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Comparative cytotoxicity of alkyl gallates on mouse tumor cell lines and isolated rat hepatocytes☆

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Abstract

Alkyl esters of gallic acid inhibited the respiration rate of mouse sarcoma 786A and mouse mammary adenocarcinoma TA3 cell lines and its multiresistant variant TA3-MTX-R more effectively than gallic acid, both in the absence and in the presence of the uncoupler CCCP. The order of inhibition of the respiration rate by gallates in intact cells was *n*-octyl- ≈ *iso*-amyl- ≈ *n*-amyl- ≈ *iso*-butyl- > *n*-butyl- > *iso*-propyl- > *n*-propyl-gallate ≫ gallic acid. Sarcoma 786A was significantly more susceptible to all seven esters than the TA3 cell line. Respiration rates of the TA3-MTX-R cell line showed almost the same sensitivity to these esters as the TA3 cell line. However, hepatocytes were significantly less sensitive than all tumor cells tested. These alkyl gallates blocked mitochondrial electron flow, mainly at the NADH-CoQ segment, preventing ATP synthesis, which would lead to cellular death. These esters also inhibited, in the same order of potencies as respiration, the growth of 786A, TA3 and TA3-MTX-R cells in culture. In mice carrying TA3 or TA3-MTX-R tumor cells, an important decrease of the tumor growth rate and an increase of survival were observed when mice were treated with *iso*-butyl gallate alone or in combination with doxorubicin. These results indicate that alkyl gallates are selectively cytotoxic to tumor cells, which may be due to the mitochondrial dysfunctions of these cells.

Keywords: Alkyl gallates; Tumor cell respiration; Oxygen consumption; Electron flow; Cytotoxicity; Cell culture growth; Solid tumor growth; Rat hepatocytes; Resistance

1. Introduction

Gallic acid (3,4,5-trihydroxybenzoic acid) is a plant phenol obtained by the hydrolysis of tannins (Inoue et al., 1995). Its alkyl esters, especially propyl gallate, octyl gallate and lauryl gallate are currently used as antioxidant additives to prevent changes in food flavor and nutritional values, due to the oxidation of unsaturated fats (Kubo et al., 2002). They are also used as antioxidants in the cosmetic and pharmaceutical industries. The lipophilic alkyl side chain of the gallic acid derivatives is important for their antioxidant potency (Nakayama et al., 1993). The structure of the alkyl chain is relevant to antimutagenicity and inhibition of CYP1A expression (Feng et al., 2003), to the suppression of mouse mammary tumor virus gene (Abe et al., 2001) and to the induction of apoptosis in tumor cells (Roy et al., 2000). The length of the lipophilic alkyl side chain seems to have a strong impact on the membrane affinity of these compounds (Feng et al., 2003; Tammela et al., 2004). Alkyl esters of gallic acid have antiviral, antibacterial and antifungal properties (Fujita and Kubo, 2002; Savi et al., 2005), specifically against Gram-positive bacteria (Kubo et al., 2002, 2003, 2004). The apoptotic activity is also dependent on the hydrophobic portion of the molecule (Inoue et al., 1995; Sakagami et al., 1997; Sakaguchi et al., 1998; Serrano et al., 1998). Cytotoxicity is not a common feature in phenolic compounds, but it is a fairly specific feature of gallic acid, where the three adjacent phenolic hydroxyl groups

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are responsible for the cytotoxicity, and the carboxyl is presumably implicated in distinguishing between normal and cancer cells (Inoue et al., 1995). On the other hand, those gallates that bear long alkyl groups bound to the carboxyl group in gallic acid are toxic to rat hepatocytes, but at higher concentrations than those found for tumor cells. It seems that mitochondrial respiration is a common target for these compounds. In isolated rat liver mitochondria they cause a concentration-dependent increase of the oxygen consumption rate in state 4, which indicates a partial uncoupling of respiration (Nakagawa and Tayama, 1995; Nakagawa et al., 1995, 1996).

A number of significant differences in the structure and function of mitochondria of normal and cancer cell have been reported, such as differences in metabolic activity, molecular composition and mtDNA sequence. Metabolic alterations associated with mitochondrial function observed in cancer cells comprise increased glutaminolytic activity, increased gluconeogenesis and reduced fatty acid oxidation. Moreover, an increased rate of aerobic glycolysis and a reduced rate of pyruvate oxidation have also been monitored (Gatenby and Gillies, 2004). Consequently, an increased production of lactic acid can be detected, which might be due to an impaired respiratory capacity in tumoral cell types. Metabolic differences between normal and malignant cells specifically associated with mitochondrial bioenergetic function have also been observed. These include differences in respiratory substrate preferences, rates of electron flow and anion transport, and the capacity to accumulate and retain calcium. Certain enzyme activities central to oxidative phosphorylation are decreased in cancer cells. For example, ATPase and cytochrome *c* oxidase activities, and the adenine nucleotide exchange function of adenine nucleotide translocase are lower in cancer cells than in normal ones. In addition, the mitochondrial membrane potential has been shown to be significantly higher in carcinoma cells than in normal epithelial cells (reviewed by Modica-Napolitano and Singh, 2004). On the other hand, their unlimited replication potential and resistance to cell death stimuli define cancer cells (Don and Hogg, 2004). Moreover, mitochondria from normal liver cells are highly organized within the cytoplasm, contrasting with a more chaotic organization of mitochondria from cancer cells (Gourley et al., 2005). The respiration rate of cancer cells is significantly lower than that of normal cells, apparently due to mitochondrial dysfunction or loss (Rossignol et al., 2004). In fact, many tumor cells show important decreases in mitochondrial mass (Penta et al., 2001). Inhibition of the already low mitochondrial activity of tumor cells may be expected to cause a profound deficit in intracellular 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 is 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 rotenone promote a strong inhibition of growth and apoptosis in HL-60 leukemia cells (Matsunaga et al., 1996). In this report, we show that alkyl gallates inhibit tumoral cell growth and mitochondrial electron transport of tumor cells significantly more than in hepatocytes, providing an opportunity to selectively target cancer cell mitochondria.

2. Materials and methods

2.1. Chemicals

Albumin (fatty acid free), 2-amino-2-hydroxymethyl-1,3-propanediol-HCl (Tris–HCl), antimycin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), collagenase (for hepatocytes isolation), dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium, doxorubicin (DOX), duroquinone, ethylene glycol bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), fetal bovine serum (FBS), gallic acid (GA), *n*-propyl gallate (nPG), *n*-octyl gallate (nOG), glutamine, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), rotenone, sodium succinate, and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Sodium borohydride was obtained from E. Merck (Darmstadt, Germany). Methotrexate (MTX) was purchased from Lemery S.A. (Mexico). The stock solution of each alkyl ester of gallic acid was prepared in ethanol or DMSO, and we did not observe any effect of these solvents at the concentrations used in our experiments. Duroquinol was prepared from duroquinone in alcoholic solution by reduction with sodium borohydride followed by recrystallization and the stock solution was dissolved in DMSO as described by Boveris et al. (1971). All other reagents were of the highest purity commercially available.

2.2. Synthesis of alkyl esters of gallic acid

The esters *iso*-amyl- (iAG), *n*-amyl- (nAG), *iso*-butyl- (iBG), *n*-butyl- (nBG) and *iso*-propyl gallate (iPG) were synthesized from gallic acid, following the method described by Christiansen (1926). Melting point determinations and thin-layer chromatography were the purity tests, and the products were further characterized by quantitative C and H elemental analyses of results within ±0.5% of calculated values. The structures of the synthesized esters were additionally established by infrared and ^{1}H NMR spectroscopy, as previously described (Gunckel et al., 1998).

2.3. Tumor cells

Sarcoma 786A and adenocarcinoma TA3 ascites tumor cell lines were grown by weekly intraperitoneal (i.p.) injection of 1.0×10^{6} cells into young adult male A Swiss and CAF 1 Jax mice, respectively (Fones et al., 1989; Pavani et al., 1994). The methotrexate-resistant cell line (TA3-MTX-R) was generated by weekly consecutive selection in the presence of MTX (Morello et al., 1995). 2.0×10^{6} cells were propagated in the same mouse strain with 2.0 mg/kg/48 h MTX administered i.p. until the day of assay (Cordano et al., 2002). 10–12 week-old mice with a mean weight of 23.5 g (CAF 1 Jax) or 27.5 g (A Swiss) were housed at 23–25 °C and 57–60% humidity. Climatization was achieved by means of air conditioning/electric heater and

ventilation system in the animal facility of the Faculty of Medicine of the University of Chile. The Faculty of Medicine's local ethics committee approved all experiments. Animals were fed with a standard laboratory chow and water ad libitum. Tumor cells were harvested 5–7 days after intraperitoneal inoculation of ascitic fluid from donors as described by Moreadith and Fiskum (1984). Tumor cells were resuspended in 150 mM NaCl, 5 mM KCl and 10 mM Tris–HCl, pH 7.4, at $58–78 \times 10^6$ cells/ml. The cells appeared to be virtually free of erythrocytes and other contaminants, such as leukocytes and fungi by microscopical examination of cell suspensions.

2.4. Isolation of rat hepatocytes

Hepatocytes were isolated according to Moldéus et al. (1978). Livers were first perfused in situ with an oxygenated washing solution (95% O_2 and 5% CO_2), free of calcium and containing 0.5 mM EGTA (40–45 ml/min at 37 °C for 5 min), followed by perfusion with Hank's modified solution, pH 7.4, containing 0.04% collagenase and 4 mM $CaCl_2$ for 10 min as described by Moldéus et al. (1978). The liver was then gently minced on a Petri dish and strained through nylon mesh. Hepatocytes were washed twice in 150 mM NaCl, 5 mM KCl and 10 mM Tris–HCl, pH 7.4 and centrifuged at 50 ×*g* for 2 min. Then, they were resuspended in the same medium at a concentration of $34–46 \times 10^6$ cells/ml. Cell viability was consistently >90% as determined by trypan blue exclusion (Cordano et al., 2002).

2.5. Cell respiration

Respiration measurements were carried out polarographically at 25 °C with a Clark electrode (Yellow Springs Instrument) using a YSI model 5300 monitor linked to a 1 V single channel recorder. The reaction mixture (1.8 mL) contained: 150 mM NaCl, 5 mM KCl and 10 mM Tris–HCl, pH 7.4, plus the following final respiratory substrate concentrations, which were at saturation conditions: 5.6 mM glutamine (tumor cells) or 10 mM glucose plus 5 mM glutamate (hepatocytes) or 1 mM duroquinol or 5 mM ascorbate plus 0.6 mM TMPD, using either about 10^7 ascites tumor cells or about 6.0×10^6 hepatocytes. Where indicated, 0.19 μM (786A, TA3 and hepatocytes) or 0.28 μM (TA3-MTX-R) CCCP was added to attain maximal uncoupling effect (Fones et al., 1989).

2.6. Growth inhibition of 786A, TA3 and TA3-MTX-R cell lines

These cell lines were cultured in the absence or presence of the alkyl gallate in Dulbecco's modified Eagle's medium with 7% fetal bovine serum, 25 mM Hepes, 44 mM $NaHCO_3$, 100 UI/ml penicillin, and 100 μg/ml streptomycin. 1.8 to 2.2×10^5 cells/ml were seeded in 20 ml of culture medium, using 125 ml culture flasks and grown at 37 °C for up to 96 h. The cells were first allowed to grow for 24 h (about 4.0×10^5 cells/ml) and alkyl gallate was then added (Pavani et al., 1994). Cell numbers were determined every 24 h with a Neubauer counting chamber and cell viability was determined by trypan blue exclusion.

2.7. Tumor growth in mice

Tumor cells, 10^6 cells per 0.1 ml of 0.9% NaCl solution, were injected into the thigh of the right hind leg 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 $[(\text{width})^2 \cdot (\text{length})/2]$ (Rush et al., 1992). The tumors were allowed to grow for four days. Then, groups of 10–20 mice were treated by i.p. injection of different drugs, as indicated on the corresponding figures.

2.8. Statistical analyses

Multiple group comparison was determined using either one- or two-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 log-rank test. Data were considered significant at P below 0.05.

Table 1
Effect of gallic acid and its alkyl esters on the respiration rates of isolated hepatocytes and tumor cell lines 786A, TA3 and TA3-MTX-R

	¶Hepatocytes		¶¶786[a]		TA3		TA3-MTX-R	
	*I_{50} (nmol/10^6 cells)		*I_{50} (nmol/10^6 cells)		*I_{50} (nmol/10^6 cells)		*I_{50} (nmol/10^6 cells)	
Compounds	–	+CCCP	–	+CCCP	–	+CCCP	–	+CCCP
Gallic acid	>400	>400	246.2±9.7[a]	205.8±9.7[a]	305.4±16.8[a]	231.7±18.2[a]	325.0±21.2[a]	205.0±8.3[a]
n-propyl ester	>400	>400	107.3±7.8[b]	67.5±8.2[b]	143.3±9.9[b]	95.5±7.7[b,c]	103.5±10.8[b,d]	99.6±9.4[b]
iso-propyl ester	>400	388.7±21.3[a]	71.3±8.3[c]	49.2±7.1[c,b]	104.5±7.1[c]	103.9±10.9[c]	157.2±13.4[c]	76.2±6.4[c]
n-butyl ester	393.8±15.2[a]	242.2±8.7[b]	46.5±5.4[d]	25.8±4.2[d,c]	67.8±4.2[d]	57.2±5.2[d]	73.4±7.5[d]	32.8±3.3[d]
iso-butyl ester	339.1±11.4[b]	286.6±9.3[c]	41.4±6.1[d]	35.2±5.1[d,c]	49.0±5.2[e]	41.2±3.9[e,d]	50.0±3.2[e]	32.0±2.0[d]
n-amyl ester	181.3±9.2[c]	71.9±6.9[d]	35.2±4.8[d]	12.2±4.8[d]	41.1±5.8[e]	24.8±4.1[e]	41.3±3.4[e]	29.9±2.5[d]
iso-amyl ester	168.4±8.9[c]	149.9±11.3[e]	33.4±5.0[d]	21.9±4.1[d,c]	43.1±3.2[e]	31.4±2.4[e,d]	48.8±7.5[e]	27.5±4.2[d]
n-octyl ester	N.D.	N.D.	N.D.	N.D.	43.9±4.2[e]	40.1±4.8[e,d]	41.4±5.8[e]	25.0±3.5[d]

*I_{50} corresponds to the nmol of compounds per 10^6 cells necessary to inhibit cell respiration by 50%, as calculated by interpolation from the respective inhibition curves. Values are means±SD_{n-1}, of at least four independent determinations. ¶Hepatocytes present significantly less sensitivity ($P<0.05$) than all tumor cell lines. ¶¶The 786A cell line shows significantly higher sensitivity than the TA3 and TA3-MTX-R cell lines. Data marked with common letters (a–e) were different from other treatments for each cell line, $P<0.05$.

3. Results and discussion

3.1. Effect of alkyl gallates on cell respiration

To examine the effect of alkyl gallate concentrations on cellular respiratory rate, tumor cells were treated with increasing concentration of gallic acid (50–850 nmol/10^6 cells) and different alkyl gallates (5–550 nmol/10^6 cells), which depended of the length and branching of the lipophilic alkyl side chain. In addition, hepatocytes were treated with alkyl gallate concentrations between 50 and 800 nmol/10^6 cells. Table 1 summarizes the effects of gallic acid and alkyl gallates on the rate of oxygen consumption of tumor cells and rat hepatocytes. The amount of these compounds per 10^6 cells required to inhibit 50% of the respiratory rate of tumor cells (I_{50}) was a function of their lipophilicity and branching, those with alkyl chain lengths between five and eight carbons being more efficient. *n*-Amyl, *iso*-amyl and *n*-octyl gallates were found to be about 4 times more effective in tumor cells than in hepatocytes, in the absence of CCCP. The I_{50} value for gallic acid was higher than that of its ester derivatives. It is important to point out that when the alkyl chain length increased from four to five carbons, the respiratory rate of hepatocytes was strongly inhibited. Consequently, the selectivity of these alkyl gallates towards tumor cells was strongly decreased. These results also suggest that the level of inhibition, although depending on the amount of alkyl gallate, is not predominantly dependent on whether respiration is coupled or not to ADP phosphorylation, since the rate of oxygen consumption is inhibited both in the absence and in the presence of CCCP (Morello et al., 1995). Consequently, the primary interaction occurs in the electron transfer pathway through the mitochondrial respiratory chain.

To establish the inhibitory site of these compounds within the mitochondrial electron transport chain of intact tumor cells, *iso*-butyl gallate was used since it showed the highest I_{50} (hepatocytes)/I_{50} (tumor) ratio. Fig. 1 shows cellular respiration

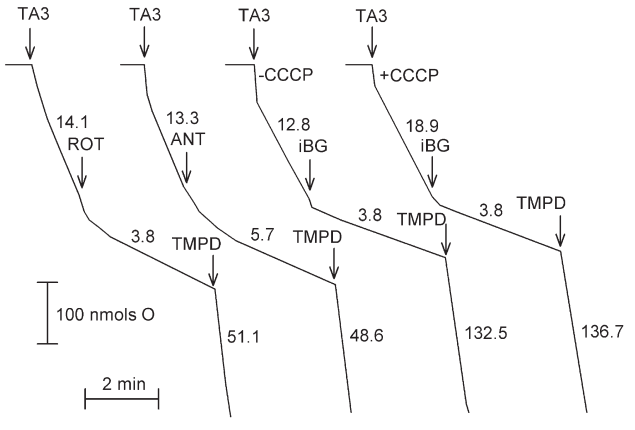

Fig. 1. Effect of TMPD on the respiratory rate of cells of the TA3 line blocked with *iso*-butyl gallate. Arrows indicate when the TA3 cells (about 10^7 cells), 1.04 μmol *iso*-butyl gallate (iBG), 2.2 μM rotenone (ROT), 0.6 μg/mL antimycin (ANT), 5.6 mM ascorbate plus 0.6 mM TMPD (TMPD) and 0.14 μM CCCP were added to the respiration medium. Numbers indicate the oxygen consumption rate as nmol of O/min/1.95×10^6 cells. Further details in the Materials and methods section.

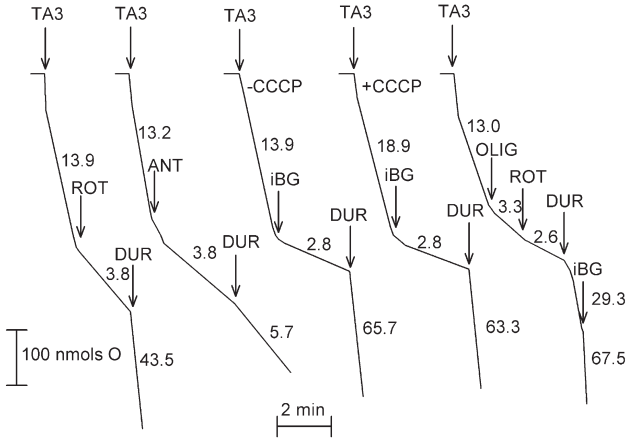

Fig. 2. Effects of *iso*-butyl gallate on duroquinol oxidation rate in the TA3 tumor cell line. Arrows indicate when TA3 cells (about 10^7 cells), 1.04 μmol *iso*-butyl gallate (iBG), 2.2 μM rotenone (ROT), 0.6 μg/mL antimycin (ANT), 0.6 mM duroquinol (DUR), 0.14 μM CCCP and 1.0 μg/mL oligomycin (OLIG) were added to the respiration medium. Numbers indicate the oxygen consumption rate as nmol O/1.95×10^6 cells. Further details in the Materials and methods section.

of TA3 cells in the presence of different mitochondrial electron flow inhibitors and iBG, whose respective concentrations were within the range of maximal inhibition. Rotenone, antimycin A and iBG inhibited the oxygen consumption rate. However, these inhibitions were bypassed by the addition of ascorbate plus TMPD, indicating that inhibition does not involve Complex IV. Similar results were found for the TA3-MTX-R and S786 cell lines. TMPD oxidation rate in the presence of iBG was higher than in the presence of rotenone or antimycin A and without CCCP, suggesting that iBG has a slight uncoupling effect on the respiratory chain.

Fig. 2 shows that the inhibition of cell respiration by rotenone and iBG was also bypassed by duroquinol, but not in the presence of antimycin A. These results not only support the conclusion that iBG inhibited electron flow at some point before ubiquinol–cytochrome *c* oxidoreductase, but they also indicate that this compound did not inhibit electron flow from Complex III to oxygen. Likewise, similarly to rotenone, iBG interfered with components of electron transfer, presumably at some site of NADH dehydrogenase (Complex I) (Pavani et al., 1994; Morello et al., 1995). Moreover, the duroquinol oxidation rate was higher in the presence of iBG without CCCP than in the presence of rotenone. It also increased the oxygen consumption rate previously inhibited by oligomycin (fifth trace) about twofold, indicating an uncoupling effect.

Considering the chemical properties of the alkyl gallate molecules, the length of the lipophilic alkyl side chain has a highly important influence on the binding to mitochondrial inner membrane of these compounds (Feng et al., 2003; Tammela et al., 2004) and the three adjacent phenolic hydroxyl groups are responsible for the cytotoxicity (Inoue et al., 1995). It is possible to conceive that one of these hydroxyl groups be an electron-donating moiety. As result of enzymatic oxidation into the Complex I — probably, by the protein-bound fast-relaxing semiquinone anion radical, which is observable only in the presence of proton-transmembrane electrochemical potential

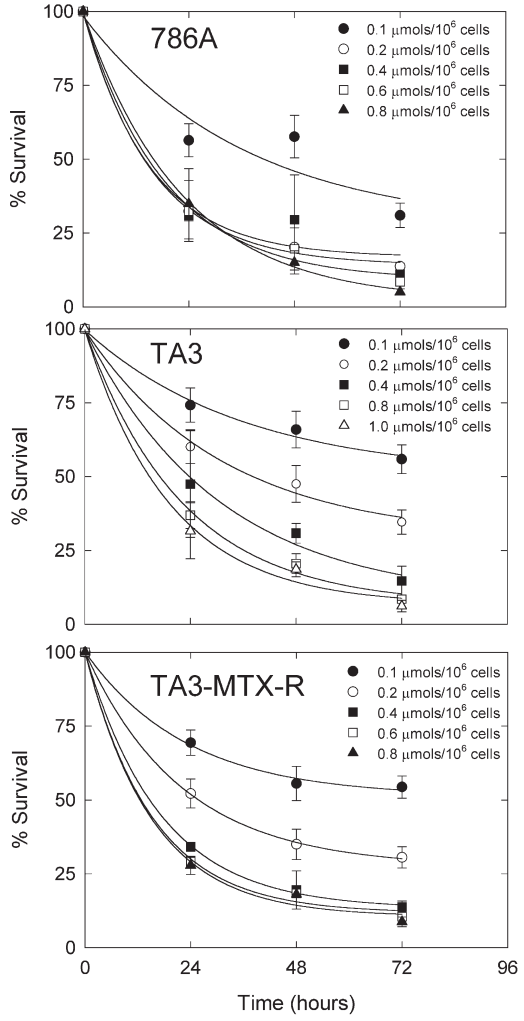

Fig. 3. Effects of *iso*-butyl gallate on 786A, TA3 and TA3-MTX-R tumor cell culture growths. Results are presented as percentage of cell survival compared with the respective control and measured every 24 h. *iso*-Butyl gallate was added in five different amounts per 10^6 cells. Each result is the average of at least three independent experiments performed in triplicate. Further details in the Materials and methods section.

(Ohnishi and Salerno, 2005) — an alkyl gallate derived free radical intermediary may be formed. This radical could undergo dismutation with another radical intermediate forming the original compound and the *o*-quinone species. Then, the *o*-quinone species could nucleophilically attack proteins of the Complex I, inhibiting the electron flow. This notion is supported by the observation that the semi-quinone form of nordihydroguaiaretic acid (NDGA) can interact with thiols groups modifying proteins (Shi and Pardini, 1995). Furthermore, it is well known that quinones are respiratory poisons (Imlay and Fridovich, 1992). In this regard, possible targets for the attack of catechols to the respiratory chain could be electron-transferring iron–sulfur clusters or flavoproteins that are rich in sulfhydryl-containing proteins present in Complexes I and II (Pritsos et al., 1982; Trumpower and Gennis, 1994). In addition, *o*-quinones can alter proteins through an attack directed to ε-amino groups, resulting in an amino-quinone adduct, then, the quinonic groups are reduced (Gut et al., 1996).

3.2. Effect of alkyl gallates on tumor cell growth

Fig. 3 shows the exponential time course effects of iBG on the growth of tumor cells culture, which are dose-dependent for all three cell lines tested. At the lowest amounts (0.1 μmol/10^6 cells) after 24 h exposure, cell viability was 57% for S786, 75% for TA3 and 69% for TA3-MTX-R cells. This effect greatly decreased after 72 h of exposure.

Table 2 summarizes the tumor cell growth inhibition by several alkyl gallates. TA3 and its multiresistant variant TA3-MTX-R were similarly sensitive towards these compounds. Amyl gallates were slightly, but significantly more active than butyl gallates, probably due to their better absorption rate and intracellular distribution. Table 2 also shows that the 786A cell line was slightly more susceptible to all the compounds tested than the TA3 and TA3-MTX-R cell lines. Results from cell growth and oxygen consumption appear to be consistent, because assays performed to determine alkyl gallate effects on respiration were done with nearly 5×10^6 cells/mL, due to the sensitivity of the oxygen electrode. Instead, cell growth determinations were done with about 0.4×10^6 cells/mL. Therefore, the amount of alkyl gallate corresponds to the number of cells. These compounds should inhibit oxidative phosphorylation, preventing ATP synthesis. This would stop cellular processes that require energy, starting a complex chain of events resulting in cell death. These findings explain, at least in part, the antineoplastic activity of the synthesized derivatives.

The major impediment to 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). The TA3-MTX-R cell line is resistant to various agents, i.e. cisplatin, doxorubicin, 5-fluorouracil, methotrexate and vinblastine (substrates of ABC transporters involved in drug resistance such as ABCC1, ABCC2, ABCC3 and ABCC11) (Cordano et al., 2002; Hernández et al., 2003; Dean et al., 2005). I_{50} values for mitochondria from tumor cell lines and hepatocytes point to a selectivity of alkyl gallates at the mitochondrial level. Thus, these derivatives might be further considered as potential antineoplastic drugs.

Table 2
Effects of alkyl gallates on the culture growth of sarcoma 786A and carcinoma TA3 and its multiresistant variant TA3-MTX-R

Compounds	Cell lines I_{50} (nmol/10^6 cells)		
	TA3	TA3-MTX-R	786A
n-butyl gallate	274±22	289±16	125±10*
iso-butyl gallate	336±26	296±17	133±12*
n-amyl gallate	166±14	173±15	133±11*
iso-amyl gallate	159±16	169±14	122±13*

I_{50} values correspond to the nmol of ester per 10^6 cells required to inhibit 50% of the culture growth. These values were calculated by interpolation from respective culture growth inhibition curves that were exposed for 24 h to the compounds. Data shown correspond to means±SD_{n-1} of three or more independent experiments. *The 786A cell line has significantly greater sensitivity ($P<0.05$) than the TA3 and TA3-MTX-R cell lines. See Materials and methods section for details.

DOX and iBG alone or in combination were tested for inhibition of TA3 and TA3-MTX-R tumor growths in mice (Fig. 4). Tumor cells were injected into the thigh of the right hind leg of the recipient mice and were allowed to develop for 4 days (time at which the tumors are clearly palpable). Then, the corresponding treatment schedules were started. Treatments were continued for up to 3 days after the tumors were clearly not palpable, but they did not exceed 35 days after tumor implantation. TA3 tumor growth in mice treated with DOX was inhibited up to 20–22 days after tumor implantation (Fig. 4A). After this period, the growth rate increased to reach a tumor size almost similar to that of the control. In contrast, TA3-MTX-R tumor growth rate in mice treated with DOX alone was only slightly less than that found in its respective control (Fig. 4B). Fig. 4A and B also shows the inhibitory responses of TA3 and TA3-MTX-R carcinoma to treatment with iBG. The combination of iBG and DOX strongly reduced the tumor growth rate and it was the best treatment against the sensitive cell line TA3, and the resistant variant, TA3-MTX-R. The inhibitory activity of *iso*-butyl gallate and DOX in combination on both rates of solid tumor growth appeared to be synergistic.

The effect of DOX and iBG on the disease-free survival of mice implanted with either TA3 or TA3-MTX-R tumor cells analyzed by the Kaplan–Meier method is also shown in Fig. 4. DOX alone had no effect on the median survival time of tumor-bearing mice implanted with either TA3 or TA3-MTX-R tumor cells, with a treated/control ratio of about 0.87 (TA3) and 1.16 (TA3-MTX-R). Treatment with iBG alone did not prolong median survival time of mice with either TA3 ($P=0.79$) or TA3-MTX-R cells ($P=0.44$), compared with the respective control. However, 10% and 20% of them, respectively, were without any vestige of tumor 200 days after implantation with either TA3 or TA3-MTX-R tumor cells. The combination of iBG and DOX did not prolong median survival time of mice with TA3 ($P=0.74$) or TA3-MTX-R cells ($P=0.47$), but it rendered 40% and 20% of the mice with TA3 or TA3-MTX-R cells tumor free, respectively.

After each mouse died its lungs and heart were removed. With the aid of a dissecting microscope, a great number of grossly visible metastatic nodules on the lung surface of mice implanted with TA3 tumor cells were observed. They were also seen on the lung and heart surfaces of tumor-bearing mice implanted with the resistant variant TA3-MTX-R cells. A decrease in the number and in the size of metastatic nodules in animals treated with either iBG or the combination of iBG and DOX was observed, but these treatments were not able to increase median survival time significantly.

In conclusion, alkyl gallates display measurable antitumor activity in mice implanted with TA3-MTX-R tumor cells (Fig. 4), which are highly refractory to various therapies. The combination of both iBG and DOX produced the best survival results and anticancer activity. iBG presumably inhibits the production of energy necessary for ABC transporter function. Consequently, a higher intracellular concentration of DOX could

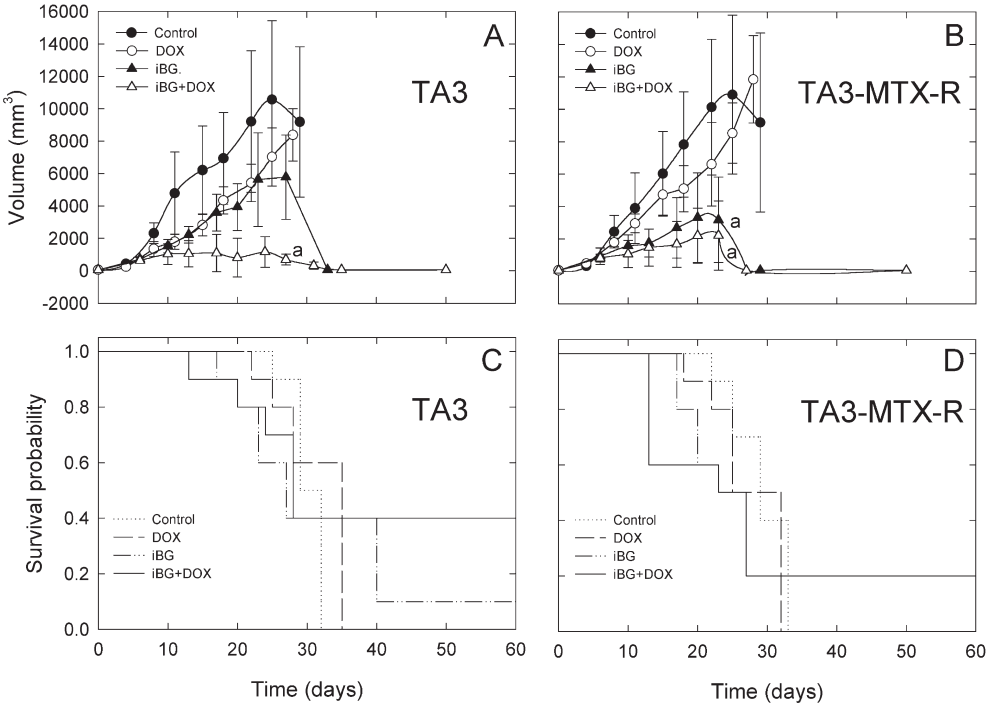

Fig. 4. Effects of DOX and *iso*-butyl gallate alone and in combination on the growth of sensitive and resistant TA3 cells and on the survival of mice implanted with these respective tumors. Two groups of twenty tumor-bearing mice were used as untreated controls implanted with TA3 cells (●, Panel A). Four groups of ten tumor-bearing mice were used as untreated controls implanted with TA3-MTX-R cells (●, Panel B). Three groups of ten mice were treated intraperitoneally starting on the fourth day after the respective tumor cells were implanted with either: (○) 1.5 mg DOX/kg on days 4, 5, 6, 7, 11, 12, 13, 14, 18, 19, 20, 21, 25, 26, 27, 28, and 32; or (▲) 60 mg iBG/kg/48 h; or (△) 60 mg iBG/kg on days 4, 5, 7, 8, 10, 11, 13, 14, 18, 19, 21, 22, 26, 27, 29, 32 and 33, combined with 1.5 mg DOX/kg on days 6, 9, 12, 15, 20, 25, 28 and 34. Survival probability (Panel C and D) and death time values of each treatment were not significantly different from control. Curves marked with letters (a) were significantly different from other treatments and from the control group, $P<0.05$.

be attained to perform its cytotoxic action. Alkyl gallates increased the accumulation of the P-glycoprotein substrates rhodamine 123 and daunorubicin by inhibiting the efflux of these substrates in P-glycoprotein-overexpressing KB-C2 cells (Kitagawa et al., 2005). Many drugs have been shown to sensitize MDR cells, but proved to be toxic in clinic trials (Dean et al., 2005). The identification of alkyl gallates as potent modulators of MDR is important. Alkyl gallates represent a new class of compounds that could be exploited for use in malignancies that display the phenomenon of MDR.

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