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Synthesis, characterization, and anti-melanoma activity of tetra-O-substituted analogs of nordihydroguaiaretic acid

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ABSTRACT

Synthesis of seven semi-synthetic analogs of NDGA is described. An approach to NDGA derivatization is described in which the *ortho*-phenolic groups are tethered together by one atom, forming a 5-membered heterocyclic ring. The analogs were evaluated for cytotoxicity in four cancer cell lines and compared to NDGA and tetra-*O*-methyl-NDGA (M4N) (**1a**). NDGA bis-cyclic sulfate (**2a**), NDGA bis-cyclic carbonate (**2b**), and methylenedioxyphenyl-NDGA (**2d**) and NDGA tetra acetate (**1b**) showed anti-cancer activity in vitro. Two compounds, (**1b**) and (**2b**), were evaluated for anticancer activity in a mouse xenograft model of human melanoma and showed dose-dependent activity.

Nordihydroguaiaretic acid (NDGA) is a lignan that is found at up to 10–15% by dry weight in the leaves and twigs of the creosote bush (*Larrea tridentata* (Sesse and Moc.) Coville, Zygophyllaceae).[1] NDGA is a 5-lipoxygenase inhibitor with a well-defined mechanism of action requiring 'free' phenolic groups for activity.[2] Tetra-substituted, 'masked' phenolic group NDGA analogs were found to be devoid of inhibitory activity against soybean, human 12, and human 15- lipoxygenase.[3] NDGA has shown many biological activities including anticancer activity. In vitro, NDGA inhibited DNA synthesis in K562 chronic myelogenous leukemia blast cells with an IC_{50} of 230 μM.[4] NDGA inhibited the proliferation of human small cell lung,[5] non-small cell (NSCLC)[6] lung, human pancreatic, and cervical cancer cells in vitro.[7] In vivo, NDGA inhibited tumor growth in esophageal adenocarcinoma[8] and NSCLC xenografts in mice.[6]

Natural[9,10] and semi-synthetic, O-methylated[11,12] analogs of NDGA have been studied for their antiviral activities. The mechanism of antiviral activity was reported to be inhibition of host Sp-1 transcription factor binding with subsequent inhibition of Sp-1-dependent viral gene expression.[12] The degree of inhibition was directly proportional to the degree of phenolic group methylation with the tetra-O-methylated NDGA (M4N) showing the strongest inhibition.[11] Tetra-acetyl[13] and tetra-glycinated[14,15] NDGA analogs were shown to have a similar antiviral mechanism. Recently, NDGA analogs have shown anti-HIV activity in an in vitro Tat-regulated secreted alkaline phosphatase assay.[16]

Heller et al. have reported that M4N arrested C3 cells, a HPV-16/ras-transformed, tumorigenic mouse embryo cell line, in the G_2 phase of the cell cycle. The growth inhibitory activity of M4N was associated with a decrease in the expression of cyclin-dependent kinase (Cdc2), an Sp-1-promoter dependent gene which progresses the cell through G_2/M. In vivo, intratumoral injections of M4N caused a decrease in C3 xenograft tumor size in mice which was correlated with an observed decrease in protein levels of CDC2.[17] M4N inhibition of Sp-1 binding was further investigated as a mechanism of anticancer activity. Sp-1 promoter binding is responsible for Cdc2 and survivin gene expression. Survivin, an inhibitor of apoptosis, is overexpressed in most cancer cells. Its expression is G_2/M specific and Sp-1 dependent. M4N-induced apoptosis in the C3 cell line was correlated to inhibition of survivin, suggesting that inhibition of survivin expression may be an underlying mechanism of action of M4N. M4N-treated C3 cells showed a decrease in CDC2 and survivin at the mRNA and protein level. A non-Sp-1 dependent promoter showed resistance to M4N-induced cytotoxic activity.[18] M4N suppressed tumor growth in Hep3B hepatocellular carcinoma, LnCaP prostate carcinoma, HT-29 colorectal carcinoma, MCF-7 breast carcinoma, and K-562 erythroleukemia in a nude mouse xenograft model. A decrease in Cdc2 and survivin gene expression was correlated to tumor growth inhibition.[19]

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M4N inhibited growth in MCF-7 human breast, A549 human lung, SW480 human colon cancer cell lines and was especially potent against A375 and ACC375 human melanoma cells with IC_{50} values of 2.5 and 5.0 μM, respectively. In the ACC375 cell line, M4N was non-schedule dependent and induced apoptosis. At 50 μM, M4N inhibited DNA synthesis by 66% after 1 h and by 95% of control after 24 h demonstrating an early event in growth inhibition. M4N arrested melanoma cells in G_1/G_0 and G_2/M phases of the cell cycle, suggesting it may affect cell cycle checkpoint proteins. In vivo, M4N inhibited tumor growth in the B16 murine melanoma model, as well as in SW480 human colon cancer and A375 human melanoma xenografts in SCID mice.[20]

Tetra-O-substituted NDGA analogs show decreased in vivo toxicity, for example, the lethal dose 50% (LD_{50}) of NDGA was found to be 75 mg/kg ip[21] M4N was well-tolerated at 1000 mg/kg ip,[18] approximately 11 times the LD_{50} of NDGA.

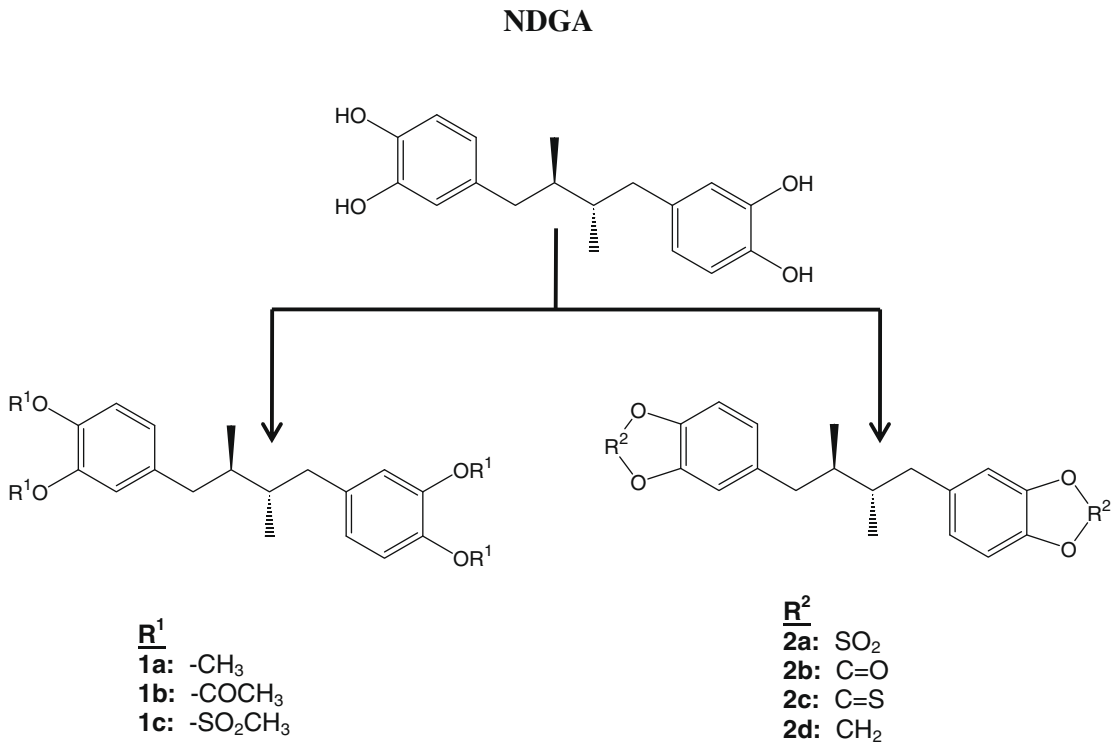
The purpose of this project was to generate new tetra-O-substituted NDGA analogs for the continued investigation of in vivo, anti-melanoma therapeutic activity demonstrated in our prior studies.[20] This work compares the in vitro cytotoxicity of NDGA and M4N to NDGA tetra-acetate and NDGA tetra-methanesulfonate (Scheme 1, R^1). In addition, we investigate an approach to tetra-O-substitution of NDGA by the tethering together of the ortho phenolic groups by a single atom forming a 5-membered heterocyclic ring (Scheme 1, R^2). Analogs from this group were evaluated for their cytotoxicity in A375, human malignant melanoma cells, HT-29, human colorectal adenocarcinoma cells, MCF-7, human mammary gland adenocarcinoma cells, and HepG2 human hepatoma cancer cell lines. Cytotoxicity was evaluated by 5-day 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assays and is summarized in Table 1. One analog from each group was evaluated in vivo using the A375 xenograft model in SCID mice.

The analogs **2a**, 2b, and **2c** were synthesized by a modification of the procedure of Tickner et al.[22] Analog **2d** was synthesized by a method described by Grazia et al.[23] Analogs **1a**, **2a**, 2d were all purified to >95% by semi-preparative HPLC and purity was assessed by three-dimensional UV scan by this method. Analogs**1b**, **1c**, **2b**, **2c** were all purified to >95% (as assessed by three-dimensional UV scan) using recrystallization from isopropanol.

The MTT assay was used to assess in vitro cytotoxicity of the test compounds. All IC_{50} determinations are for 5-day treatment times. Compounds **1a**, **1b**, and NDGA showed the highest potency in the MTT assay against the panel of cell lines with IC_{50} values ranging from 8.5 to 79.4 μM (Table 1). Compounds **2a** and **2b** had moderate inhibitory activity against MCF-7 cells. Compounds **1c** and **2d** were 2.2–116-fold less potent than NDGA against the panel. Compound **2c** showed no significant cytotoxicity against any of the cell lines tested.

The cytotoxicity of the analogs against A375 cells was further evaluated by determining the inhibitory effects of the analogs against DNA, RNA, and protein synthesis by a method described by Mayr et al.[24] The results are shown in Table 1. There was relatively good agreement between cytotoxicity and inhibition of macromolecular synthesis inhibition with the exception of **1c**. This compound was inactive below 1000 μM in the MTT assay but showed strong inhibitory activity against RNA and DNA synthesis at 85 and 100 μM, respectively. Further studies of this discrepancy are needed.

Effects of the test compounds on tumor growth were determined in two studies. In study 1, **1b**, **2a**, and **2b** were suspended in 100% Tween 80 (Sigma–Aldrich, St. Louis, MO.). Mice were implanted sc with 10×10^6 A375 human melanoma cells in 100 μl phosphate buffer saline in the rear right flank. The mice were implanted on day 0 and were dosed with test compounds on days 1, 5, and 9. Controls ($n = 4$) received 100% Tween 80. The method for study 2 was identical to study 1, except there were eight mice per group and the analogs were suspended in 100% PEG 300. Controls ($n = 4$) received 100% PEG 300. Tumor volumes were measured 3



Scheme 1. Reagents and conditions **1a**: potassium carbonated, acetone, dimethylsulfate, reflux **1b**: acetyl chloride, pyridine, methylene chloride **1c**: methanesulfonyl chloride, pyridine **2a**: 1,1′-sulfuryldiimidazole, *N,N′*-dimethylacetamide, KF **2b**: *N,N′*-carbonyldiimidazole, *N,N′*-dimethylacetamide, KF **2c**: 1,1′-thiocarbonyldiimidazole, pyridine, paradioxane **2d**: bromochloromethane, DMF, cesium carbonate.

Table 1
Cytotoxicity and macromolecular synthesis inhibition by NDGA analogs in cancer cell lines

Compound ID	5-day MTT IC_{50} (μM) ± SD				Macromolecular synthesis inhibition (% of control ± SD)		
	HT-29	MCF-7	HepG2	A375	DNA	RNA	Protein
NDGA	51.3 ± 0.6	8.5 ± 0.2	54.1 ± 1.0	54.3 ± 1.0	75 ± 18	72 ± 6	74 ± 6
1a	61.5 ± 0.9	42.4 ± 0.3	44.5 ± 0.7	16.6 ± 0.8	23 ± 8	31 ± 3	70 ± 6
1b	25.6 ± 0.6	24.0 ± 0.2	51.3 ± 1.0	79.4 ± 0.8	77 ± 18	68 ± 16	81 ± 7
1c	343 ± 0.6	103.4 ± 0.2	542.2 ± 0.8	>1,000 ± NA	43 ± 12	23 ± 5	81 ± 6
2a	105.4 ± 0.7	51.4 ± 0.3	98.5 ± 0.6	124.5 ± 1.0	102 ± 31	75 ± 11	55 ± 3
2b	72.3 ± 0.7	41.1 ± 0.3	164.5 ± 0.6	150.3 ± 0.8	184 ± 54	115 ± 11	76 ± 3
2c	>1000 ± NA	>1000 ± NA		>1000 ± NA	85 ± 6	100 ± 12	98 ± 28
2d	89.9 ± 0.4	102.7 ± 0.3	175.3 ± 1.0	235.5 ± 1.0	89 ± 33	98 ± 25	84 ± 2
[a]Doxorubicin					34 ± 7		
[b]Actinamycin D						0.7 ± 02	
[c]Cyclohexamide							3 ± 0.2

Doses of positive controls: [a]10 μg/ml, [b]50 μg/mL, [c]10 μg/mL.
NA = not applicable.
Cytotoxicity was determined by the MTT assay. Inhibition of DNA, RNA and protein synthesis was determined in A375 human melanoma cells at a concentration of 50 μM for each test compound to compare to the IC_{50} of NDGA and to maintain analog solubility.

times/week and were determined based on the following formula: $V(mm^3)$ = length × $(width)^2/2$. The in vivo tumor growth inhibition model data was analyzed by determining % T/C as described by Bissery et al.[25] and are summarized in Table 2. A percent T/C between 42% and 10% is considered moderate tumor growth inhibition. Both **1b** and **2b** showed moderate growth inhibitory effects against xenograft tumors in the first experiment, however in the second study, tumor growth inhibition was observed only for **1b** at the 300 mg/kg dose. This may be the result of lower solubility of **2b** in a PEG 300 formulation compared to the Tween 80 formulation in study 1. In study 2, PEG 300 was used as the vehicle because Tween 80 displayed some tumor growth inhibition compared to untreated controls in study 1 (data not shown). Compounds **1b** and **2b** were well-tolerated at doses of 100–300 mg/kg and no sign of general toxicity was observed compared to control mice. Compound **2a** displayed significant toxicity after a single injection at the doses tested and studies of this compound were discontinued.

In conclusion, among the tetra-substituted analogs, **1a** and **1b** were clearly more potent in vitro than **1c**, although **1c** was rather insoluble in vitro above 100 μM. The compounds in Scheme 1, R^2, showed markedly reduced cytotoxicity compared to Scheme 1, R^1, with the exception of analogs **2a** and **2b** which showed IC_{50} values similar to Scheme 1, R^1 in the MCF-7 cell line. Moreover, compounds **1b** and **2b** demonstrated significant antitumor activity in vivo and were well-tolerated at doses 2.6 and 3.4 times the LD_{50} of NDGA. The fused-ring heterocyclic derivatives of NDGA, such as **2b**, represent a new class of derivatives that should be further investigated for their potential antitumor activity and the possible underlying mechanisms.

Table 2
A375 tumor growth inhibition parameters

Analog	Dose (mg/kg)	N	%T/C[a]	T–C[b]	Td[c]	TCK[d]
Study 1						
1b	100	4	34	2.5	7.4	0.1
1b	200	4	16	3.5	7.4	0.1
2b	100	4	21	3.5	7.4	0.1
2b	200	4	18	3.5	7.4	0.1
Study 2						
1b	300	8	44	1	2.6	0.1
2b	300	8	68	1	2.6	0.1

[a] Percent T/C value = median tumor volume of the treated/median tumor volume of the control × 100 when median control tumor volumes are between 750 and 1100 mm^3.
[b] Tumor growth delay (T–C value) was calculated by comparing the median time in days for the treated and control group tumors to reach a predetermined volume.
[c] Tumor doubling time (Td) was calculated from the line of best fit from a log(*y* or tumor volume) versus linear (*x* or time) plot when the control tumors were in exponential growth between 100 and 1000 mm^3.
[d] Tumor cell kill (TCK) were approximated by the following formula: Log_{10} cell kill = T–C value in days/3.32 × Td.

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Supplementary data

Supplementary data (materials, methods and results of synthesis, purification characterization and bioassay) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.06.063.

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