozen at −80 °C. After thawing, $MgCl_2$ (15 mM) was added and the cells were treated with DNase (1.0 μg/ml) to eliminate viscosity. The lysed cell suspension was then centrifuged at 17,000 × *g* for 30 min. The pellet containing the protein was resuspended in lysis buffer using a Dounce homogenizer and the lysozyme treatment was repeated. After centrifugation the pellet was washed once with lysis buffer without EDTA or Triton X-100. The resultant protein pellet was dissolved in 6.0 M urea, 50 mM Tris–HCl, pH 7.8, 1.0 mM DTT to give a total protein concentration of 10–15 mg/ml. The solution was clarified by centrifugation for 1 h at 27,000 × *g*. The solubilized protein was diluted to 5.0 mg/ml with the same urea solution and dialyzed for 18 h at 4 °C against 2.0 M urea, 20 mM Tris–HCl, pH 7.8, 1.0 mM EDTA, 1.0 mM DTT. The Sp1 protein was cleaved from the GST moiety by incubation with thrombin (10 u/mg protein, Amersham Pharmacia Biotech) in 2.0 M urea, 20 mM Tris–HCl, pH 7.8, 1.0 mM EDTA, 1.0 mM DTT for 16 h at 25 °C and stored at −80 °C for use in EMSAs.

2.3. Electrophoretic mobility-shift assay (EMSA)

The DNA template used in all electrophoretic mobility-shift assays was a double-stranded 48 bp oligonucleotide spanning nucleotides −87 to −40 of the HIV LTR (Gnabre et al., 1995a) which was labeled with $dATP^{32}$ by filling in a 3′ recessed end using the Klenow fragment of DNA polymerase. The reaction buffer for all shift assays consisted of 10 mM Tris–HCl, pH 7.5, 0.70 mM HEPES–KOH, pH 7.7, 30 mM KCl, 1.0 mM EDTA, 0.80 mM $MgCl_2$, 0.60 mM $ZnSO_4$, 10% glycerol, 5.0 mM DTT and 1.0 ng of labeled DNA template was used per each 15 μl reaction. Before the addition of the Sp1-167C protein, BSA (0.86 μl) was added to give a final concentration of 0.50 mg/ml.



Scheme 1.

A fresh stock of G_4N was made from dried G_4N powder for each experiment. For blocking Sp1 binding, G_4N was added to reaction tubes containing template to give the appropriate final G_4N concentration for the reaction volume of 15 μl. After a 30 min incubation at 25 °C, Sp1 was added and incubation continued for another 30 min prior to electrophoresis. For G_4N displacement of Sp1 bound to template, Sp1 was first incubated with the template in a 15 μl reaction volume for 30 min. Concentrated stocks of G_4N were prepared that gave the appropriate final concentration when added to the reaction. Incubation was continued for another 30 min prior to electrophoresis. For displacement of template-bound G_4N by Sp1, the template was first incubated for 30 min in 1.2 mM G_4N. Then increasing amounts of Sp1 were added and the incubation was continued for an additional 30 min prior to electrophoresis.

2.4. Inhibition of HIV Tat transactivation using the secreted alkaline phosphatase (SEAP) assay

The method for the inhibition of HIV Tat transactivation using the SEAP assay applied here has been previously described (Gnabre et al., 1995a, 1996). Cos cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum and antibiotics. A day prior to cell cotransfection with plasmid DNAs, triplicate cell samples were seeded at a density of approximately 5×10^4 cells per well in a 48-well plate. The subsequent 70% subconfluent cells were cotransfected with plasmids pBC12/HIV/SEAP (0.60 μg per well) and pBC12/CMV/t2 (encoding for Tat function, 0.30 μg per well) using the Fugene-6 transfection reagent (Roche). Five hours later the transfection cocktail was aspirated and cells were replenished with 500 μl of fresh IMDM. The cultures were incubated for an additional 12 h, after which various concentration of drug were added to the test wells. After 48 h, a 250 μl aliquot of the culture supernatant was removed from each well for SEAP analysis. The absorbance of the reaction products was read at 405 nm in 5 min intervals over the course of 60 min using a Bioteck EL-340I microplate reader (Bio-teck Instuments). The percent inhibition of SEAP expression was calculated as previously described (Gnabre et al., 1996). Each point represents the mean of six determinations from two independent experiments.

2.5. Cells and viruses

H9 cells were grown and infected with HIV-1_{RTMF} (an AZT-resistant strain) as described recently (Abd-Elazem et al., 2002). H9 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 u/ml penicillin and 100 μg/ml streptomycin. H9 cells were suspended in culture medium at 1×10^5 cells/ml and infected with HIV at a multiplicity of infection of 0.1. After infection, the cells were washed with PBS followed by culture medium. The cell suspension (100 μl) was added to each well of a 96-well plate and then various concentrations of G_4N (1.5–100 μM) were added. After a 4-day incubation at 37 °C, the H9 cells were replenished with fresh culture medium containing G_4N and further incubated until 8 days post-infection. Viral replication was determined by the amount of HIV-1 p24 antigen in culture supernatants using the HIV/p24 monoclonal antibody assay (Veronese et al., 1985).

In experiments with SIV, 174 × CEM cells (1.0×10^7) were mixed with a 24 h harvest stock of SIV_{mac239} (4.0 ng of p27) at a multiplicity of infection of 0.05 for 2 h at 37 °C. The virus was removed and the infected cells were resuspended in culture medium and 1.0×10^5 cells were added to each well of a 96-well plate. Various concentrations of G_4N from a freshly made stock were prepared and then added to each of six designated wells. Culture supernatants were collected after 4 and 8 days for virus production analysis. Virus production was assayed by a modified p27 capsid protein antigen capture ELISA. Each point represents average of data from two duplicate studies with differences within 1–2% of each other.

2.6. Cytotoxicity assay

The cytotoxicity of G_4N against H9 cells and 174 × CEM cells was analyzed using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based assay (Sigma) (Uckun et al., 1998). Briefly, exponentially growing cells were seeded in 96-well plates at a density of 3×10^4 cells per well. Twenty-four hours later a series of concentrations of freshly made G_4N (0, 12.5, 25, 50 and 100 μM) were added and the cells were incubated for an additional 8 days. To assay cytotoxicity, 50 μl of MTT (1 mg/ml final concentration) was added to each well and the plates were incubated at 37 °C for 4 h. The resultant formazan crystals were solubilized with DMSO and the absorbance of the contents of each well was measured in a microtiter reader at optical density of 540 nm.

3. Results

3.1. Inhibition of Sp1 binding activity by G_4N in a electrophoretic mobility-shift analysis

Sp1 family proteins induce bends toward the major groove of DNA upon binding (Sjottem et al., 1997). The zinc finger domain of the Sp1 protein is responsible for the binding DNA at the GC box enhancer sequence 5′-GGGCGG-3′. To determine whether G_4N can serve as an Sp1 blocker as well as an Sp1 displacer, we performed Sp1/enhancer interaction studies in the presence or absence of G_4N by electrophoretic mobility-shift analysis using only the DNA binding domain of Sp1 for testing. In the blocking experiment, different concentrations of G_4N were first incubated with ^{32}P-labeled DNA in the binding buffer for 30 min at 25 °C. The DNA

binding domain of a recombinant Sp1 protein (Sp1-167C) was next added and incubated for additional 30 min in the presence of a large excess of BSA protein. In the displacement study, the recombinant protein (Sp1-167C) was first allowed to bind DNA and then G_4N was added at the second step of the incubation. The G_4N and Sp1-167C concentrations, incubation times and gel electrophoresis conditions were identical in both studies (Section 2). As shown in Fig. 1, in either case, G_4N was able to keep DNA from interacting with the Sp1-167C protein. When only the DNA binding domain of Sp1 alone was tested, G_4N appeared to be more efficient in displacement of the bound Sp1 than blocking Sp1 from binding to the enhancer, as shown by the electrophoretic mobility-shift analysis (Fig. 1A, B and D). We have also examined whether bound G_4N can be replaced by Sp1-167C. In this study, the DNA template was incubated with G_4N at a concentration (1.2 mM) which yielded a 40–50% template shift (Fig. 1C, lanes 2 and 5). When the

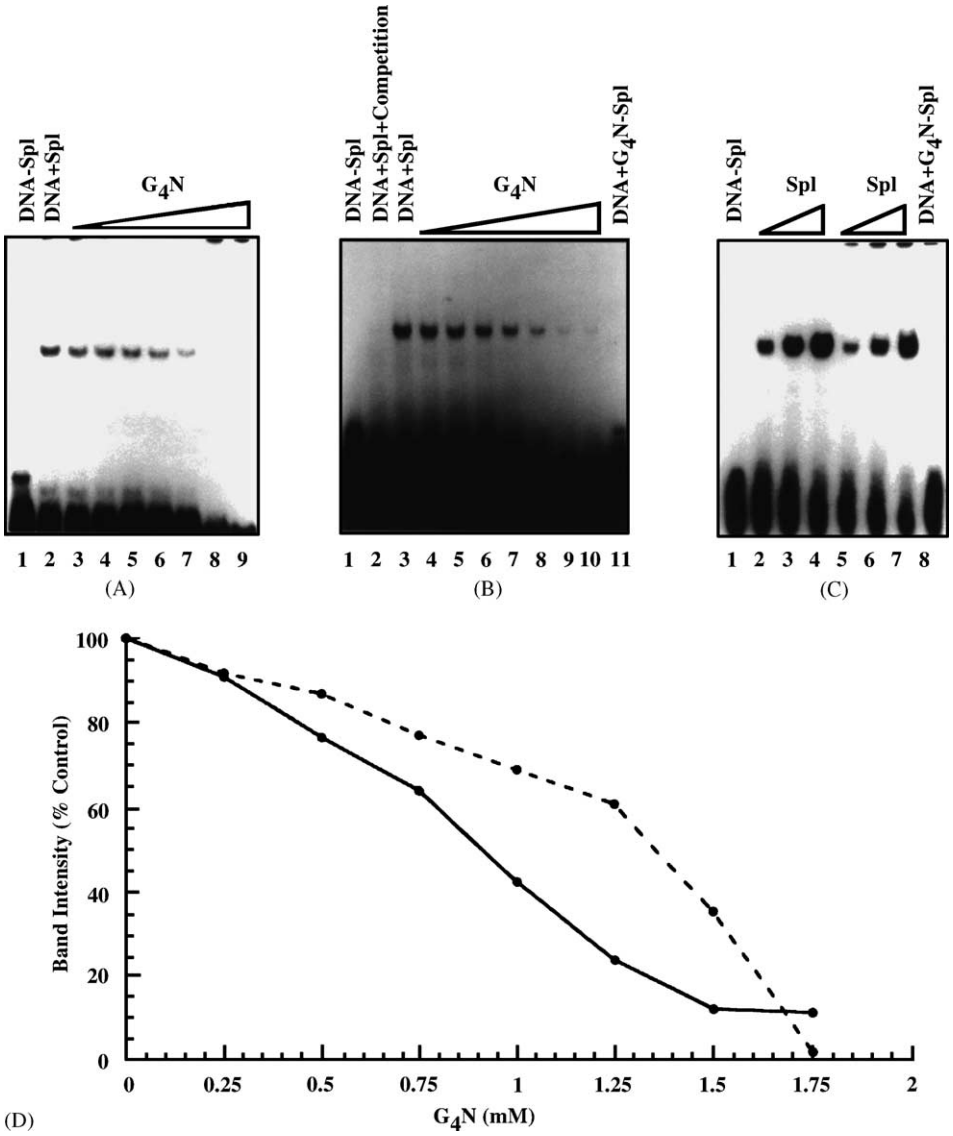


Fig. 1. Electrophoretic mobility-shift analysis (EMSA) of G_4N interaction with the HIV Sp1 binding sites (−87 to −49). (A) G_4N inhibition of Sp1-167C binding to ^{32}P-labeled HIV Sp1 DNA template. Lane 1, template alone; lane 2, template plus 0.10 μg Sp1-167C; lanes 3–9, template incubated with increasing concentrations of G_4N (0.25–1.75 mM) prior to the addition of 0.10 μg Sp1-167C. (B) G_4N displacement of Sp1-167C bound to the HIV template. Lane 1, template alone; lane 2, template plus 0.10 μg Sp1-167C plus 100-fold excess of unlabeled template; lane 3, template plus 0.10 μg Sp1-167C; lanes 4–10, Sp1–DNA complex challenged with increasing concentrations of G_4N (0.25–1.75 mM); lane 11, template incubated in reaction buffer containing 1.75 mM G_4N. (C) Sp1-167C displacement of G_4N bound to template. Lane 1, template alone; lanes 2–4, template plus increasing amounts of Sp1-167C (0.075, 0.150, 0.300 μg); lanes 5–8, template incubated in reaction buffer containing 1.2 mM G_4N followed by challenge with increasing amounts of Sp1-167C (0.075, 0.150, 0.300 μg). Lane 8 received no Sp1-167C. (D) Plot of diminishing Sp1-167C DNA complex band intensities in response to increasing concentrations of G_4N used in (- - -) blocking and (—) displacement. The gels used were 5% non-denaturing polyacrylamide with each lane receiving 5.0 μl of each reaction volume as described in Section 2.

G_4N-bound template was challenged with Sp1-167C, we observed a displacement of G_4N by Sp1-167C from the template and a dosage dependent increase of the band intensities of the Sp1-167C/DNA complex (Fig. 1C, lanes 6 and 7).

3.2. Inhibition of Sp1-regulated Tat transactivation of HIV promoter activity by G_4N

As reported previously, methylated NDGA derivatives blocked Sp1 binding to the enhancer sites of a variety of viral promoters including the LTR of HIV, *ICP4* of HSV and E6/E7 of HPV and inhibited viral gene expression and replication (Gnabre et al., 1995a; Chen et al., 1998; Craigo et al., 2000). We further tested the effect of G_4N on the Tat transactivation of HIV promoter activity in Cos cells using the SEAP reporter gene assay. The basal level of HIV LTR-driven SEAP expression was previously found to be barely detectable in Cos cells. There is a 60-fold or more increase in SEAP expression when Cos cells are co-transfected with the CMV promoter driven Tat gene that has been shown to be inhibited by 3′-*O*-methyl-NDGA (Gnabre et al., 1995a). Such inhibition of Tat-driven transactivation of the HIV LTR promoter resulted from the effect of drug on suppression of basal proviral transcription, not from its binding to Tat protein during transactivation (Gnabre et al., 1995a; Hwu et al., 1998). In the presence of G_4N, we also observed inhibition of HIV transactivation in a dose-dependent fashion (Fig. 2). An average IC_{50} value of 36 μM for G_4N was comparable to that of 3′-*O*-methyl-NDGA, Mal.4 (IC_{50} 25 μM), and somewhat higher than that of tetra-*O*-methyl-NDGA, M_4N (IC_{50} 11 μM). The differences perhaps are due to the chemical nature of the test compounds affecting drug uptake into the cells.

3.3. Inhibition of SIV and HIV production in cell cultures by G_4N

Both HIV and SIV are retroviruses that require integration into the host genome to complete their replication. Both rely on host transcription factors for proviral transcription. Sp1 plays a central role in the expression of these two viruses which share an almost identical mode of transcription regulation. In anticipation of using SIV-infected rhesus monkeys as an animal model for testing the antiviral effect of G_4N, we have studied and compared the inhibitory effect of G_4N on the replication of SIV in 174 × CEM cells with that of HIV in H9 cells. Cellular toxicities of G_4N in these two cell lines were also examined. For the SIV inhibition study, 174 × CEM cells were mixed with a high titer stock of SIV_{mac239} and different concentrations of G_4N were added. Culture supernatants were collected every 4 days post-infection (p.i.) and afterward the cells were replenished with fresh drug-containing medium. Viral production was assayed by a modified p27 core antigen capture ELISA. As shown in Fig. 3, SIV production was completely inhibited

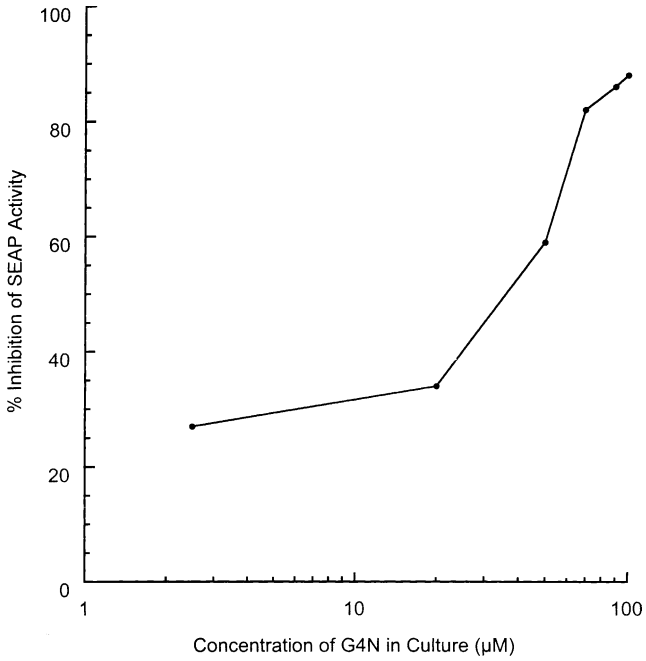


Fig. 2. Inhibition of HIV Tat-regulated transactivation in Cos cells by G_4N. Cos cells cotransfected with a Tat-producing plasmid and a plasmid containing the HIV LTR linked to the SEAP reporter gene were treated with a range of concentrations of G_4N. After 48 h aliquots of media were removed and analysis of SEAP activity was performed. The percent inhibition of SEAP activity by G_4N was calculated by comparison to the SEAP activity present in the medium of untreated cells.

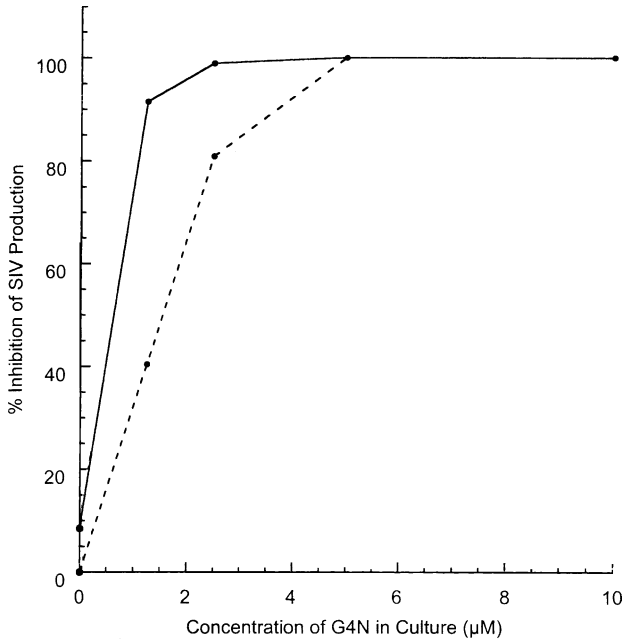


Fig. 3. Inhibition of SIV production in 174 × CEM cells by G_4N. Percent inhibition of SIV production was calculated by comparing the amount of p27 antigen produced in cultures treated with G_4N to the amount produced by untreated 174 × CEM cells after 8 days of infection; (—) day 4 and (- - -) day 8.

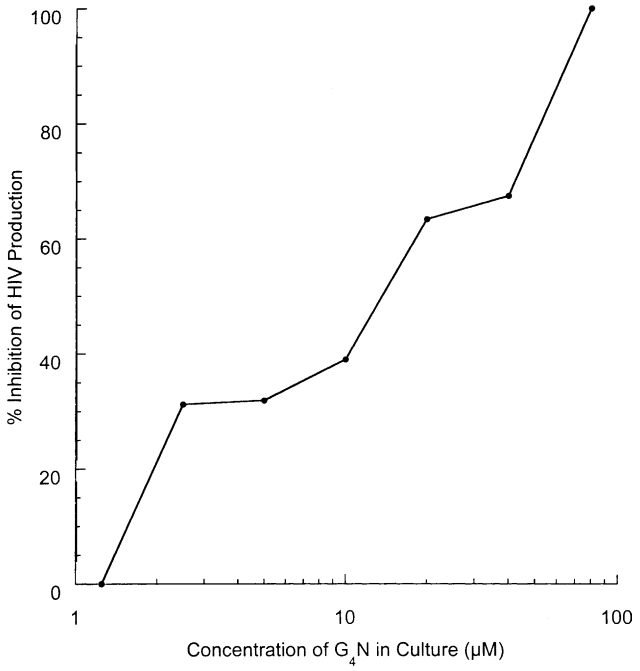


Fig. 4. Inhibition of HIV p24 antigen production in H9 cells by G_4N. Percent inhibition of HIV production by G_4N was calculated by comparing the p24 level from an average of two duplicate cultures of G_4N-treated and -untreated H9 cells 9 days following viral infection with an AZT-resistant HIV strain, HIV-1_{RTMF}.

in cells treated with G_4N in concentrations above 5.0 μM. At G_4N concentrations below 2.5 μM, SIV production was detected in culture supernatants from 4 and 8 days p.i., but at levels below that observed in the absence of the drug. A similar experiment was also carried out to study the inhibition of HIV-1 by G_4N in H9 cells. The H9 cells were infected with an AZT resistant strain of HIV-1 (HIV-1_{RTMF}) and G_4N at different concentrations was added 2 h after infection. Fresh drug-containing medium changes were made every 4 days. Cell growth in the presence of G_4N was monitored carefully during the 9-day experimental period and viral production was assayed by a p24 core antigen capture ELISA. As shown in Fig. 4, a G_4N concentration of 80 μM completely inhibited HIV production in H9 cells. An IC_{50} of 12 μM G_4N for the inhibition of HIV-1_{RTMF} was found (Fig. 4) and G_4N (100 μM) showed no toxic effects on uninfected 174 × CEM cells and H9 cells as determined by a MTT-based cytotoxicity assay (Weislow et al., 1989) (Table 1).

Table 1
Cytotoxicity of G_4N towards H9 cells and 174 × CEM cells was analyzed using an MTT-based assay as described in Section 2

G_4N (μm)	OD_{540}	
	H9 cells	174 × CEM cells
0	2.04	1.15
12.5	2.14	1.08
25	2.19	1.28
50	2.31	0.95
100	2.26	1.156

The absorbance of each well at 540 nm was a measurement of formazan crystals in DMSO from metabolically active cells. Data represent an average from quadruplicate wells.

4. Discussion

In this communication we showed that NDGA derivative G_4N, like *O*-methylated-NDGAs (Gnabre et al., 1995a,b, 1996; Hwu et al., 1998; Chen et al., 1998; Craigo et al., 2000), can block Sp1-regulated viral gene expression and replication. We have also shown that G_4N is relatively non-toxic to H9 cells and 174 × CEM cells in culture (Table 1). In animal studies, there was little toxicity toward mice following either subcutaneous or intravenous daily injection of G_4N at dosages as high as 375 mg/kg for a total of 6 days (Huang et al., 2002). Topical application of G_4N to dorsal cutaneous regions of guinea pigs (six regions per animal, at 24 mg per region, twice daily for 6 days) also showed no toxicity to animals based upon body weights monitoring and organ (kidney, liver, spleen and region of skins) histology from biopsied samples taken on the sixth day of treatment (Park, 2002).

The binding efficiency of G_4N to the GC box in the promoters of cellular genes versus viral genes in infected hosts are difficult to assess. The requirement of Sp1 is strictly essential in the several virus systems that we have studied. Its requirement for endogenous gene expression in culture and in animals, however, has not been extensively studied. From gene microarray analysis using cDNA sequences as detection probes, we have found that the expression of most of the cellular genes containing Sp1 sites in their promoters is not affected by NDGA-related compounds. Chromatin structure, as well as transcription factors occupying sites neighboring GC box sequences must greatly influence the related NDGA/template interactions. In this regard, we have found that M_4N is extremely effective at inhibiting tumor cell growth by blocking cell division at the G_2/M phase of the cell cycle, but without harming the adjacent tissues. The cell arrest by M_4N was found to correlate with inhibition of Sp1-regulated *CDC*$_2$ gene expression (Heller et al., 2001). We have further shown that such M_4N-induced cell arrest is reversible upon drug removal from the culture. Furthermore, the same cells are able to escape cell cycle arrest in the presence of the drug when they have been transfected with the *CDC*$_2$ gene expressed from the non-Sp1-regulated CMV promoter (Heller, 2002).

During the past decade a series of very potent viral inhibitors have been chemically synthesized. The list includes different nucleoside analogs (Squires, 2001) and compounds that specifically interact with viral proteins (Tavel, 2000). Few of them are currently used in clinics. Many are being evaluated in clinical trials. In short-term cultures of infected cells, most of these compounds are highly potent and have

extremely impressive therapeutic indices (Mewshaw et al., 2002; Yoshimura et al., 2002). Long-term use of these types of antiviral agents however invariably will generate drug resistant viruses resulting from the high rate of mutation in rapidly growing viral populations. Compounds such as the NDGA derivatives, which target not at viral proteins, but at host factors that are required for viral growth, possess some distinct advantages. These host factors are synthesized under no selective pressure, they are structurally stable and viruses are not likely to become drug resistant to them quickly. For example, we have found that the concentration of tetra-*O*-methyl-NDGA, M_4N, required for suppression of HSV replication remained constant during 10 serially propagated generations. Conversely, the required concentration of acyclovir, which targets viral DNA synthesis via HSV thymidine kinase to suppress HSV replication, gradually increased and reached the 100-fold level by the 10th viral generation (Chen et al., 1998). Thus, G_4N is a mutation-insensitive viral inhibitor while acyclovir is a mutation-sensitive drug.

In the present study, we have focused on wild type SIV and AZT-resistant HIV and found that the IC_{50} of G_4N is approximately 10 μM for HIV and 2.5 μM for SIV in infected cells in culture. Although these concentrations are much less potent than those reported for many other viral inhibitors, they are mutation insensitive, and fairly non-toxic to the host. G_4N in aqueous solution undergoes slow hydrolysis to generate a series of water soluble, yet still active glycylated NDGA isomers, each containing one to four glycine molecules. Assuming this is also the case in vivo, perhaps G_4N can be used as a prodrug to complement known mutation-sensitive drugs as one component of a treatment cocktail. Including G_4N in such a cocktail can help to eliminate a small population of mutant viruses at the onset from becoming resistant to the other inhibitors present in the cocktail. Long-term efficacy for treatment using such a multidrug scheme is currently being explored in our laboratory.

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