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Chemosensitizing Effect of Nordihydroguaiaretic Acid and its Tetra-acetylated Derivative on Parental and Multiresistant TA3 Mouse Mammary Adenocarcinoma Cells

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Abstract. *Background: Multidrug resistance (MDR) continues being the major obstacle for successful anticancer chemotherapy. Materials and Methods: The action of nordihydroguaiaretic acid (NDGA) and its tetra-acetylated derivative (NDGATA) on TA3 mouse mammary adenocarcinoma cells and their ability to restore doxorubicin (DOX), cisplatin (CPT) and methotrexate (MTX) sensitivity of the multiresistant variant TA3-MTX-R was examined. Results: Both NDGA and NDGATA synergistically enhanced the cytotoxicity of DOX, CPT and MTX, with a more evident effect in the TA3-MTX-R than in the TA3 cells. NDGATA was more effective than NDGA, as analyzed by the isobologram method. The combination of NDGATA and DOX also reduced the tumor growth rate in mice. Although it did not prolong the median survival time, 30% of mice showed no vestiges of tumor 200 days after implantation with either TA3 or TA3-MTX-R cells. Moreover, NDGA and NDGATA increased the accumulation of DOX and rhodamine (RHO) 123 in both cell lines. Conclusion: NDGA and NDGATA are able to chemosensitize tumor cells and combination therapy with NDGATA and DOX is effective at inhibiting tumor growth in mice.*

Tumours often exhibit intrinsic or inherent resistance to chemotherapeutic agents. In addition, this resistance may also be developed during the course of chemotherapy after showing an initial response (acquired resistance), frequently accompanied by cross-resistance against a variety of anticancer drugs. In consequence, tumors become insensitive to a range of different antineoplastic agents, including a large variety of them that do not share a common structure or a common cytotoxic intracellular target. Such multidrug resistance (MDR) has been a major obstacle to effective cancer chemotherapy. In various cancer cells, one of the major mechanisms of MDR is a net decrease in intracellular drug accumulation through increased efflux, increased sequestration into intracellular organelles or decreased uptake. It is frequently associated with the over-expression of various multidrug resistance-associated proteins which mediate bidirectional nucleocytoplasmic transport of a wide range of substrates, including anticancer drugs (1, 2). Moreover, a variety of ATP-binding cassette (ABC) transporters operate as drug-efflux pumps. At least, 14 ABC transporters involved in MDR have been described (3).

The identification of agents able to synergistically modulate anticancer activity may be useful in overcoming resistance at non-toxic doses, especially those capable of circumventing cross-resistance to a large number of unrelated chemotherapeutic agents. Among these chemosensitizers, nordihydroguaiaretic acid (NDGA) and its derivatives may be a class of great interest. The combination of NDGA with diverse antineoplastic drugs has shown synergistic interactions against both human lung and breast cancer cells (4, 5), and also sensitized both prostate and colorectal cancer cells to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (6). A wide variety of cellular processes are affected by NDGA. Many of them are ascribable to its action as an antioxidant or free radical scavenger (7-9). In addition, NDGA induces apoptosis dependently and independently of its activity as a lipoxygnase inhibitor (6, 10-12). NDGA also inhibits tumor cell proliferation, metastasis and angiogenesis

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(13-16), but relatively high concentrations are required to achieve efficacy in most cases. NDGA inhibits glycolysis and respiration (17-20), and it also causes Golgi disassembly (21). However, NDGA causes kidney toxicity in rats and there is evidence that chronic NDGA consumption would provoke liver toxicity in humans by probably undergoing biotransformation to a reactive quinone species (22). 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. The non-toxic NDGA derivative, tetra-*O*-methyl nordihydroguaiaretic acid (M_4N), which itself is a potent antineoplastic agent, acted synergistically with doxorubicin (DOX) and paclitaxel in inhibiting cell growth of multidrug-resistant NCI/ADR-RES and drug-sensitive MCF-7 cells, whereas, no such synergism was observed when M_4N was used in combination with cisplatin (CPT) (23). Therefore, in designing potential antineoplastic drugs, compounds that are also able to inhibit simultaneously a wide range of MRP activities could prove promising. We have previously reported (20) that the tetra-acetylated derivative of NDGA (NDGATA) inhibited the cell growth, oxygen consumption, ATP synthesis and viability of mouse mammary adenocarcinoma TA3 and the multiresistant variant TA3-MTX-R cells. It also inhibited the growth of both intramuscularly implanted tumor cells (20). A quantitative alteration in energy-dependent activities would be expected to occur, decreasing drug extrusion mediated by ATP-dependent transporters 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. Based on this assumption, the possibility of using NDGATA to sensitize multidrug-resistant cells, thereby increasing the effectiveness of the cytotoxic drugs DOX, CPT and methotrexate (MTX) was explored. Moreover, because of independent antitumor properties of NDGATA, combination therapy of NDGATA with DOX, CPT or MTX could possibly be effectively carried out at lower doses of both agent; consequently, reducing or eliminating toxic side-effects.

Materials and Methods

Chemicals. Dulbecco's modified Eagle's medium, DOX, fluorescein isothiocyanate (FITC)-labeled rabbit anti-goat serum, glutamine, Hepes, NDGA, rhodamine 123 (RHO) and Tris-HCl were purchased from Sigma Chemical Co. (St Louis, MO., USA). Anti-P-glycoprotein$_{170}$ (P-gp$_{170}$) goat serum was obtained from Santa Cruz Biotechnology (Santa Cruz, CA., USA). CPT was obtained from Pharmachemie B.V. (Haarlem, Holland). Fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco Laboratories (Santa Clara, CA., USA). MTX was purchased from Lederle Parenterals Inc. (Carolina, Puerto Rico). NDGATA was synthesized as described previously (20). The stock solutions of NDGA and NDGATA were 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.

Tumor cells. Adenocarcinoma TA3 and its multiresistant variant TA3-MTX-R ascites tumor cells were propagated until the day of assay by weekly *i.p.* injection into young adult male CAF 1 Jax mice as described previously (18-20, 24, 25). The TA3-MTX-R cells were generated by weekly consecutive selection with 2.0 mg MTX/kg/48 h administered *i.p.* in mice until the day of assay (24, 25) and showed MTX resistance and also exhibited cross-resistance to CPT, DOX, 5-flourouracil and vinblastine (20). Mice were housed and fed under the same conditions indicated before in the animal facility of the Faculty of Medicine of the University of Chile (25). The local ethics committee of the Faculty of Medicine approved all the experiments. Tumor cells were harvested 5-7 days after *i.p.* inoculation of ascitic fluid from donors as described by Moreadith and Fiskum (26) and resuspended in 150 mM NaCl, 5 mM KCl and 10 mM Tris - HCl, pH 7.4, at 58-78×10^6 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.

Growth inhibition of tumor cells. The cell lines were cultured in Eagle's medium modified by Dulbecco, which was supplemented with 10% FBS, 25 mM Hepes, 44 mM $NaHCO_3$, penicillin (100 units/ml) and streptomycin (100 μg/ml). For the experiments, 1.8-2.2×10^5 cells/ml were seeded in 20 ml of culture medium, using 100-ml culture flasks, and grown at 37˚C for up to 72 h. The cells were allowed to grow for 24 h (about 4.0×10^5 cells/ml), and then antineoplastic drugs and or NDGA or NDGATA were added. The cell numbers were determined with a Neubauer counting chamber, as described before (18-20). The analysis of the effect of the drug combinations was carried out by the isobologram method described by Singh and Moorehead (27).

Cellular doxorubicin accumulation. Tumor cells (10^6/ml) were shaken at 37˚C (75 oscillations/min) in PBS (pH 7.4), 2 mM ethylene glycol-*bis*(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), 10 μM DOX supplemented with 5 mM glutamine and 10 mM glucose as energy substrates, in the presence or absence of NDGA or NDGATA for 3 h, under the same conditions described previously for cellular ATP level determination (19, 20). Aliquots of the cell suspension (1 ml) were retrieved, immediately centrifuged for 30 s at 11,000 xg and washed three times with ice-cold medium. The cell pellets were resuspended in 0.3 N HCl in 50% ethanol (28), and mixed thoroughly in a vortex mixer. They were centrifuged for 120 s at 11,000 xg. The DOX content in the supernatant was determined from standard curves prepared by dissolving a stock solution of DOX in 0.3 N HCl in 50% ethanol for each assay in a Shimadzu RF-540 spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan). The results were expressed as nmol/10^6 cells. Excitation and emission wavelengths of 470 and 585 nm were used respectively (29).

Cellular accumulation and efflux of rhodamine 123. Tumor cells (5×10^5/ml) were incubated at 37˚C with 1 μM RHO in the presence or absence of 30 μM NDGA or 7 μM NDGATA for 60 min, under the same conditions described above for DOX uptake. Following this period of RHO accumulation, cells were centrifuged at 100 xg for 5 min and then resuspended in RHO-free medium in the absence or presence of

NDGA or NDGATA and incubated again at 37˚C. During both the drug accumulation phase and the efflux phase of the experiment, aliquots of the cell suspension (1 ml) were retrieved at 15 min intervals. The aliquots were immediately centrifuged for one min at 11,000 xg and washed three times with ice-cold PBS containing 1% BSA and 0.05% sodium azide (28). The cell pellets were mixed thoroughly in a vortex mixer with distilled water; under these conditions, the cells lyse and all the RHO leaks out (30). The samples were centrifuged for 2 min at 11,000 xg. The RHO content in the supernatant was determined from standard curves, prepared by dissolving a stock solution of RHO in distilled water for each spectrofluorometric assay and expressed as nmol/5×10^5 cells. Excitation and emission wavelengths of 495 and 535 nm were used, respectively (30).

ABCB1 membrane expression by fluorescence-activated cell sorting (FACS) analysis. Cell suspensions (1.5×10^6 viable cells/ml) were fixed and permeabilized prior to the experiment by 40-min incubation at 4˚C with 3.7% freshly prepared paraformaldehyde in PBS, pH 7.2. They were washed twice in PBS supplemented with 2% FBS. Subsequently, the cells were incubated for 6 h at 8-12˚C in 1 ml of a solution containing 125 μl of anti P-gp_{170} goat serum (200 μg/ml). The cells were pelleted and washed twice with 1 ml of the same buffer. Specific antibody binding was detected using FITC-labeled rabbit antigoat-serum (1:50) with incubation for another 1 h at room temperature and then the cells were washed three times with the same buffer. The samples were analyzed on a FACS Star flow cytometer (Becton Dickinson, San Jose, CA., USA), using the Fluorescence-activated Cell Analyzer Lysys II Program. As control of the specificity of the reaction paraformaldehide fixed cells and cells with only the FITC-conjugate were used.

Tumor growth in mice. The tumor cells, 10^6 cells per 0.1 ml of 0.9% NaCl solution, were injected into the right thigh of the recipient mice. The tumors were allowed to grow for four days by which time they were clearly palpable. Groups of 10 mice were they treated by *i.p.* injection with 1.0 mg DOX/kg on days 4-7, 11-14, 18-21, 25-28, and 32, or 60 mg NDGATA/kg/48 h; or 60 mg NDGATA/kg on days 4, 5, 7, 8, 10, 11, 13, 14, 18, 19, 21, 22, 26, 27, 29, 32 and 33, combined with 1.0 mg DOX/kg on days 6, 9, 12, 15, 20, 25, 28 and 34. The treatments were continued up to 3 days after tumors were clearly not palpable, but they did not exceed 35 days after tumor implantation. The minimum and maximum tumor diameters were measured in millimeters twice a week and used to calculate the tumor size index according to the formula [$(width)^2$ x (length)/2] (20, 25).

Statistical analyses. Paired comparison was conducted by Student's *t*-test analyses. Multiple group comparisons were made using one-way analysis of variance (ANOVA). The distributions of survival and death times were estimated using the Kaplan-Meier method followed by a log-rank test. The data were considered significant at a *p*-value 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

Growth inhibition. NDGA, NDGATA and DOX inhibited the growth of the TA3 and TA3-MTX-R cells in a dose-dependent manner. The concentration resulting in a 50% inhibition (IC_{50}) of the culture growth when the TA3-MTX-R cells were exposed to either NDGA or NDGATA for 24 h was 15.8±2.7 μM or 11.1±1.9 μM, respectively, while the IC_{50} value was 373.1±29.2 nM for DOX alone (Table I). In contrast, the IC_{50} values for NDGA and NDGATA against the sensitive TA3 cells were 20.2±2.9 μM and 2.5±0.4 μM for NDGA and NDGATA, respectively, and was 50.8±8.8 nM for DOX, rendering a relative drug resistance (RDR) value of 7.3 for DOX (Table I).

Table I. *Effect of NDGA and NDGATA on DOX sensitivity in TA3 and TA3-MTX-R cells*.

Drugs	TA3		TA3-MTX-R		
	IC_{50} (nM)[a]	DMF[b]	IC_{50} (nM)[a]	DMF[b]	RDR[c]
DOX	50.8±8.8		373.1±29.2		7.3
DOX + 0.25 μM NDGA	18.4±1.8	2.8	19.2±2.4	19.4	
DOX + 0.50 μM NDGA	10.8±1.6	4.7	10.7±1.7	34.9	
DOX + 1.00 μM NDGA	4.8±0.7	10.6	8.5±0.9	43.9	
DOX + 0.25 μM NDGATA	6.5±1.8	7.8	3.4±2.6	109.7	
DOX + 0.50 μM NDGATA	2.8±0.5	18.1	2.6±0.8	143.5	
DOX + 1.00 μM NDGATA	1.5±0.8	33.9	2.1±0.6	177.6	
DOX + 1.50 μM NDGATA	1.3±0.5	39.1	1.6±0.8	233.2	

[a]IC_{50}=concentration resulting in a 50% inhibition of the culture growth when cells were exposed to DOX for 24 h and NDGA or NDGATA for 3 h. [b]DMF=dose modifying factor (IC_{50} DOX/IC_{50} DOX + NDGA or NDGATA). [c]RDR=relative drug resistance (IC_{50} DOX TA3-MTX-R/IC_{50} DOX TA3). At least two independent determinations were performed in triplicate.

To test the effectiveness of combination chemotherapy the cells were grown in the presence of varying concentrations of DOX for 21 h. NDGA or NDGATA were then added, and 3 h later, cell survival was assessed. Consequently, the cells were treated with DOX for a period of 24 h and with NDGA or NDGATA for 3 h. Table I shows that both NDGA and NDGATA enhanced the DOX toxicity in a dose-dependent manner in both cell lines. The combinations of DOX with NDGATA provided greater inhibition of the growth of both tumor cells and the effect was more evident in the TA3-MTX-R cells than in the parental cells. In fact, NDGATA used at fixed concentrations (0.25, 0.50, 1.00 and 1.50 μM) in combination with variable DOX concentrations produced a dose-dependent decrease of DOX IC_{50}, as indicated by the increased dose-modifying factor (DMF) (233.2 for TA3-MTX-R *versus* 39.1 for TA3 with 1.50 μM NDGATA) (Table I). Both NDGA and NDGATA, which themselves are potent antitumoral agents, acted synergistically with DOX in inhibiting the growth of the cells in culture allowing significant dose decreases of DOX (Figure 1), as indicated by the plots of the IC_{50} values of the respective combinations falling below the line that joins the IC_{50} values of the two single agents.

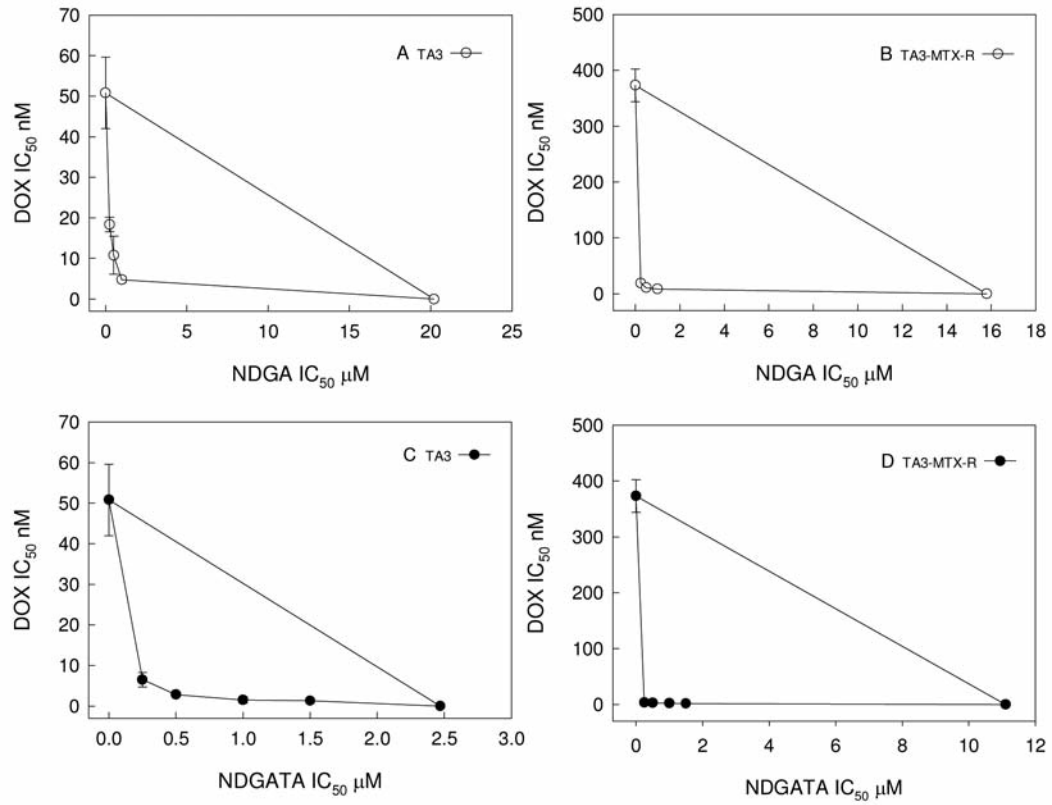

Figure 1. *Effect of DOX combinations with either NDGA or NDGATA on the growth inhibition of TA3 and TA3-MTX-R tumor cells. The line that joins the IC_{50} of the two drugs used independently indicates the form the plot would take if the effects of the two drugs were additive. IC_{50} corresponds to the concentration required to inhibit 50% of the culture growth when cells were exposed to DOX for 24 h and NDGA or NDGATA for 3 h. Each value is the mean±SD of four or more independent experiments with each assay performed in triplicate.*

As with the combination of NDGATA with DOX, NDGATA similarly enhanced both CPT and MTX in a dose-dependent manner (0.25, 0.5, 1.0, 1.5 or 2.0 μM), with a more evident effect in the TA3-MTX-R cells than in the parental cells (Table II), as indicated by the increase in the respective DMF (476.9 *versus* 19.3) for CPT with 1.5 μM NDGATA and 880.5 *versus* 49.6 for MTX with 2.0 μM NDGATA. Moreover, analysis of the data by the isobologram method (27) showed that both the combination of CPT with NDGATA and MTX with NDGATA produced synergistic antiproliferative activity in both cell lines (data not shown).

Doxorubicin accumulation. An attempt was made to determine the relationship between cellular drug levels and the cytotoxicity of DOX and the potential role for NDGA and NDGATA in modulating these effects. In the absence of both NDGA and NDGATA, the net cellular accumulation of DOX after 3-h incubation in the sensitive cell line, TA3, was only two-fold higher than that observed in the similarly treated resistant variant TA3-MTX-R (Figure 2A); but, this difference was statistically

Table II. *Effect of NDGATA on CPT and MTX sensitivities in TA3 and TA3-MTX-R cells.*

Drugs	TA3		TA3-MTX-R		
	IC_{50} (nM)	DMF	IC_{50} (nM)	DMF	RDR
CPT	3.50±0.80		76.3±7.8		22.0
CPT + 0.25 μM NDGATA	1.00±0.50	3.5	0.74±0.4	103.1	
CPT + 0.50 μM NDGATA	0.29±0.09	12.0	0.34±0.2	224.4	
CPT + 1.00 μM NDGATA	0.25±0.03	13.9	0.27±0.1	282.6	
CPT + 1.50 μM NDGATA	0.18±0.03	19.3	0.16±0.1	476.9	
MTX	96.8±20.00		1717±40.3		17.7
MTX + 0.25 μM NDGATA	14.0±0.8	6.9	21.1±1.3	81.3	
MTX + 0.50 μM NDGATA	5.9±0.5	16.5	4.9±0.9	352.2	
MTX + 1.00 μM NDGATA	5.5±1.9	17.5	2.3±0.4	753.1	
MTX + 1.50 μM NDGATA	3.6±2.4	27.0	2.0±0.5	880.5	
MTX + 2.00 μM NDGATA	2.0±0.5	49.6	2.0±0.5	880.5	

IC_{50}=concentration resulting in a 50% inhibition of the culture growth when cells were exposed to either CPT or MTX for 24 h and NDGA or NDGATA for 3 h. DMF=dose modifying factor (IC_{50} drug/IC_{50} drug + NDGA or NDGATA). RDR=relative drug resistance (IC_{50} drug TA3-MTX-R/IC_{50} drug TA3). At least two independent determinations were performed in triplicate.

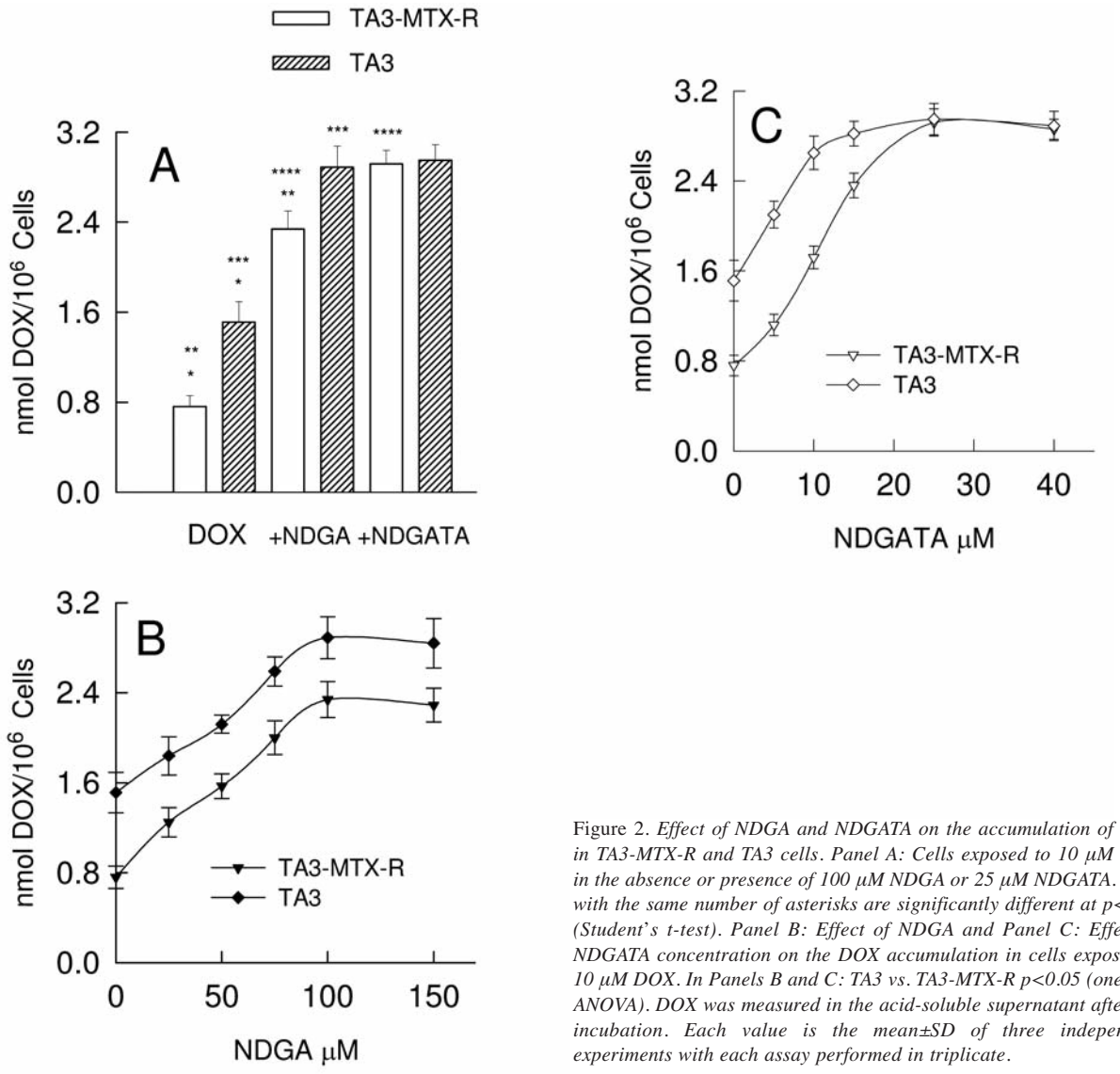

Figure 2. *Effect of NDGA and NDGATA on the accumulation of DOX in TA3-MTX-R and TA3 cells. Panel A: Cells exposed to 10 μM DOX in the absence or presence of 100 μM NDGA or 25 μM NDGATA. Data with the same number of asterisks are significantly different at p<0.05 (Student's t-test). Panel B: Effect of NDGA and Panel C: Effect of NDGATA concentration on the DOX accumulation in cells exposed to 10 μM DOX. In Panels B and C: TA3 vs. TA3-MTX-R p<0.05 (one-way ANOVA). DOX was measured in the acid-soluble supernatant after 3 h incubation. Each value is the mean±SD of three independent experiments with each assay performed in triplicate.*

significant (p<0.004). When NDGA or NDGATA were added to the medium, the total accumulation of DOX increased significantly to about 2-fold in the TA3 cells. In contrast, the DOX accumulation increased 3-fold in the presence of NDGA and 4-fold in the presence of NDGATA in the TA3-MTX-R cells. As shown in Figure 2B and C, the accumulation of DOX by these cells was dependent on the concentration of NDGA or NDGATA, reaching a plateau at about 100 μM NDGA and 25 μM NDGATA. The DOX accumulations in the TA3 cells were slightly, but significantly higher than in the TA3-MTX-R cells. Consequently, NDGATA was more effective, because similar DOX accumulation was attained in both tumor cell lines at lower concentrations of NDGATA than with NDGA (Figure 2B and C).

Accumulation and efflux of rhodamine 123. Figure 3 shows the time course of cellular accumulation and retention of RHO in the absence and in the presence of either NDGA or NDGATA in the TA3 and TA3-MTX-R cells. In the absence of both NDGA and NDGATA, the net cellular accumulation of RHO reached the steady state after 30 min incubation in the resistant variant cells. In contrast, RHO continued accumulating in the cells and the level did not plateau during the 60 min incubation in the sensitive TA3 cell line. The TA3 cells accumulated more RHO than the TA3-MTX-R cells at all the time-points. Using the 60 min time-points, the TA3 to TA3-MTX-R RHO accumulation ratio was 2.4±0.1; this difference was statistically significant (p<0.01). When the cells were incubated in medium containing 30 μM NDGA, the time course of RHO accumulation was increased and the

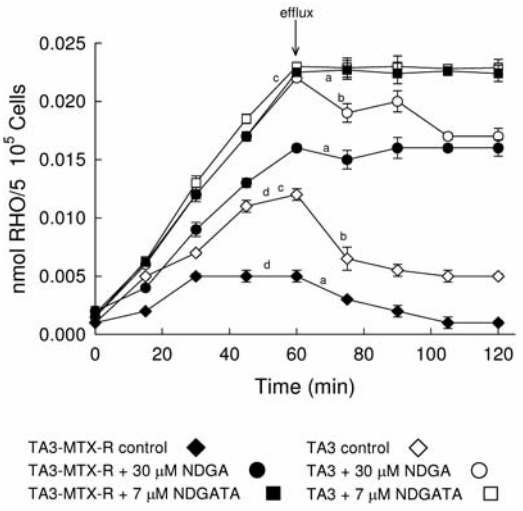

Figure 3. *Effect of NDGA and NDGATA on the accumulation and efflux of RHO. Sensitive TA3 and resistant TA3-MTX-R cells were incubated with 1 μM RHO in the presence or absence of NDGA or NDGATA at 37°C for 60 min (accumulation phase), then washed and resuspended in RHO-free medium containing NDGA or NDGATA (efflux phase). Cellular RHO concentration was measured during the accumulation and efflux phases of the experiment. Each point is the mean±SD of at least four independent experiments. Curves marked with the same letters were significantly different from each other and from the control group, p<0.05, by one-way ANOVA.*

level did not plateau during the 60 min exposure in both cell lines. After 1 h incubation, the accumulation of RHO was up to 3-fold higher ($p<0.02$) in the TA3-MTX-R cells and 2-fold higher ($p<0.03$) in the TA3 cells in the presence of NDGA compared to the controls. In the presence of NDGA, the TA3 cells accumulated slightly more RHO than the TA3-MTX-R cells at all the time-points. Using the 30-60 min time-points, the TA3 cells accumulated only 30 percent more RHO than the TA3-MTX-R cells. This difference was not statistically significant. In contrast, NDGATA was more efficient, because with only 7 μM NDGATA similar RHO accumulation level was attained in both cell lines. When NDGA or NDGATA were absent from the accumulation phase (control), there was a rapid depletion of the cellular RHO level within 30 min in the efflux phase. In contrast, Figure 3 also demonstrates that NDGA and NDGATA added in the accumulation phase markedly inhibited the efflux of RHO from both the resistant and sensitive cells, which was independent of whether the RHO-free medium in the efflux phase was with or without NDGA or NDGATA (data not shown).

ABCB1 expression. In the sensitive TA3 cells, 27.7±6.2% of the total cells expressed ABCB1, which is not a transporter for RHO. In the TA3-MTX-R cells 42.8±2.8% expressed ABCB1 (Figure 4).

Tumor growth in mice. Both the TA3 and TA3-MTX-R tumor growth rates in the mice treated with DOX were initially slightly inhibited but then, on the one hand, the TA3 tumors increased until almost reaching sizes similar to those found in the respective controls and, on the other hand, the TA3-MTX-R tumors subsequently increased until reaching the sizes found in the controls (Figure 5). Figure 5 also shows the respective responses of the TA3 and TA3-MTX-R tumors to treatment with NDGATA. As indicated by the mean tumor sizes in the both control and treated mice, NDGATA exhibited a marked inhibition of both tumor growth rates. The combination of NDGATA and DOX reduced the growth rate more strongly and it was the best treatment against the sensitive cell line (TA3) and the resistant variant (TA3-MTX-R). The inhibitory activity of the NDGA and DOX combination appeared to be synergistic in both cell lines. The effects of DOX and NDGATA on the disease-free survival of the mice implanted with either TA3 or TA3-MTX-R tumor cells analyzed by the Kaplan-Meier method are also shown in Figure 5. None of the treatments tested prolonged the median survival time of the mice with either the TA3 or TA3-MTX-R tumors ($p>0.05$), compared with the respective control. However, 20% of the mice treated with NDGATA alone and 30% of those treated with the combination of DOX and NDGATA showed no vestiges of tumor 200 days after implantation with either TA3 or TA3-MTX-R cells.

Discussion

Surprisingly, both the TA3 and TA3-MTX-R cells were sensitive to NDGA and NDGATA. The results showed that NDGA and NDGATA not only restored sensitivity to DOX in the TA3-MTX-R cells in a synergistic manner, but also increased sensitivity to DOX in the TA3 cells (Table I and Figure 1). NDGA and NDGATA produced maximum modulation without displaying intrinsic toxicity at concentrations of about 1.5 μM. Moreover, NDGATA showed a synergistic chemosensitizing effect for MTX (substrate for ABCC1-4 and ABCG2 transporters), and CPT (substrate for ABCC2 transporter) in both tumor cell lines (Table II) (32).

To explain the acquired resistance towards this heterogeneous group of antineoplastic drugs, those transporters would have to be induced. Reduced drug accumulation appears to be a primary reason for resistance (32). DOX influx occurs by simple Fickian diffusion and efflux is an active transport process, mediated by various ABC transporters (ABCB1, ABCC1, ABCC2 and ABCG2 transporters) (3, 31, 32). The findings suggested that there are small differences between these two cell lines in regard to intracellular drug accumulation as a net balance of uptake and efflux, and that this slightly increased concentration of

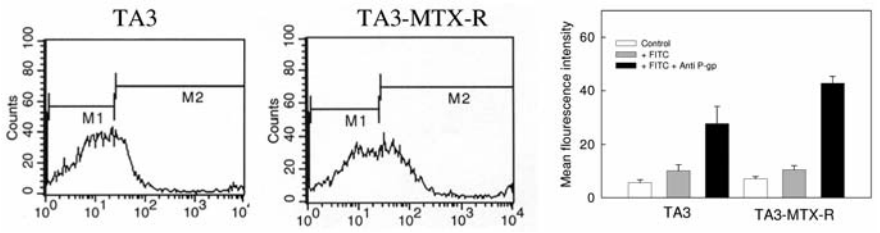

Figure 4. *Representative ABCB1 expression of TA3 and TA3-MTX-R cell lines. FACS analysis (10,000 cells per assay) was carried out after incubation with anti-Pgp170 goat serum and FITC-labeled anti-goat serum and control untreated. Results correspond to the mean percentage±SE of the total cells expressing ABCB1 (M2 section) from five independent experiments. Student's t-test: p<0.05 TA3 vs. TA3-MTX-R cells.*

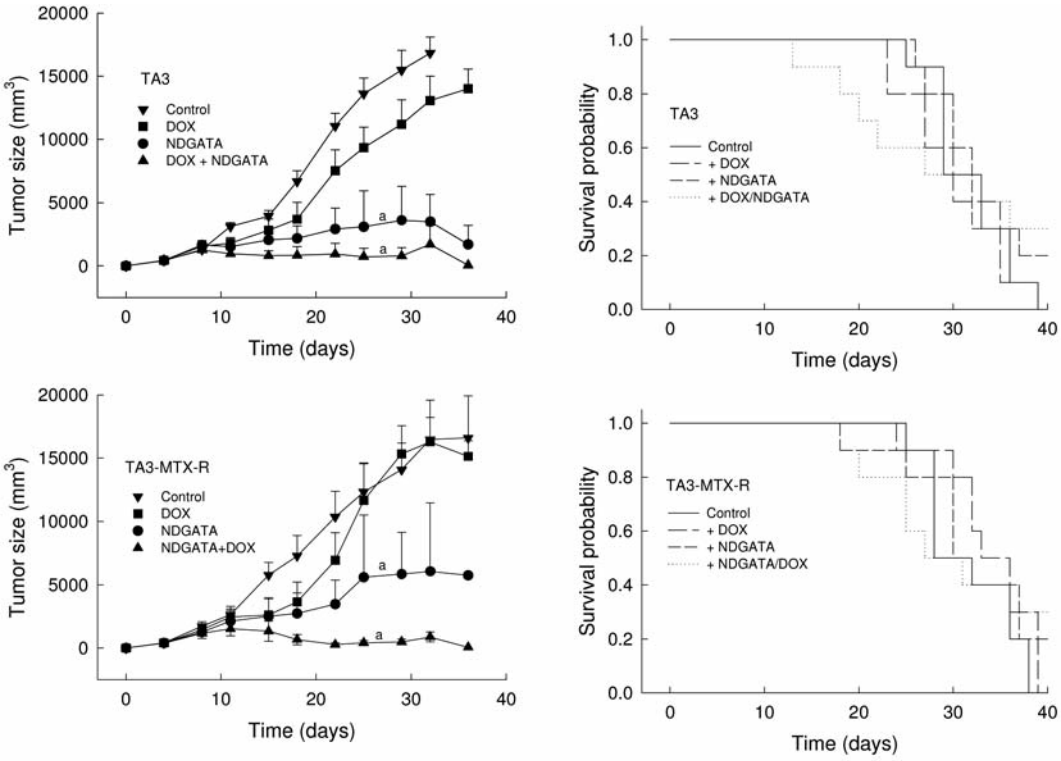

Figure 5. *Effects of DOX, NDGATA and the combination of NDGATA and DOX on the growth of sensitive TA3 and resistant TA3-MTX-R cell tumors and on the survival of implanted mice. From the fourth day after the tumor cell implantation groups of 10 mice were treated with DOX, NDGATA or DOX and NDGATA or untreated (control). Curves were assessed statistically using one-way ANOVA. Those marked "a" were significantly different from DOX treatment and from the control group, p<0.05.*

DOX present in both resistant and sensitive cells after exposure to NDGA or NDGATA would be only in part associated with the strong increase in toxicity. To test whether NDGA and NDGATA modulate ABCC1 and ABCG2 mediated drug transport, the effect of these compounds on the unidirectional RHO efflux was measured, the RHO efflux is ABCC1 and ABCG2 transporter mediated, virtually all the RHO efflux can be blocked by verapamil. However, RHO influx is possibly mediated by solubility-diffusion in response to negative-inside plasma and mitochondrial transmembrane potentials and insensitive to verapamil (31). The ability of NDGA and NDGATA to increase the accumulation of RHO and DOX as well as to block the efflux of RHO (Figure 3), strongly supported the conclusion that NDGA and its derivative act by interfering with a process associated with those activities. NDGA

increased the DOX accumulation by 2-fold, but it sensitized the TA3-MTX-R cells by 44-fold; NDGATA increased DOX accumulation by 3-fold (Figure 2) and it sensitized these cells by 233-fold (Table I). Disproportion between drug accumulation and cytotoxicity is frequently observed (28), but remains incompletely understood. Decreased sequestration into intracellular organelles far from the therapeutic target could occur. NDGATA also displayed noticeable antitumor activity in the mice implanted with TA3 and TA3-MTX-R cells, although the latter cell line is highly refractory to various therapies. The combination of both NDGATA and DOX produced the best survival results and anticancer activity (Figure 5).

We have no evidence to support a specific action of NDGA and NDGATA on the ABC transporter activities. As the inhibition of mitochondrial electron flow by these compounds resulted in a pronounced reduction of cellular ATP levels (20), a quantitative alteration in energy-dependent activities would be expected to occur. For instance, drug extrusion mediated by ABC transporter and other enzymatic activities, including those that participate in drug detoxification processes and in the compartmentalization of drugs away from target sites might decrease. Additionally NDGA and NDGATA also able to inhibit the phosphorylation of growth factor receptors, consequently, deactivating signal transducers, activators of transcription and mitogen-activated protein kinase (33).

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. Accumulated evidence indicates that oxidation of glutamine to CO_2, not glucose, is the major energy source for tumor cells, even in the presence of physiological levels of glucose (34). Hexokinase (HK) bound to the mitochondria of tumor cells has preferred access to the mitochondrially-generated ATP relative to cytosolic ATP (35). But when oxygen consumption is deeply compromised and mitochondrially-generated ATP is insufficient to maintain high HK activity, glycolysis also may be depressed. Consequently, the mitochondria of tumor cells would be the most important site of ATP synthesis.

While many drugs have been shown to sensitize multidrug resistant cells, most have proven to be unacceptably toxic when used in clinically (32). NDGATA is a more efficient and selective respiratory inhibitor than NDGA, since mouse liver mitochondria were about 7-fold less sensitive than those from tumor cells (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. NDGATA might be used to target cellular system that are not only relevant in a specific phase of the cell cycle, but which are 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 analogs of NDGA have been either isolated or synthesized and are available for study.

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References

1 Sugawara I, Akiyama S, Scheper RJ and Itoyama S: Lung resistance protein (LRP) expression in human normal tissues in comparison with that of MDR1 and MRP. Cancer Lett *112*: 23-31, 1997.

2 Tucci M, Quatraro C, Dammacco F and Silvestris F: Role of active drug transporters in refractory multiple myeloma. Curr Top Med Chem *9*: 218-224, 2009.

3 Tao L, Liang Y, Wang F, Chen L, Yan C, Dai C and Fu L: Cediranib (recentin, AZD2171) reverses ABCB1- and ABCC1-mediated multidrug resistance by inhibition of their transport function. Cancer Chemother Pharmacol: 3 March 2009. doi: 10.1007/s00280-009-0949-1

4 Soriano AF, Helfrich B, Chan DC, Heasley LE, Bunn PA Jr and Chou TC: Synergistic effects of new chemopreventive agents and conventional cytotoxic agents against human lung cancer cell lines. Cancer Res *59*: 6178-6184 1999.

5 Rowe DL, Ozbay T, Bender LM and Nahta R: Nordihydroguaiaretic acid, a cytotoxic insulin-like growth factor-I receptor/HER2 inhibitor in trastuzumab-resistant breast cancer. Mol Cancer Ther *7*: 1900-1908, 2008.

6 Yoshida T, Shiraishi T, Horinaka M, Nakata S, Yasuda T, Goda AE, Wakada M, Mizutani Y, Miki T, Nishikawa A and Sakai T: Lipoxygenase inhibitors induce death receptor 5/TRAIL-R2 expression and sensitize malignant tumor cells to TRAIL-induced apoptosis. Cancer Sci *98*: 1417-1423, 2007.

7 Siddique YH, Beg T and Afzal M: Protective effect of nordihydroguaiaretic acid (NDGA) against norgestrel induced genotoxic damage. Toxicol In Vitro *20*: 227-233, 2006.

8 Floriano-Sánchez E, Villanueva C, Medina-Campos ON, Rocha D, Sánchez-González DJ, Cárdenas-Rodríguez N and Pedraza-Chaverrí J: Nordihydroguaiaretic acid is a potent *in vitro* scavenger of peroxynitrite, singlet oxygen, hydroxyl radical, superoxide anion and hypochlorous acid and prevents *in vivo* ozone-induced tyrosine nitration in lungs. Free Radic Res *40*: 523-533, 2006.

9 Yam-Canul P, Chirino YI, Sánchez-González DJ, Martínez-Martínez CM, Cruz C, Villanueva C and Pedraza-Chaverri J: Nordihydroguaiaretic acid attenuates potassium dichromate-induced oxidative stress and nephrotoxicity. Food Chem Toxicol *46*: 1089-1096, 2008.

10 Deshpande VS and Kehrer JP: Oxidative stress-driven mechanisms of nordihydroguaiaretic acid-induced apoptosis in FL5.12 cells. Toxicol Appl Pharmacol *214*: 230-236, 2006.

11 Wu X, Biswal SS and Kehrer JP: Roles of 5-lipoxygenase activating protein in cell proliferation and apoptosis. Cell Biol Toxicol *19*: 135-43, 2003.

12 Tong WG, Ding XZ, Witt RC and Adrian TE: Lipoxygenase inhibitors attenuate growth of human pancreatic cancer xenografts and induce apoptosis through the mitochondrial pathway. Mol Cancer Ther *1*: 929-935, 2002.
13 Ansar S, Iqbal M and Athar M: Nordihydroguaiaretic acid is a potent inhibitor of ferric-nitrilotriacetate-mediated hepatic and renal toxicity, and renal tumour promotion, in mice. Carcinogenesis *20*: 599-606, 1999.
14 Hagmann W: Lipoxygenase in human tumor cells. Pathol Oncol Res *3*: 83-88, 1997.
15 Timár J, Rásó E, Döme B, Li L, Grignon D, Nie D, Honn KV and Hagmann W: Expression, subcellular localization and putative function of platelet-type 12-lipoxygenase in human prostate cancer cell lines of different metastatic potential. Int J Cancer *87*: 37-43 2000.
16 Shureiqi I and Lippman SM: Lipoxygenase modulation to reverse carcinogenesis. Cancer Res *61*: 6307-6312, 2001.
17 Peng Q, Moan J and Cheng LS: The effect of glucose administration on the uptake of photofrin II in a human tumor xenograft. Cancer Lett *58*: 29-35, 1991.
18 Fones E, Amigo H, Gallegos K, Guerrero A and Ferreira J: *t*-Butyl-4-hydroxyanisole as an inhibitor of tumor cell respiration. Biochem Pharmacol *38*: 3443-3451, 1989.
19 Pavani M, Fones E, Oksenberg D, Garcia M, Hernandez C, Cordano G, Muñoz S, Mancilla J, Guerrero A and Ferreira J: Inhibition of tumoral cell respiration and growth by nordihydroguaiaretic acid. Biochem Pharmacol *48*: 1935-1942, 1994.
20 Plaza C, Pavani M, Faundez M, Maya JD, Morello A, Becker MI, De Ioannes A, Cumsille MA and Ferreira J: Inhibitory effect of nordihydroguaiaretic acid and its tetra-acetylated derivative on respiration and growth of adenocarcinoma TA3 and its multiresistant variant TA3MTX-R. In Vivo *22*: 353-361, 2008.
21 Arasaki K, Tani K, Yoshimori T, Stephens DJ and Tagaya M: Nordihydroguaiaretic acid affects multiple dynein-dynactin functions in interphase and mitotic cells. Mol Pharmacol *71*: 454-460, 2007.
22 Billinsky JL, Marcoux MR and Krol ES: Oxidation of the lignan nordihydroguaiaretic acid. Chem Res Toxicol *20*: 1352-1358, 2007.
23 Chang CC, Liang YC, Klutz A, Hsu CI, Lin CF, Mold DE, Chou TC, Lee YC and Huang RC: Reversal of multidrug resistance by two nordihydroguaiaretic acid derivatives, M4N and maltose-M3N, and their use in combination with doxorubicin or paclitaxel. Cancer Chemother Pharmacol *58*: 640-653, 2006.
24 Cordano G, Pezoa J, Muñoz S, Rivera E, Medina J, Núñez-Vergara LJ, Pavani M, Guerrero A and Ferreira J: Inhibitory effect of vanillin-like compounds on respiration and growth of adenocarcinoma TA3 and its multiresistant variant TA3-MTX-R. Eur J Pharm Sci *16*: 255-263, 2002.
25 Frey C, Pavani M, Cordano G, Muñoz S, Rivera E, Medina J, Morello A, Maya JD and Ferreira J: Comparative cytotoxicity of alkyl gallates on mouse tumor cell lines and isolated rat hepatocytes. Comp Biochem Physiol A Mol Integr Physiol *146*: 520-527, 2007.
26 Moreadith RW and Fiskum G: Isolation of mitochondria from ascites tumor cells permeabilized with digitonin. Anal Biochem *137*: 360-367, 1984.
27 Singh G and Moorehead R: Mitochondria as a target for combination cancer chemotherapy. Int J Oncol *1*: 825-829, 1992.
28 Hait W, Gesmonde JF, Murren JR, Yang J, Chen H and Reiss M: Terfenadine (seldane®): a new drug for restoring sensitivity to multidrug resistant-cancer cells. Biochem Pharmacol *45*: 401-406, 1993.
29 Ganapathi R, Grabowski D, Rouse W and Riegler F: Differential effect of the calmodulin inhibitor trifluoperazine on cellular accumulation, retention, and cytotoxicity of anthracyclines in doxorubicin (adriamycin)-resistant P388 mouse leukemia cells. Cancer Res *44*: 5056-5061, 1984.
30 Altenberg GA, Young G, Horton J, Glass D, Belli JA and Reuss: Changes in intra- or extracellular pH do not mediate P-glycoprotein-dependent multidrug resistance. Proc Natl Acad Sci USA *90*: 9735-9738, 1993.
31 Rogan AM, Hamilton TC and Young RC: Reversal of adriamycin resistance by verapamil in human ovarian cancer. Science *224*: 994-996, 1984.
32 Dean M, Fojo T and Bates S: Tumor stem cells and drug resistance. Nat Rev Cancer *5*: 275-284, 2005.
33 Meyer AN, McAndrew CW and Donoghue DJ: Nordihydroguaiaretic acid inhibits an activated fibroblast growth factor receptor 3 mutant and blocks downstream signaling in multiple myeloma cells. Cancer Res *68*: 7362-7370, 2008.
34 Yuneva M: Finding an "Achilles' heel" of cancer: the role of glucose and glutamine metabolism in the survival of transformed cells. Cell Cycle *7*: 2083-2089, 2008.
35 Mathupala SP, Ko YH and Pedersen PL: Hexokinase-2 bound to mitochondria: cancer's stygian link to the "Warburg Effect" and a pivotal target for effective therapy. Semin Cancer Biol *19*: 17-24, 2009.

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