**Abstract.** The effects of nordihydroguaiaretic acid (NDGA) and its tetraacetylated derivative (NDGATA) on the growth, oxygen consumption, adenosine 5'-triphosphate (ATP) level and viability of mouse mammary adenocarcinoma TA3 and its multiresistant variant TA3-MTX-R cell lines were determined. NDGA inhibited mitochondrial carbonyl cyanide m-chlorophenylhydrazone (CCCP)-stimulated oxygen consumption in mouse liver and tumor cells when glutamate plus malate or succinate was added as substrate. The effects were considerably weaker when respiration was supported by duroquinol, indicating that NDGA inhibited primarily mitochondrial electron flow located at some point before ubiquinone. Although NDGATA only inhibited the electron flow through complex I, it was more efficient and selective than NDGA because mouse liver mitochondria were significantly less sensitive to it than both tumor cell lines tested. NDGA and NDGATA inhibited mitochondrial ATP synthesis and, consequently, cell viability and growth rate were also decreased. NDGA and NDGATA inhibited the growth of intramuscularly implanted tumor cells, indicating that NDGATA was also antineoplastic in vivo. In conclusion, NDGATA is cytotoxic to tumor cells, provoking selective induction of mitochondrial dysfunctions, which could be interesting as potential antitumoral agent.

A wide variety of cellular processes are affected by nordihydroguaiaretic acid (NDGA). Many of these effects are ascribable to its action as an antioxidant or free radical scavenger, such as its protective action on human lymphocytes against norgestrel-induced genotoxic damage *in vitro* (1), its potent *in vitro* scavenging of ONOO−, O2−, OH′, O2•− and HOCl and its ability to prevent lung tyrosine nitration *in vivo* (2), and its aging-delaying and significant survival-improving actions in male mice (3). This compound has also been used commercially as a food additive to prevent changes in flavor and nutritive values due to oxidation of fat-containing products, but it has been banned in some countries due to its toxicity in rat hepatocytes (4), and it is known to have hepatotoxic and nephrotoxic effects in rats and mice (5, 6). NDGA is best known as a general lipoxygenase (LOX) inhibitor preventing the conversion of arachidonic, linoleic and other polyunsaturated fatty acids into biologically active metabolites that influence the induction of carcinogenesis, tumor cell proliferation, metastasis and angiogenesis (7-10), although relatively high concentrations are required to achieve efficacy in most cases. NDGA can inhibit tumor growth by inhibiting receptor tyrosine kinase phosphorylation (11). In addition, NDGA induces apoptosis dependently and independently of its activity as a LOX inhibitor, either sensitizing malignant tumor cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis through DR5 up-regulation or through the mitochondrial pathway (12-14). On the other hand, it has been reported that NDGA is an inhibitor of glycolysis (15, 16) and respiration of a wide variety of malignant cells and tissues both *in vitro* and *in vivo* (15, 17). It primarily blocks the mitochondrial electron flow in intact tumor cells between NADH-dehydrogenase and ubiquinone (18). Moreover, mitochondrial Complex I and II activities from beef heart, rat liver mitochondria and carcinoma cells were also inhibited by NDGA, which was independent of the oxygen concentration (19, 20). NDGA likely undergoes biotransformation to a reactive quinone species, either an orthoquinone or a paraquinone methide.
which is responsible for its toxicity (21). The multiplicity of NDGA’s effects may, in some cases, enhance its antitumoral actions, but may also antagonize its therapeutic effects or increase the undesirable side-effects in non-tumor sites. In designing potential antineoplastic drugs, compounds able to selectively inhibit tumor mitochondrial electron flow could prove promising, since many significant differences in the structure and function of mitochondria of normal and cancer cells have been reported. The respiration rate of cancer cells is significantly lower than that of normal cells, apparently due to mitochondrial dysfunction or loss (22). In fact, many tumor cells show important decreases in mitochondrial mass (23). Inhibition of the already low mitochondrial activity of tumor cells may be expected to cause a profound deficit in intracellular adenosine 5’-triphosphate (ATP). These changes in the oxidative phosphorylation system of tumor cells offer a useful pharmacological strategy for the development of selective agents which could inhibit respiration. Therefore, it may be possible to trigger a complex chain of events leading eventually to cancer cell death, while normal cells should be able to recover from such a treatment. Thus, relatively low doses of vanillin-like compounds and alkyl gallates promote a strong inhibition of growth and respiration rate of mouse tumor cell lines (24, 25). The development of NDGA analogs with more specific potencies for this cellular target is expected to result in an enhancement of its anticancer properties. In this report, we investigate the effect of the tetra-acetylated derivative of NDGA, NDGATA, on tumor cell growth and mitochondrial electron transport in tumor cells.

Materials and Methods

Chemicals. ATP, carbonyl cyanide m-chlorophenylhydrazone (CCCP), a diagnostic kit for determination of ATP levels, digitonin, Dulbecco’s modified Eagle’s medium (DMEM), doxorubicin (DOX), duroquinone EGTA, 5-fluorouracil (5-FU), glutamate, HEPES, malate, NDGA, succinate and Tris-HCl were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cisplatin (CPT) was obtained from Pharmachemie B.V. (Haarlem, Holland). Fetal bovine serum was obtained from Gibco Laboratories (Santa Clara, CA, USA). Methotrexate (MTX) was purchased from Lederle Parenterals Inc. (Carolina, Puerto Rico). Sarcoma 786A and adenocarcinoma TA3 were gently purchased by Dr. Gacic about 20 years ago. Vinblastine (VIN) was obtained from Lemery S.A. de C.V. (Mexico). Duroquinol was prepared from duroquinone in alcoholic solution by reduction with sodium borohydride followed by recrystallization and the stock solution was dissolved in dimethyl sulfoxide (DMSO) as described by Boveris et al. (26). The stock solution of NDGA was prepared in ethanol or DMSO. No effects of these solvents were observed at the concentrations used in our experiments. All other reagents were of the highest purity commercially available.

NDGATA synthesis. Two drops of concentrated sulfuric acid were added to a mixture of 302.4 g (1.0 mole) of NDGA and 611.9 g (565.2 ml, 6.0 moles) of freshly redistilled acetic anhydride in a 1-L Erlenmeyer flask. The mixture was stirred gently by hand, upon which it warmed up very rapidly and the NDGA dissolved. The reaction was allowed to proceed for 30 min at 60°C in a water bath, and then left standing overnight at room temperature to complete the formation of the tetra-acetylated derivative. The clear solution was poured onto approximately 800 ml of crushed ice. Immediately after all the ice had melted, the light butter-colored crystalline solid which separated was collected on a Büchner filter and washed with 11 of water. The filter cake was pressed occasionally to facilitate the removal of water. The solid was dried to constant weight over phosphorous pentoxide in a vacuum desiccator. Recrystallization from 70% ethanol permitted an 85-90% recovery of the compound melting at 82.1-82.9°C. Melting point determinations and thin-layer chromatography were used to test for purity, and the product was further characterized by quantitative C and H elemental analyses which gave results within ±0.5% of calculated values. The structure of the synthesized ester was additionally established by infrared and 1H NMR spectroscopy (24). Stock solutions of NDGATA were prepared in ethanol or DMSO.

Tumor cells. Sarcoma 786A and adenocarcinoma TA3 and its multiresistant variant TA3-MTX-R ascites tumor cell lines were propagated until the day of assay by weekly i.p. injection into young adult male A Swiss and CAF 1 jax mice, respectively, as described elsewhere (17, 18, 24, 25). Mice strains were housed and fed in the animal facility of the Faculty of Medicine of the University of Chile under the same conditions indicated before (25). The Faculty of Medicine’s local Ethics Committee approved all experiments. Tumor cells were harvested 5-7 days after i.p. inoculation of ascitic fluid from donors as described by Moreadith and Fiskum (27). Tumor cells were resuspended in 150 mM NaCl, 5 mM KCl and 10 mM Tris-HCl (pH 7.4) at 58-78×10⁶ cells/ml. The cells appeared to be virtually free of erythrocytes and other contaminants, such as leukocytes and fungi, by microscopic examination of cell suspensions.

Preparation of mitochondria. Mitochondrial suspensions of approximately 40-50 mg protein/ml were prepared from tumor cell (27) and from mouse liver (28), with the following minor modifications: mitochondrial fractions were washed twice at 12,000 ×g for 10 min and resuspended in a minimal volume of their respective medium in the absence of bovine serum albumin to eliminate the adsorption of hydrophobic compounds. Protein concentration was determined by the Lowry reaction and standardized with serum albumin (29).

Growth inhibition of tumor cell lines. Cell lines were cultured in DMEM which was supplemented with 10% fetal bovine serum, 25 mM Heps, 44 mM NaHCO₃, penicillin (100 units/ml) and streptomycin (100 μg/ml). For the experiments, 1.8-2.2×10⁵ cells/ml were seeded in 20 ml of culture medium, using 100-ml culture flasks, and grown at 37°C for up to 96 h. The cells were allowed to grow for 24 h (approx. 4.0×10⁵ cells/ml), and then NDGA, NDGATA or antineoplastic drugs were added. Cell numbers were determined with a Neubauer counting chamber, as described elsewhere (18).

Assay of oxygen consumption. The rates of oxygen consumption were measured polarographically at 25°C with a Clark electrode No. 5331 (Yellow Springs Instrument, Yellow Spring, OH, USA) and using a YSI model 53 monitor (Yellow Springs Instrument, Yellow Spring, OH, USA) linked to a 100 mV single channel Goerz RE.
511 recorder (Vienna, Austria). For tumor cells, the 2.0 ml reaction mixture contained 150 mM NaCl, 5 mM KCl and 10 mM Tris-HCl (pH 7.4) plus 5 mM glutamine as substrate and 2.5 mg of protein/ml (about 4.9x10⁹ cell/ml) of ascites tumor cells (24). For mitochondrial suspensions, the 2.0 ml reaction medium contained final concentrations of 200 mM sucrose, 50 mM KCl, 3 mM Hepes (pH 7.4), 0.5 mM EGTA, 3 mM potassium phosphate, 2 mM MgCl₂, and 1.0-1.3 mg/ml of mitochondrial protein. Substrate concentrations were: 2.5 mM glutamate + 2.5 mM malate, or 5 mM succinato, or 1 mM duroquinol. The system was equilibrated with mitochondria at 25°C for 2 min; then NDGA or NDGATA were added and all measurements were made after a 3-min preincubation with the compound, when 0.06 nmol CCCP was added (24).

Cellular ATP levels. Tumor cells (10⁷/ml) were shaken at 37°C (75 oscillations/min) in phosphate buffered saline (PBS) (pH 7.4), containing 5% fetal bovine serum, 2 mM EGTA and supplemented with 5 mM glutamine and 10 mM glucose as substrates, either with or without NDGATA, as described elsewhere (18). Aliquots of cell suspension (1.0 ml) were removed at different times for processing and spectrophotometric assay of cellular ATP content (18). At the same time, 0.2 and 2.0 ml aliquots of cell suspension were removed to determine viability and cellular respiration, respectively, as described above.

Tumor growth in mice. Tumor cells, 10⁶ cells per 0.1 ml of 0.9% NaCl solution, were injected into the right thigh of the recipient mice. The minimum and maximum tumor diameters were measured in millimeters twice a week and used to calculate tumor size index according to the formula [(width)² x (length)/2]. The tumors were allowed to grow for four days. Groups of 10-20 mice were then treated by i.p. injection of 40 mg NDGA/kg/48 h, or 60 mg NDGATA/kg/48 h, or 0.5 mg MTX/kg/48 h (25).

Statistical analyses. Multiple group comparison was made using either a sigmoidal dose response curve, sigmoidal inhibitory curve fitted to the Hill equation, or one-way analysis of variance (ANOVA) followed by Tukey’s test. The distributions of survival and death times were estimated using the Kaplan-Meier method followed by a log-rank test. Data were considered significant at p below 0.05. The analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.comprisma) or SigmaStat 2.01 (SPSS Inc., Chicago, IL, USA).

Results

The multiresistant variant TA3-MTX-R cell line was generated from TA3 ascites tumor in vivo, using a stepwise selection process, starting at an initial MTX dose of 0.1 mg/kg/48 h. The tumor growth rate in mice (expressed as body weight increase) was slow initially, but stabilized at the normal growth rate for the TA3 cell line after five transplants and remained constant thereafter; the corresponding group of mice were then treated with the next higher dose of MTX during five more successive transplants. Ascites tumor cells were sequentially exposed to MTX until doses of 2.0 mg/kg/48 h were attained with an apparent growth rate very similar to the TA3 ascites tumor growth rate (24, 25).

Table 1. Drug resistance of TA3 cell lines.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC₅₀ (nM)</th>
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<tr>
<td></td>
<td>TA3 (WT)</td>
</tr>
<tr>
<td>CPT</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>DOX</td>
<td>22.5±1.9</td>
</tr>
<tr>
<td>5-FU</td>
<td>1,900.0±360</td>
</tr>
<tr>
<td>MTX</td>
<td>65.6±7.7</td>
</tr>
<tr>
<td>VIN</td>
<td>7.6±2.1</td>
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WT: Wild-type, CPT: cisplatin, DOX: doxorubicin, 5-FU: 5-fluouracil, MTX: methotrexate, VIN: vinblastine. IC₅₀ is the concentration required to inhibit 50% of the culture growth when 4.0x10⁵ cells/ml were exposed to drugs for 48 h. Each value is the mean±SD n-1 of three or more independent experiments with each assay performed in triplicate. Relative resistance is expressed as the IC₅₀ of the resistant line divided by the IC₅₀ of TA3 (parental, drug-sensitive) cells. Significance was determined by comparing the respective TA3 IC₅₀ value with that of the TA3-MTX-R selected cells, as in Figure 1 (*p<0.05).

The cell growth was better examined in culture. TA3-MTX-R cells had a doubling time and a cell density similar to that seen in the TA3 cell line (23.2±0.6 and 24.±0.4 h, respectively). To estimate the relative resistance of the TA3-MTX-R cell line, the IC₅₀ values were calculated from the survival curves after treatment of both cell lines with different concentrations of MTX for 48 h (Table I). These cells exhibited a 10-fold increase in resistance compared to the parental TA3 cell line. The cross-resistance profile of four structurally and functionally unrelated drugs was also studied. The results of the cross-resistance experiments are also summarized in Table 1. Significant cross-resistance was observed towards CPT, DOX, 5-FU and VIN. Moreover, the TA3-MTX-R cell line was more resistant to CPT than MTX. The effects of NDGA and NDGATA on growth of the TA3 and TA3-MTX-R cell lines in culture were also examined (Figure 1). A dose-dependent decrease in cell growth was observed with a minimal effective concentration of 5 μM NDGA both for TA3 and TA3-MTX-R cell lines when the cells were exposed to this compound for 48 h (Figure 1A). Strong growth inhibition was observed starting at a concentration of 10 μM NDGA, and both TA3 and TA3-MTX-R were equally sensitive to NDGA. The concentrations of NDGA required to inhibit culture growth by 50% relative to untreated controls (IC₅₀) were 11.1±1.8 and 17.1±1.9 μM NDGA for TA3 and TA3-MTX-R cells, respectively. These values were calculated by interpolation from the respective growth inhibition curves for cultures that were exposed for 48 h to the chemical. While both cell lines were equally sensitive to NDGATA, they were significantly more susceptible to the inhibitory action of this compound. The calculated IC₅₀ values were 3.0±0.8 and 6.1±0.7 μM NDGATA for TA3 and TA3-MTX-R cells, respectively.
Addition of NDGATA to the incubation medium of ascites tumor cell lines resulted in a number of related metabolic effects, which increased with the length of exposure to this chemical. Figure 2 shows the effect of NDGATA on the time course of oxygen consumption, ATP level and viability of TA3 tumor cells. An exponential decay of the oxygen consumption was seen (Figure 2A). One hour after addition of 200 μM NDGATA, cellular respiration had already fallen to 65%, and it decreased to 3.6% of the respective control values at 4 h. However, only a slight decrease of the ATP level and viability were observed one hour after NDGATA was added (Figure 2B and C). Later, both ATP level and viability strongly decreased to 42% (2 h) and 30% (3 h) of the control, respectively. It seems likely that the complex chain of events which results in cell death requires a certain period of time. Between 3-4 h incubation with NDGATA, ATP level and viability slightly decreased to 26% and 25%, respectively. Moreover, these NDGATA effects on TA3-MTX-R were very similar to those seen on the parental TA3 cell line. These results also indicate that NDGATA is able to affect mitochondrial oxidative phosphorylation of the latter cells, which could explain, in part, its stronger cytotoxic effect than that of NDGA (18).

To establish more precisely the inhibitory sites of NDGA and NDGATA on the oxidative phosphorylation process, we performed experiments to study the dose-dependent effect of these compounds on the rate of oxygen consumption when well characterized substrates that donate electrons to the energy-conserving sites 1 and 2 were added (Figure 3). Figure 3A shows the effect of NDGA concentration on CCCP-stimulated oxidation of glutamate + malate. A sigmoidal inhibitory curve was observed when the NDGA concentration was increased in the assay system, and almost complete inhibition was attained with 100 μM in TA3-MTX-R ascites cell mitochondria. NDGA similarly inhibited ADP-stimulated oxidation of glutamate + malate, indicating that NDGA inhibits uncoupled- or ADP-stimulated NAD+-linked electron transport. The effect of NDGA on the oxidation of succinate, which donates electrons into the energy-conserving site 2 of the respiratory chain through succinate dehydrogenase, was also examined (Figure 3A). CCCP-stimulated oxidation of succinate, although it was inhibited by NDGA, was noticeably less sensitive than glutamate + malate. The action of NDGA on the cytochrome b-c1 complex was studied by using duroquinol as electron donor to the energy-conserving site 2 of the respiratory chain. Figure 3A also shows that CCCP-stimulated duroquinol oxidation was practically insensitive to NDGA. The inhibition of oxygen consumption by NDGA was dependent on the nature of the respiratory substrate used. Thus, these results suggest not only that NDGA does not perceptibly inhibit electron flow from cytochrome b to oxygen, but also that the predominant inhibitory sites of NDGA are located at some point before ubiquinone. Moreover, no noticeable variation in the IC50 values of NDGA was found in mitochondria from mouse liver and from the ascites tumor cells 786A, TA3 and TA3-MTX-R (Table II). Figure 3B shows the effect of NDGATA concentration on CCCP-stimulated oxidation of glutamate + malate in mitochondria from TA3 tumor cells. A hyperbolic inhibitory curve was observed when NDGATA concentration was increased in the assay system, and almost complete inhibition was attained by 30 μM. NDGATA also inhibited ADP-stimulated oxidation of glutamate + malate, which indicates that NDGATA inhibits uncoupled- or ADP-stimulated NAD+-linked mitochondrial electron flow. Figure 3B also shows that CCCP-stimulated succinate oxidation was practically insensitive to NDGATA. Thus, these results suggest...
that the predominant inhibitory site of NDGATA is located at some point of the mitochondrial Complex I (NADH dehydrogenase). Moreover, these results suggest not only that NDGATA is a more efficient respiratory inhibitor than NDGA, but also that it is more selective, since mouse liver mitochondria were approximately 7-fold less sensitive than those from the tumor cells tested (Table II).

MTX, NDGA and NDGATA were tested for inhibition of the growth of TA3 and TA3-MTX-R tumors in mice (Figure 4). Tumor cells were injected into the right thigh of the recipient mice. The mice were allowed to develop the tumor for 4 days (time at which it is clearly palpable). The corresponding treatment schedules were then started. Treatments were continued up to 3 days after tumors were clearly not palpable but did not exceed 35 days after tumor implantation. TA3 tumor growth rate in mice treated with MTX was inhibited up to 20-23 days after tumor implantation (Figure 4A). After this period, the growth rate increased with tumors reaching a size similar to those found in respective controls. On the other hand, TA3-MTX-R tumor growth rate in mice treated with MTX was slightly higher than that found in its respective controls (Figure 4B). Figure 4A and B also show the respective responses of TA3 and TA3-MTX-R carcinoma to treatment with NDGA and NDGATA. As indicated by the mean tumor sizes in both control and treated mice, NDGA and its ester derivative exhibited marked inhibition of both tumor growth rates.

The effects of MTX, NDGA and NDGATA on the disease-free survival of mice implanted with either TA3 or TA3-MTX-R tumor cells analyzed by the Kaplan-Meier method are also shown in Figure 4. MTX did not prolong the median survival time of mice with either TA3 (p=0.49) or TA3-MTX-R cells (p=0.55), compared with the respective control, nor did NDGA prolong the median survival time of mice with TA3 (p=0.07) cells. However, the survival time of mice implanted with TA3-MTX-R cells was prolonged by

<table>
<thead>
<tr>
<th>Mitochondria</th>
<th>IC$_{50}$ NDGA (μM)</th>
<th>IC$_{50}$ NDGATA (μM)</th>
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<tbody>
<tr>
<td></td>
<td>GLUT + MAL SUCC</td>
<td>GLUT + MAL SUCC</td>
</tr>
<tr>
<td>786A</td>
<td>24.4±2.2</td>
<td>76.5±8.7</td>
</tr>
<tr>
<td>TA3</td>
<td>38.9±3.2</td>
<td>80.1±5.3</td>
</tr>
<tr>
<td>TA3-MTX-R</td>
<td>40.3±3.6</td>
<td>84.7±4.8</td>
</tr>
<tr>
<td>Mouse liver</td>
<td>36.9±4.8</td>
<td>82.6±5.2</td>
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</tbody>
</table>

IC$_{50}$ is the concentration required to inhibit oxygen consumption rate by 50%, as calculated by interpolation from the respective inhibition curves. Values are means±SD, of at least four independent determinations. *Mouse liver mitochondria presented significantly less sensitivity to NDGATA (p<0.05) than did the tumor cells tested.

Figure 2. Time course for the decrease of the rate of oxygen consumption, ATP content and viability of TA3 ascites cells caused by NDGATA. Cells were incubated at 37°C in the absence or presence of 200 μM NDGATA. Determinations were made after incubation for the lengths of time indicated. See Materials and Methods for further details. Results were expressed as mean percentage of the respective control ± SD of four to six independent experiments. At zero time, the oxygen consumption rate of control cells was 36±2 nmol O2/min/10⁷ cells; ATP content was 124.8±22.0 nmol ATP/10⁷ cells. Viability was expressed as percentage of trypan blue excluding cells vs. total number of cells present in 1 ml. (○) Viability in the absence of NDGATA. At zero time, the cell viability was 9.8±1.0×10⁶±1.6×10⁵. Very small differences in these parameters were found as an effect of incubation. They were assessed statistically using one-way ANOVA and Tukey post hoc test. Data marked with different letters (a-c) were significantly different from other times post-NDGATA treatment and from the control group at the same time points, p<0.05.
NDGA ($p=0.004$), compared with the respective controls. In addition, 40% and 20% of them, respectively, showed no vestiges of tumor 200 days after implantation with either TA3 or TA3-MTX-R tumor cells. NDGATA did not prolong the median survival time of mice with TA3 ($p=0.81$) or TA3-MTX-R cells ($p=0.18$), but it rendered 20% of both groups of mice TA3 or TA3-MTX-R tumor free.

**Discussion**

A major hurdle in the successful treatment of neoplastic diseases is that tumors often exhibit intrinsic or inherent resistance to chemotherapeutic agents, or they may develop resistance to treatment after showing an initial response (acquired resistance). Thus, tumors become insensitive to a range of different chemotoxic agents, including a large variety of them that do not share a common structure or a common cytotoxic intracellular target. The TA3-MTX-R cell line showed MTX resistance and it also exhibited cross-resistance to CPT, DOX, 5-FU, MTX and VIN (Table I). To explain the acquired resistance towards this heterogeneous group of antineoplastic drugs, more than one type of ABC transporter would have to be induced, such as ABCC1, ABCC2, ABCC3, ABCC4 or ABCC2, but induction of ABCB1 and ABCC11 would also be required (30). Surprisingly, both TA3 and TA3-MTX-R cell lines were similarly sensitive to NDGA and NDGATA, but NDGATA was a better growth inhibitor than NDGA (Figure 1). We have no evidence in support of a specific action of NDGA and NDGATA on ABC transporter activities. As the inhibition of mitochondrial electron flow by these compounds resulted in a pronounced reduction of cellular ATP levels, a quantitative alteration in energy-dependent activities would be expected to occur. This would result in reduced drug extrusion mediated by ABC transporter and other enzymatic activities that are components of different cellular defense systems against damage that might be caused by antineoplastic drugs, including those that participate in drug detoxification processes and in the compartmentalization of drugs away from target sites.

ATP is required by living organisms to drive the majority, if not all, of their biosynthetic pathways, as well as for maintenance of the intracellular ion balance and for specialized functions, such as muscle contraction and nerve transmission. Cancer cells are characterized by a high rate of glycolysis which serves as an important energy generating pathway. The molecular basis of this high rate of glycolysis involves a number of genetic and biochemical events, including overexpression of mitochondrial bound isoforms of hexokinase (HK-I and HK-II). By binding to the mitochondrial outer membrane, HK gains preferential access to mitochondrially generated ATP, thereby leading to greatly increased rates of aerobic glycolysis. In addition, elevated levels of mitochondria-bound HK result in apoptosis being evaded, thereby allowing the cells to continue proliferating. In the presence of ADP or ATP, HK stabilizes the permeability transition pore in the closed conformation (31-33). As one hour after the addition of NDGATA cellular respiration was already strongly reduced, while the ATP level and viability remained almost constant, glycolysis probably serves as a sufficient energy generating pathway. Nonetheless later, when oxygen consumption was deeply compromised and mitochondrially-generated ATP was...
insufficient to maintain high HK activity, glycolysis was also
depressed. Consequently, the ATP level strongly decreased and
the cytotoxic effect of NDGATA was observed (Figure 2).

The amounts of NDGA and NDGATA required to inhibit
50% (IC\textsubscript{50}) of the respiratory rate were related to their
lipophilicity, NDGATA being more efficient and selective
towards tumor cells at the mitochondrial level. Variations in
IC\textsubscript{50} may be due to the greater rigidity of the inner
mitochondrial membrane from tumor cells due to its lower
polyunsaturated phospholipid content (34). It is important to
underline that the effects of these compounds are dependent
not only on their concentration in the culture medium but also
on the relative cell density used. NDGATA is a highly
hydrophobic compound that partitions into hydrophobic
cellular compartments, thereby increasing the effective dosage
to the cell. Cellular accumulation of high quantities of this
hydrophobic compound may be most pronounced in
experiments in which cells are perfused with a constant stream
of drug-containing culture medium. Under these conditions,
cells might accumulate large quantities of drugs even when the
extracellular drug concentrations are maintained at a low level.

NDGA undergoes biotransformation to an orthoquinone,
which is responsible for its toxicity (21) and could explain
the oxidant activity and toxicity of NDGA as measured by
the peroxidation of membrane lipids and DNA double-strand
breaks (4). This oxidation could also be effected by the
mitochondrial respiratory chain. This o-quinone could then
interact with and deplete the thiol groups of the sulfhydryl-
rich proteins present in Complex I and Complex II electron-
transferring iron-sulfur clusters and flavoproteins (20),
inhibiting mitochondrial respiration in an oxygen-
independent fashion as a result. In contrast, NDGATA is
unable to undergo this biotransformation, resulting in lower
hepatotoxicity and nephrotoxicity than NDGA.

Figure 4. Effects of MTX, NDGA and NDGATA on the growth of sensitive and resistant TA3 cells and on the survival of mice implanted with these
respective tumors. Four groups of ten tumor-bearing mice were used as untreated controls implanted with either TA3 cells (\textbullet{}, Panel A) or TA3-MTX-R
cells (\textblacktriangle{}, Panel B). Three groups of ten mice were treated intraperitoneally starting on the fourth day after the respective tumor cells were implanted
with either: (\textblacktriangle{} 0.5 mg MTX/kg/48 h, or (○) 40 mg NDGA/kg/48 h, or (●) 60 mg NDGATA/kg/48 h. Survival probability (Panel C and D) is also
shown. Curves marked with letters (a) were significantly different from other treatments and from the control group, p<0.05.
NDGA and NDGATA also display noticeable antitumor activity in mice implanted with TA3 and TA3-MTX-R tumor cells, although the latter cell line is highly refractory to various therapies. While many drugs have been shown to sensitize multidrug-resistant (MDR) cells, most have proven to be unacceptably toxic when transferred to the clinic (30). NDGA is relatively non-toxic (15, 20). It was used as a human and animal food additive to prevent oxidative rancidity and was a constituent of the American diet until 1967 (20). The identification of NDGA and its ester derivative as potent modulators of MDR is important for several reasons. First, they represent a new class of compound that could be exploited for use in patients harboring malignancies that display MDR, since it might be used in order to target a different system in cells than is not only relevant in a specific phase of the cell cycle but which is essential in cellular energy metabolism during the whole cell cycle. Second, it is possible to search for more potent and effective derivatives for future evaluations, since many congeners of NDGA have been either isolated or synthesized and are available for study. An example of this is tetra-O-methyl nordihydroguaiaretic acid (M4N), a global transcription inhibitor, which induces cancer cell death and is also able to reverse the MDR phenotype of tumor cells, besides having potent antiviral action (35-39). In conclusion, NDGATA is cytotoxic to tumor cells, provoking selective induction of mitochondrial dysfunctions, which could be interesting as potential antitumoral agent.

Acknowledgements

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