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Derivatives of alkyl gallate triphenylphosphonium exhibit antitumor activity in a syngeneic murine model of mammary adenocarcinoma

Liliana Peredo-Silva [a], Sebastián Fuentes-Retamal [a], Cristian Sandoval-Acuña [b], Mario Pavani [a], Juan D. Maya [a], Vicente Castro-Castillo [c], Matías Madrid-Rojas [d], Solange Rebolledo [d], Ulrike Kemmerling [e], Eduardo Parra [f], Jorge Ferreira [a,*]

[a] Clinical and Molecular Pharmacology Program, Institute of Biomedical Sciences (ICBM), Faculty of Medicine, University of Chile, Av. Independencia 1027, Santiago 8380453, Chile
[b] Institute of Biotechnology, Czech Academy of Sciences, Prúmyslová 595, Vestec, 25250, Prague, Czech Republic
[c] Department of Organic and Physical Chemistry, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, Santos Dumont 964, Santiago 8380494, Chile
[d] Department of Chemistry, Faculty of Basic Sciences, Metropolitan University of Educational Sciences, Av. José Pedro Alessandri 774, Santiago 7760197, Chile
[e] Program of Anatomy and Developmental Biology, Institute of Biomedical Sciences (ICBM), Faculty of Medicine, University of Chile, Av. Independencia 1027, Santiago 8380453, Chile
[f] School of Medicine, Faculty of Health Sciences, University of Tarapacá, Av. General Velásquez 1775, Arica 1000007, Chile

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ABSTRACT

We previously demonstrated that alkyl gallates coupled to triphenylphosphine have a selective and efficient antiproliferative effect by inducing mitochondrial uncoupling *in vitro* due to the increased mitochondrial transmembrane potential of tumor cells. Therefore, in this work, the *in vivo* antitumor activities of alkyl gallate triphenylphosphonium derivatives (TPP^+C_8, TPP^+C_{10} and TPP^+C_{12}) were evaluated in a syngeneic murine model of breast cancer. We found that TPP^+C_{10} increased the cytosolic ADP/ATP ratio and significantly increased the AMP levels in a concentration-dependent manner in TA3/Ha murine mammary adenocarcinoma cells. Interestingly, TPP^+C_{10} induced a decrease in the levels of cellular proliferation markers and promoted caspase-3 activation in tumor-bearing mice. Additionally, TPP^+C_{10} inhibited tumor growth in the syngeneic mouse model. Importantly, 30 days of intraperitoneal (i.p.) administration of the combination of TPP^+C_{10} (10 mg/kg/48 h) and the antibiotic doxycycline (10 mg/kg/24 h) completely eliminated the subcutaneous tumor burden in mice ($n = 6$), without any relapses at 60 days post-treatment. This enhancement of the individual activities of TPP^+C_{10} and doxycycline is due to the uncoupling of oxidative phosphorylation by TPP^+C_{10} and the inhibition of mitochondrial biogenesis by doxycycline, as demonstrated by loss of mitochondrial mass and overexpression of PGC1-α as an adaptive response. Moreover, i.p. administration of TPP^+C_{10} (10 mg/kg/24 h) to healthy mice did not produce toxicity or damage in organs important for drug metabolism and excretion, as indicated by hematological, biochemical and histological assessments. These findings suggest that the combination of TPP^+C_{10} with doxycycline is a valuable candidate therapy for breast cancer management.

1. Introduction

Gallic acid (GA; 3,4,5-trihydroxybenzoic acid) is a well-known polyphenol found abundantly in tea, grapes, berries and other fruits. GA has various biological activities, such as antibacterial, anti-melanogenic, antiviral and anti-inflammatory activities (Kang et al., 2008; Kratz et al., 2008; Yoon et al., 2013), in addition to its antineoplastic activity in various cancer cell types (Ji et al., 2009; Maurya et al., 2011), which merit attention in the development of drugs against cancer. GA and its alkyl derivatives, the propyl, octyl and lauryl gallates, have been widely

Abbreviations: $\Delta\Psi_m$, mitochondrial membrane potential; ADP, adenosine diphosphate; AMP, adenosine monophosphate; AP, alkaline phosphatase; AST, aspartate aminotransferase; ATP, adenosine triphosphate; BUN, blood urea nitrogen; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; CK, creatine kinase; CK-MB, creatine kinase MB; DAB, diaminobenzidine; DMEM HG, Dulbecco's modified eagle medium-high glucose; DMSO, dimethyl sulfoxide; FITC, 4-fluorescein isothiocyanate; GA, gallic acid; HE, hematoxylin and eosin; HK-II, hexokinase 2; IIA, iodoacetate; IMM, inner mitochondrial membrane; LDH, lactate dehydrogenase; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCT, monocarboxylate transporter; MCV, mean corpuscular volume; OXPHOS, oxidative phosphorylation; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PGC1-α, peroxisome proliferator-activated receptor-γ coactivator 1α; ROS, reactive oxygen species; TPP^+, triphenylphosphonium; TPP^+C_8, (8-((3,4,5-trihydroxybenzoyl)oxy)octyl) triphenylphosphonium; TPP^+C_{10}, (10-((3,4,5-trihydroxybenzoyl)oxy)decyl) triphenylphosphonium; TPP^+C_{12}, (12-((3,4,5-trihydroxybenzoyl)oxy)dodecyl) triphenylphosphonium; VDAC, voltage-dependent anion channel.

* Corresponding author.
E-mail address: jferreir@med.uchile.cl (J. Ferreira).

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used as antioxidant additives in the food and pharmaceutical industries, and these compounds have demonstrated significantly low toxicities both *in vitro* and *in vivo* (Fiuza et al., 2004; Verma et al., 2013). Alkyl gallates have shown various biological activities, including cardiovascular protection, tyrosine kinase inhibition, and antibacterial, antifungal, anti-inflammatory, and anticancer activities, that in many cases are greater than those exhibited by GA itself (Locatelli et al., 2008, 2009). Additionally, it has been demonstrated that *n*-alkyl gallates can elicit the following effects: a) decrease mitochondrial membrane potential ($\Delta\Psi_m$); b) induce mitochondrial permeability transition pore opening; c) promote cytochrome *c* and apoptosis-inducing factor (AIF) release and procaspases and endonuclease G activation; and d) upregulate the expression of Bcl-2-associated X protein (BAX) and caspase-3, -4 and -9, subsequently causing DNA fragmentation (Yeh et al., 2011). It has also been reported that increasing concentrations of GA esters generate greater uncoupling of the oxidative phosphorylation (OXPHOS) system, as well as inhibit electron flow through the mitochondrial respiratory chain (at higher concentrations), mainly at NADH-CoQ oxidoreductase. These effects of GA esters prevent ATP synthesis and ultimately lead to cell death. Moreover, although the structure and lipophilicity of the alkyl side chain is important for the antitumor activity of these compounds, a "cutting" effect has been reported with increasing length of the alkyl chain of GA esters, leading to a decrease in their effects on a range of biological activities (Losada Barreiro et al., 2013).

Mitochondria play an important role in regulating energy metabolism, the cytosolic calcium concentration, ROS production and apoptosis. Importantly, mitochondria exhibit significant differences in OXPHOS between tumor and non-tumor cells; the inner mitochondrial membrane (IMM) of tumor cells has a $\Delta\Psi_m$ of approximately 150–180 mV, in which the potential is more negative on the matrix side, thus causing an increased $\Delta\Psi_m$. The $\Delta\Psi_m$ in tumor cells is much higher than the membrane potential of any other cell organelle and is higher than the $\Delta\Psi_m$ in other tissues and non-tumor cells (Modica-Napolitano and Singh, 2004). Furthermore, the activities of important enzymes involved in establishing the $\Delta\Psi_m$ are decreased in tumor cells (Lopez-Rios et al., 2007; Putignani et al., 2008), in association with a low respiration rate, which is possibly due to mitochondrial dysfunction (Modica-Napolitano and Singh, 2004); however, the latter point is under debate. In addition, tumor cells have increased expression of several proteins involved in glucose metabolism. In some cases, aerobic glycolysis may contribute >50% of total ATP synthesis and thus serve as the main source of energy in tumor cell (Pedersen, 2007). Nevertheless, many studies have confirmed the importance of OXPHOS in ATP production for tumor cells. This process enables direct delivery of newly synthesized ATP to hexokinase 2 (HK-II), an enzyme attached to the outer mitochondrial membrane through the voltage-dependent anion channel (VDAC) that is upregulated in tumor cells. Thus, HK-II uses the ATP synthetized *via* OXPHOS to phosphorylate and convert glucose into glucose-6-phosphate, one of the limiting steps in glycolysis (Rosano, 2011). This evidence suggests that mitochondria are excellent targets for antitumor therapies.

Because tumor cells have the highest $\Delta\Psi_m$, small molecules can be selectively targeted to tumor cell mitochondria (Coulter et al., 2000). To enhance the cytotoxic effect of GA esters, we synthesized delocalized lipophilic cations, in which GA with different alkyl chain lengths were conjugated to the triphenylphosphonium (TPP^+) moiety (Jara et al., 2014). These compounds, guided by the $\Delta\Psi_m$, selectively accumulate in tumor cells by binding directly to the phospholipid bilayer due to their large hydrophobic surface area. This interaction reduces the activation energy required to capture the TPP^+ group (Murphy and Smith, 2007), resulting in the accumulation of the target in the IMM at a nearly 500-fold higher concentration than that in the cytosol. We previously determined that alkyl gallate TPP^+ derivatives provoked mitochondrial uncoupling in mouse mammary adenocarcinoma cells (TA3/Ha), leading to a decrease in OXPHOS-mediated ATP synthesis and ultimately causing cell death (Jara et al., 2014). Also, we have tested the effectiveness of alkyl gallates in several human breast cancer cell lines, which differ in their expression of estrogen and epidermal growth factor receptors, as well as in their metabolic profile (Sandoval-Acuna et al., 2016). Because of these promising *in vitro* results, we evaluated the first *in vivo* antitumor activities of alkyl gallate-TPP^+ derivatives.

It is important to note that the "no observed adverse effect level" (NOAEL) of GA is approximately 120 mg/kg/day for rats and that the NOAEL of its alkyl gallate derivatives is approximately 1000 mg/kg/day in mice (van der Heijden et al., 1986; Lu et al., 2006). Furthermore, studies using phosphonium salts as contrast agents for diagnostic imaging (Kim et al., 2008) have elucidated two key points concerning these compounds: 1) they preferentially accumulate within tumor cells, and 2) the phosphonium cation itself does not impart cytotoxicity.

Because of the genetic complexity of cancer cells and the lack of a specific therapeutic target enabling personalized treatment, it is necessary to resort to an alternative therapy that focuses on a common trait of tumor cells. One of these features is the strict dependence on mitochondrial biogenesis for the anchorage-independent clonal expansion and survival of cancer stem cell populations. Considering this dependence, many classes of FDA-approved antibiotics inhibit mitochondrial biogenesis or OXPHOS as a mild side effect, and their ability to eradicate cancer stem cells has been studied (Lamb et al., 2015). Among these drugs, doxycycline represents an attractive new anticancer agent. Doxycycline is a broad-spectrum antibiotic of the tetracycline class that inhibits protein synthesis by preventing the binding of activated aminoacyl-tRNAs to the A site of the 30S subunit of bacterial ribosomes. Importantly, the 30S subunit of the bacterial ribosome is homologous to the 28S subunit of the mitochondrial ribosome; as a consequence, tetracycline-based antibiotics inhibit mitochondrial biogenesis. Moreover, doxycycline has been used in human tumor xenograft and other animal models to significantly reduce the tumor burden and even suppress metastatic cancer cell growth (Duivenvoorden et al., 2002; Shen et al., 2010; Chang et al., 2014).

Importantly, combination therapies are known to be more clinically effective than individual therapies against cancer. Thus, based on the properties of alkyl gallate TPP^+ derivatives described above and the evidence of their cytotoxicity *in vitro*, we investigated the antitumor effects of these compounds (chemical structures in Supplementary Fig. 1) in a syngeneic murine model. In addition, according to the results from studies of doxycycline in different animal models of breast cancer (Lamb et al., 2015), the effect of the combination of TPP^+C_{10} and doxycycline on mitochondrial biogenesis and the capacity of this combination to reduce tumor growth were analyzed. The results revealed that this combined treatment decreased the mitochondrial mass and consequently increased the levels of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1-α), the master regulator of mitochondrial biogenesis, as compensatory effect. Moreover, combined doxycycline and TPP^+C_{10} treatment completely eliminated the tumor burden *in vivo*, without recurrence at 60 days post-treatment. This combination treatment might be a beneficial therapeutic strategy for the management of breast cancer.

2. Materials and methods

2.1. Drugs used in this study

The compounds used in the present study were synthesized according to Jara et al. (2014) and were numbered based on the size of the carbon chain:

TPP^+C_8: triphenyl (8-((3,4,5-trihydroxybenzoyl)oxy)octyl) phosphonium bromide;
TPP^+C_{10}: triphenyl (10-((3,4,5-trihydroxybenzoyl)oxy)decyl) phosphonium bromide; and
TPP^+C_{12}: triphenyl (12-((3,4,5-trihydroxybenzoyl)oxy)dodecyl) phosphonium bromide.