JOURNAL OF THE TENNESSEE ACADEMY OF SCIENCE 67(4):77-80 OCTOBER1992

A COMPARISON OF THE CYTOTOXICITY OF NORDIHYDROGUAIARETIC ACID AND ITS DERIVATIVES

JOHN M. ZAMORA, EMILIO C. MORA, AND EDWARD J. PARISH

Department of Biology, Middle Tennessee State University, Murfreesboro, TN 37132
Department of Poultry Science and Alabama Agricultural Experiment Station, Auburn University, AL 36849-5416
Department of Chemistry, Auburn University, AL 36849

ABSTRACT--Both natural and synthetic nordihydroguaiaretic acid (NDGA) were highly cytotoxic. The methylated NDGA derivatives showed no cytotoxicity against the HEp-2 and Vero cell lines. A hexabromo-derivative of NDGA was cytotoxic. A podocarpic acid (Podp Br) derivative was tested for cytotoxicity in order to compare the NDGA microtiter test assay system to the standard KB cell tissue culture assay. It was concluded that NDGA and one NDGA derivative are potentially useful cytotoxic agents.

Nordihydroguaiaretic acid (NDGA; Fig. 1) is a component of the resinous exudate of some plants (Stecher, 1983). It was isolated from Larrea tridentata Cav. (creosote bush) in 1942, and, for many years, the creosote bush was the sole source of NDGA (Waller, 1942; Waller and Gisvold, 1945; Oliveto, 1972). Nordihydroguaiaretic acid was used mainly as an antioxidant in fats and oils (Lundberg et al., 1944; Gisvold and Thaker, 1974). Biochemical tests indicated that NDGA was a competitive inhibitor of lipoxidase, catechol o-methyl transferase, liver and serum esterases, phenylalanine hydrolase, NADH oxidase, and succinoxidase (Burba and Becking, 1969; Oliveto, 1972).

Nordihydroguaiaretic acid was shown to be toxic to several cancer cell lines in vitro and in vivo. It inhibited the aerobic and anaerobic glycolysis and respiration of K2-ascites, Ehrlich carcinoma, and L1210 (MeGAG resistant) mouse leukemia cells (Burk and Woods, 1963). A combination of NDGA and ascorbic acid reduced Ehrlich ascite tumors in mice. Nordihydroguaiaretic acid was believed to be the active ingredient of "chaparral" tea, a preparation that reduced the malignant melanoma of a patient (Smart et al., 1970). In plants, NDGA inhibited the growth of tumors caused by *Agrobacterium tumefaciens*. It inhibited complexes I and II (NADH-coenzyme Q reductase and succinate-coenzyme Q reductase) of the mitochondrial electron transport system (Pardini et al., 1970). Phenolic compounds similar in structure to NDGA have shown inhibition of mitochondrial electron transport (Pardini et al., 1971).

In view of the biological properties of NDGA, it was pertinent to test NDGA and synthetic NDGA-derivatives for cytotoxicity. Cytotoxicity testing is a preliminary technique used for the screening of anticancer agents (Suffness and Dourous, 1982). All but two of the important anticancer agents were discovered using the KB cell cytotoxicity test (Perdue, 1982).

MATERIALS AND METHODS

Natural NDGA was isolated and purified by the method described by Waller and Gisvold (1945). The diethyl ether extract of *L. tridentata* was charcoal filtered, concentrated, and dissolved in hot $CHCl_3$. The NDGA was recrystalized from hot $CHCl_3$-diethyl ether. The melting point (mp), mass spectrum, optical rotation, proton nuclear magnetic resonance (^{1}Hnmr), ultraviolet spectrum, and thin layer chromatography (TLC) of NDGA were used to confirm the crystals as NDGA.

Synthetic NDGA obtained from Sigma Chemical Company, Lot no. 42-F-0666 (mp 186 to 187°C uncorr.) and Lot no. 53-F-0749 (mp 181 to 184°C uncorr.), were tested for cytotoxicity and used in the synthesis of the derivatives. The melting point, mass spectrum, optical rotation, ^{1}Hnmr, ultraviolet spectrum, and TLC of NDGA were taken of these two synthetic lots and compared to natural NDGA.

Derivatives of NDGA were prepared according to Bradford (1985). Derivatives obtained were NDGA tetramethyl ether, dibromo NDGA tetramethyl ether, hexabromo NDGA tetramethyl ether, and hexabromo NDGA (Fig. 2).

The bromoketone of podocarpic acid (Podo Br) was used as a standard (Fig. 3). The Podo Br derivative was tested for biological activity at the Cancer Chemotherapy National Service Center, Bethesda, Maryland. The general procedures, protocols, and interpretive data were those of the National Cancer Institute (E. J. Parish and P. H. Miles, in litt.).

Maintenance medium for the cell lines consisted of Eagle's minimal essential medium with Earl's salts, with L-glutamine with gentamycin 50 μg/ml, and $NaHCO_3$, pH 7.2. Growth medium consisted of maintenance medium with 5.0% fetal calf serum.

The Vero ATCC CCL 81 and HEp-2 ATCC CCL 23 cell lines were passaged by trypsinization of confluent monolayers of cells with a buffered 0.05% trypsin and 0.02% ethylene diamine tetraacetate disodium salts solution (EDTA-trypsin). Growth medium was removed from 25-cm^2 flasks, and 2 ml of EDTA-trypsin were added to each flask for 1 min and then removed. The flasks were then incubated for 15 min at 37°C. The flasks were agitated until the monolayers detached; then 10 ml of growth medium were added to each flask to suspend the cells, and the cell suspensions were poured into 125-ml flasks and diluted with 40 ml of growth medium. This final suspension was used for the microtiter plate ($TCED_{50}$ (tissue culture effective $dose_{50}$) tests.

The $TCED_{50}$ is a modification of the method described by Van den Berghe et al. (1978) and Giambrone (1980). A stock suspension of each compound was made by adding a known weight of the compound to 10

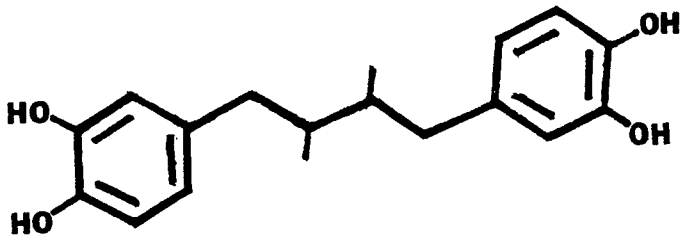

FIG. 1. Nordihydroguaiaretic acid (NDGA).

ml of Earle's balanced salt solution (EBSS), and the suspensions were heated to 100°C for 5 min and stored at -20°C until used. Two-fold serial dilutions of each suspension were made in sterile screw-capped test tubes, and 25µl of each dilution were placed in a corresponding well of a 96-flat well microtiter plate. A cell suspension of either HEp-2 or Vero cells in 125 µl of growth medium was added to each well. Two rows of wells per plate were used as controls, and the microtiter plates were covered with plastic tape and incubated for 2 days. Media in the wells were then decanted, and the cells were fixed for 10 min with cold methanol (-20°C), then stained with 2% crystal violet (Hucker Modification; Benson, 1990) for 2 min, and then washed. The end point was the lowest concentration of plant extract that prevented the cells from growing. The $TCED_{50}$ was calculated after five replicates were run on each sample using a method described by Reed and Meunch (1938).

The tissue-culture-lethal-dose$_{50}$ ($TCLD_{50}$) assay was a modification of the method described by Van den Berghe et al. (1978). A 125-µl cell suspension was added to each well of an 96-flat well microtiter plate. The wells were covered with plastic tape and incubated at 37°C until the Vero and HEp-2 cells were a semiconfluent monolayer. Growth media were removed from the wells, and 150 µl of the appropriate dilution of NDGA or its derivatives was added to each well. Control wells contained 150 µl of maintenance medium, and the plates were sealed with plastic tape and incubated for 2 days. Then the medium was decanted, and the cells were fixed with cold methanol and stained with 2% crystal violet. Cells were examined with an inverted phase microscope for morphological changes associated with cytopathic effects, such as vacuolization, nuclear condensation, granularization, rounding of cells, and loss of monolayer. The end point was the lowest concentration that caused these morphological changes, and the $TCLD_{50}$ was calculated after five toxicity tests using the method described by Reed and Meunch (1938).

RESULTS AND DISCUSSION

Cytotoxicity testing was used as a screening procedure in order to separate the active compounds from the inactive compounds. Figure 4 shows a microtiter $TCED_{50}$ assay for natural and synthetic NDGA and the methyl ether of NDGA after staining with crystal violet. The darkened wells show where the cells grew and attached to the wells. The clear wells indicate inhibited growth or cell death. The $TCLD_{50}$ assay is a subjective evaluation of cell death.

The Vero cell line is a cell line derived from normal African green monkey kidney cells. The HEp-2 cell line is derived from human epidermoid carcinoma (ATCC, 1988). The purpose of using a cancer cell line and a seminormal cell line was to see if there was any differential cell killing or growth inhibition (Wininger and Ross, 1981). There were very little differences in toxicities of the compounds between the two cell lines. This would show that neither the NDGA, the NDGA derivatives, nor the podocarpic acid derivative were capable of differentially killing cancer cells.

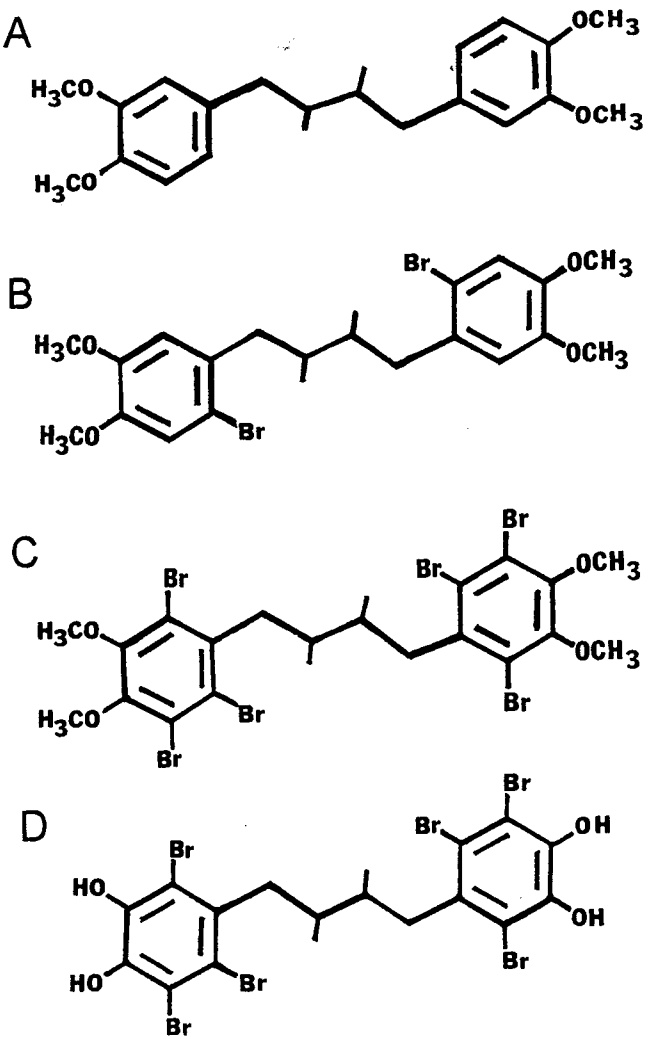

FIG. 2. Derivatives of nordihydroguaiaretic acid. A. NDGA tetramethyl ether. B. NDGA dibromotetramethyl ether. C. NDGA hexabromotetramethyl ether. D. NDGA hexabromo.

Natural NDGA obtained from *L. tridentata* and synthetic NDGA Lot no. 42-F-0666 and Lot no. 53-F-0749 had approximately the same toxicity (Table 1). Methylation of NDGA (Fig. 2) resulted in loss of cytotoxicity, indicating that the catechol group was probably responsible for cytotoxic activity. Methylated dibromo and hexabromo compounds also had poor cytotoxic activities. Hexabromo NDGA had moderate activity but was not as active as the NDGA. Although bromination of

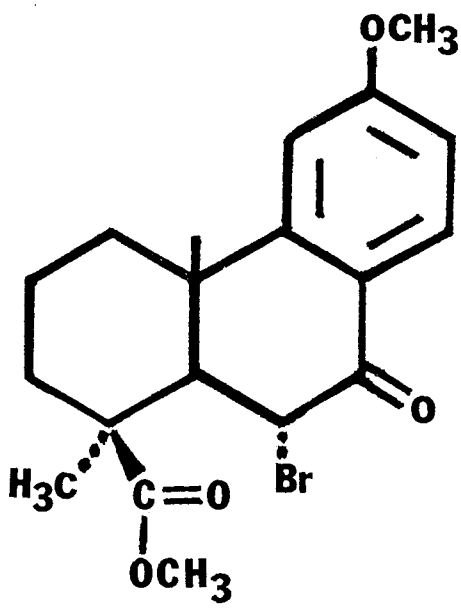

FIG. 3. Bromoketone (podocarpic acid).

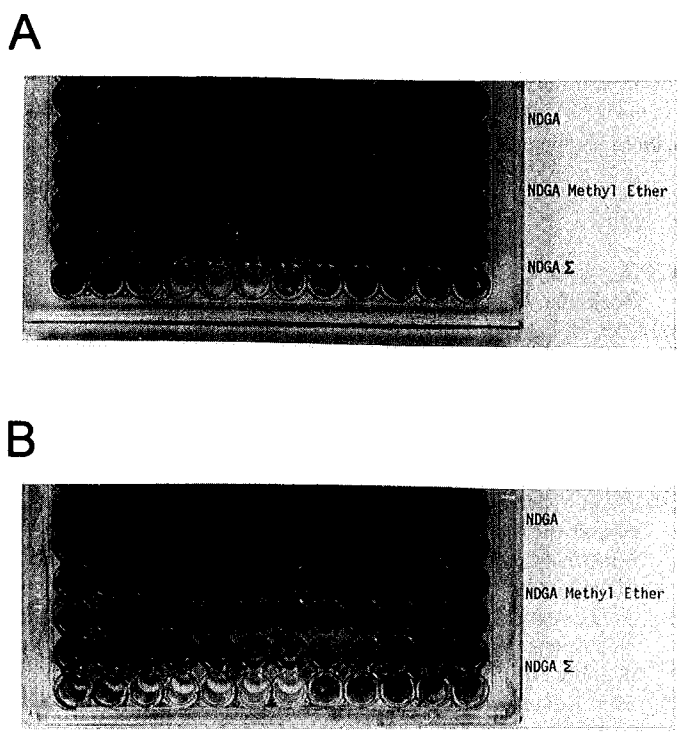

FIG. 4. Microtiter tissue-culture-effective-dose assay ($TCED_{50}$) of HEp-2 (A) and Vero (B) cell lines. The natural nordihydroguaiaretic acid fraction (NDGA), the methyl ether of NDGA, and synthetic NDGA were assayed. The wells in columns 11 and 12 contained untreated HEp-2 and Vero cells.

phenolics usually increases biological activity, it often results in decreased solubility in water. It is possible that the six bromine molecules made the hexabromo NDGA too large to enter the cells. The biological activity of a natural product compound requires an ED_{50} of 20 μg/ml (Cancer Chemotherapy National Service Center, 1972).

The Podo Br derivative was included in the study to determine how the microtiter cytotoxicity correlated with other standard tests. The Podo Br derivative was tested for biological activity at the Cancer Chemotherapy National Service Center, Bethesda, Maryland. The general procedures, protocols, and interpretive data were those of the National Cancer Institute (E. J. Parish and P. H. Miles, in litt.). In order for a synthetic compound to be considered biologically active, it requires an ED_{50} of 4 μg/ml (Cancer Chemotherapy National Service Center, 1972). This compound has an ED_{50} of 3.1 μg/ml against the KB cell line using N.N-dimethylformamide as a vehicle (Bradford, 1985). At this point, the Podo Br was considered toxic enough to warrant evaluation as a possible antitumor agent. Higher concentrations of Podo Br were needed to detect cytotoxicity in the microtiter $TCED_{50}$ and $TCLD_{50}$ assays (Table 1), partly because no vehicles were used in the microtiter assays. Values of $TCLD_{50}$ for Podo Br were four times greater that those of $TCED_{50}$, indicating Podo Br may have greater activity on rapidly dividing cells.

TABLE 1. Cytoxicity of NDGA, derivatives of NDGA, and podocarpic acid. Fifty percent end point calculated by Reed and Meunch (1938) method; 95% confidence limit calculated by Pizzi method (Woolf, 1968).

Compound	$TCED_{50}$[1] (μg/ml)		$TCLD_{50}$[2] (μg/ml)	
	HEp-2	Vero	HEp-2	Vero
NDGA (natural)	17.0 ± 6.0	18.0 ± 7.0	19.0 ± 8.0	19.0 ± 9.0
NDGA (Lot 42-F-0666 Sigma)	8.6 ± 2.6	9.1 ± 4.1	22.0 ± 16.0	26.0 ± 16.0
NDGA (Lot 53-F-0749 Sigma)	19.0 ± 6.0	16.0 ± 4.0	14.0 ± 6.0	9.1 ± 3.7
NDGA Me (tetramethyl ether)	>531	>313	>1024	>908
NDGA $Me(Br)_2$ (dibromo tetramethyl ether)	>589	>589	>589	>589
NDGA $Me(Br)_6$ (hexabromo tetramethyl ether)	>549	>589	>589	>589
NDGA $(Br)_6$ (hexabromo)	129.0 ± 64.0	135.0 ± 76.0	232.0 ± 120.0	199.0 ± 95.0
Podocarpic acid (bromoketone)	84.0 ± 39.0	104.0 ± 53.0	354.0 ± 207.0	465.0 ± 289.0

[1]Tissue culture effective dose.
[2]Tissue culture lethal dose.

The purpose of the microtiter assay was to screen for possible cytotoxic compounds using an assay that is comparatively inexpensive. This assay indicates the cytotoxicities of NDGA and the bromo derivative of NDGA. It is prudent that further assays and evaluations be made of these compounds.

LITERATURE CITED

ATCC. 1988. Catalogue of cell lines and hybridomas. ATCC, Rockville, Maryland, 6408:16,49.

BENSON, H. J. 1990. Microbiological applications. William C. Brown Publishers, Dubuque, Iowa.

BRADFORD, S. 1985. An investigation of potential antitumor derivatives of podocarpic and nordihydroguaiaretic acids and a steroid analysis of selected teleost livers. MS thesis, Auburn Univ., Auburn, Alabama.

BURBA, J. V., AND G. C. BECKING. 1969. Effect of the antioxidant nordihydroguaiaretic acid on the *in vitro* activity of catechol o-methyl transferase. Arch. Internat. Pharmacodyn. Therap., 180:323-329.

BURK, D., AND M. WOODS. 1963. Hydrogen peroxide, catalase, glutathione peroxidase, quinones, nordihydroguaiaretic acid, and phosphopyridine nucleotides in relation to x-ray action on cancer cell. Radiation Res. Suppl., 3:212-246.

CANCER CHEMOTHERAPY NATIONAL SERVICE CENTER. 1972. Protocols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemotherapy Rept., Part 3, 3(2):1-53.

GIAMBRONE, J. J. 1980. Microculture neutralization test for serodiagnosis of three avian viral infections. Avian Dis., 24:284-287.

GISVOLD, O., AND E. THAKER. 1974. Lignans from *Larrea*. J. Pharm. Sci., 6:1905-1907.

LUNDBERG, W. O., H. O. HALVORSON, AND G. O. BURR. 1944. Antioxidant properties of nordihydroguaiaretic acid. Oil and Soap, 21:33-35.

OLIVETO, E. P. 1972. Nordihydroguaiaretic acid, a naturally occurring antioxidant. Chem. Industry, 1972:677-679.

PARDINI, R. S., J. C. HEIDKER, AND D. C. FLETCHER. 1970. Inhibition of mitochondrial electron transport by nordihydroguaiaretic acid (NDGA). Biochem. Pharm., 9:2695-2699.

PARDINI, R. S., J. C. CATLIN, J. C. HEIDKER, AND K. FOLKERS. 1971. Specificity of inhibition of coenzyme systems by lipoidal benzoquinone derivatives. J. Med. Chem., 14:195-197.

PERDUE, R. E. 1982. KB cell culture. I. Role in discovery of antitumor agents from higher plants. J. Nat. Prod., 45:418-426.

REED, L. J., AND H. MUENCH. 1938. A simple method for estimating fifty percent endpoints. Amer. J. Hygiene, 27:493-497.

SMART, C. R., H. H. HOGLE, H. VOGEL, A. D. BROOM, AND D. BARTHOLOMEW. 1970. Clinical experience with nordihydroguaiaretic acid--"Chaparral tea" in the treatment of cancer. Rocky Mtn. Med. J., November:39-43.

STECHER, P. G. (ED.). 1983. The Merck index. Tenth ed. Merck and Company, Rahway, New Jersey.

SUFFNESS, M., AND J. DOUROUS. 1982. Current status of the NCI plant and animal product program. J. Nat. Products, 45:1-14.

VAN DEN BERGHE, D. A., M. IEVEN, F. MERTENS, A. J. VLIETINCK, AND E. LAMMENS. 1978. Screening of higher plants for biological activity. II. Antiviral activity. Lloydia, 41:463-471.

WALLER, C. W. 1942. A phytochemical study of *Larrea divaricata*. PhD dissert., Univ. Minnesota, Minneapolis.

WALLER, C. W., AND O. GISVOLD. 1945. A phytochemical investigation of *Larrea divaricata* Cav. J. Pharm. Assoc., 34:78-81.

WININGER, M. T., AND W. D. ROSS. 1981. Screening of natural products for potential antitumor activity by an *in vitro* clonal cytotoxicity assay using human carcinoma cells. In vitro, 17:260.

WOOLF, C. M. 1968. Principles of biometry. D. Van Nostard Company, Inc., Princeton, New Jersey.