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Inhibition of human papillomavirus type 16 gene expression by nordihydroguaiaretic acid plant lignan derivatives

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Abstract

Several methylated derivatives of a plant lignan, nordihydroguaiaretic acid (NDGA) were found to be potent anti-viral agents by suppressing Sp1 regulated transcription within the sexually transmitted viruses human immunodeficiency virus (HIV) and herpes simplex virus (HSV). A prominent Sp1 DNA binding site within many human papillomavirus (HPV) promoters has been noted to play an active role in HPV gene expression. In this report it is shown that the three NDGA derivatives, Mal.4, M_4N, and tetra-acetyl NDGA can also inhibit gene expression from the early promoter P_{97} of HPV16. The drug activity on gene expression was measured after DNA transfection of recombinant vector constructs linking the viral promoter and enhancer elements to the luciferase reporter gene. Using the specific luciferase activity as the indicator of gene expression, Mal.4 and M_4N were found to be active in a dose dependent manner that is in the same range of concentrations reported for the promoters of HIV, HSV, and simian virus 40 (SV40) while tetra-acetyl NDGA was much more active in suppression of the HPV P_{97} promoter activity than Mal.4 and M_4N. The drugs showed limited to no effect on gene expression driven by the adenovirus major late promoter and the cytomegalovirus (CMV) promoter. Hence, such drug derivatives may be significant in the therapy of papillomavirus infections and their associated induced human cancers.

Keywords: *Larrea tridentata*; Plant lignan; Human papillomavirus

1. Introduction

A plant lignan, 3′-O-methyl nordihydroguaiaretic acid (Mal.4), isolated from the creosote bush (*Larrea tridentata*) and several derivatives of its parent compound, nordihydroguaiaretic acid (NDGA) have previously been found to be potent

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inhibitors of viruses such as human immunodeficiency virus (HIV-1) and herpes simplex virus (HSV) (Gnabre et al., 1995a,b, 1996; Hwu et al., 1998). Studies on the mechanism of action of these drugs determined that they inhibited the cellular transactivator Sp1 protein from binding to sites on the viral promoters and interfered with viral gene expression thus inhibiting viral replication (Gnabre et al., 1995b). These derivatives form part of a new class of anti-viral agents in that they are active against a cellular protein functioning on a viral promoter and inhibit its activity and virus reproduction well before they damage the cell itself (Daelemans et al., 1999).

Human papillomaviruses (HPVs) are small DNA tumor viruses of which specific genomic types have been found to be involved in the induction of uterine cervical and other cancers worldwide (Howley, 1991; zur Hausen, 1991, 1996). The HPV types closely associated to cervical cancer and ano-genital warts have a highly conserved Sp1 DNA binding site within their major early promoter region (O'Conner et al., 1995). This site has been previously shown to be important in HPV early gene expression (Gloss and Bernard, 1990; Parker et al., 1997) and so it became of interest to test if the lignan derivatives could interfere with HPV replication. However, since the systems to study HPV in vitro (Meyers et al., 1992; Parker et al., 1997) are technically difficult and not convenient for screening the utility of potential anti-viral drugs at an early stage of development, other simpler methods were used in this study. The experiments reported here show the effects of the lignan derivatives on HPV gene expression as measured by viral promoter luciferase reporter recombinant vector constructs and DNA transfection into cells grown in tissue culture.

The methodology used to determine drug effects on viral gene expression as measured by a luciferase reporter gene was also tested on non-papillomavirus viral promoters isolated from viruses previously known to have viral replication inhibited by the lignan derivatives (Gnabre et al., 1995b). The results indicated that the drugs were inhibitory in a dose dependent manner on the tested viral promoters that also inhibited viral replication in tissue culture. These experiments suggested that the studies using the papillomavirus early promoter luciferase reporter constructs would be a reasonable approach to screen for potential anti-viral and anti-cancer activities of the lignan derivatives.

The results in this paper show that the isolated plant lignan 3′-O-methyl NDGA (Mal.4) and two other derivatives of its parent compound, NDGA (M_4N and tetra-acetyl NDGA) are substantial inhibitors of the HPV type 16 early promoter, P_{97}. These results further suggest that such drugs may be useful anti-viral agents against the sexually transmitted papillomaviruses and their induced tumors.

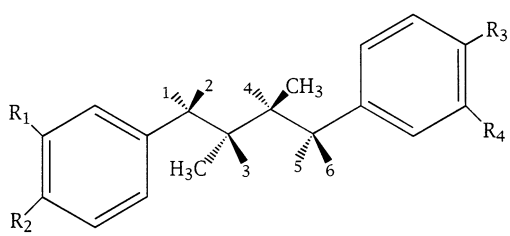

Fig. 1. Molecular structures of a plant lignan, nordihydroguaiaretic acid and its derivatives. NDGA = nordihydroguaiaretic acid 1,4-bis-(3,4-dihydroxyphenyl)-2,3-dimethyl butane, $C_{18}H_{22}O_4$, mol. wt. 302.37; R_1, R_2, R_3, R_4 are all OH; M_4N = derivative of NDGA; R_1, R_2, R_3, R_4 are all CH_3O; Tetra acetyl NDGA = R_1, R_2, R_3, R_4 are all $CH_3(C{=}O)O$; Mal.4 = derivative of NDGA R_1, R_2, R_3, are independently OH, R_4 is CH_3O.

2. Materials and methods

2.1. Reagents

The main reagents used in this study were plant lignan derivatives of NDGA that have been isolated and synthesized as described previously (Gnabre et al., 1995a, 1996; Hwu et al., 1998). The drugs used in this study are: 3-O-methyl NDGA (Mal.4), tetra methyl NDGA (M4N), and tetra acetyl NDGA (Fig. 1). Pure drug compounds were typically dissolved in 40–100% dimethyl sulfoxide (DMSO). Dilutions of the stock solutions were made in Dulbecco's modified Eagles (DME) medium and added to the cell culture growth DME medium with the final DMSO concentration being

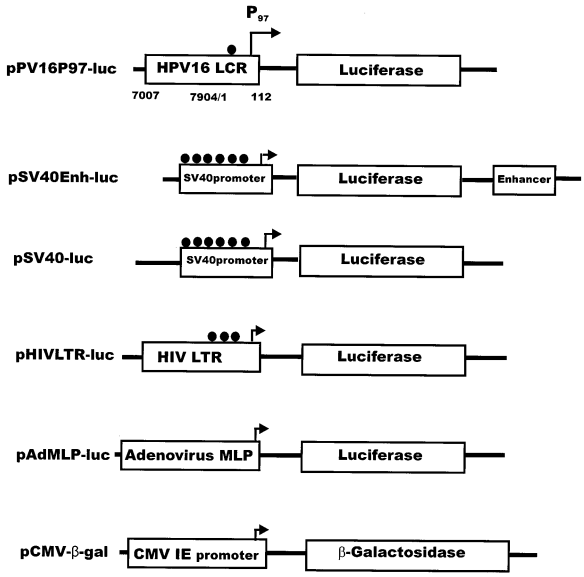

Fig. 2. Reporter expression vectors. PV16P97 contains the critical sequences for early gene expression and the entire enhancer element for human papillomavirus (HPV) 16. Simian virus 40 (SV40)Enh contains the SV40 early promoter, Sp1 DNA binding sites, and enhancer elements. SV40 contains only the early promoter and the Sp1 DNA binding sites. HIVLTR contains the cDNA of the U3 and R regions of the 3′ human immunodeficiency virus (HIV) long terminal repeat (LTR) and is known to be responsive to the tat protein. Adenovirus major late promoter (AdMLP) contains all the essential sequences for the adenovirus 2 major late promoter. Cytomegalovirus (CMV)-β-gal contains the sequences for the immediate early promoter and enhancer for hCMV. The ● symbol represents the location of the Sp1 DNA binding sites within the viral promoter and enhancer elements.

2%. Nordihydroguaiaretic acid (NDGA) and DMSO were purchased from Sigma, St. Louis, MO. Other molecular biological reagents such as restriction endonuclease enzymes and T4 DNA ligase were from New England Biolabs and Promega. Plasmid DNA was isolated and purified from bacterial cells by a procedure using alkaline lysis and column chromatography (Clontech).

2.2. Cells

The major cell line used in these studies is C33A, derived from a cervical carcinoma, and were obtained from the American Type Culture Collection (ATCC no. HTB31). The cells were grown and maintained in DME supplemented with 5% fetal bovine serum, 0.075% sodium bicarbonate, and 50 μg/ml gentamicin sulfate (Sigma).

2.3. Recombinant plasmids

Several of the recombinant plasmids used in this study were constructed in the laboratory (see Fig. 2). The reporter vector containing the luciferase gene into which the various promoters were inserted was pGL2 (Promega). Briefly, the HPV 16 long control region (LCR) containing the P_{97} promoter and the enhancer elements was subcloned out of a plasmid containing the entire genome of HPV16 (gifts from Drs zur Hausen and Dürst) by using restriction enzymes EcoO109/PstI and ligated into pGL2. The HIV-1 3′ long terminal repeat (LTR) was removed from pU3R-III CAT (AIDS Research & Reference Reagents Program no. 128) by XhoI and HindIII and was then ligated into pGL2. The adenovirus type 2 major late promoter was removed from pADβ (Clontech) by XhoI and SmaI and ligated into pGL2. The SV40 promoter (pGL2-promoter) and the SV40 promoter/enhancer (pGL2-control) constructs were from Promega. They were used without any modifications. The pCMV-β-galactosidase containing vector was from Clontech.

2.4. DNA transfection and drug addition

Purified recombinant plasmid DNA containing the various viral promoter luciferase reporter constructs were introduced into tissue culture cells by the calcium phosphate method (Ausubel et al., 1994). The cells to be transfected were seeded at 5×10^5 cells in a 35 mm well of a six well culture plate (Falcon). Generally 1–3 μg of DNA and $CaCl_2$ solution were added to HEPES buffered saline and the resulting precipitate was permitted to sit at room temperature for 5 min. The DNA phosphate precipitate was distributed over the cells and not removed until 18 h later. As the DNA was removed, various drug derivatives were

added at different concentrations with fresh medium. At 30 h post DNA removal the cells were lysed, cellular debris was centrifuged, and the supernatant was assayed for luciferase activity. The basic transfection experiments were completed at least three times with each drug concentration completed in triplicate. Protein measurements were made by the Bradford method (Biorad) and the luciferase activity was divided by protein concentration for a specific activity measurement. Further separate experiments were conducted with a co-transfection of a control plasmid pCMV-β-galactosidase (assayed as described by methods from Promega) in order to normalize for any apparent transfection efficiency differences and it was determined that repeated trials without co-transfection of control DNA gave comparable results to the normalized results.

2.5. Luciferase assay

The luciferase activity in the cell lysate was measured with the addition of substrate luciferin and the resulting light production was measured with the use of a scintillation counter (Nguyen et al., 1988). Each luciferase assay is the average of at least three transfections and each experiment was repeated at least three times. Protein measurements were accomplished by the Bradford method (BioLabs) and divided into the luciferase activity to provide specific luciferase activity. A number of experiments were conducted to examine for any transfection efficiency differences between cells in separate wells as measured by the addition of a second plasmid containing the β-galactosidase gene. The cell lysate was measured for luciferase, protein, and β-galactosidase. However, the main results of the drug effects were derived from repeated experiments and not normalized for transfection efficiency for all the data.

Purified luciferase (Roche) was used to determine the linear range of counting for the scintillation counter. Also, purified luciferase was used in experiments to rule out any effect of the drug derivatives interfering directly on enzyme activity under the conditions that are used to measure luciferase in the cell lysate.

2.6. Cell toxicity studies

C33A cells were exposed to various concentrations of drug derivatives dissolved in DMSO. Treated cell cultures were observed and trypan blue dye exclusion assays were performed on the cell cultures (Strober, 1997). Cell viability was determined and as some cell death ($< 15\%$) was noted at concentrations of 100 μg/ml the experiments were all conducted at levels below this concentration. An additional measurement of cell viability was determined by the use of the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma). The assay for the product formazan produced by living cells when treated with MTT was performed as in Carmichael et al. (1987). Spectrophotometric readings were performed using a plate reader, Spectra Max (Molecular Devices).

3. Results

3.1. Effect of Mal.4 on the HIV-1 LTR promoter activity

In order to attempt to compare the results of the plant lignan derivatives on HPV gene expression with the previous viral studies which showed effects on HIV and SV40 growth cycles, several viral reporter recombinants were made and tested in transfection experiments in the presence and absence of drugs. The HIV LTR fused to the luciferase gene within the pGL2 plasmid was found to have very low promoter activity as measured by the resulting luciferase activity when it was transfected into C33A cells. However, when this vector was co-transfected with an expression vector containing the HIV *tat* (AIDS Research and Reference Reagents Program) gene then the luciferase activity being driven by the LTR promoter was easily measured with a 150-fold increase over basal LTR activity without the *tat* gene expression vector (data not shown). When the NDGA derivative, Mal.4, was added to the co-transfected cells there resulted a considerable decline in the LTR promoted luciferase activity (see Fig. 3A). At the maximum amount of Mal.4

tested there was at least a 10-fold decrease in luciferase activity compared to controls with no drug added. Control experiments (data not shown) with pCMV-β-galactosidase and pCMV-luciferase (gift from Aldevron) determined that the CMV promoter is affected by Mal.4 less than 2-fold at the maximum concentration of drug used on transfected C33A cells.

3.2. Effect of Mal.4 on the SV40 early promoter

The SV40 early promoter contains six DNA binding sites for the cellular Sp1 transactivator and its strong promoter activity depends on their presence. The construct containing the SV40 early promoter and enhancer (pSV40Enh-luc) when transfected into C33A cells resulted in considerable luciferase activity (see Fig. 3B). This time when Mal.4 was added to the transfected cells, a 20-fold decrease in luciferase reporter activity was observed over the concentrations of Mal.4 used in the experiment. A second SV40 promoter luciferase reporter vector (pSV40-luc) lacking the enhancer elements was also used and transfected into the C33A cells. The luciferase activity as expected was less than that for the promoter enhancer construct (see Fig. 3C). Mal.4 again greatly inhibited the luciferase activity driven by the promoter, however, the final fold decrease was less than that observed for the promoter and enhancer vector (see Fig. 3B, C). It was further determined that there was no significant effect of the derivatives on the luciferase reaction at the maximum drug level that could be expected in the cell lysate (see Table 1).

3.3. The effect of lignan derivatives on the early promoter for human papillomavirus type 16, P_{97}

The above results indicated that the luciferase measurements could accurately reflect the activity of the promoter under the influence of the drug addition after DNA transfection. A series of lignan derivatives were tried out on the major early

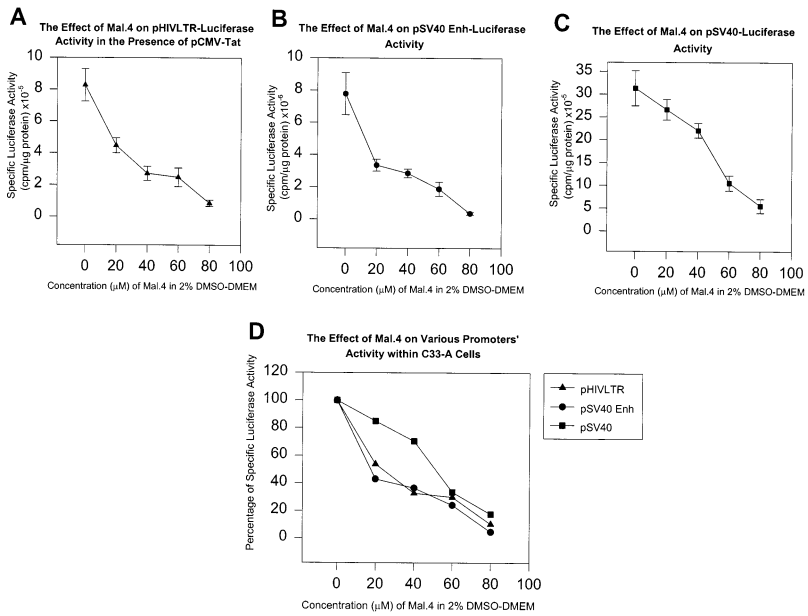

Fig. 3. The effect of Mal.4 on non-human papillomavirus (HPV) viral promoters. The viral promoter reported gene construct was transiently transfected into human C33A cells. Eighteen hours after transfection media and DNA were removed and fresh media containing various concentrations of drug in a final concentration of DMSO of 2% were added to the cells. Thirty hours after drug addition, the cells were lysed and asayed for luciferase activity and protein concentration (A) pHIVLTR-luciferase activity and the effect of Mal.4 in the presence of a co-transfection of an expression vector for Tat protein, pCMV-Tat. (B) pSV40ENH-luciferase activity on Mal.4 as a percentage of the reporter activity without any drug addition.

Table 1
The effect of Mal.4 on the luciferase reaction[a]

Mal.4 concentration (μM)	0	6	12
Luciferase activity (cpm × 10^6)	8.1	7.0	6.4

[a] Purified firefly luciferase enzyme was diluted and directly incubated with Mal.4 and DMSO. The concentrations of the drug were determined to be potential maximal amounts that could be present within the cell lysate during the actual luciferase reported assay carried out in the experiments. The experimental activities are the averages of triplicate determinations.

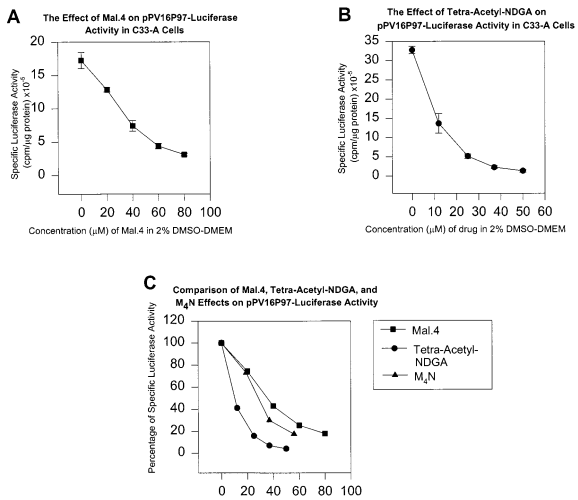

Fig. 4. The effect of three plant lignan derivatives of nordihydroguaiaretic acid (NDGA) on the early human papillomavirus (HPV) 16 P_{97} promoter and enhancer. (A) Mal.4 at various concentrations and its effect on the transfected promoter luciferase reporter vector in C33A cells. (B) Tetra-acetyl NDGA at various concentrations and the effect on transfected HPV promoter luciferase reporter vector in C33A cells. (C) Comparison of the three derivatives, tetra-acetyl -●-; Mal.4 -■-; M_4N -▲-; as a percentage of specific luciferase activity without drug addition. IC_{50}s were determined as: Mal.4 = 37 μM; M_4N = 28 μM; and tetra-acetyl NDGA = 11 μM.

promoter for the high risk oncogenic human papillomavirus type 16. The construct has the entire long control region (LCR) of the virus which includes the promoter P_{97}, and the enhancer elements (Gloss and Bernard, 1990; O'Conner et al., 1995). The recombinant which fuses the promoter to the luciferase gene was determined to have adequate activity within the C33A cells used in the drug studies. After DNA transfection and drug addition the cells were incubated in culture for 48 h. The cells were then lysed and assayed for luciferase activity and protein levels. Some cultures were also co-transfected with pCMV-β-galactosidase which was measured to assess transfection efficiencies among the various transfected cultures. Mal.4 inhibits the P97 promoter in a dose dependent manner with a greater than 5-fold reduction in activity as measured at the highest amount of drug tested (see Fig. 4A). When the M_4N derivative was used there also was a considerable drop in luciferase activity over the amounts of drug tested (see Fig. 4C). Tetra acetyl NDGA (see Fig. 4B) appears within this simple assay system to be the most potent as an inhibitor of HPV gene expression. There is an initial drop in promoter activity at the smaller concentrations as compared to the other derivatives. The main conclusion is that all of the derivatives show inhibition of promoter activity that warrants further investigation (Fig. 4C).

3.4. The effect of Mal.4 on the adenovirus major late promoter

Several control plasmids containing the luciferase gene or the gene for β-galactosidase were used to determine the specificity of the lignan derivatives. One recombinant reporter vector links the adenovirus major late promoter to the luciferase gene in pGL2. After plasmid DNA transfection and drug addition it was observed that the viral promoter was the least sensitive to the lignan derivative compared to all the others used in this study. There was less than a 2-fold decrease in promoter activity at the highest amount of drug tested (see Fig. 5).

3.5. Toxicity of lignan derivatives on the C33A cell cultures

Toxicity of the lignan derivatives on the C33A cell line used in this study was assessed by two independent methods. In Fig. 6 the results of the tetrazolium salt, MTT assay (Carmichael et al., 1987) are shown. The results measuring cell viability by the conversion of MTT to formazan by living cells surviving after the drug addition to the

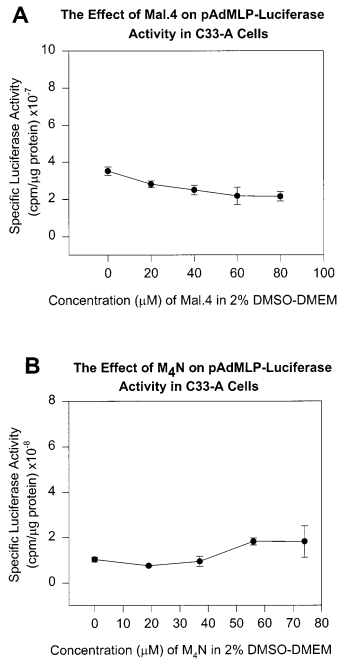

Fig. 5. The effect of Mal.4 and M_4N on the adenovirus type 2 major late promoter in C33A cells. The specific luciferase activity was measured after DNA transfection and various drug concentration addition.

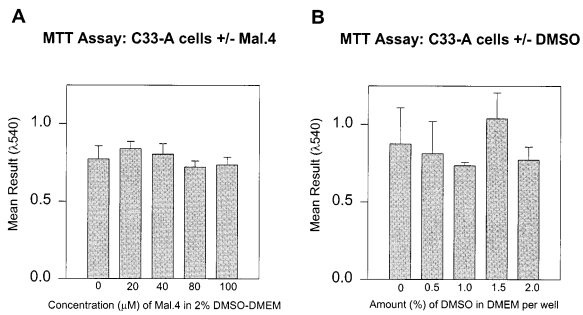

Fig. 6. The effect of Mal.4 and DMSO on C33A cell viability. C33A cells were seeded onto 96 well plates and treated with various amounts of Mal.4 in a final concentration of 2% DMSO (A) or with various concentrations of DMSO alone (B). Thirty hours after drug or DMSO addition, MTT solution was directly added to the media in each well. The cells were incubated at 37°C, 5% CO_2 for 4 h followed by removal of the media and MTT reagent. The resulting formazan product crystals produced by living cells were solubilized in 100% DMSO. Absorbance at λ 540 was determined and recorded. The results are an average of five replicates.

cells show (Fig. 6A) limited cell toxicity at the concentrations of drug tested. The effect on cell viability for different concentrations of DMSO on the cells was also measured (Fig. 6B) and determined to have limited impact on cell viability.

Trypan blue dye exclusion experiments were used to determine the effect of lignan derivatives on the cell cultures used in the DNA transfection and promoter studies. The NDGA derivatives were dissolved in DMSO (final concentration 2%) and added to tissue cultured cells at various concentrations from 10 to 250 µg/ml and left on the cells for 48 h. The dead cells were then counted after the addition of the dye. Approximately 10% of the cells within a culture were counted to be dead when 100 µg/ml of Mal.4 was added to the culture. At the maximum concentration of drug used in the effect on promoter studies no significant loss of cell viability was observed. A similar toxicity pattern was observed for M_4N, and tetra acetyl NDGA (data not shown).

The data suggest that for the C33A cell line there is a good correlation between the MTT assay and the dye exclusion assay in measuring cell viability at the drug concentrations used in the DNA transfection experiments.

4. Discussion

Previous work has indicated how potent the plant lignan NDGA derivatives are as anti-viral reagents against HIV, HSV, and SV40 (Gnabre et al., 1995a,b, 1996; Chen et al., 1998). The work also showed that the mechanism of action of inhibition by the drugs involves the cellular Sp1 transactivator protein and Sp1 DNA binding sites on the viral promoters. The results suggest that the compounds actually bind to DNA that prevents Sp1 interaction and thus interferes with Sp1 protein transactivation function in gene transcription. The LCR of human papillomaviruses contains just upstream of the major early promoter, a single Sp1 DNA binding site. Other studies using retroviruses to infect keratinocytes with HPV genes and regulatory regions have proven the importance of the Sp1 site for viral gene expression. Mutations in the site have a considerable effect on the high risk oncogenic HPV type 18

gene expression when tested in such a system whereas there is less of an effect found in the low risk virus HPV type 11 (Parker et al., 1997; Zhao et al., 1997). Hence, it became of great interest to examine the effects of the NDGA derivatives on HPV early gene expression. Since current in vitro systems for the HPV replication are not very convenient (Kreider et al., 1987; Meyers et al., 1992) for initial drug inhibitory studies, a cell system that depends on DNA transfection, drug addition, and subsequent luciferase reporter assay was developed in order to easily and quickly screen for potential drug effects on HPV gene expression.

At drug concentrations below those which appear stressful to the cell there is a potent and specific inhibition of HPV early gene expression. The inhibition of promoter activity with the most active drug derivative (tetra acetyl NDGA) is over 20-fold. That the drug inhibitory action on the HPV promoter and reporter system reflected on a good system to study such effects was suggested by the results on the viral promoters from viruses that are known to be inhibited by the drugs. The HIV experiments were slightly complicated in that they required the cotransfection of a Tat expression plasmid to obtain robust HIV LTR promoter function in the absence of any drugs. When the derivative Mal.4 was added there resulted in a sharp decline of 10-fold in HIV LTR function. In separate control experiments the effect of Mal.4 on the CMV promoter driving the Tat gene expression was less than 2-fold suggesting that the main effect of the drugs is on the LTR promoter function as shown with the viral studies. The next set of control vectors used were the SV40 promoter and enhancer elements or just the SV40 early promoter itself linked to the luciferase reporter gene. Both reporter constructs were highly repressed by the Mal.4 derivative. Here there are no complicating factors and these results further confirm the utility of the assay system in regard to being useful to screen for anti-papillomavirus activity.

The results with the Mal.4 derivative used with the SV40 promoter vectors also further supported previous work on the mechanism of drug action and also on Sp1 function. Sp1 is known to transactivate promoters that contain its GC rich DNA binding site. However, it may be especially active as an intermediary between upstream transcription factors and the RNA polymerase complex forming at the promoter (Tjian, 1994; Apt et al., 1996). The results reported here observed an increased effectiveness against the SV40 promoter that contained the enhancer elements as compared to the inhibition of the reporter being driven just from the SV40 early promoter itself. Since many viral promoters have enhancer elements near a viral promoter, those that use Sp1 as an intermediary from such upstream factors to interact with transcription complexes may be more sensitive to the drug action than those promoters with just the Sp1 sites acting as independent transactivating sites.

The adenovirus major late promoter (AdMLP) was originally used to help show the specificity of the inhibitory activity of the lignan derivatives toward the HPV early promoter. Although there was less than a 2-fold effect on the adenovirus promoter at the highest concentration of Mal.4 used, this was still surprising. Only very recently have the GC rich regions within the AdMLP been found to interact with Sp1 protein (Parks and Shenk, 1997). It was also determined that these sites are responsible for the effect of Sp1 protein on the promoter activity. Sp1 protein added to the AdMLP was found to stimulate the promoter a few fold, however, in the presence of the E1A protein a considerable activation of 200-fold could be detected. Thus the slight effect of drug on the AdMLP should be expected and the effect of the derivatives on AdMLP in the presence of E1A protein is currently being tested. The results reported here would also suggest that a promoter just having the DNA sites to bind Sp1 protein are not enough for large transactivation effects nor necessarily permit potent drug inhibition of promoter activity. The C33A cell line, unlike most cell lines derived from uterine cervical tumors, has been found not to contain any HPV DNA. The cells do contain mutations in

the tumor suppressor gene, p53 (Scheffner et al., 1991). C33A cells also have increased levels of Sp1 protein compared to fibroblasts or primary keratinocytes (Apt et al., 1996). While increased levels of Sp1 protein may have made higher levels of drug necessary to inhibit the HPV P_{97} promoter, the cell line is also very resistant to genotoxic assault which permitted large amounts of plant lignans to be added to the cells to show early gene inhibition. These cells were previously observed to be resistant to mitomycin C, cisplatin, and UV irradiation (Butz et al., 1995). However, the effect of the plant lignans on HPV16 early gene expression is not specific to the C33A cells as a very similar dose dependent inhibition of gene expression was observed in COS-1 monkey transformed kidney cells (data not shown).

Most cervical carcinoma tumors and derived cervical tumor cell lines have integrated viral DNA genomes into the host cell chromosomes with continuous expression of the E6 and E7 oncogenes (Schwarz et al., 1985; Yee et al., 1985; Smotkin and Wettstein, 1986; Howley, 1991). This continued unregulated expression of these genes is considered a crucial factor in cancer induction and maintenance of the transformed phenotype (von Knebel Doeberitz et al., 1992; Jeon and Lambert, 1995; zur Hausen, 1996). Early studies on such tumors and cells established that the early promoter region containing P97 remained intact within the viral DNA that had integrated into the cellular chromosomes (Smotkin and Wettstein, 1986). This previous work combined with the results presented in this study strongly suggest that the drug inhibitory activity may also be directed at tumor cells actively producing HPV gene products (Stanley, 1994).

The results in this study show that the plant lignans belonging to a new class of viral inhibitory reagents may be active anti-viral and anti-tumor drugs against human papillomaviruses and their induced tumors. The results also indicate that the cell based assay for HPV gene expression can be useful for more extensive testing of drug concentrations, time course of activity, and still more derivatives to provide for the best reagents to use in further animal tumor model systems and human therapy studies.

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