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Suppression of TNF-α induced NFκB activity by gallic acid and its semi-synthetic esters: possible role in cancer chemoprevention

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Suppression of TNF-α induced NFκB activity by gallic acid and its semi-synthetic esters: possible role in cancer chemoprevention

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Gallic acid (3,4,5-trihydroxybenzoic acid), found in many plants either in free-form or part of tannins, is known to possess anti-microbial, antioxidant and cytotoxic properties. NFκB regulates the expression of several genes involved in carcinogenesis. These include anti-apoptotic, cytokines and cell cycle-regulatory genes. It is well established that the transcriptional factor NFκB is deregulated in many forms of cancer. Thus, agents that can suppress NFκB activation have the potential of suppressing carcinogenesis. In the present investigation, gallic acid was isolated from *Alchornea glandulosa* (Euphorbiaceae) and eight esters were synthesised. These compounds were evaluated against TNF-α-induced NFκB activation with stably transfected 293/NFκB-Luc human embryonic kidney cells. Gallates with IC_{50} values in a range of 10–56 μM mediated inhibitory activity higher than gallic acid (IC_{50} 76.0 ± 4.9 μM). In addition to inhibiting NFκB activation, gallic acid mediated a modest cytotoxic effect, and some of the gallates affected cell viability at the tested concentrations. Based on these results, suppression of NFκB activation by gallate esters could play a chemopreventive role in carcinogenesis.

Keywords: gallic acid; NFκB pathway; cytotoxicity; 293/NFκB cells; *Alchornea glandulosa*; cancer chemoprevention

1. Introduction

Phenolic acids are natural products which possess a wide spectrum of biological activity (Nazaruk, Czechowska, Markiewicz, & Borawska, 2008).

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Gallic acid (3,4,5-trihydroxybenzoic acid), a secondary metabolite accumulates in nearly all plants, either in free form or part of tannins, is known to possess antimicrobial, antiviral, anti-inflammatory, antioxidant and cytotoxic properties (Aruoma, Murcia, Butler, & Halliwell, 1993; Fiuza et al., 2004; Kratz et al., 2008; Kroes, van den Berg, van Ufford, van Dijk, & Labadie, 1992; Kubo, Fujita, K. Nihei & A. Nihei, 2004). It is commonly used in the pharmaceutical industry as salts and esters. Some ointments used to treat psoriasis and external hemorrhoids contain gallic acid (García de Hombre & Pérez Peñate, 2006; Hatton, 2000). Gallic acid has two major functional groups, hydroxyls and a carboxylic acid, which can yield numerous esters and salt derivatives. Some galloyl compounds are used as antioxidants in food and can exhibit important roles in prevention of inflammatory and atherosclerosis responses by blocking activation of NFκB (Murase et al., 1999).

NFκB consists of homo and heterodimeric protein complexes acting as a transcription factor, and is found in many animal cell types. It is involved in regulation of immune response and inflammation, cell lineage development, cell apoptosis, cell-cycle progression and oncogenesis due to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidised LDL and microbial antigens, and has been shown to regulate the expression of several genes, including bcl-2, bcl-xl, cIAP, survivin, TRAF, COX-2, MMP-9, iNOS and cell cycle-regulatory components, whose products are involved in tumourigenesis (Aggarwal, Sethi, Nair, & Ichikawa, 2006; Chen, Castranova, & Shi, 2001; Melisi & Chiao, 2007; Thanos & Maniatis, 1995). Most carcinogens, proinflammatory cytokines such as TNF and IL-1, or tumour promoters, including cigarette smoke, phorbol esters, okadaic acid, H_2O_2, have been shown to activate NFκB, and resulting tumours have misregulated NFκB activity (Baldwin, 2001). It is now well established that aberrant regulation of NFκB, and the signalling pathways that control its activity, are involved in cancer development and progression, as well as in drug resistance, especially during chemotherapy and radiotherapy (Inoue, Gohda, Akiyama, & Semba, 2007). Blocking NFκB can cause tumour cells to stop proliferating, undergo apoptosis, or to become more sensitive to the action of antitumour agents (Frydrych & Mlejnek, 2008; Yamamoto & Gaynor, 2001). Thus, agents that can suppress NFκB activation have the potential to suppress carcinogenesis or tumourigenesis.

In the present investigation, gallic acid was isolated from *Alchornea glandulosa* Poepp and Endl. (Euphorbiaceae), and eight semi-synthetic esters were prepared. All the compounds were evaluated for cytotoxicity as well as potential to inhibit TNF-α-induced NFκB activation with stably transfected 293/NFκB-Luc human embryonic kidney cells.

2. Materials and methods

2.1. *Materials*

Methanol, *n*-hexane, ethyl acetate and *p*-dioxane were of high performance liquid chromatography (HPLC) grade (JT Baker). Column chromatography was carried out over silica gel (0.06–0.20 mm, Acros Organics). Gel permeation chromatography (GPC) was performed using Sephadex LH-20 (Pharmacia Biotech). Preparative HPLC was carried out using a Varian Prep-Star 400 system and a Phenomenex C-18 (250 mm × 21.2 mm) column. The monodimensional NMR experiments were

recorded on a Varian INOVA 500 spectrometer (11.7 T) at 500 MHz (^{1}H) and 125 MHz (^{13}C), using $CDCl_3$ and DMSO-d_6 as solvent (Aldrich). Alkyl alcohols and DCC were purchased from Aldrich. Dimethyl sulphoxide (DMSO) and sulphorhodamine B (SRB) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM), antibiotic–antimycotic, hygromycin B, and MEM sodium pyruvate were purchased from Invitrogen Co. (Carlsbad, CA). Reporter Lysis Buffer, Luciferase Assay System was purchased from Promega (Madison). Tumour Necrosis Factor-α (TNF-α) was purchased from Calbiochem (Gibbstown, NJ). Alcohol was purchased from J.T. Baker (Phillipsburg, NJ).

2.2. *Isolation of gallic acid*

The leaves of *A. glandulosa* were collected in the Biological Reserve and Experimental Station at Mogi Guaçu, São Paulo State, Brazil, in March 2005. A voucher specimen (SP319257) has been deposited in the herbarium of the Botanic Institute (São Paulo-SP, Brazil).

The shade-dried plant material (1.5 kg) was ground and defatted with *n*-hexane (3.5 L × 3) at room temperature and exhaustively extracted by maceration with MeOH (4.2 L × 3). The crude extract was concentrated under reduced pressure to yield 3.8 g of a syrup residue, which was diluted with MeOH : H_2O (4 : 1) and then successively partitioned with EtOAc and *n*-BuOH. After evaporation under reduced pressure, the partition phases yielded 2.5 g and 0.8 g, respectively. The part of EtOAc residue (2.0 g) was chromatographed by gel permeation over Sephadex LH-20 eluted with methanol to afford 11 fractions (A1–A11). Fraction A2 (730 mg) was purified by RP-HPLC [MeOH : H_2O : HOAc (7.5 : 92 : 0.5), UV detection at 265 nm; flow rate 15 mL min^{-1}] to give compound **1** (335 mg). Compound **1** was identified as gallic acid based on analysis of the ^{1}H and ^{13}C NMR data, as well as by comparison with authentic material in our laboratory (Figure 1).

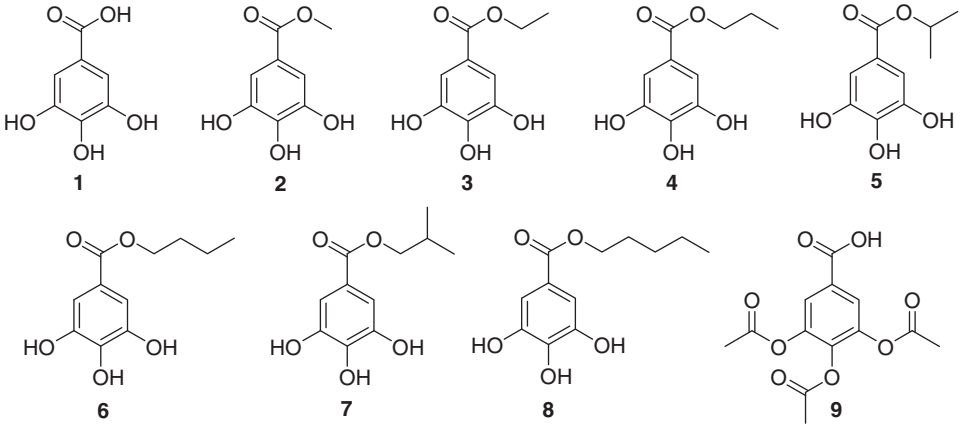

Figure 1. Molecular structures of gallic acid (**1**) and semi-synthetic esters (**2**–**9**).

2.3. *Synthesis of esters 2–9*

After being cooled at 5°C, the mixture of gallic acid (0.2 mmol) and alcohol (20 mmol) in *p*-dioxane (6.0 mL) was added to a solution of *N*,*N*′-dicyclohexylcarbodiimide (DCC, 1.0 mmol) in *p*-dioxane (3.0 mL). The solution was stirred for 48 h, and the solvent was removed under reduced pressure. The residue was partitioned three times with EtOAc and then filtered. The filtrate was washed successively with saturated aqueous citric acid solution (three times), saturated aqueous $NaHCO_3$ (three times), water (two times), dried over $MgSO_4$, and evaporated under reduced pressure. The crude products were purified over a silica gel column using an isocratic system of $CHCl_3$–MeOH (98 : 2) to give gallate esters **2–8**. Structures of the semi-synthetic esters, methyl gallate (**2**), ethyl gallate (**3**), *n*-propyl gallate (**4**), isopropyl gallate (**5**), *n*-butyl gallate (**6**), isobutyl gallate (**7**), *n*-pentyl gallate (**8**) and 3,4,5-triacetoxybenzoic acid (**9**), were established by ^{1}H and ^{13}C NMR spectral analysis (Figure 1).

For synthesis of ester **9**, gallic acid (20 mmol) was dissolved in dry pyridine (5.0 mL) and anhydride acetic (5.0 mL) under hydrogen atmosphere. The mixture was stirred for 48 h at room temperature, carried to dryness under reduced pressure, and purified by column chromatography with a mixture of $CHCl_3$–MeOH (85 : 15) to give a product (**9**). The NMR spectroscopic data of **9** were compatible with 3,4,5-triacetoxybenzoic acid.

2.4. *NFκB luciferase assay*

NFκB inhibition assay was conducted using a luciferase reporter gene bioassay as previously described (Homhual et al., 2006). Human embryonic kidney cells (293/NFκB-Luc) were used to monitor alteration of the NFκB pathway. This cell line is stably transfected with a luciferase reporter construct regulated by the NFκB response element. Transcription factor can bind to the response element when stimulated by TNF-α, allowing transcription of the luciferase gene. Luciferase reacts with substrate, emitting light that was detected using a luminometer. Cells were seeded (10^5 cells mL^{-1}) on sterile white walled 96-well plates and grown to approximately 80% confluence by incubating for 48 h. Gallic acid and alkyl gallates were tested at 20 μg mL^{-1} following 1 : 3 serial dilutions. After treatment with tested compounds for 10 min, cells were incubated for an additional 6 h with or without TNF-α (5 ng mL^{-1}). The cells were gently washed with PBS and kept at −80°C with luciferase lysis buffer (Promega Corporation). The luciferase assay was performed using the Luc assay system from Promega following manufacturer instructions. Luciferase activity was monitored (LUMIstar Galaxy BMG), dose-response curves were constructed, and results were expressed as IC_{50} values (i.e. concentration required to inhibit TNF-α induced NFκB activity by 50%).

2.5. *Cytotoxicity assay*

Cytotoxicity was determined by the method of Skehan et al. (1990). The cells were fixed with trichloroacetic acid and stained for 30 min with 0.4% (w/v) sulphorhodamine B (SRB) prepared in 1% acetic acid. Unbound dye was removed by washing with 1% acetic acid, and protein-bound dye was extracted with 10 mM Tris base

[tris (hydroxymethyl)aminomethane] for determination of optical density in a computer-interfaced, 96-well microtiter plate reader.

2.6. *Statistical analysis*

Statistical analysis was performed using GraphPad Prism® Version 5.01 (GraphPad Software Inc.). Results were subjected to one-way analysis of variance (ANOVA), and differences between means were located using Tukey's multiple comparison test. Differences between means at the 5% ($p < 0.05$) level were considered significant. The data are expressed as means ± standard error.

3. Results and discussion

Herein we report cytotoxic properties and suppression of TNF-α induced NFκB activation of gallic acid (**1**) and eight esters (**2–9**) determined with stably transfected 293/NFκB-Luc human embryonic kidney cells. Table 1 shows the concentrations of gallates **1–9** required to inhibit NFκB-induced activation in 50% (IC_{50}). *N*-Tosyl-L-phenylalaninyl-chlormethylketone (TPCK) was adopted as positive control, exhibiting 98.5% ± 0.5% NFκB inhibition at 30 μM, with an IC_{50} value of about 5 μM. At the highest concentration tested (20.0 μg mL^{-1}), gallic acid mediated a moderate cytotoxic effect (52.9% ± 3.5% survival), but none of the gallate esters significantly affected cell viability. The most cytotoxic compound evaluated was **6**, with 48.9% ± 3.3% cell survival.

Among inhibitors of NFκB, salicylates, aspirin (Kopp & Ghosh, 1994), apigenin and curcumin (Gerritsen et al., 1995; Singh & Aggarwal, 1996) were reported to suppress activation of NFκB by preventing the degradation of the NFκB inhibitor (IκB), and therefore NFκB was retained in the cytosol.

The serine protease inhibitor TPCK can cause a complete block to NFκB activation. TPCK inhibits NFκB subunits processing and IκB degradation (Mellitis, Hay, & Goodbourn, 1993). It has also been demonstrated that TPCK inhibits DNA replication by G_0–G_1 growth arrest and thus promotes apoptosis by other cytotoxic stimuli (Kirillova, Chaisson, & Fausto, 1999).

Table 1. Inhibition of TNF-α-induced NFκB activity by gallic acid (**1**) and gallate esters (**2–9**).

Compound	IC_{50} (μM)
1	76.0 ± 4.9
2	10.3 ± 1.1
3	54.6 ± 5.1
4	29.7 ± 3.1
5	31.1 ± 2.0
6	56.1 ± 5.9
7	37.2 ± 4.0
8	38.3 ± 4.1
9	48.0 ± 5.9
TPCK	5.09 ± 2.14

The present study provides evidence that some gallates can act as inhibitors of NFκB. Methyl gallate (**2**) demonstrated the greatest activity. The inhibitory actions of gallates do not appear to result from non-specific cellular toxicity but rather are specific for NFκB-dependent gene transcription. In another study, it has been demonstrated that gallates can inhibit TNF-α-induced nuclear translocation of NFκB by way of a mechanism independent of IκB degradation (Murase et al., 1999).

More recently, prevention of atherosclerosis, cancer, anti-plasmodial and antioxidant activity of a group of galloyl compounds was described, as well as structure-activity relationships yielding cytotoxic properties (Fiuza et al., 2004; Jagan, Ramakrishnan, Anandakumar, Kamaraj, & Devaki, 2008; Locatelli et al., 2008; Murase et al., 1999; Na et al., 2006; Petrônio et al., 2007; Saxena et al., 2008). Based on these encouraging intracellular responses, we suggest suppression of NFκB activation by gallate esters could play a chemopreventive role in carcinogenesis, and further investigations related to the actions of gallates, especially in NFκB signaling pathway and *in vivo* models, may provide new insights into the cancer chemopreventive ability.

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