

METHOD FOR TREATMENT OF TUMORS USING NORDIHYDROGUAIARETIC ACID DERIVATIVES

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BACKGROUND OF THE INVENTION

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1. Field of the Invention

The invention relates to the use of nordihydroguaiaretic acid derivatives, in particular derivatives containing substituents of naturally occurring amino acids, for the treatment of tumors and viral infections.

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2. Background Information

Carcinogenesis is a multistage event affected by a variety of genetic and epigenetic factors and is typified by the outbreak of uncontrolled cell growth originated from different tissues. A universal goal for anticancer research lies in the development of a clinical treatment that is highly effective in curtailment of tumor growth, non-toxic to the host, and is affordable for most patients. Drugs that focus on the inhibition of targets that are unique to dividing cells should be effective chemotherapeutic agents without the risk of substantial side effects.

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Cells pass through many checkpoints as they proceed through the cell cycle. Certain criteria must be met in order to pass each of these checkpoints. In the G2/M transition, the most essential regulator is the cyclin-dependent kinase CDC2. This kinase binds tightly to the regulatory protein cyclin B, and this complex, also called the maturation promoting factor (MPF), is responsible for stimulating a myriad of events that lead to the cell's entry into early prophase (1): Not surprisingly, the loss or deactivation of either component of the MPF will block cellular progression out of G2.

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The expression and activity of the MPF is regulated at different levels. Cyclin B protein levels slowly rise through the G1 and S phases of the cell cycle, peak during the G2 to M phase transition, and drop sharply during mitosis (2). The CDC2 protein, on the other hand, is always present during the cell cycle, although levels rise slightly in the last stages of the G2 phase (3). The activity of the protein is dependent on the association with the appropriate cyclin, as well as on the dephosphorylation of its inhibitory sites by the phosphatase CDC25C (4,5). It has been shown that the failure of this dephosphorylation initiates G2 arrest in response to DNA damage by radiation or chemical action. Recent evidence also suggests that any remaining active CDC2 may be transported outside the nucleus following DNA damage (6).

A number of naturally occurring derivatives of the plant lignan nordihydroguaiaretic acid (NDGA) have been shown to block viral replication through the inhibition of viral transcription. This earlier work has shown that NDGA derivatives, originally isolated from *Larrea Tridentata* and subsequently synthesized chemically, can inhibit the production of HIV (7,8), HSV (9), and HPV transcripts (10) by the deactivation of their Sp1-dependent promoters. Unexpectedly, one of these derivatives, tetra-O-methyl NDGA, appears to also induce cell cycle arrest in mammalian cell lines. The evidence presented hereinbelow demonstrates that M₄N is capable of inducing G2 arrest in mammalian cells without detected toxicity, and supports the view that this arrest is due to the inhibition of the cyclin-dependent kinase CDC2.

Human papillomavirus (HPV) infection causes unregulated cell growth in many types of squamous epithelial cells, resulting in afflictions ranging from benign pallilomae (warts) to cervical, penile and mouth cancer. The strong association of these cancers with HPV and the widespread occurrence of infection denotes the importance of developing an anti HPV therapy.

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 the inhibition of HIV-1 have been reviewed (11).

Applicants reported earlier that 3'-O-methylated NDGA (i.e. Ma1.4),
5 isolated from Creosote bush (*Larrea tridentata*) can specifically block basal HIV transcription, Tat-regulated transactivation, and HIV replication in human cell culture(8, 12, 13). Ma1.4 exerts its effects by interfering with the binding of transcription factor Sp1 to the promoter of the HIV proviral template. The target of Ma1.4 is mapped to nucleotides -87 to -40, the Sp1 binding sites of
10 the HIV long terminal repeat (LTR). The unmodified NDGA, *in vitro*, does not inhibit HIV transcription and has no effect on Sp1 binding (8).

Isolation and purification of plant lignans, however, is labor intensive and costly. In anticipation of the possible clinical use of plant lignans in controlling Sp1-regulated viral and tumor growth in humans, nine different
15 methylated NDGA activities were synthesized chemically using unmethylated NDGA as the parent substrate in large quantities with low cost (7). At drug concentrations below 30 μ M, tetra-O-methyl NDGA was found to be most effective in the control of replication HIV via inhibition of Sp1 regulated proviral transcription and transactivation (7). This study has since been
20 extended to the control of the growth of Herpes simplex virus (HSV-1 and HSV-2) (9). Herpes simplex immediate early (IE) ICP4 gene is essential for HSV replication (14). Its promoter region possesses eight Sp1 consensus binding sites (15), five of which are required for ICP4 gene expression. It thus makes the ICP4 gene a good candidate for such testing. Applicants have found
25 that both 3-O-methyl NDGA (Mal. 4) and tetra-O-methyl NDGA (M₄N) are effective transcriptional inhibitors for HSV ICP4 gene expression in Vero cells via the blocking of Sp1 protein binding to the ICP4 promoter as shown by the electrophoretic mobility shift assay (9).

When the anti-HSV activities of M₄N and Mal. 4 were tested and
30 compared to that of acycloguanosine (acyclovir, ACV) in infected Vero cells, Applicants observed that the IC₅₀ for M₄N varied between 11.7 μ M to 4 μ M for 10 passages of HSV-1 and 4 passages of HSV-2 without obvious uprisings

trend for requirement of higher drug concentration. However, the IC_{50} for ACV increased from 7 μM for the first viral passage to 444 μM for the tenth passage of HSV-1 and to >88 μM for the fourth passage of HSV-2 indicating their rapid build-up of drug resistance against ACV in Vero cells.

- 5 Consequently, while the selective index, S.I. (TC_{50}/IC_{50}) remained relatively stable for M_4N , the S.I. for ACV dropped 60 fold following the viral passages in Vero cells (9). Thus M_4N is a mutation insensitive drug. It can inhibit ACV resistant HSV effectively (9).

Due to the fact that Sp1 is an important cellular transcription factor
10 (16), the possible inhibitory effect of this class of compounds on the expression of Sp1-regulated cellular genes should be addressed. $Ma1.4$ cannot displace Sp1 once it is stably bound to its binding sites (8). It therefore seemed likely that NDGA derivatives would have a greater effect on Sp1-regulated genes in proliferating cells than on the expression of
15 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 already stably bound at their promoters. This, in fact, has been shown
20 to be the case. As will be demonstrated below, by using gene array studies with 9600 expressed genes, Applicants found products of most Sp1 regulated genes remained at similar levels, and not affected by the drug treatment of cervical cancer cells C3 in culture (Figure 5). Even so, the relatively low selective index of M_4N certainly limits its use to the lowest effective
25 concentration if the drug must be used systemically. On the other hand, human papilloma virus induces solid cervical and oral tumors initially through the Sp1 regulated expression of HPV E_6E_7 genes (17). Applicants reasoned that if drug can be delivered *in situ*, and be kept only in the tumor area, the drugs of high concentration may be used to effectively destroy the tumor with
30 little damage to the patients.

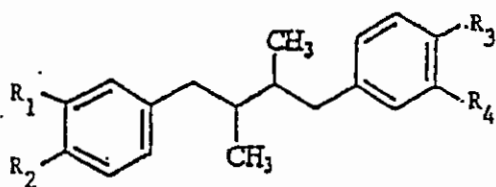
Survivin is an inhibitor of apoptosis that is abundantly expressed in many human cancers (35), but not in normal adult human tissue, and is considered a

possible modulator of the terminal effector phase of cell death/survival. (36).
 Survivin is expressed in G₂-M in a cell cycle-dependent manner, binding directly
 to mitotic spindle microtubules. It appears that survivin phosphorylation on Thr34
 may be required to maintain cell viability at cell division (37), and expression of a
 5 phosphorylation-defective survivin mutant has been shown to trigger apoptosis in
 several human melanoma cell lines (38). Phosphorylated survivin acts on the
 caspase pathway to suppress the formation of caspase-3 and caspase-9, thereby
 inhibiting apoptosis. (Ref. 39, page 10 presents an outline of apoptosis signalling
 pathways.) Thus, compounds that reduce the expression of survivin will be
 10 expected to increase the rate of apoptosis and cell death. CDC-2 has been shown
 to be necessary for survivin phosphorylation (37).

SUMMARY OF THE INVENTION

Accordingly, it is one object of the invention to provide compounds and
 15 compositions for use in the treatment of cancerous and noncancerous tumors
 in animals, particularly in mammals, and most particularly in humans.
 According to this aspect of the invention, novel nordihydroguaiaretic acid
 derivatives are provided that inhibit tumor growth.

By nordihydroguaiaretic acid derivatives is meant compounds of the
 20 structure



(I)

wherein R₁, R₂, R₃ and R₄ independently represent -OH, -OCH₃,
 -O(C=O)CH₃, or an amino acid residue, but are not each -OH
 25 simultaneously. Amino acid substituents are intended to include, *inter alia*,
 alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine,
 glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine,

proline, serine, threonine, tryptophan, tyrosine, valine, 5-hydroxylysine, 4-hydroxyproline, thyroxine, 3-methylhistidine, ϵ -N-methyllysine, ϵ -N,N,N-trimethyllysine, aminoadipic acid, γ -carboxyglutamic acid, phosphoserine, phosphothreonine, phosphotyrosine, N-methylarginine, and N-acetyllysine.

5 Particularly preferred compounds for use according to the invention are M_4N and G_4N , which are shown in Figure 1.

 It is a further object of the invention to provide a method for treating cancerous and noncancerous tumors by the use of these novel derivatives, and by similar derivatives that are known in the art, but have not heretofore
10 been used for the treatment of tumors. The method should be especially effective against rapidly proliferating cell types containing the cyclin dependent kinase CDC2. It is a further object of the invention to provide a method of inhibiting CDC2 in a eukaryotic cell cycle, particularly in an animal cell, more particularly in a mammalian cell, and most particularly in a human
15 cell.

 Tumors to be treated include any tumor that is sensitive to the above-mentioned compounds used according to the methods of the invention. In particular, this includes rapidly dividing cancerous and benign tumors that are sensitive to inhibition of the cyclin-dependent kinase CDC2 cycle.

20 The term "cancerous tumor" is intended to include any malignant tumor that may or may not have undergone metastasis. The term "noncancerous tumor" is intended to include any benign tumor. These terms are used as customarily understood by persons of skill in the art.

 Examples of benign and malignant tumors which may be treated by the
25 compositions and methods of the invention can be found in Table 1-1 of Cancer Biology (Raymond W. Ruddon, Cancer Biology, 3rd Ed., Oxford Univ. Press, 1995, incorporated herein by reference). Tumors to be treated include those that are known to be of viral origin, as well as those that are not of viral origin. The compositions and methods of the invention are expected to be
30 particularly useful in the treatment of solid tumors.

 It is yet another object of the invention to provide a method of inhibiting the cyclin-dependent kinase CDC2 cycle. This method will be useful in

inhibiting cell proliferation, particularly in rapidly dividing cell types.

In a preferred embodiment, the compounds and compositions described herein are used in the treatment of HPV-induced tumors. HPV-induced tumors include in particular, but are not limited to, cervical, oral,
5 penile and head and neck cancers that are associated with HPV infection.

The method comprises local application of nordihydroguaiaretic acid derivatives, in particular tetra-O-methylnordihydroguaiaretic acid (M₄N) and tetraglycinal nordihydroguaiaretic acid (G₄N), to cancerous and non-cancerous HPV-induced tumors.

10 It is yet another object of the invention to provide a method of inhibiting viral replication and growth by the administration of the compounds of formula I containing amino acid substituents. Preferred for use in this method are compounds in which the amino acid substituents R₁, R₂, R₃ and R₄ are identical.

It is a further object of the invention to provide a method of inhibiting
15 survivin production in a eukaryotic cell cycle in a cell that expresses survivin, particularly in a cancer cell. The inventors have found that the nordihydroguaiaretic acid derivatives of the invention downregulate survivin mRNA and protein levels and activate both CDC-2 and the caspase pathway, thereby increasing the level of apoptosis in cell populations where survivin is
20 expressed. This method should provide a treatment for cancers where survivin is expressed by suppressing or eliminating survivin expression, thereby increasing the rate of apoptosis.

It is contemplated that M₄N, G₄N and other derivatives will be administered by local injection into the tumors, generally along with
25 pharmaceutically acceptable diluents, excipients and carriers. In preferred embodiments, M₄N is injected into tumors in the form of a DMSO solution, and G₄N is administered in PBS solution. The use of G₄N will complement the use of M₄N, particularly in larger tumors (> 2 cm³), due to its water solubility, which allows it to spread to a larger region of the tumor. Other
30 water-soluble and water-insoluble nordihydroguaiaretic acid derivatives can be similarly employed, according to the invention. These may also be employed in lipid based formulations for systemic delivery, as known and

used in the art.

By pharmaceutically acceptable diluents, excipients and carriers is meant such compounds as will be known to persons of skill in the art as being compatible with M₄N, G₄N and other similar derivatives and suitable for local
5 administration to a human or other mammal according to the invention.

Although the examples hereinbelow describe administration by means of local injection, other means of local administration, such as topical application or targeted delivery to the tumor site, may also be used.

The amount of compound administered to obtain the desired treatment
10 effect will vary but can be readily determined by persons of skill in the art. The amount of dosage, frequency of administration, and length of treatment are dependent on the circumstances, primarily on the size and type of tumor. However, dosages of from 10 mg to 20 mg of either M₄N alone or with similar
15 amounts of G₄N per gram tumor weight at intervals from daily to weekly or less frequently may be mentioned for purposes of illustration. Administration of 50 μ l to 100 μ l of M₄N dissolved in DMSO at a concentration of 200 mg/ml, either alone or in combination with G₄N, is expected to be effective in many cases for tumors of 1-1.5 cm³.

20 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Structures of M₄N and G₄N

Figure 2A. HPV-16 LCR showing region of E₆/E₇ promoter (pPV16P97) and the
25 binding site for Sp1 protein.

Figure 2B. The effect of M₄N on the E₆/E₇ promoter activity in C-33A cells. (Inhibition of E₆/E₇ promoter driven luciferase gene transcription by different concentration of M₄N)

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Figures 3A-3C. Inhibition of Viral E₆ and E₇ RNA Transcripts by 40 μ M M₄N. Total RNA isolated from C₃ cells treated with either 40 μ M M₄N or

DMSO alone in growth media for 71 hours was subjected to relative RTPCR. The RTPCR samples were removed after increasing cycles of amplification and resolved on an agarose gel. The gel photographs (3A and 3B) indicate these cycles, the presence of (+) or absence (-) of M₄N in the growth media, and two digests of a pGMT vector used as size markers. The amplification map (2C) indicates the two expected size products of the amplification, resulting from the alternate splicing of the early viral RNA transcript.

Figure 4A. Inhibition of C3 Cell Growth by M₄N.

Figure 4B. Inhibition of C3 Cell Growth Following the Removal of M₄N.

Figures 5A-5B. Effect of M₄N on gene expression in C3 cells as examined by the GENE Assay analysis. 5A. GENE expressed in C3 cells after > 2 hours of DMSO treatment (C3 DMSO). 5B. GENE expressed in C3 cells after > 2 hours of M₄N treatment using DMSO as solvent (C3 M₄N).

Figures 6A-6B. Visual observations of tumor-bearing mice following M₄N treatment. 6A. Mice bearing single tumors were treated with *in situ* injection of DMSO (#3) or M₄N (#7). *In situ* injection of M₄N was also made to one of the two tumors grown in mouse #9. 6B. M₄N treated tumor (white scar) with untreated tumor from the same mouse, #9 as described in Table 2.

Figure 7. Histopathology Effect of M₄N and M₄N/G₄N on Tumor Growth in Mice. First column from the panel presents the large size of tumors from mouse #4, 10, 12, following DMSO treatment (CON) as compared to the relatively small drug treated (M₄N or M₄N/G₄N) lesions from mouse #12, 10, 27 and 20 (M₄N). The subsequent photographs are examples of these tumors examined at 100X magnification (A, B, C, DMSO treated, D untreated, E, F, G, H, M₄N or M₄N/G₄N treated) mice (Table 1 and Table 2).

Figure 8. HSV-1 replication in the absence of drugs (HSV-C, HSV-SC), in the

presence of ineffective drugs (ABDS₁ ["HSV-ABDS₁"], ABDS₂ ["HSV-ABDS₂"]) and in the presence of effective drugs (M₄N ["HSV-4N"] and ACV ["HSV-ACV"]).

- 5 Figure 9. M₄N Causes Growth Arrest in Mammalian Cells. (a-d) C3, CEM-T4, C33a, and TC-1 cells were treated with different concentrations of M₄N. The number of cells present at the initiation of the experiment is indicated as Day 0. After three days the number of viable cells were counted and plotted versus the M₄N concentration. (e) C3 cells were split into T-25
10 flasks with 5×10^3 cells per flask and given either M₄N in 1% DMSO in media or 1% DMSO in media alone (first media change). After 3 days, one-half of the M₄N treated cells were given fresh media containing only 1% DMSO (M-D), while the rest of the cells were given fresh media with the same conditions (second media change). The cells were counted daily and plotted versus the
15 time of treatment.

- Figure 10. Cells Treated With M₄N Arrest in G2/M. C3 cells (a), C33a cells (b), CEM-T4 cells (c), and TC1 cells (d) were grown for three days in media containing either 1% DMSO or 1% DMSO with M₄N (M₄N). The cells
20 were trypsinized, fixed with ethanol, stained with propidium iodide, and were subsequently analyzed by flow cytometry. The data is displayed as number of cells ($3-5 \times 10^4$ total cells) versus propidium iodide stain intensity. The indicated stages of the cell cycle are labeled and correspond to the relative cellular DNA complement as determined by staining intensity.

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- Figure 11. C3 Cells Treated With 40 μ M M₄N Demonstrate G2 Cell Structures. C3 cells were grown on coverslips for three days in media containing either 1% DMSO (Control) or 1% DMSO with 40 μ M M₄N (M₄N). Samples were fixed with ethanol and incubated with antibodies against α (green) and γ (orange) tubulin (a)
30 or with the DAPI DNA stain (b). Cells were examined by fluorescence microscopy.

Figure 12. CDC2 and Viral Oncogenes are Reduced by M₄N. C3 cells were grown for different amounts of time (numbers are in hours) in media containing either 1% DMSO (D) or 1% DMSO with 40 μ M M₄N (M). After the specified times, total protein or total RNA was isolated from the cells.

- 5 Western blots (a - top two panels) were performed using antibodies against CDC2 or cyclin B with the same nitrocellulose filter. Kinase assays (a - bottom two panels) were performed, following immunoprecipitation with antibodies to cyclin B, by incubation with γ -³²P ATP and histone H1. The coomassie stain of the PAGE gel is included as control for loading.
- 10 assays for 24 and 72 hour drug treatments were performed separately. Northern blots (b) were performed on total RNA extracts. Filters were incubated overnight with random-primed ³²P-labeled DNA for CDC2 or GAPDH, washed, and exposed to film for three days. The same filter was used to test CDC2 and GAPDH RNA. rtPCR analysis (c) was performed on total RNA extracts with
- 15 primers hybridizing to regions within either HPV-16 E7 or GAPDH. Both primer pairs were used in the same reactions, and the products were analyzed by agarose gel electrophoresis.

- Figure 13. Electrophoretic mobility shift assay (EMSA) of G₄N interaction with the HIV Sp1-binding sites (-87 to -49). (A) G₄N inhibition of Sp1-167D binding to ³²P labeled HIV Sp1 DNA template. Lane 1, template alone; lane 2, template plus 0.1 μ g Sp1-167D; lanes 3-9, template incubated with increasing concentrations of G₄N (0.25 to 1.75 mM prior to the addition of 0.1 μ g Sp1-167D. (B) G₄N displacement of Sp1-167D bound to HIV template. Lane 1, template alone; lane 2, template plus 0.1 μ g Sp1-167D plus 100-fold excess of unlabeled template; lane 3, template plus 0.1 μ g Sp1-167D; lanes 4-10, Sp1/DNA complex challenged with increasing concentrations of G₄N (0.25 to 1.75 mM); lane 11, template incubated in reaction buffer containing 1.75 mM G₄N. (C) Sp1-167D displacement of G₄N bound to template. Lane 1, template alone; lanes 2-4, template plus increasing amounts of Sp1-167D (0.075, 0.150, 0.300 μ g); lanes 5-8, template incubated in reaction buffer containing
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 - 25
 - 30

1.2 mM G₄N followed by challenge with increasing amounts of Spl-167D (0.075, 0.150, 0.300 µg), lane 8 received no Spl-167D. (D) Plot of diminishing Spl-167D/DNA complex band intensities in response to increasing concentrations of G₄N used in (A) ---•--- and (B) —•—. The gels used were 5% non-denaturing polyacrylamide with each lane receiving 5 µl of each reaction volume as described in experimental section and Ref. [1].

Figure 14. Inhibition of HIV Tat-regulated transactivation in Cos cells by G₄N.

Figure 15. SIV production with presence of G₄N. 10⁷ 174 x cells, a human T-cell lymphoma cell line, were mixed with a 24 hrs. harvest stock of SIV mac 239 (4 ng of p27) for two hours at 37. Cells were resuspended and 1 x 10⁵ cells in 100 µl medium were added to each well of three 96-well plates. Various concentrations of G₄N from a freshly made stock were prepared and added to each of the six designed well. Culture supernatants were collected after four and eight days for viral production analysis. Viral production was assayed by a modified p27 capsid protein antigen capture ELISA as described in experimental section.

Figure 16. Inhibition of HIV p24 antigen production in H9 cells by G₄N.

Inhibition in percentage was calculated by comparing p24 level from an average of two duplicate cultures of G₄N treated and not treated H9 cells 9 days following viral infection with a AZT resistant HIV strain, HIV-1RTMF.

Figure 17. RT-PCR Analysis of Survivin Gene Expression. (a) Top: Survivin gene expression in C3 cells treated with 40 µM M₄N for 24 hours and 72 hours, respectively (lanes 3 and 4) and in the untreated controls (lanes 1 and 2). Bottom: the corresponding GAPDH controls. Band intensities were quantitated with Scion Image. (b) Survivin RT-PCR product signals were normalized to those of the GAPDH controls and plotted.

Figure 18. Drug concentration-dependent down-regulation of survivin protein. (a) C3 cells were incubated with various concentrations of M₄N for 72 hours and the

total cell lysate was immunoblotted against survivin. (b) Relative band intensities were quantitated by Scion Image and plotted against M₄N concentration.

Figure 19. Immunoblot analysis of caspase-3 cleavage in C3 cells treated with M₄N for 72 hours. (a) Western blot of caspase-3 showed cleavage of the 32 KD procaspase-3 and the formation of the active 20 KD cleaved product. (b) Band intensities were quantitated and plotted against M₄N concentration.

DETAILED DESCRIPTION OF THE INVENTION

Experimental Methods

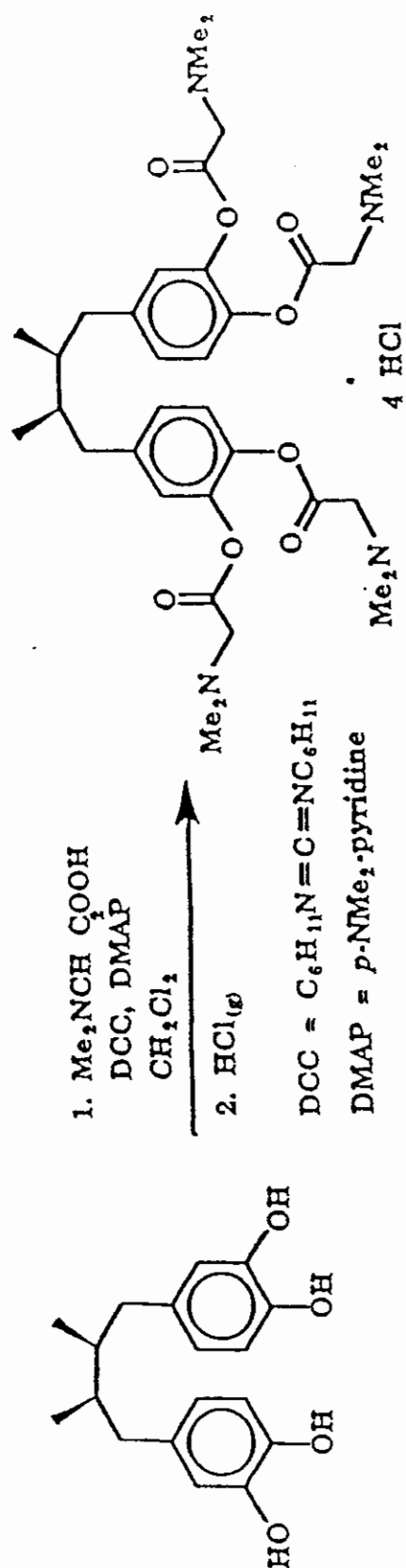
NDGA derivatives were synthesized chemically (7). Cell line C3 is a HPV16E + L plus activated Ras transformed cell line of C57 BL/6kh origin provided by W. Martin Kast of Loyola University Medical Center, Chicago, Illinois, U.S.A. It is maintained and cultivated as described by Greenstone et al. (18) and Feltkamp et al. (19, 20).

Synthesis of G₄N:

Standard Procedure for the Preparation of *meso*-1,4-Bis [3,4 - (dimethylaminoacetoxymethyl) phenyl]- (2R,3S)- dimethylbutane Hydrochloride Salt Tetraglycinyl NDGA, G₄N. To a dichloromethane (250 ml) solution containing NDGA (12.8 g, 42.3 mmol, 1.0 equiv) and N,N, -dimethylglycine (26.2 g, 254 mmol, 6.0 equiv) were added DCC (52.4 g, 254 mmol, 6.0 equiv) and DMAP (2.32 g, 18.9 mmol, 1.0 equiv). The reaction mixture was stirred for 24 h under nitrogen at room temperature. After the reaction mixture was filtered, the solution was concentrated under reduced pressure. Acetone (250 ml) was then added into the reaction flask and the solution was bubbled with excess HCl(g). The water-soluble precipitate was dissolved in H₂O and re-precipitated twice at room temperature from acetone to give (1) (29, 2 g, 36.8 mmol) as a white solid in 87% yield. Proton NMR spectra were obtained on a Varian Unity-400 (400 MHz) spectrometer by use of D₂O solvent and TSP as Internal standard. Carbon-13 NMR spectra were obtained on a Varian Unity-

400 (400 Mhz) spectrometer by use of D₂O as solvent. Carbon-13 chemical shifts are referenced to the TSP singlet (δ0.0 ppm).

The synthesis is depicted in Scheme 1.



General Procedure. All reactions were carried out in oven-dried glassware (120°C) under an atmosphere of nitrogen, unless as indicated otherwise.

Acetone, dichloromethane, 1,4-dioxane, ethyl acetate, hexanes, and

5 tetrahydrofuran were purchased from Mallinckrodt Chemical Co. Acetone was dried with 4A molecular sieves and distilled. Dichloromethane, ethyl acetate, and hexanes were dried and distilled from CaH_2 . 1,4 -Dioxane and tetrahydrofuran were dried by distillation from sodium and benzophenone under an atmosphere of nitrogen. Nordihydroguaiaretic acid was purchased
10 from Fluka Chemical Co. N,N' -Dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), morpholine, triethylamine, and potassium carbonate were purchased from Merck Inc. 1 -Bromo-3-chloropropane, N,N -dimethylglycine, and methylphosphorodichloridate were purchased from Aldrich Chemical Co.

15 Analytical thin layer chromatography (TLC) was performed on precoated plates (silica gel 60 F-254), purchased from Merck Inc. Gas chromatographic analyses were performed on a Hewlett-Packard 5890 Series II instrument equipped with a 25-m cross-linked methyl silicone gum capillary column (0.32 mm i.d.). Nitrogen gas was used as a carrier gas and the flow rate was kept
20 constant at 14.0 ml/min. The retention time (t_R) was measured under the following conditions: injector temperature 260°C, isothermal column temperature 280°C. Gas chromatography and low resolution mass spectral analyses were performed on a Hewlett-Packard 5890 Series II instrument equipped with a Hewlett-Packard 5971A Mass Selective Detector and a
25 capillary HP-1 column. Separations by medium-pressure liquid chromatography (MPLC) were performed at a flow rate of 120 ml/h by use of a Jasco Model 880-PU intelligent HPLC pump. The MPLC packing material, Reversed Phase Silica Gel C18 (particle size 0.035-0.070 mm), was purchased from Knauer Co. Purification by gravity column chromatography was carried out
30 by use of Merck Reagents Silica Gel 60 (particle size 0.063-0.200 mm, 70-230 mesh ASTM).

Infrared (IR) spectra were measured on a Bomem Michelson Series

FT-IR spectrometer. The wave numbers reported are referenced to the polystyrene 1601 cm^{-1} absorption. Absorption intensities are recorded by the following abbreviations: s, strong; m, medium; w, weak. Proton NMR spectra were obtained on a Varian Unity-400 (400 MHz) spectrometer by use of D₂O as solvent and 3-(trimethylsilyl)propionic acid, sodium salt as internal standard. Carbon-13 NMR spectra were obtained on a Varian Unity-400 (100 MHz) spectrometer by use of D₂O as solvent. Carbon-13 chemical shifts are referenced to the center of the 3-(trimethylsilyl)propionic acid, sodium salt singlet (6 0.0 ppm). Multiplicities are recorded by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; J, coupling constant (hertz). High-resolution mass spectra were obtained by means of a JEOL JMS-HX110 mass spectrometer.

meso-1,4Bis[3,4(dimethyleminoacetoxy)phe 3S-dimethylbutane Hydrochloride Salt (2). To a solution of NDGA (1, 12.81 g, 42.37 mmol, 1.0 equiv) and N,N-dimethylglycine (26.21 g, 254.2 mmol, 6.0 equiv) in dichloromethane (250 ml) was added DCC (52.45 g, 254.2 mmol, 6.0 equiv) and DMAP (5.176 g, 42.37 mmol, 1.0 equiv). The reaction mixture was stirred for 24 h under nitrogen at room temperature. After dicyclohexylurea in the reaction mixture was filtered off, the resultant solution was concentrated under reduced pressure. Acetone (250 ml) was then added into the residue and the resultant solution was bubbled with excess HCl (g). The precipitate was dissolved in water and re-precipitated twice by use of acetone at room temperature to give 2 (28.97 g, 36.86 mmol) as a white solid in 87% yield: ¹H NMR (D₂O, 400 MHz) δ 0.78 (d, J = 6.0 Hz, 6 H, 2 x CH₃), 1.73 (m, 2 H, 2 x CH), 2.38 (dd, J=13.2, 9.6 Hz, 2 H, 2 x ArCH), 2.78 (dd, J = 13.2, 4.4 Hz, 2 H, 2 x ArCH), 3.03 (s, 24 H, 8 x CH₃N), 4.53 (s, 8 H, 4 x CH₂N), 7.22 (m, 4 H, 4 x ArH), 7.29 (d, J= 8.4 Hz, 2 H, 2 x ArH); ¹³C NMR(D₂O, 100 MHz) δ 18.11, 40.82, 41.73, 46.75, 59.59, 125.79, 126.58, 131.63, 140.66, 142.47, 146.11, 167.84; IR (KBr) 3461 (br), 2963 (m), 1777 (s, C=O), 1620 (m), 1478 (m), 1377 (m), 1210(m), 1106 (m), 961 (w), 852 (w) cm^{-1} ; MS (FAB) of (2-4 HCl) m/z (relative intensity) 643 (M⁺, 30), 600 (20), 558 (43), 515 (20), 473 (42), 430 (13), 388 (26), 185 (18), 93 (38), 58 (100), 44 (22); HRMS (FAB) of

(2 - 4 HCl) calcd for $C_{34}H_{50}N_4O_8$ 642.3628, found 642.3614; Anal. Calcd for $C_{34}H_{54}N_4O_8Cl_4$: C, 51.78; H, 6.90; N, 7.10; O, 16.23. Found: C, 51.70; H, 6.85; N, 7.05; O, 16.21.

It will be appreciated that by suitable substitution of other N,N-dimethyl-substituted amino acids, additional amino acid substituted compounds of the invention can be synthesized.

Example 1

Effect of M_4N and several other NDGA derivatives of SP1-regulated HPV E_6/E_7 promoter activity.

The effect of M_4N and several other NDGA derivatives of SP1-regulated HPV E_6/E_7 promoter activity was examined using luciferase as a reporter. The assay depends upon DNA transfection of the HPV16 LCR (P_{97} promoter) fused to the luciferase reporter gene into C33A cells by calcium phosphate methods. C33A is a cervical tumor cell line (ATCC accession no. HTB-31) that does not contain any integrated HPV DNA, but has transcription factors necessary for a robust expression of the HPV early gene promoter. One day following DNA transfection various drug concentrations dissolved with the help of dimethyl sulfoxide (DMSO) were added to the cells. Thirty hours after drug treatment (so that the assay is complete within the standard forty-eight hours for transient transfection experiments), the cells were lysed and specific luciferase activity was determined (Luciferase Assay Systems, Promega, U.S. Pat. No. 5,283,179). As the M_4N drug concentration was increased the specific luciferase activity decreased.

The results (shown in Figure 2) demonstrate that M_4N dramatically reduces Sp1 regulated transcription initiation at the HPV E_6/E_7 promoter in luciferase assay.

Example 2

Inhibition of E_6/E_7 mRNA synthesis following M_4N treatment

Inhibition of E_6/E_7 mRNA synthesis following M_4N treatment was

measured by RT-PCR in cervical cell line C3. Relative RT-PCR was performed with quantities of total cellular RNA standardized to the cell numbers counted. The RT-PCR product was analyzed on a 2% agarose gel. The results are shown in Fig. 3. The RT-PCR results indicated that the amplified cDNAs of the expected size for E7 (321 bp) and E6 (204 bp) were detected in the DMSO treated cells as early as cycle 22 of amplification. These same products were barely detectable in the drug treated RNA extracts following 30 cycles of amplification. No amplified products were detected for the no template PCR control or from total RNA extracts of the HPV16-negative C33a cell line.

Example 3

Inhibition of cervical C3 cell growth by M₄N treatment

HPV-16 transformed immortal mouse epithelial cells (C3 cells) were plated at a density of 10^5 cells per vial. After 24 hours, $\frac{1}{2}$ of the vials were given growth media containing 40 μ M M₄N dissolved in 1% DMSO while the other half were given growth media containing only 1% DMSO. The results are shown in Figure 4A. Within 24 hrs a difference in cell morphology between drug treated and control C3 cells was observed. The growth and division of the drug treated cells was markedly reduced in comparison to the untreated control, while the fraction of viable cells compared to the total cell count remained constant for both drug treated and DMSO only control cells. This indicates that M₄N dramatically reduces cell division.

The effect on C3 growth following removal of M₄N from the medium was also examined. C3 cells were plated at a density of 10^4 cells per vial. At time=0, $\frac{2}{3}$ of the vials were given growth media supplemented with 40 μ M M₄N in 1% DMSO. The remaining vials were given growth media containing only 1% DMSO. After 73 hours, $\frac{1}{2}$ of the vials that had received M₄N in their growth media were washed and media containing only 1% DMSO was added. The other $\frac{2}{3}$ of the cell vials were washed and replaced with the same media administered before. The results, shown in Figure 4B, indicate that the rate of cell growth was not notably increased in M₄N treated sample following the

change to drug-free media, indicating that M₄N continues to significantly reduce cell division even after its removal from the extracellular environment.

Example 4

- 5 Analysis of cellular gene expression in C₃ cells before and after 72 hrs of drug treatment.

Gene expression with 9600 gene arrays was studied (Fig. 5). Five micrograms each of poly A⁺ RNA from 72 hrs. M₄N (40 μm) treated (C₃ M₄N) and non-treated (C₃ DMSO) was used in a pair of human 9600 gene array
 10 hybridization study according to the procedure described in Genomics 51, 313-324 1998. The hybridization image was captured by a color video camera with a Nikon 55 mm AF micro Niko lens and digitized by a Macintosh LC630 computer. Such detection via enzyme substrate reaction of color-forming enzymes in either single or dual-color mode is reproducible and
 15 extremely sensitive (can detect <5 copies of transcript per cell with RNAs from 10⁷ cells).

The computer print outs showing differentially expressed genes (C₃ M₄N/C₃ DMSO >10 and C₃ DMSO/C₃ M₄N >10) were listed for examination. Image files in TIFF format and data files in MS excel format are kept on ZIP
 20 diskette. Gene names and clone ID numbers are available for obtaining Image clones for future northern blot confirmation.

Among a group of genes that are either up-regulated or down-regulated 72 hrs after M₄N treatment, the following are those specifically related to cell division and apoptosis. Several other cell cycle
 25 related genes are also greatly upregulated in response to M₄N. In addition to cyclin-dependent kinase CDC2 (Example 11), for example:

	Increase
Cyclin-dependent kinase inhibitor	(100X)
Apoptosis (APO-1) antigen	(100X)
30 Death Domain Three DR ₃	(100X)

Ras-related protein RAP-1 (60X)

Human Map Kinase (40X)

The following cell cycle related genes are greatly downregulated in response to M₄N:

5		Treated	Untreated
	Cyclin-dependent kinase 7	(5%)	100%
	Human cytokine receptor	(2%)	100%
	Proliferating cell nuclear antigen, PCNA	(1%)	100%
	Human TNF-related Apoptosis AP0 ₂	(3%)	100%
10	Cysteine protease	(7%)	100%

At earlier time points, such as after one hour drug treatment, E₆/E₇ level was found to be similar with those in control cells while after 4.5 hrs, E₆/E₇ were no longer detectable by RT-PCR (10). Gene expressions with 9600 gene arrays can be repeated with RNA isolated from these short-time treated cells (1 hour and 5 hours) in order to further pin down the initial cellular effects of the drug.

Example 5

20 Targeting C3 tumor growth in mice by local injection of M₄N

Thirty six C57bl-16 NCR mice were injected with 5×10^5 C3 cells between the shoulders on the backs of the mice. Twenty four of the mice developed tumors within 20 days. Daily injection (50 μ l-100 μ l of M₄N or M₄N/G₄N) (200 mg/ml M₄N in DMSO, 200mg/ml G₄N in PBS) showed profound effect in tumor growth in animals, as shown in Tables 1 and 2, Fig. 6 and 7.

Table 1. M₄N and G₄N Effect on Growth of Single Tumors Developed in Mice

Mouse #	Treatment Period Days 1-16	Lesion Size (mm)			Wt. Of Excised Lesion (g)		Body wt. (g)		
		Day 1	Day 7	Day 21	Day 16	Day 24	Day 1	Day 16	Day 24
1	DMSO*	3x8x3.3	—	5x7x4	—	0.3	18.8	—	20.2
2	DMSO	4.4x6x3.5	10x12x8	—	1.56	—	19.6	20.5	—
3	DMSO	0.8x0.8x1	—	10.5x11x9	—	1.14	18.2	—	16.1
4	DMSO	2.8x3.8x2.5	—	18x11x9	—	2.9	17.6	—	20.2
6	Days 1-16 M ₄ N	—	9x8x5	—	0.2	—	19	19.2	—
7	M ₄ N	—	6x7x7	—	—	0.1	18.2	—	20.4
11	M ₄ N	1x1.3x1	9.5x10x9	—	—	0	19.5	—	20.2
14	M ₄ N	3.8x3.8x3.5	8x9x6	—	0.4	—	17	17.6	—
15	M ₄ N	—	5x4x4	—	0.1	—	18.9	20.0	—
16	M ₄ N	2.8x2.8x2.8	9x6x4	—	0	—	17.2	17.6	—
17	M ₄ N	2.3x2.3x2.3	6x6x4	—	0.2**	—	17.3	—	—

	Days 1-10	Days 9-17								
18	M ₄ N	G ₄ N	3x2.8x3	8x7x5	—	—	1.0***	18.8	—	21.1
19	M ₄ N	G ₄ N	—	5x5x5	—	0.2	—	18.2	19.9	—
21	M ₄ N	G ₄ N	1.8x1.8x1.8	9x10x5	—	0.2	—	17.3	19.2	—
22	M ₄ N	G ₄ N	—	7x7x5	—	—	0	17.9	—	19.5
27	M ₄ N	G ₄ N	2.5x5x2.5	9x6x6	—	—	1.8***	20	—	20.7
28	M ₄ N	G ₄ N	2.8x2.3x2.8	5x5x4	—	0.17	—	18.1	19.8	—
29	M ₄ N	G ₄ N	2.8x2.5x2.8	5x6x4	—	—	0.2	18.8	—	19.6

*DMSO = Vehicle for Drug

** Taken on Day 15

*** Lesion contained mostly necrotic cells as also found in lesions from mouse 6, 7, 11, 14, 15, 17, 19, 21, 28, 19 (Fig. 6,7). There were no lesions left in mouse #11 and #22 following drug treatments. Tumors found in control mouse #1, 2, 3, 4 contained growing cells (Fig. 2).

Experimental Procedures:

36 C57bl-16NCR mice were injected with 5×10^5 C3 cells/mouse. Injections were 100 μ L made subcutaneously between the shoulders on the backs of the mice. Cells were suspended in low-salt HBSS and suspension uniformity was maintained by gentle vortexing.

24 mice developed tumors. Their lesion sizes were measured by dial caliper. These mice were shaved, weight and treatment begun (Day 1). Four mice were sequestered as controls. Control mice received 50 μ l DMSO injected intratumorally daily. Experimental mice (10) received 50 μ l M₄N dissolved in DMSO(200mg/mL). An additional 10 mice received M₄N treatments for 8 days followed by G₄N treatments (50 μ l, 200mg/ml in PBS) daily for 8 days. Injections were made to several regions of tumor. Mice

anesthetized with ether or metaphane prior to injection.

Table 2. M₄N and G₄N Effect on Growth of Treated Lesions in Mice Carrying Multiple Tumors

Mouse #	Treatment Period		Lesion Size (mm)		Wt. Of Excised Lesion (g)		Body wt. (g)	
	Days 1-16		Day 1	Day 7	Treated*	Not Treated**	Day 1	Day 24
9	M ₄ N		1.3x5x0.75	7x9x8	0.25	0.6	20.2	17.9
10	M ₄ N		2.3x2.5x2.3	9.5x10x9	0.1	2.9	17.5	22.1
12	M ₄ N		2.5x2.5x2.5	8x9x6	0.11	1.82	17.8	20.0
	Days 1-9	Days 10-18						
20	M ₄ N	G ₄ N	1.8x1.8x1.8	9x10x5	0.1	0.2	17	20.2
24	M ₄ N	G ₄ N	—	7x9x6	0	1.7	17.2	20.8
26	M ₄ N	G ₄ N	5x3.3x2.5	7x7x7	0.2	1.9	19.3	20.6

*Drug in DMSO was injected directly to the tumor regions

20

** From adjacent tumors deprived of drug

Table 3. Toxicity Studies of G₄N in Mice

	Group	# of Mice	Route	Treatment per day	Days of Injection	Mortality
1	187.5mg/kg	3	Subcutaneous	2X	6	0/3
2	375mg/kg	3	Subcutaneous	1X	6	0/3
3	750mg/kg	4	Subcutaneous	1X	6	1/4
4	375mg/kg	2	IV	2X	6	0/2

C57BL-16NCR female mice from NCI were used in this experiment.

35 Tetraglycinal NDGA (G₄N) was freshly made everyday in PBS in concentrations of 75mg/ml. Injections of 0.05 ml for group 1, 0.1 ml for groups

2 and 4, and 0.2 ml for group 3 per treatment were made for a period of 6 days. Experiments lasted seven days. Body weights were determined before and after six days of injection. No significant weight changes were observed during the experimental period.

5

All treated mice, controls (mouse numbers 1-4) and experimental mice (mouse numbers 6, 7, 9, 10, 11, 12, 14, 15, 16, 17 M₄N numbers 18-22, 24, 26-29 M₄N/G₄N) exhibited swelling. Measurements of lesion sizes were made by dial caliper. Some mice experienced mild bleeding due to injection.

10 The treatment regimen and results were as follows:

Day 10: Mice weighed again. All mice exhibited growth up to two grams.

Day 12: No treatments made.

Day 13: All mice have raised skin but to very different degrees. The skin of one M₄N treated mouse (#7) has split open through which the “dried-out

15 tumor” fell out.

Day 14: Injection volume raised to 100 μ l.

Day 15: One M₄N treated mouse (#17) died due to overdose of anesthesia/handling. The skin at the lesion site of #17 cracked with the “dried-out tumor” showing. It was dissected, and lesion excised and weighed. Day

20 16: Four more M₄N treated mice (#6, 14, 15, 16), three M₄N/G₄N treated mice (#19,21,28) and one control mouse (#2) were euthanized, dissected and weighed. Remaining control mice (#1, 3, 4) were examined non-invasively and were carrying tumors.

Day 21: Tumor sizes from control mice were measured by dial caliper.

25 Observation: The skin at the lesion sites of mouse #10 and #12 (M₄N treated regions) cracked with the “dried-out tumor” showing.

Day 24: Mouse #7 skin recovered completely. The experiment was terminated on this date. All remaining mice, M₄N treated (#7, 9, 10, 11, 12) and M₄N/G₄N treated (#18, 20, 24, 26, 29) were euthanized, dissected, examined and

30 weighed.

The effects of M₄N and M₄N/G₄N on C3 tumor growth in mice are summarized in Tables 1 and 2 and Figures 5 and 6. Table 1 shows the drug

effect on C3 cell growth in mice carrying single tumors. The average weight of four excised tumors of the control group was 1.48 g while weights of lesions from M₄N treated and M₄N/G₄N treated were 0.142 and 0.51 g respectively. Drug treated lesions consisted mainly of dried out necrotic cells (Fig. 6). Tumors from the control group appeared homogenous and contained actively growing cells. Table 2 shows the drug effect on C3 tumor growth in mice carrying multiple tumors. In this study, drug was injected into one of the tumors. The average weight of untreated tumors was 1.77 g while that of M₄N treated lesions was 0.15 g. Similar results were obtained following M₄N/G₄N injection—the average weight of untreated tumors was 1.27 g, while that of the drug treated lesions was only 0.103 g. The body weight changes of all mice during the entire experimental period appeared insignificant (Table 1 and 2).

Example 6

Drug treated (M₄N) and DMSO vehicle-treated or untreated tumors (CON) from two groups of mice were prepared for histopathology examination. The excised tumors were immediately fixed and then stored in 4% formaldehyde in phosphate buffered saline. The fixed tissue was then dehydrated through a series of graded alcohols and xylene and embedded in paraffin. The paraffin tissue blocks were thin sectioned and stained for microscopy with hematoxylin and eosin. Histopathology studies showed that the control tumors were unaffected by DMSO treatment and continued to grow. They show the high nuclear/cytoplasmic ratio, pleomorphic nuclear changes, high mitotic figures, spindle like sarcoma shape, and infiltration into the surrounding tissue characteristic of cancer cells. In contrast, those tumors receiving M₄N treatment discontinued growth shortly after treatment began. They demonstrate significant necrosis and are no longer viable. There is a small amount of drug precipitate visible at higher magnification, and focal areas show chronic inflammation and fibrosis. This healing effect leads to the shedding of these deceased tumor cells from the area. The same results are seen with M₄N/G₄N treatment as with the M₄N treatment alone. However, since G₄N is water-soluble, it

can spread to a larger area of the tumor than M₄N. It is expected that G₄N when used with M₄N synergistically may be more effective in treating tumors of large sizes (i.e. greater than 2 cm³).

5 Example 7

Effect of M₄N on HSV-1 Skin Infection in Guinea pig

The drug M₄N was also tested in inhibition of HSV-1 replication in skin infections in guinea pigs. Guinea pig skin was pinched with needles and HSV-1 suppression was applied topically to infect each pricked area. M₄N
10 was then applied to the pricked infected area following infection daily for 6 days.

Six areas of bared back skin of a guinea pig were punched sterily with a 5=DIN needle. Two areas were infected with HSV-1 (HSV-C, culture supernatant, or isolated HSV in saline, HSV-SC). The other four areas were
15 infected with HSV-SC. Fifteen minutes after infection, 30 µl of test compounds (ABDS₁, ABDS₂, ACV and M₄N (4N) in 60 mg/ml of DMSO were applied to each punched infected region of an area, five times per day for six days. ABDS₁ and ABDS₂ were included as negative controls. The photograph in Figure 8 was taken at day 6 and shows the extent of HSV-1
20 replication in the absence of drugs (HSV-C, HSV-SC), in the presence of ineffective drugs (HSV-ABDS₁, HSV-ABDS₂) and in the presence of effective drugs (HSV-M₄N and HSV-ACV). It can be seen that six large confluent blisters were developed in areas treated by HSV-C, HSV-SC, HSV-ABDS₁, HSV-ABDS₂, while no blisters were observed in infected areas following M₄N
25 (4N) and ACV treatments. Clearcut results that M₄N can block HSV replication were obtained in this model system as shown by the disappearance of the skin lesions and by no shedding of the virus 4 days after the drug treatment. Initial animal studies also showed M₄N to be non-toxic to mice at concentrations as high as 300mg/kg when given intraperitoneally, and as high as 375 mg/kg when given
30 either subcutaneously or by IV (Table 3) (6).

Example 8

M₄N for Clinical Treatment using *in situ* Injection

Administration of M₄N directly into tumors as a drug delivery route provides several distinctive advantages. 1) M₄N is a hydrophobic compound and is exceedingly soluble in DMSO (200 mg/ml). Therefore only a small volume of the drug solution is needed for injection in order to achieve effective dosage of the drug. In the mouse study described in Example 5, above, daily injection of 50 μ l to 100 μ l for several days was sufficient to completely stop tumor growth in mice. There have been several previous studies on the use of large dosages (30 ml IV per treatment) of DMSO for treating diseases (21). The results were not conclusive (22). However, since tens of millions of people have been safely tested with large amounts of DMSO worldwide in the past, it appears that DMSO should be safe as a vehicle for drug delivery when only small volume of it will be used (23).

2) By injection *in situ*, a majority of the drug residue remains insoluble and concentrated in the tumor areas, and does not enter the circulatory system, thus whole body toxicity is avoided. In addition, since enough drug remains within the tumor to suppress its growth, continued injection of drug is unnecessary after relatively few treatments. In the mouse study of Example 5, tumor cells continued to die even after discontinuation of M₄N injections. Thus when drug is directly targeted, tumor size becomes the determining factor for the required amount of drug to be administered. The difference between whole body weights of a human vs. a mouse becomes irrelevant. In the mouse tumor studies, 20 mg/day for 10 days were more than sufficient to eliminate tumors. There should be no reason to use a higher dosage than this for treating a human tumor of comparable size (1-1.5 cm³). This should reduce the risk considerably in human trials.

Example 9

M₄N Treatment of Cells Blocks Cellular Proliferation

Our previous research on M₄N indicated that it could inhibit viral transcription by deactivation of Sp1-dependent promoters. Many mammalian

cell cycle genes also contain essential Sp1 promoters and M₄N may therefore block their transcription. This hypothesis was tested by examining the antiproliferative effect of M₄N on a number of different cell lines. Low concentrations (10 μ M) of the parent compound, NDGA, have previously been shown to induce apoptosis in mammalian cells (24). This effect, however, can be circumvented by blocking one of the catechol oxygens or the addition of a hydrophilic group to NDGA (25). Increasing amounts of the NDGA derivative M₄N were tested on cultures of the HPV-16/ras transformed C3 cell line (26) to determine the optimal concentration required to inhibit proliferation (Figure 9a). The cells respond well to M₄N, ceasing division after 72 hours over the range of concentration from 40 to 60 μ M. After three days at these concentrations the number of cells remained equal to the count at the initiation of treatment (day 0, Fig 9). A more modest reduction in cell growth was observed at lower concentrations of the drug and some cell death was seen at concentrations greater than 60 μ M.

The antiproliferative effect of M₄N on the C3 cell line is not solely due to the drug's ability to deactivate the Sp1-dependent HPV-16 E6/E7 oncogene promoter, as similar growth inhibition was observed in the HPV-16 transformed TC-1 cell line whose E₆/E₇ oncogenes are under control of a non-Sp1 dependent retroviral promoter(27) (Figure 9d). In addition, growth of the C33a cell line (Figure 9c), an HPV-negative human cervical cancer cell line, and the CEM-T4 line (Figure 9b), a human leukemia cell line (28), was also blocked by treatment with M₄N. In the four cell lines that were treated with the drug, nearly all (>95%) of the arrested cells were viable until the concentration of M₄N exceeded a "threshold" value (60 μ M for C3 cells, 40 μ M for TC-1 cells, etc.). Above these concentrations the percentage of viable cells decreases precipitously. Interestingly, arrested cells maintained >95% viability even after prolonged exposure to the drug. The C3 cells exhibited no increase in cell death after eight days of treatment with 40 μ M M₄N (Figure 9e).

Example 10

Cells Treated With M₄N Arrest in G2 Phase

Once it was established that cells treated with M₄N cease proliferation yet remain viable, analysis of cellular DNA content and fluorescence

5 examination of cell structures were used to determine the point in the cell cycle where the cells arrest. Cells exposed to M₄N for 72 hours demonstrated increased G2/M DNA content relative to the controls (Figure 10a-d). The most extreme responses were seen from the C3 and CEMT4 cell lines, in which >90% of the cells show G2/M DNA content.

10 In order to distinguish between an arrest in G2 or a mitotic block, antibodies against α tubulin (green) and γ tubulin (red) were used to determine the status of the centrosomes in the C3 cell line following 72 hours M₄N treatment. As shown in Figure 11a, the centrosomes of M₄N treated cells are duplicated but still located next to each other in the nucleus of the
15 cell. Since centrosomes separate during early prophase, it can be concluded that these cells have not begun mitosis. In contrast, the gamma tubulin staining of the control cells has the diffuse pattern characteristic of G1 or S phase (29). A lack of chromatin condensation in the M₄N treated cells was also observed with DAPI staining (Figure 12b), additional evidence that the
20 cells have not moved forward out of G2 phase (30).

Example 11

Production of CDC2 is Inhibited by 40 μ M M₄N

Since progression of cells out of G2 is dependent on the production of
25 the MPF, the status of its protein components was examined in C3 cells treated with 40 μ M M₄N. Asynchronous cells were grown for 24 or 72 hours in media containing either M₄N in 1% DMSO, or 1% DMSO alone. The cells were harvested, and equal amounts of total cellular protein were analyzed by western blotting. A marked reduction in the amount of CDC2 was observed
30 after 72 hours treatment with M₄N (Figure 12a). However, levels of cyclin B, detected by stripping and reprobing the same membrane, were found to be unchanged. These results indicate that, under these conditions, the arrest is

not likely a response to p53 since it has been shown that overexpression of p53 leads to a decrease in cyclin B (31, 32). Consistent with the results of the western analysis, CDC2 kinase activity was eliminated by 72 hours of M₄N treatment (Figure 12a). These experiments support the view that the drug
5 acts by inhibiting the production of the CDC2 protein, resulting in a loss of activity of the MPF.

Our previous studies demonstrating the ability of M₄N to block Sp1-dependent viral transcription suggest reduction of CDC2 mRNA levels as a possible mechanism for the decrease of CDC2 protein. This is consistent
10 with the finding that the cyclin B protein, whose gene does not require Sp1 for its expression, is produced at normal levels while the CDC2 protein, whose gene has two essential Sp1 sites in its promoter, is substantially reduced in quantity. To test this hypothesis, northern blot analysis was performed on RNA harvested from C3 cells treated with 40 μ M M₄N for 5 to 72 hours. As
15 shown in Figure 12b, the amount of CDC2 mRNA is reduced after only 24 hours treatment with M₄N and nearly eliminated after 72 hours. Production of the non-Sp1 regulated housekeeping gene GAPDH was used as an RNA loading control, and its levels were not effected by 40 μ M M₄N.

The use of the C3 cell line allows us an additional control for analysis
20 of the mechanism of M₄N mediated cell cycle arrest since other Sp1-dependent gene promoters are also likely to be inhibited by M₄N treatment. This possibility was examined in C3 cells by analyzing the effect of M₄N on transcription from the Sp1 dependent HPV-16 E₆/E₇ promoter. rtPCR analysis of RNA isolated from C3 cells treated with 40 μ M M₄N for 5 to 72
25 hours demonstrated a clear reduction in the levels of the E₇ transcript (Figure 12c). GAPDH was again used as an internal control in this experiment, and its levels were unaffected by drug treatment. These results provide additional evidence that M₄N reduces the transcripts of Sp1 regulated promoters.

30 Example 12

Inhibition of Sp1-Binding Activity by G₄N in a Gel Mobility-Shift Analysis.

Sp1 family proteins induce bends toward the major groove of DNA upon

binding (33). The zinc finger domain of the Sp1 protein is responsible for the binding of the GC Box sequence 5'-GGGGCGGGG-3'. From computational analysis, it was determined that G₄N, the aminoester derivative of NDGA, could form a stable complex with such a sequence in the major groove. To
5 determine whether G₄N can serve as an Sp1 blocker as well as an Sp1 displacer, we performed Sp1/enhancer interaction studies in the presence or absence of G₄N by the gel mobility-shift analysis using only the DNB binding domain of Sp1 for testing. In the blocking experiment, different concentrations of G₄N were first incubated with ³²P-labelled DNA in the binding buffer for 30
10 min at 25°C. DNA binding domain of recombinant Sp1 protein (Sp1-167D) 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 SP1-167D was first allowed to bind DNA, G₄N was then added at the second step of the incubation. The G₄N and Sp1-167D concentrations and, the incubation and
15 gel electrophoresis conditions were identical in both studies (experimental section). As shown in Figure 13, in either case, G₄N was found to be able keep DNA from interacting with Sp1-167D protein. When only the DNA binding domain of Sp1 alone was tested, G₄N appeared to be more efficient in displacement of the bound Sp1 than blocking Sp1 from binding to the
20 enhancer, as shown by the gel mobility-shift analysis (Figure 13, A,B,D). We have also examined whether the bound G₄N can be replaced by Sp1-167D. In this study, the inhibition of Sp1-167D binding by G₄N was first established by the mobility-shift analysis (Fig 13C, lanes 2 and 5). When the G₄N bound template was challenged with additional Sp1-167D, we observed a dosage
25 dependent increase of the band intensities of the Sp1-167D/DNA complex (Fig 6C, Lanes 6,7) indicating the displacement of G₄N by Sp1-167D from the template.

Example 13

30 Inhibition of Sp1 Regulated Tat-Transactivation of HIV Promoter Activity by G₄N.

As reported previously, methylated NDGA derivatives can block Sp1

binding to the enhancer sites of a variety of viral promoters including HIV, ICP4 of HSV, E₆/E₇ gene of HPV (8, 9, 10). We further tested the G₄N effect on the Tat-transactivation of HIV promoter activity in Cos cells by the SEAP assay as previously described. Basal level of the HIV LTR driven SEAP expression was previously found to be barely detectable in Cos cells. There were 60-fold or more increase in SEAP expression when Cos cells were cotransfected with the CMV promoter driven Tat gene (8). Such Tat-driven transactivation of the HIV LTR promoter activity was previously shown to be Sp1 regulated (7, 8). In the presence of G₄N, we observed inhibition of HIV transactivation in a dose-dependent fashion (Figure 14). An average value IC₅₀ value of 36 μ M for G₄N was comparable to that of 3-O-methyl NDGA, Ma1.4 (IC₅₀ 25 μ M) and somewhat higher than that of tetra-methyl NDGA, M₄N (IC₅₀ 11 μ M). The differences perhaps are due to the chemical nature of the test compounds affecting the drug uptake to the cells.

15

Example 14

Inhibition of SIV-1 and HIV-1 Production in Cell Cultures by G₄N.

Both HIV-1 and SIV are retroviruses that require integration into the host genome to complete their replication. Both rely on host transcription factors for their proviral transcriptions. Sp1 plays a central role for such expression in these two viruses sharing an almost identical mode of transcription regulation. In anticipation of using SIV infected rhesus monkeys as animal model for testing the antiviral effect of G₄N, we have studied and compared the G₄N effect in inhibition of SIV in 174 x CEM cells with that of HIV in H9 cells. Cellular toxicities of G₄N in these two cell lines were also examined. For SIV inhibition study, 10⁷ 174 x CEM cells were mixed with high titer stock of SIVmac 239 at 37°C for two hours and then washed twice with cold PBS buffer to remove the unabsorbed virus. Cell suspension was aliquoted into each well of three 96 well plates. Various concentrations of the G₄N solutions were made from freshly prepared stock and aliquoted separately and each to six wells in a column of one 96 well plate. Culture supernatants were collected every four days post infection. (P.I.) and fresh medium containing appropriate concentrations of the drug were added to the culture following supernatant collections. Viral production was assayed by a modified p27 core antigen capture ELISA as shown (Figure 15). There was no SIV production detected using G₄N in concentrations above 5 CIM. At G₄N concentrations below 2.5 CIM, SIV production was detected (Fig. 15) in culture supernatants from 4th and 8th days post infected cultures as compared to viral production in the absence of the drug. G₄N (250 μM or less) showed no toxic effect on uninfected 174 x CEM cells, as determined by the MTT assay (34).

A similar experiment was also carried out for the study of inhibition of HIV-1

by G₄N in H9 cells. The H9 cells were subcultured at 1 x 10⁵/ml and were infected with an AZT resistant strain of HIV-1(HIV-1RTMF). G₄N in different concentrations was added two hours after infection. Fresh medium change was made every four days. Cell growth in the presence of G₄N was monitored carefully during the nine-day experimental period. Viral production was

assayed by a p24 core antigen capture ELISA. As shown (Figure 16), G₄N concentration of 80 μ M completely inhibited HIV replication in H9 cells. An IC₅₀ of 12 μ M CIM G₄N for the inhibition of HIV-1 RTMF was found. Again, there

- 5 was no detectable toxicity to uninfected H9 cells within the range of the assay (and below 250 μ M).

Example 15

Effect of M₄N Treatment on Survivin Gene Expression in C3 Cells

10 Materials and methods

Cell Culture. C3 cells were grown as monolayers in Iscove's Modified Dulbecco's Medium (GIBCO BRL) supplemented with 5% fetal bovine serum (GIBCO BRL) and maintained in a humid incubator at 37°C in a 5% CO₂ environment.

- 15 M₄N Treatment. C3 cells (5×10^6) were seeded in 150-mm plates and allowed to attach to the plates. Twenty-four hours after seeding, cultures were washed twice with PBS and treated with M₄N dissolved in 1% DMSO mixed with the growth medium.

- Cell extracts and immunoblotting. Cells were lysed in lysis buffer
20 containing 50 mM HEPES pH 7, 250 mM NaCl, 0.1% (v/v) Nonidet P-40, 10% glycerol, 1mM DTT, and 50 μ l/ml protease inhibitor cocktail (Sigma). Protein concentration of the extract was determined by the Bradford assay (Bio-Rad Laboratories), and then 50 μ g of protein was separated by SDS-PAGE and electrotransferred to nitrocellulose membrane (ECL). Membranes were incubated
25 with primary antibodies against Survivin (Santa Cruz Biotechnology) and caspase-3 (Santa Cruz Biotechnology). Blots were then incubated with anti-rabbit biotin-conjugated secondary antibody and then with Avidx-AP™ streptavidin-alkaline phosphatase, and detected with CSPD® substrate (Tropix).

- RT-PCR analysis. mRNA was isolated by the guanidinium thiocyanate and
30 phenol method from the cultured cells as described in Molecular Cloning (40). A 343-base pair RT-PCR product was generated by using the survivin-sense

oligonucleotide primer 5'-GCATCGCCACCTTCAAGAACTGGCCC-3' and the survivin-antisense oligonucleotide primer 5'-CGGGTAGTCTTTGCAGTCTCTTCAAACCTC-3'. GAPDH sense and anti-sense primers 5'-GAATCTACTGGCGTCTTCACC-3' and 5'-GTCATGAGCCCTTCCACGATGC-3' were used to generate a 238-base pair RT-PCR product as a control. mRNA aliquots were incubated in 20 µl reaction buffer containing 1 U of rRNAsin and DNase at 75 °C for 5 minutes followed by reverse transcription reaction with MMLV (Promega). The c-DNA products obtained were amplified under the PCR conditions: 55°C for 55 seconds, 60°C for 55 seconds, and 72°C for 1 minute for 30 cycles. The PCR products were separated by electrophoresis on a 1.8% agarose gel containing ethidium bromide and photographed under UV. The bands were quantitated by Scion Image and the signal intensities of the survivin PCR reaction products were normalized to those of the GAPDH PCR products to generate a survivin gene down-regulation graph.

To determine whether the Spl-regulated survivin gene expression in C3 cells is reduced by M4N treatment, we treated the cells with 40 µM M4N for 24 hours and 72 hours. As shown in Figure 17, treatment of cells with M4N resulted in a significant decrease in survivin gene expression in a time-dependent manner. Treatment with 40 p2d M4N for 24 hours and 72 hours resulted in 65% and 80% reduction in survivin expression, respectively. Untreated cells did not show any reduction in survivin gene expression.

Survivin protein was also shown by immunoblotting to be downregulated by 72 hours of M4N treatment. This downregulation was dosage-dependent (Figure 18).

Example 16

Induction of Apoptosis with M4N treatment

Because our data showed that M4N resulted in survivin mRNA and protein reduction, we investigated whether this reduction induces apoptosis since survivin has anti-apoptosis function. As shown by immunoblotting of caspase-3 (Figure 19), treatment of M4N for 72 hours resulted in caspase-3 activation. This activation

would be expected to result in an increase in an increase in apoptosis in cells treated with M_4N .

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5 incorporated by reference.

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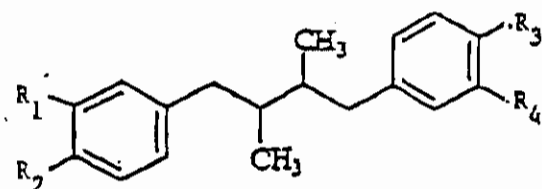
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Claims for Divisional Application (Singapore)

1. A pharmaceutical composition for treating a tumor, said pharmaceutical composition comprising an effective amount of a compound of formula



wherein R₁, R₂, R₃, and R₄ independently represent -OH, -OCH₃, -O(C=O)CH₃, or an unsubstituted or substituted amino acid residue or salt thereof, but are not each -OH simultaneously; wherein the effective amount is any amount greater than 0 μM.

2. The pharmaceutical composition of claim 1, wherein said tumor is present in a mammal.
3. The pharmaceutical composition of claim 2, wherein said tumor is malignant.
4. The pharmaceutical composition of claim 2, wherein said tumor is benign.
5. The pharmaceutical composition of claim 2, wherein said tumor is selected from the group consisting of papilloma, teratoma and adenoma.
6. The pharmaceutical composition of claim 2, wherein said tumor is a solid tumor.
7. The pharmaceutical composition of claim 2, wherein said mammal is a human.
8. The pharmaceutical composition of claim 2, wherein said tumor is derived from transformed cells.

9. The pharmaceutical composition of claim 2, wherein said compound is administered along with at least one pharmaceutically excipient or carrier.
10. The pharmaceutical composition of claim 9, wherein said excipient or carrier is dimethylsulfoxide.
11. The pharmaceutical composition d of claim 9, wherein said compound is tetra-O-methyl nordihydroguaiaretic acid or tetraglycinylnordihydroguaiaretic acid.
12. A pharmaceutical composition for suppressing Sp1 regulated promoter activity in a cell comprising tetra-O-methyl nordihydroguaiaretic acid (M_4N); wherein the concentration of M_4N is a number greater than 0 μM .
13. The pharmaceutical composition of claim 12, wherein the M_4N concentration is selected from the group consisting of: at least about 10 μM , at least about 20 μM , at least about 30 μM , at least about 40 μM , at least about 50 μM and at least about 60 μM .
14. A pharmaceutical composition for suppressing Sp1 regulated promoter activity in a cell comprising tetra-glycinylnordihydroguaiaretic acid (G_4N), wherein the concentration of G_4N is a number greater than 0 μM .
15. The pharmaceutical composition of claim 14, wherein the G_4N concentration is selected from the group consisting of: at least 20 μM , at least 40 μM , at least 60 μM , at least 80 μM and at least 100 μM .
16. The pharmaceutical composition of claim 14, wherein the G_4N concentration is selected from the group consisting of: at least about 0.25 mM, at least about 0.5 mM, at least about 0.75 mM, at least about 1.0 mM, at least about 1.25 mM, at least about 1.5 mM, and at least about 1.75 mM.

17. A pharmaceutical composition for suppressing tumor cell growth comprising tetra-O-methyl nordihydroguaiaretic acid (M_4N), wherein the concentration of M_4N is a number greater than 0 μM .
18. The pharmaceutical composition of claim 17, wherein the concentration of M_4N is selected from the group consisting of at least about 10 μM , at least about 20 μM , at least about 30 μM , at least about 40 μM , at least about 50 μM , at least about 60 μM , at least about 70 μM , at least about 80 μM , at least about 90 μM , and at least about 100 μM .
19. The pharmaceutical composition of claim 17, wherein the M_4N is formulated for exposing the tumor cell for a number of hours greater than 0.
20. The pharmaceutical composition of claim 19, wherein the number of hours is selected from the group consisting of at least about 24 hr, at least about 48 hr, and at least about 70 hr.
21. The pharmaceutical composition of claim 19, wherein the number of hours is at least 72.
22. The pharmaceutical composition of claim 1, wherein the concentration of the compound is selected from the group consisting of: at least 20 μM , at least 40 μM , at least 60 μM , at least 80 μM and at least 100 μM .
23. The pharmaceutical composition of claim 1, wherein the concentration of compound is selected from the group consisting of at least about 10 μM , at least about 20 μM , at least about 30 μM , at least about 40 μM , at least about 50 μM , at least about 60 μM , at least about 70 μM , at least about 80 μM , at least about 90 μM , and at least about 100 μM .
24. A pharmaceutical composition for inhibiting expression of a Sp1-regulated eukaryotic

gene, wherein the Sp1-regulated eukaryotic gene comprises a Sp1-regulated promoter, comprising an inhibitor of Sp1-regulated gene expression, wherein the inhibitor is a derivative of NDGA.

25. The pharmaceutical composition of claim 24, wherein the Sp1-regulated gene is present in a cell.

26. The pharmaceutical composition of claim 25, wherein the cell is an animal cell.

27. The pharmaceutical composition of claim 26, wherein the animal cell is a mammalian cell.

28. The pharmaceutical composition of claim 27, wherein the mammalian cell is a human cell.

29. A pharmaceutical composition for stimulating apoptosis in a cell expressing survivin comprising an inhibitor of survivin expression, wherein the inhibitor is a derivative of NDGA.

30. The pharmaceutical composition for claim 29, wherein the cell is an animal cell.

31. The pharmaceutical composition for claim 30, wherein the animal cell is a mammalian cell.

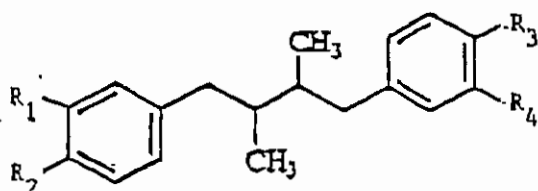
32. The pharmaceutical composition of claim 31, wherein the mammalian cell is a human cell.

33. The pharmaceutical composition of claim 25, wherein the cell is a rapidly dividing cell.

34. The pharmaceutical composition of claim 33, wherein the rapidly dividing cell is a

cancer cell.

35. The pharmaceutical composition of claim 24, wherein the derivative of NDGA comprises a formula:



wherein R₁, R₂, R₃, and R₄ independently represent --OH, --OCH₃, --O(C=O)CH₃, or an amino acid residue, or a salt thereof, but are not each --OH simultaneously.

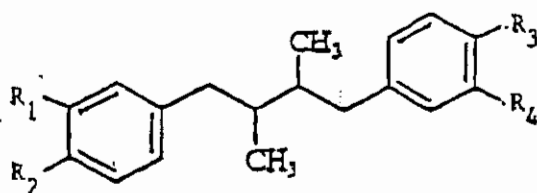
36. The pharmaceutical composition of claim 35, wherein the NDGA derivative is tetra-O-methyl NDGA.

37. The pharmaceutical composition of claim 35, wherein the NDGA derivative is meso-1,4-Bis[3,4-(dimethylaminoacetoxylphenyl]-(2R,3S)-dimethylbutane hydrochloride.

38. The pharmaceutical composition of claim 24, wherein the Sp1-regulated eukaryotic gene is selected from the group consisting of: cyclin-dependent kinase 7, cdc 2 kinase, human cytokine receptor, proliferating cell nuclear antigen, human TNF-related apoptosis APO₂, cysteine protease and survivin.

39. The pharmaceutical composition of claim 24, wherein the Sp1-regulated eukaryotic gene is survivin.

40. The pharmaceutical composition of claim 29, wherein the derivative of NDGA comprises a formula:



wherein R_1 , R_2 , R_3 , and R_4 independently represent $-OH$, $-OCH_3$, $-O(C=O)CH_3$, or an amino acid residue, or a salt thereof, but are not each $-OH$ simultaneously.

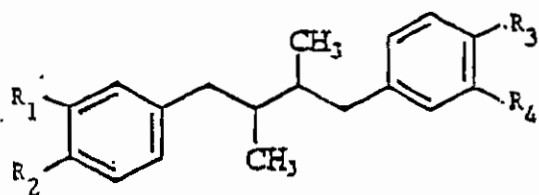
41. The pharmaceutical composition of claim 39, wherein the NDGA derivative is tetra-O-methyl NDGA.

42. The pharmaceutical composition of claim 39, wherein the NDGA derivative is meso-1,4-Bis[3,4-(dimethylaminoacetoxymethyl)phenyl]-(2R,3S)-dimethylbutane hydrochloride.

43. A pharmaceutical composition for arresting cell growth comprising an inhibitor of expression of a Sp1-regulated gene, wherein the Sp1-regulated gene is necessary for cell division; wherein the inhibitor is a derivative of NDGA.

44. The pharmaceutical composition of claim 43, wherein the Sp1-regulated gene is selected from the group consisting of: cyclin-dependent kinase 7, cdc 2 kinase, human cytokine receptor, proliferating cell nuclear antigen, human TNF-related apoptosis APO₂, and cysteine protease.

45. The pharmaceutical composition of claim 43, wherein the derivative of NDGA comprises a formula:



wherein R_1 , R_2 , R_3 , and R_4 independently represent $-OH$, $-OCH_3$, $-O(C=O)CH_3$, or an amino acid residue, or a salt thereof, but are not each $-OH$ simultaneously.

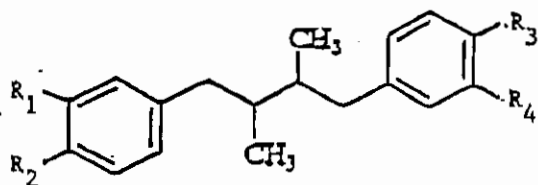
46. The pharmaceutical composition of claim 45, wherein the NDGA derivative is tetra-O-methyl NDGA.

47. The pharmaceutical composition of claim 45, wherein the NDGA derivative is meso-1,4-Bis[3,4-(dimethylaminoacetoxyl)phenyl]-(2R,3S)-dimethylbutane hydrochloride.

48. A pharmaceutical composition for inhibiting HPV replication in a cell comprising an inhibitor that inhibits binding of Sp1 to a promoter of HPV.

49. The pharmaceutical composition of claim 48, wherein the inhibitor is a derivative of NDGA.

50. The pharmaceutical composition of claim 49, wherein the NDGA derivative comprises a formula:



wherein R_1 , R_2 , R_3 , and R_4 independently represent $-OH$, $-OCH_3$, $-O(C=O)CH_3$, or an

amino acid residue, or a salt thereof, but are not each --OH simultaneously.

51. The pharmaceutical composition of claim 48, wherein step of contacting the promoter of HPV with the inhibitor comprises contacting the cell that comprises the HPV with the inhibitor.

52. The pharmaceutical composition of claim 51, wherein the HPV promoter is selected from the group consisting of promoter of E₆ and promoter of E₇.

53. The pharmaceutical composition of claim 48, wherein the inhibition of HPV replication comprises inhibition of HPV mRNA synthesis.

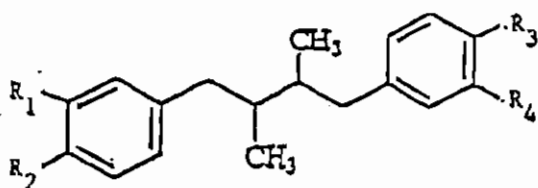
54. The pharmaceutical composition of claim 50, wherein the inhibitor is tetra-O-methyl NDGA.

55. The pharmaceutical composition of claim 50, wherein the inhibitor is meso-1,4-Bis[3,4-(dimethylaminoacetoxymethyl)phenyl]-(2R,3S)-dimethylbutane hydrochloride.

56. A pharmaceutical composition for inhibiting HPV transcription in a cell comprising an inhibitor of binding of Sp1 to an HPV promoter.

57. The pharmaceutical composition claim 56, wherein the inhibitor is a derivative of NDGA.

58. The pharmaceutical composition of claim 57, wherein the NDGA derivative comprises a formula:



wherein R_1 , R_2 , R_3 , and R_4 independently represent $-\text{OH}$, $-\text{OCH}_3$, $-\text{O}(\text{C}=\text{O})\text{CH}_3$, or an amino acid residue, or a salt thereof, but are not each $-\text{OH}$ simultaneously.

59. The pharmaceutical composition of claim 58, wherein the NDGA derivative is tetra-O-methyl NDGA.

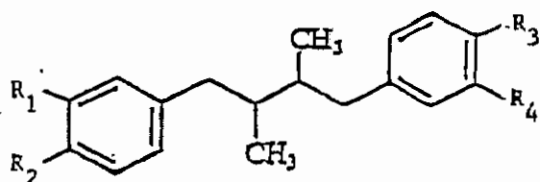
60. The pharmaceutical composition of claim 58, wherein NDGA derivative is meso-1,4-Bis[3,4-(dimethylaminoacetoxyl)phenyl]-(2R,3S)-dimethylbutane hydrochloride.

61. The pharmaceutical composition of claim 56, wherein the step of contacting the HPV promoter with the inhibitor comprises contacting the cell comprising the HPV with the inhibitor.

62. A pharmaceutical composition for treatment of HPV infection in a subject in need of such treatment comprising an inhibitor of HPV replication or transcription.

63. The pharmaceutical composition of claim 62, wherein the inhibitor of HPV replication is a derivative of NDGA.

64. The pharmaceutical composition of claim 63, wherein the NDGA derivative comprises a formula:

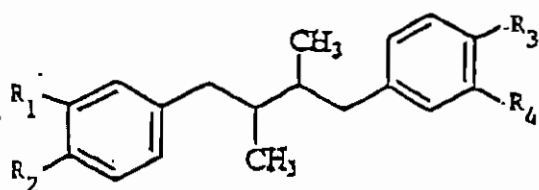


wherein R_1 , R_2 , R_3 , and R_4 independently represent $-\text{OH}$, $-\text{OCH}_3$, $-\text{O}(\text{C}=\text{O})\text{CH}_3$, or an amino acid residue, or a salt thereof, but are not each $-\text{OH}$ simultaneously.

65. The pharmaceutical composition of claim 64, wherein the NDGA derivative is tetra-O-methyl NDGA.

66. The pharmaceutical composition of claim 64, wherein the NDGA derivative is meso-1,4-Bis[3,4-(dimethylaminoacetoxymethyl)phenyl]-(2R,3S)-dimethylbutane hydrochloride.

67. A pharmaceutical composition for treating tumor in an animal, comprising an effective amount of at least one compound of the formula:



wherein R₁, R₂, R₃, and R₄ independently represent -OH, -OCH₃, -O(C=O)CH₃, or an unsubstituted or substituted amino acid residue or salt thereof, but are not each -OH simultaneously.

68. The pharmaceutical composition of claim 67, wherein the unsubstituted or substituted amino acid residue is selected from the group consisting of alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, 5-hydroxylysine, 4-hydroxyproline, thyroxine, 3-methylhistidine, ε-N-methyllysine, ε-N,N,N-trimethyllysine, aminoadipic acid, γ-carboxyglutamic acid, phosphoserine, phosphothreonine, phosphotyrosine, N-methylarginine, and N-acetyllysine.

69. The pharmaceutical composition of claim 67, wherein the animal is a mammal.

70. The pharmaceutical composition of claim 67, wherein the animal is a human.

71. The pharmaceutical composition of claim 67, wherein the salt is a hydrochloride.
72. The pharmaceutical composition of claim 67, wherein the compound is tetra-O-methyl nordihydroguaiaretic acid.
73. The pharmaceutical composition of claim 67, wherein the compound is tetraglycinylnordihydroguaiaretic acid.
74. The pharmaceutical composition of claim 73, further comprising tetra-O-methylnordihydroguaiaretic acid.
75. The pharmaceutical composition of claim 67, wherein the compound is formulated for administration along with at least one pharmaceutically acceptable excipient or carrier.
76. The pharmaceutical composition of claim 75, wherein the excipient or carrier is dimethylsulfoxide.
77. The pharmaceutical composition of claim 75, wherein the excipient or carrier is phosphate buffered saline.
78. The pharmaceutical composition of claim 67, wherein the tumor is selected from the group consisting of a cancerous tumor and a non-cancerous tumor.
79. The pharmaceutical composition of claim 67, wherein the tumor is cervical tumor, an oral tumor, a penile tumor, or a head and neck tumor.
80. The pharmaceutical composition of claim 67, wherein the compound is formulated for injection into the tumor on a daily basis for a certain period of time.
81. The pharmaceutical composition of claim 67, wherein the compound is formulated

for injection into the tumor weekly for a certain period of time.

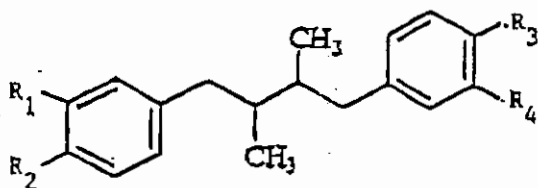
82. The pharmaceutical composition of claim 67, wherein the compound is formulated for administration at a dose of from 10 mg to 20 mg per gram tumor weight.

83. The pharmaceutical composition of claim 67, wherein the compound is formulated for injection into the tumor for up to 10 days.

84. The pharmaceutical composition of claim 67, wherein the compound is formulated for injection into the tumor for up to 16 days.

85. The pharmaceutical composition of claim 67, wherein the compound is formulated for injection into the tumor for 9 days.

86. A pharmaceutical composition of inhibiting growth of rapidly proliferating cells containing cyclin dependent kinase CDC2, comprising at least one compound of the formula:



wherein R₁, R₂, R₃, and R₄ independently represent -OH, -OCH₃, -O(C=O)CH₃, or an unsubstituted or substituted amino acid residue or salt thereof, but are not each -OH simultaneously.

87. The pharmaceutical composition of claim 86, wherein the unsubstituted or substituted amino acid residue or substituted amino acid residue is selected from the group consisting of alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine,

histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, 5-hydroxylysine, 4-hydroxyproline, thyroxine, 3-methylhistidine, ϵ -N-methyllysine, ϵ -N,N,N-trimethyllysine, aminoadipic acid, γ -carboxyglutamic acid, phosphoserine, phosphothreonine, phosphotyrosine, N-methylarginine, and N-acetyllysine.

88. The pharmaceutical composition at claim 86, wherein the salt is a hydrochloride.

89. The pharmaceutical composition of claim 86, wherein the compound is tetra-O-methyl nordihydroguaiaretic acid.

90. The pharmaceutical composition of claim 86, wherein the compound is tetraglycyl nordihydroguaiaretic acid.

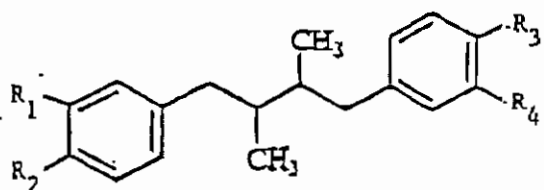
91. The pharmaceutical composition of claim 90, additionally comprising tetra-O-methyl nordihydroguaiaretic acid.

92. The pharmaceutical composition of claim 86, wherein the compound is formulated for administration along with at least one pharmaceutically acceptable excipient or carrier.

93. The pharmaceutical composition of claim 92, wherein the excipient or carrier is dimethylsulfoxide.

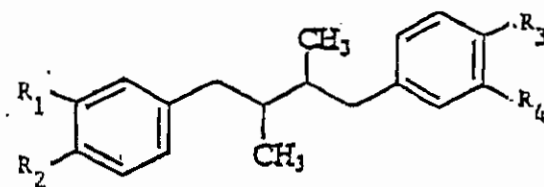
94. The pharmaceutical composition of claim 92, wherein the excipient or carrier is phosphate buffered saline.

95. A pharmaceutical composition for inhibiting Sp1 regulated promoter activity in a proliferating cell comprising at least one compound of the formula:



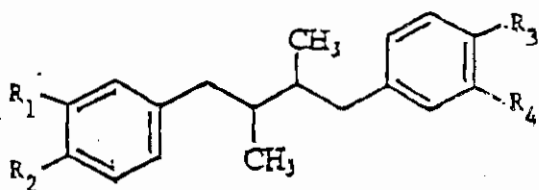
wherein R_1 , R_2 , R_3 , and R_4 independently represent $-OH$, $-OCH_3$, $-O(C=O)CH_3$, or an amino acid residue or substituted amino acid residue or salt thereof, but are not each $-OH$ simultaneously, to said cell.

96. A pharmaceutical composition for up-regulating the expression of a cell cycle related gene in a cell, wherein the cell cycle related gene is selected from the group consisting of: cyclin-dependent kinase inhibitor, apoptosis antigen APO-1, death domain three DR₃, ras-related protein RAP-1, and human Map Kinase, comprising at least one compound of the formula:



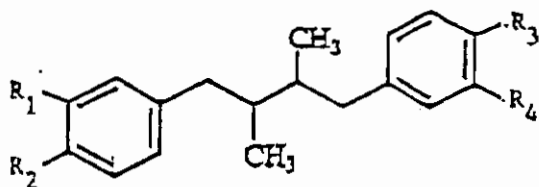
wherein R_1 , R_2 , R_3 , and R_4 independently represent $-OH$, $-OCH_3$, $-O(C=O)CH_3$, or an unsubstituted or substituted amino acid residue or salt thereof, but are not each $-OH$ simultaneously.

97. A pharmaceutical composition for down-regulating the expression of a cell cycle related gene in a cell, wherein the cell cycle related gene is selected from the group consisting of cyclin-dependent kinase 7, cytokine receptor, proliferating cell nuclear antigen PCNA, TNF-related apoptosis APO₂, and cysteine protease, comprising a compound of the formula:



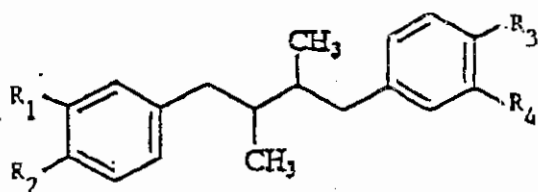
wherein R₁, R₂, R₃, and R₄ independently represent -OH, -OCH₃, -O(C=O)CH₃, or an unsubstituted or substituted amino acid residue or salt thereof, but are not each -OH simultaneously.

98. A pharmaceutical composition for inhibiting cyclin-dependent kinase CDC2 activity in a cell comprising an effective amount of a compound of the formula:



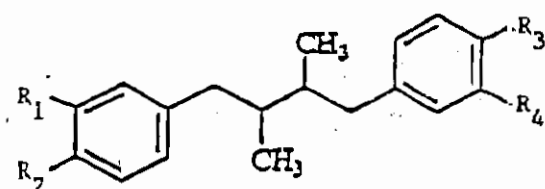
wherein R₁, R₂, R₃, and R₄ independently represent -OH, -OCH₃, -O(C=O)CH₃, or an unsubstituted or substituted amino acid residue or salt thereof, but are not each -OH simultaneously.

99. A pharmaceutical composition for treating tumor in an animal comprising an effective amount of at least one compound, wherein the compound has the formula:



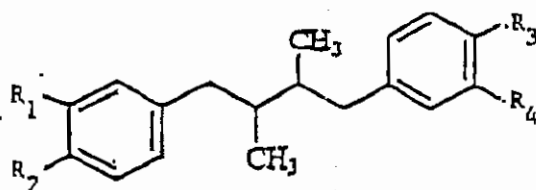
wherein R_1 , R_2 , R_3 , and R_4 independently represent $-OH$, $-OCH_3$, $-O(C=O)CH_3$, or an unsubstituted or substituted amino acid residue or salt thereof, but are not each $-OH$ simultaneously, wherein said composition is formulated for injection.

100. A pharmaceutical composition for treating tumor in an animal comprising an effective amount of at least one compound formulated for systemic delivery to the subject, wherein the compound has the formula



wherein R_1 , R_2 , R_3 , and R_4 independently represent $-OH$, $-OCH_3$, $-O(C=O)CH_3$, or an unsubstituted or substituted amino acid residue or salt thereof, but are not each $-OH$ simultaneously.

101. A pharmaceutical composition for treating tumor in an animal comprising an effective amount of at least one compound, wherein the compound has the formula:



wherein R_1 , R_2 , R_3 and R_4 are identical and represent an unsubstituted or substituted amino acid residue or salt thereof, wherein the substituted or unsubstituted amino acid residue is selected from the group consisting of alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, 5-hydroxylysine, 4-hydroxyproline, thyroxine, 3-methylhistidine, ϵ -N-methyllysine, ϵ -N,N,N-trimethyllysine, aminoadipic acid, γ -carboxyglutamic acid, phosphoserine, phosphothreonine, phosphotyrosine, N-methylarginine, and N-acetyllysine.

102. The pharmaceutical composition of claim 101, wherein the compound is a salt.

103. The pharmaceutical composition of claim 102, wherein the salt is a hydrochloride.

104. The pharmaceutical composition of claim 101, wherein the compound is formulated for injection.

105. The pharmaceutical composition of claim 101, wherein the compound is formulated for injection into the tumor of the animal.

106. The pharmaceutical composition of claim 101, wherein the animal is a human.

107. The pharmaceutical composition of claim 101, wherein the compound is formulated for administration more than once.

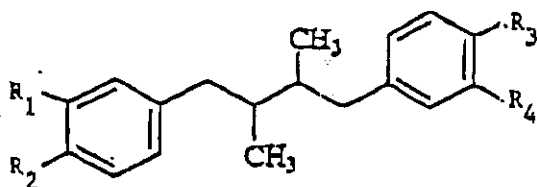
108. The pharmaceutical composition of claim 101, wherein the compound is formulated

for administration daily for a certain period of time.

109. The pharmaceutical composition of claim 101, wherein the compound is formulated for administration once daily for a certain period of time.

110. The pharmaceutical composition of claim 101, wherein the compound is formulated for administration weekly for a certain period of time.

111. Use of a compound for the manufacture of a medicament for the treatment of a tumour in which survivin is expressed, wherein the compound has the following structural formula:

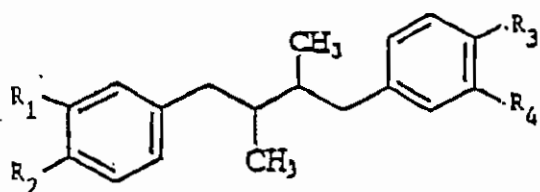


wherein R₁, R₂, R₃, and R₄ independently represent -OH, -OCH₃, -O(C=O)CH₃, or an amino acid residue, but are not each -OH simultaneously.

112. Use according to claim 111 wherein said tumour is a malignant or benign tumour.

113. Use according to claim 111, wherein the treatment induces, or increases the rate of, apoptosis in the tumour cells.

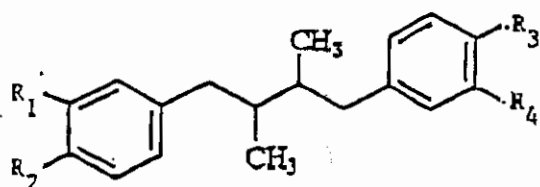
114. Use of a compound for the manufacture of a medicament for the induction of apoptosis, wherein the compound has the following structural formula:



wherein R₁, R₂, R₃, and R₄ independently represent -OH, -OCH₃, -O(C=O)CH₃, or an amino acid residue, but are not each -OH simultaneously.

115. Use according to claim 114 wherein said induction of apoptosis is for treatment of a tumour.

116. A compound of formula:

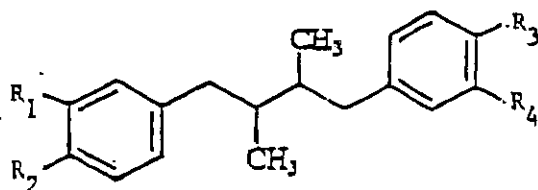


wherein R₁, R₂, R₃, and R₄ independently represent -OH, -OCH₃, -O(C=O)CH₃, or an amino acid residue, but are not each -OH, simultaneously, for use in the treatment of a tumour in which survivin is expressed.

117. A compound for use according to claim 116 wherein said tumour is a malignant or benign tumour.

118. A compound for use according to claim 117 or 118, wherein the treatment induces, or increases the rate of, apoptosis in the tumour cells.

119. A compound of formula:



wherein R₁, R₂, R₃, and R₄ independently represent -OH, -OCH₃, -O(C=O)CH₃, or an amino acid residue, but are not each -OH simultaneously, for use in induction of apoptosis.

120. A compound for use according to claim 119 wherein induction of apoptosis is for the treatment of cancer.

121. A compound for use according to any one of claims 119 and 120 wherein the compound is a solid.

122. A compound for use according to any one of claims 119 and 120 wherein the compound is in solution.