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

Christian Frey [a], Mario Pavani [b], Gianni Cordano [a], Sergio Muñoz [a], Enrique Rivera [a], Jorge Medina [a], Antonio Morello [b], Juan Diego Maya [b], Jorge Ferreira [b,*]

[a] *Department of Organic and Physical Chemistry, Faculty of Chemical and Pharmaceutical Sciences, Olivos 1007, Santiago-7, Chile*
[b] *Program of Molecular and Clinical Pharmacology, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Independencia 1027, P.O. Box 70086, Santiago-7, Chile*

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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.
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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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* Corresponding author. Tel.: +56 2 978 6069; fax: +56 2 735 5580.
E-mail address: jferreir@med.uchile.cl (J. Ferreira).

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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

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