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Ester derivatives of gallic acid with potential toxicity toward L1210 leukemia cells

Claudriana Locatelli,[a,c] Rober Rosso,[a] Maria C. Santos-Silva,[b] Camila A. de Souza,[b] Marley A. Licínio,[a] Paulo Leal,[d] Maria L. Bazzo,[b] Rosendo A. Yunes[d] and Tânia B. Creczynski–Pasa[a,*]

[a]*Departamentos de Ciências Farmacêuticas, Universidade Federal de Santa Catarina, CP 476 Florianópolis, 88040-900 SC, Brazil*
[b]*Análises Clínicas, Universidade Federal de Santa Catarina, CP 476 Florianópolis, 88040-900 SC, Brazil*
[c]*Curso de Farmácia, Universidade do Contestado, Campus Caçador, 89500-000 Caçador, SC, Brazil*
[d]*Química, Universidade Federal de Santa Catarina, CP 476 Florianópolis, 88040-900 SC, Brazil*

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Abstract—Gallic acid and gallates with the same number of hydroxyl groups and varying the length of the side carbon chain, with respective lipophilicity being defined through the C log *P* values, were examined for their ability to induce apoptosis (through the DNA ladder fragmentation pattern), mitochondrial and cytoplasmic GSH depletion and NF-κB activation in murine lymphoblastic L1210 leukemia cells. A relationship between cytotoxic effect and a limited degree of lipophilicity was observed.

1. Introduction

The greatest problem associated with acute leukemia is the resistance to systemic methods of treatment and despite advances in the therapies 20–30% of the patients die.[1] Leukemia therapy with anticancer compounds is based on cell growth inhibition, induction of cell death through apoptosis or leukemic blast differentiation.[1,2] Cell death through apoptosis can result from several cellular events, occasioned frequently by oxidative metabolism alteration,[3] and glutathione, the most important intracellular antioxidant complex, is directly involved in this process.[4] The last step of apoptosis, cell death, is promoted by the activation of caspases, leading to changes characteristic of apoptotic cells such as DNA fragmentation, chromatin condensation, cell shrinkage, and membrane blebbing.[1]

During oxidative or nitrosative stress, the decrease in glutathione (GSH) and increase in oxidized glutathione (GSSG) intracellular concentrations are associated with the upregulation of γ-glutamylcysteine synthetase (γ-GCS) expression,[5] which is mediated by NF-κB activation.[6] The phosphorylation of IκB leads to NF-κB activation with the consequent liberation of p50 and p65 subunits, resulting in anti- or pro-apoptotic protein synthesis in the nucleus.[7]

Several recent studies have shown that the alteration of mitochondrial function is a key event in apoptotic cascade. Antitumoral drugs may damage mitochondria since they induce an increase in the permeability of the mitochondrial membrane.[8] These changes are associated with the collapse of the membrane potential, promoting intracellular ATP depletion and inhibiting ATP synthesis. The damage on the mitochondrial membrane is triggered by the generation of reactive oxygen species (ROS) and/or depletion of intracellular and intramitochondrial GSH.[9] These events alter the mitochondrial redox ratio and intracellular pH and induce the translocation of cytochrome *c* to the cytosol.[8,9]

Gallic acid and its analogs have been shown to be involved in a wide variety of biological actions. Gallic acid is an intermediary component of plant metabolism.[10–15] The biological activity of gallic acid and its analogs has

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* Corresponding author. Tel.: +55 48 3721 8057; fax: +55 48 3721 9542; e-mail: taniac@mbox1.ufsc.br

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been described as antioxidant,[16] antifungal,[17] antibacterial,[18] antimalarial,[19] antitumoral,[20] and antiherpetic.[15] However, the main interest in gallic acid derivatives is related to its antitumoral activity. Lauryl, methyl, and propyl gallate induce apoptosis in tumor cell lines and inhibit lymphocyte proliferation.[20] Other previous studies suggest that apoptosis induced by gallic acid and its analogs is associated with oxidative metabolism alterations, mitochondrial dysfunction,[21] an increase in intracellular Ca^{2+} levels (causing caspase activation)[21,22] and DNA fragmentation.[23]

The objective of this study was to investigate the effect of 14 gallic acid derivatives toward a lymphoblastic leukemia cell line, monitoring the oxidative stress induced through GSH content depletion. The influence of the side carbon chain length (C log *P*) of the compounds on cytotoxic activity was also analyzed. Additionally, some biochemical and molecular mechanisms possibly related to the cytotoxicity of these compounds are discussed.

2. Results

In order to screen and compare the cytotoxicity induced by the 14 ester derivatives of gallic acid the compounds were assayed with the L1210 cell line at 100 μM for 24 h. Concentration–time–response curves were obtained for the more active compounds. The compound concentrations analyzed were 0, 1, 10, 25, 50, 75, and 100 μM. The cell death was evaluated after 24, 48, and 72 h of incubation with the compounds by monitoring the mitochondrial activity. Figure 1(A–F) shows the time–concentration–response versus percent of viable cells for the compounds that promoted more than 50% of cell death. It is possible to observe that for all compounds minimum incubation time to reach their maximum effect was about 24 h. Figure 1 shows also that compounds **10**, **11**, **12**, and **14** reached their maximum effect in 48 h of incubation.

Following the procedure described for L1210, all compounds were also tested with CEM cells, a human leukemia cell line. Figure 2 shows the concentration–response curve versus percent of viable cells for the more active compounds and in Table 2 are summarized the IC_{50} values for cell toxicity for both cell lines.

In Figure 3 the relation between C log *P* and cell death is shown. The effective compounds have C log *P* values between 3.32 and 6.09. The C log *P* values of all compounds are also listed in Table 1.

In order to investigate whether cell death was induced through apoptosis, the cells of both cell lines were incubated with compounds **0**, **6**, **7**, **8**, **10**, **11**, **12**, **14**, and **16** at 30 μM and 100 μM for 24 h. After incubation the DNA was extracted and analyzed. As shown in Figure 4, all of the *n*-alkyl-esters of gallic acid tested induced the internucleosomal breakdown of DNA chromatin (in both cell lines), resulting in ladder-like agarose electrophoretic patterns of degraded DNA products.

In a previous work, the gallic acid derivatives were tested for their cytotoxicity and genotoxicity toward a non-tumoral cell line (VERO)[15] and the IC_{50} values for both activities were found to be much greater than those for L1210 and CEM cell lines cytotoxicity evaluated in this work. The cytotoxic and genotoxic concentrations for VERO cells were above 1 mM and 200 μM, respectively.

Figure 5 (left) and (right) show that compounds **0**, **7**, **8**, **10** and **11**, **12**, **14**, **16**, respectively, were able to decrease GSH levels significantly. Furthermore, compound **14** was able to decrease the intracellular GSH level in a concentration-dependent manner. This compound started to induce a significant reduction in cell GSH content at concentrations relatively high ($\geqslant 50$ μM). Compound **8** decreased the GSH concentration by approximately 50% at 100 μM, whereas with the other compounds the decrease in GSH levels reached 75–90% at the same concentration.

As can be seen in Figure 6 (left and right), compounds **8** and **10**, and compounds **11**, **12**, **14**, **16**, respectively, were also able to reduce the mitochondrial GSH content, although only at high concentrations, between 75 and 100 μM. In particular, compound **8** depleted the mitochondrial GSH content more intensively than the cytoplasmic GSH, and compound **7** did not alter the mitochondrial GSH content at all.

In order to access whether NF-κB p65 was involved, its expression was evaluated. The results of the immunoblotting are shown in Figure 7, where it can be observed that only compound **8** (*n*-octyl gallate) was able to increase significantly the peptide expression.

3. Discussion

Although several types of pharmacological activity have been reported for gallic acid and its derivatives, there have been no systematic studies addressing the issue of the relationship between the lengths of the side carbon chain and antitumoral activity, particularly against leukemia.

The gallic acid ester derivatives showed cytotoxic effect toward the L1210 cells in a concentration-dependent manner (Fig. 1). It was possible to observe that the major difference between these molecules considering the variation in the cytotoxic potential is due to the carbon chain length. The compounds with C log *P* values between 3.32 and 6.09 were more active (Fig. 3), in agreement with previous results obtained for the antioxidant activity of these compounds.[15] However, in a previous study, the greatest cytotoxic effect on human adenocarcinoma cells (HeLa) was obtained for propyl gallate, with a C log *P* of 1.7.[12] In our study, a relation between cytotoxicity and alkyl chain length was observed. Compounds **8**, **10**, **11**, **12**, **14**, displayed a considerably more pronounced cytotoxic effect (Fig. 1). It has been previously proposed that the lipophilicity of these compounds increases with alkyl chain length, increasing

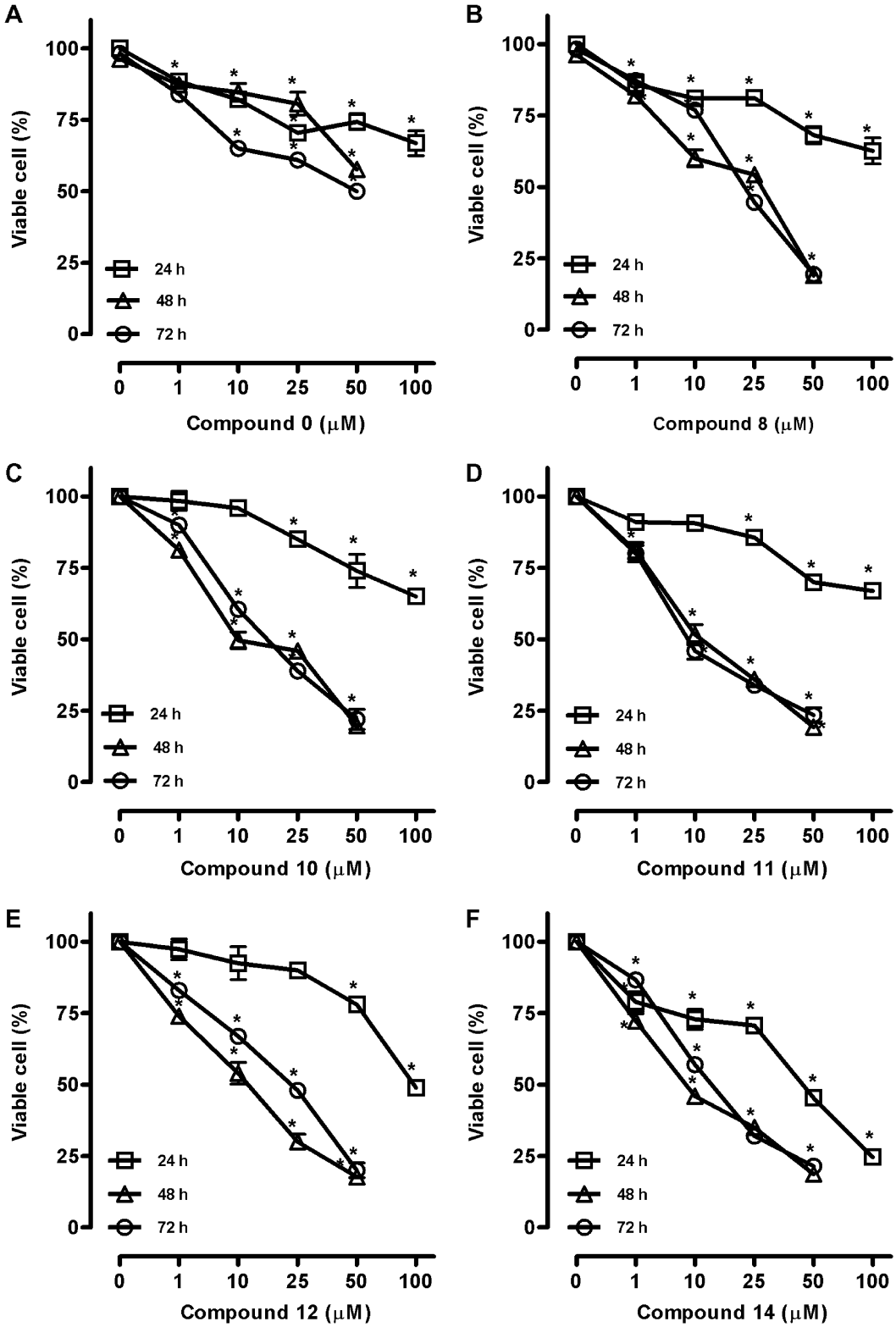


Figure 1. Concentration and time–response curve × cell viability for gallic acid derivatives using L1210 cell line. Cell viability was assayed by MTT. Results are expressed as percentage of viable cells of drug-treated samples in comparison to the control samples (100%). $^{*}p < 0.05$.

their affinity for the lipid bilayer of the cellular membrane, thus influencing their interaction and/or entrance into the cells.[12] Moreover, the hydroxyl groups in the phenolic ring are essential for the biological activity, since 100 μM of trimethoxy benzoic acid and trimethoxy decyl gallate, both gallic acid derivatives, did not show toxicity toward L-1210 cells (results not shown).

Figure 3 shows the ladder-like agarose electrophoretic pattern of the DNA extracted from L-1210 cells treated

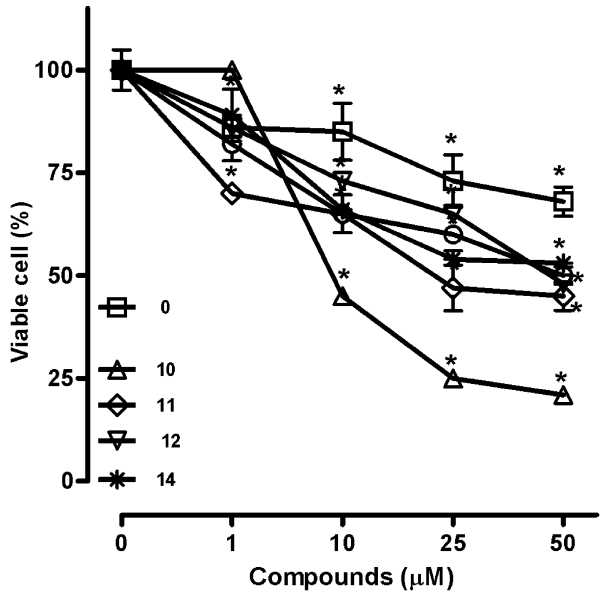


Figure 2. Concentration–response curve × cell viability for gallic acid derivatives using CEM cell line. The cells were incubated for 48 h with compounds **0** and **8**–**14**. Cell viability was assayed by MTT. Results are expressed as percentage of viable cells of drug-treated samples in comparison to control samples (100%). $^{*}p < 0.05$.

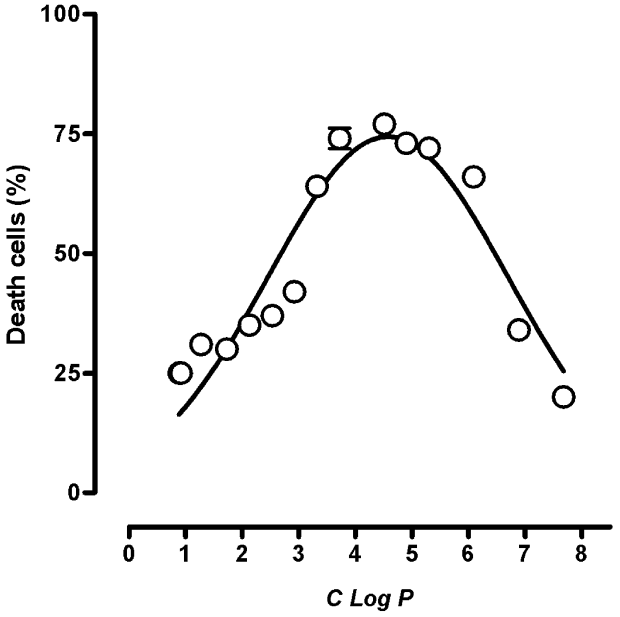
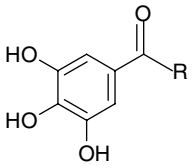


Figure 3. Relationship between cytotoxic activity and compound lipophilicity. The line is simply a visual guide.

with gallic acid and its derivatives. The fact that hydrophobicity is so important is highlighted by the finding that seven of the fourteen compounds were able to induce DNA damage. This may be due to a better permeation through the cell membrane by this type of compound or/and a better interaction of the compound with the membrane receptor(s) or membrane enzyme(s).[20] These results suggest that the less lipophilic compounds were less efficient at reaching the interior of the cell or sending a signal to the interior of the cell

Table 1. Chemical structures and Clog *P* values of gallic acid *n*-alkyl ester derivatives



No	Gallates	–R	Clog *P*
0	Gallic acid	–OH	0.89
1	Methyl	$-CH_3$	0.92
2	Ethyl	$-(CH_2)-CH_3$	1.27
3	Propyl	$-(CH_2)_2-CH_3$	1.73
4	Butyl	$-(CH_2)_3-CH_3$	2.13
5	Pentyl	$-(CH_2)_4-CH_3$	2.53
6	Hexyl	$-(CH_2)_5-CH_3$	2.92
7	Heptyl	$-(CH_2)_6-CH_3$	3.32
8	Octyl	$-(CH_2)_7-CH_3$	3.72
10	Decyl	$-(CH_2)_9-CH_3$	4.51
11	Undecyl	$-(CH_2)_{10}-CH_3$	4.90
12	Dodecyl	$-(CH_2)_{11}-CH_3$	5.30
14	Tetradecyl	$-(CH_2)_{13}-CH_3$	6.09
16	Hexadecyl	$-(CH_2)_{15}-CH_3$	6.89
18	Octadecyl	$-(CH_2)_{17}-CH_3$	7.68

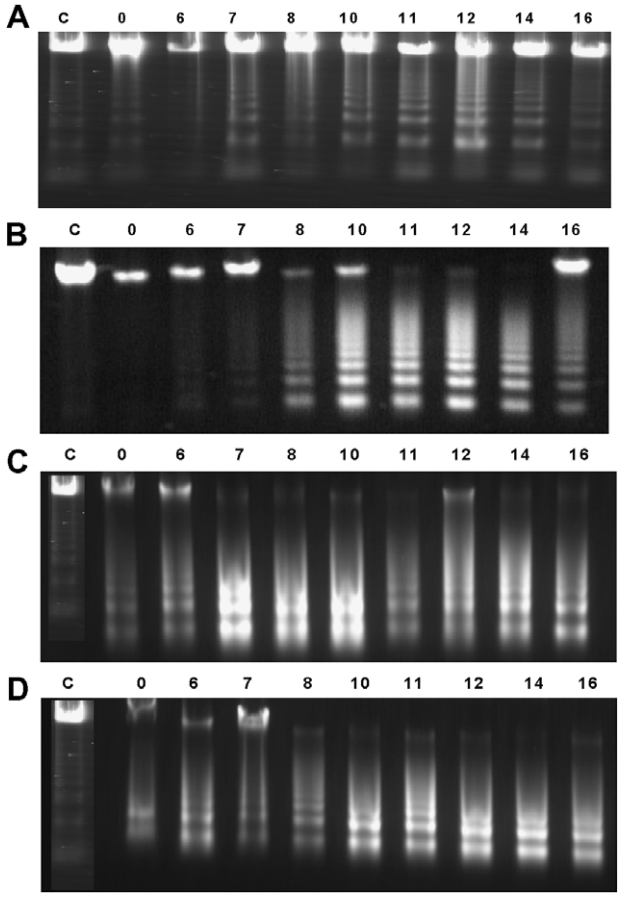


Figure 4. DNA fragmentation in L1210 and CEM cells induced by gallic acid derivatives. L1210 cells (5×10^6) were treated with the compounds (A) 30 μM and (B) 100 μM for 24 h. CEM cells (5×10^6) were treated with the compounds (C) 30 μM and (D) 100 μM for 24 h. The DNA was analyzed by agarose gel electrophoresis as described in Section 5.

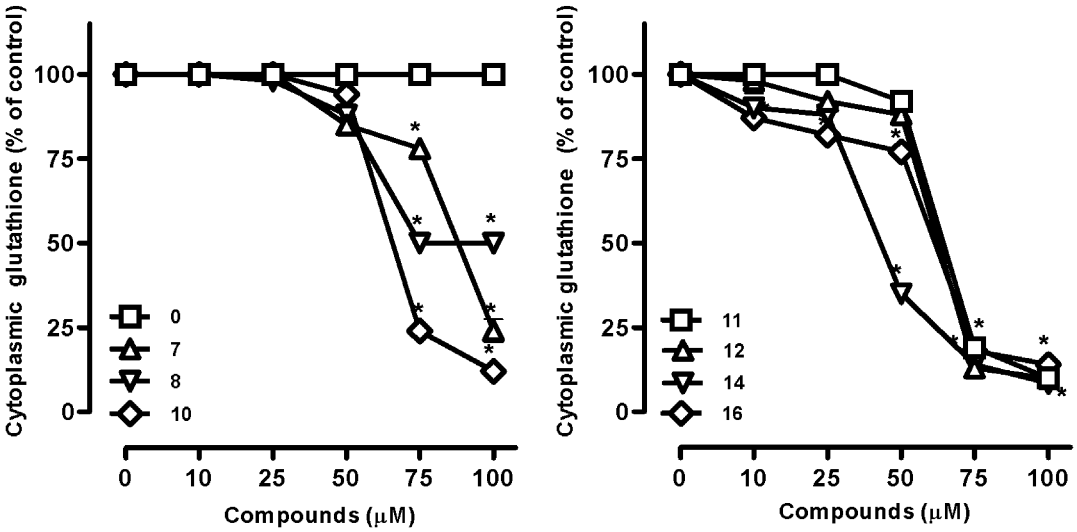


Figure 5. Effect of gallic acid and its derivatives on cytoplasmic glutathione content in L1210 cells. The samples were processed for GSH determination spectrophotometrically as described in Materials and Methods. The values were expressed as percentage of glutathione in relation to the control (100% of glutathione = 0.756 ± 0.015 μmol of GSH). $^{*}p < 0.05$.

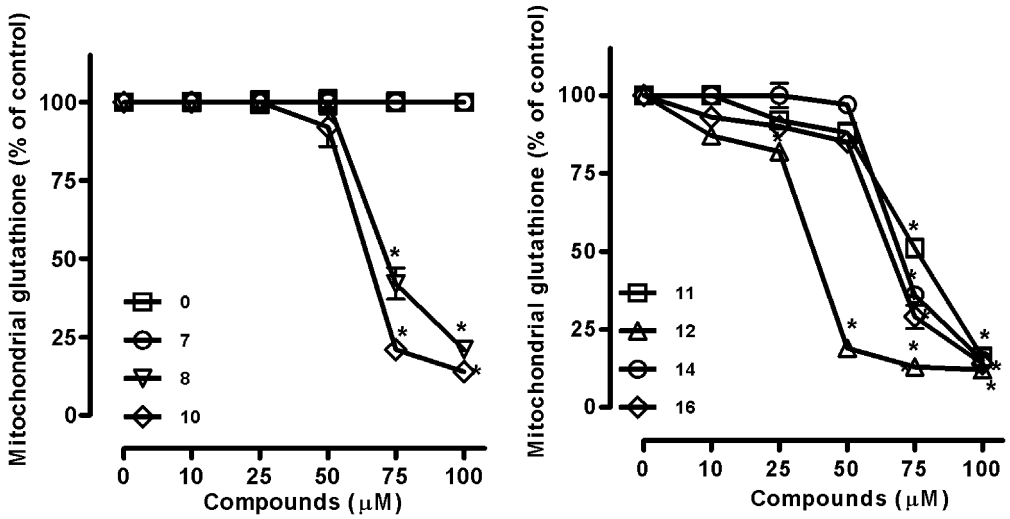


Figure 6. Effect of gallic acid and its derivatives on mitochondrial glutathione content in L1210 cells. The samples were processed for GSH determination spectrophotometrically as described in Section 5. The values were expressed as percentage of glutathione in relation to the control (100% of glutathione = 1.657 ± 0.026 μmol of GSH). $^{*}p < 0.05$.

inducing the cell death, because the more lipophilic compounds, with C log *P* values above 2.9, had a greater potential for DNA fragmentation in comparison with compounds with C log *P* values below 2.9. Even so, there is an upper limit of lipophilicity for a compound to be able to reach the DNA, or to reach any other target that can induce DNA fragmentation.

Assuming that gallic acid is also able to induce apoptosis in tumor cells,[23] we promoted chemical alterations in the molecule and observed that the changes could influence the cytotoxicity. These simple structural modifications to the gallic acid molecule induced changes in its physico-chemical properties, including the solubility and partition coefficient (C log *P*). Besides changes in the diffusion potential through the lipid membranes can also affect the interactions of the molecules with their intracellular targets, since the orientation of the head group, as well as total lipophilicity, is the determinant for the pharmacological activity.[24]

The drug interaction with the cell membrane allows the activation of caspases which may start the apoptotic process and cause DNA fragmentation, a possible mechanism of action of the compounds tested in this work. This suggestion came from the observation that for the same compounds there were different relationships between their lipophilicity and the biological activity. However, some assays were carried out with an enzyme preparation and some with a reaction medium for HOCl scavenging. Consequently, the compounds did not need to cross the bilayer barrier of

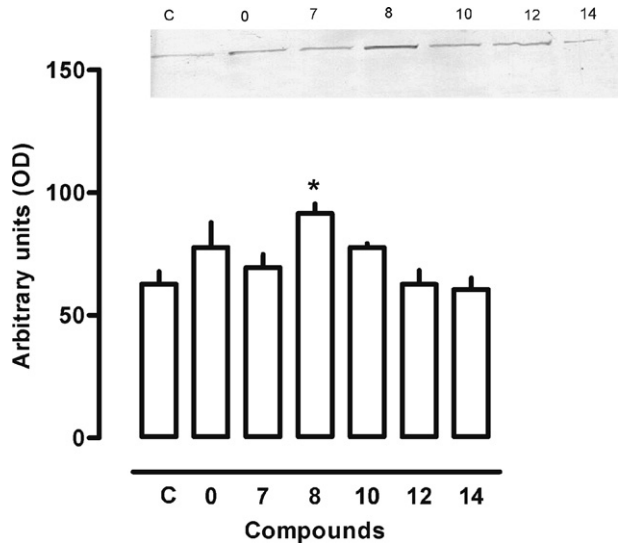


Figure 7. Expression of NFκB in L1210 cells induced by gallic acid derivatives. The samples were analyzed by immunoblotting as described in Section 5. Lane 1, control cells; compounds **0**, **7**, **8**, **10**, **11**, **16** correspond to lanes 2, 3, 4, 5, 6, 7. The bands were quantified by densitometry, using Scion Image Software, $n = 3.^{*}p < 0.05$.

a whole cell; the compounds with C$\log P$ between 0.92 and 2.92, (methyl to hexyl gallates) were the strongest inhibitors of myeloperoxidase activity and all of them were active scavengers of HOCl.[25] Kobayashi et al.[26,27] suggested a similar mechanism for other gallic acid derivatives. Furthermore, the alkyl chain size appears to be very important for the membrane interactions with gallates. It has been demonstrated, using the Caco-2 cell membrane as a model of permeability, that methyl (C$\log P = 0.9$) and propyl (C$\log P = 1.73$) gallates were able to cross the cell membrane and octyl gallate (C$\log P = 3.72$) was not.[28]

In general, the GSH depletion increases the sensitivity of tumor cells to the cytotoxic effects of alkylating compounds and ionizing radiation.[29] The compounds tested here promoted cytoplasmic GSH depletion (Fig. 5). This result suggests that an irreversible binding of these substances with glutathione may occur without the formation of GSSG. During oxidation, the decrease in GSH levels are associated with the upregulation of γ-GCS expression,[29,30] which is mediated by NF-κB activation.[31,32]

The compounds were also able to deplete the mitochondrial GSH content (except heptyl galate) and, although observed only with high concentrations, they may be implicated in a redox potential alteration inducing cell death through apoptosis (Fig. 4), probably through cytochrome c release. Compound **12** was the most active in terms of mitochondrial GSH depletion perhaps because it is the most active compound toward both cell lines (Table 2). Compound **7** probably induces cell death through apoptosis via another mechanism.

Our results demonstrated that only compound **8** increased the constitutive activity of NF-κB in leukemia cells (Fig. 7). Moreover, the compounds that did not influence the NF-κB activation were stronger inducers of GSH depletion. These results suggest that these compounds impede the glutathione synthesis provoking an imbalance in intracellular oxidative metabolism and cell death by apoptosis. Thus, we suggest that compound **8** may induce oxidative stress and consequently GSH depletion, since it decreases the cell viability, although it promotes the recovery of GSH concentration by NF-κB activation. However, other mechanisms of action, such as the induction of pro-apoptotic protein expression,[33] cannot be discarded.

Several studies have shown that NF-κB activation occurs via compounds that induce oxidative stress and are able to activate Fas and TNF-induced AP-1 binding is inhibited.[33–35] Some chemotherapy compounds, such as doxorubicin and its analogs, activate NF-κB, and this is essential in terms of the cytotoxic effects.[36] Perhaps gallic acid derivatives act differently in the apoptotic process and compound **8** may promote cell death through Fas activation or inhibition of TNF-induced Ap-1 binding which is able to promote NF-κB activation. The NF-κB activation promotes the downregulation of bcl-2 translocation of bax, and upregulation of p-53 induced apoptosis in human aortic endothelial cells.[34] Concerning Fas activation, gallic acid apoptosis induction in 3T3 pre-adipocytes via a mitochondrial pathway has been described.[37] Compounds **10**, **11**, **12**, and **14** tested here seem to promote apoptosis through GSH depletion. On the other hand, since gallic acid derivatives can promote intracellular Ca^{2+} changes,[22] the apoptosis induction may occur through the alteration of the mitochondrial permeability, resulting in the unlocking of the pore, followed by the release of cytochrome c and activation of caspases.

Table 2. Comparison between the IC_{50} values of the *n*-alkyl gallates for cell viability in L1210 and CEM cell lines

Compound	L1210 IC_{50} (μM)		CEM IC_{50} (μM)	
	48 h	72 h	48 h	72 h
0	>50	50	>50	>50
8	22	22	50	43
10	12.2	15.5	9.8	20
11	11.33	8.12	21	31.8
12	10.9	21	47.4	22
14	8.5	12.5	>50	28.6

4. Conclusions

In general, new anticancer drugs are expected to induce at least cell death induction through apoptosis. In the carcinogenesis process the cell cycle is deregulated and apoptosis is suppressed; these are the minimal conditions for neoplastic progression.[38] Although the mechanism by which these gallic acid derivatives induce apoptosis was not precisely defined, membrane perturbation and generation of reactive species which promote cytoplasmic GSH and mitochondrial GSH depletion may be involved. Further studies are being developed by our group targeting the mechanism of action of the five active compounds shown here, as well as complementary aspects regarding the structure–activity relationship with analogs modified in the ring. The results described here together with those reported in the literature indicate that the compounds may act on different targets, as observed for compounds **7** and **8**. Compound **8** was the only one able to increase the NFκB expression; lauryl gallate (our compound **12**) acts by blocking the tyrosine phosphorylation[39]; and the alkyl length and degree of hydroxylation of gallates drive their interaction with the cell membranes.[28,37]

5. Experimental

5.1. Chemicals

The cell culture media, serum, and antibiotics were purchased from GIBCO (São Paulo, Brazil), the rabbit polyclonal NF-κB p65 antibody from Santa Cruz Biotechnology (San Diego, CA) and all other reagents from Sigma Chemical Co. (St. Louis, MO). Rainbow markers and PVDF membranes were obtained from Hybond-P, Amersham Biosciences, San Francisco, CA. The compounds were synthesized and characterized as described elsewhere[15] and numbered according to the size of the carbon chain, as specified in Table 1, as 0 (gallic acid) and from 1 to 18 C (compounds with **9**, **13**, **15**, and **17** C were not obtained). The compounds were dissolved in 100% of dimethyl sulfoxide (DMSO, Merck), and diluted in cell culture medium to a final concentration of 0.01% of DMSO.

5.2. C log *P* determination

The C log *P* values were obtained using the commercially available software Tsar 3D version: 3.3 from Oxford Molecular Ltd.[40]

5.3. Cell culture

Two cell lines of lymphoblastic leukemia were used namely L-1210, of murine origin, and CEM, of human origin (ATCC, Manassas, VA). Both cell lines were cultured in DMEM supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mM Hepes, pH 7.4, at 37 °C, in a 5% CO_2 humidified atmosphere. Every 2–3 days, cells were passaged by removing 90% of the supernatant and replacing it with fresh medium.

5.4. Viability assay

The cell incubations with the compounds were carried out for 24, 48, and 72 h with cell densities (cell/0.2 mL) of 3×10^5, 7.5×10^4, and 5×10^4, respectively. The amounts of the cells for each incubation time were determined taking into account 100% of viability under each condition. Cell viability was monitored using the MTT (3-(4,5-dimethiazol-zyl)-2-5-diphenyltetrazolium bromide) assay.[41] A control was run in parallel to monitor the influence of DMSO on the assays. The control with solvent did not show a statistical difference from the control with cells alone.

5.4.1. Analysis of DNA fragmentation by agarose gel electrophoresis. The isolation of apoptotic DNA fragments was based on the method of Han.[42] For this, 2×10^6 L1210 cells were treated with the compounds for 24 h. The cells were then washed with cold PBS and incubated with lyses buffer (10 mM EDTA, 50 mM Tris–HCl pH 8.0, 0.25% NP-40, 0.5 g/L proteinase K) at 50 °C for 2 h. The DNA was then precipitated with 2.5 vol of ethanol at 25 °C overnight and dried in air. After washing with ice-cold 70% ethanol, the pellets were dissolved in TE buffer containing 10 mM Tris–HCl pH 8.0, 1 mM EDTA, and 0.6 g/mL RNase A and further incubated at 37 °C for 1 h. Horizontal electrophoresis was performed at 200 V in 1.5% agarose gel with TAE (Tris–acetic acid and EDTA) as the running buffer. The gel was stained with ethidium bromide and visualized by 2UV Transilluminator (MacroVue UV-20 Hoefer) for ladder formation.

5.4.2. Glutathione measurement. Total glutathione (GSH + GSSG) was measured using the glutathione reductase method.[43] Briefly, 1×10^6 cells were washed in PBS, resuspended in chilled Milli-Q water containing 1 mM EDTA and sonified for 10 s. About 20 μL of each homogenate was then transferred to a 96-well plate followed by the addition of the solution (75 μM 5,5′-dithiobis-2-nitrobenzoic acid, DTNB; 120 μM NADPH, 1 U/mL glutathione reductase, and 10 mM EDTA in 100 mM phosphate buffer, pH 7.4). The absorbance was measured after 15 min at 412 nm using a Microwell Systems (Organon Teknika). The values were expressed as percentage of the control. The control absorbance considered as 100% was that for glutathione (0.756 ± 0.015 μmol GSH/1.10^6 cells/0.5 mL).

5.4.3. Mitochondrial glutathione measurement. Briefly, 1×10^6 cells were washed in PBS, homogenized in a buffer containing 10 mM Tris–HCl, pH 7.5, and 250 mM sucrose. After that the cells were incubated on ice for 15 min and centrifuged at 1500*g* for 10 min, at 4 °C. The mitochondrial extract was resuspended in chilled Milli-Q water containing 1 mM EDTA and sonified for 10 s. The total mitochondrial glutathione (GSH + GSSG) was measured using the glutathione reductase method[43] as explained above. The control absorbance considered as 100% was that for glutathione (1.657 ± 0.026 μmol GSH/1.10^6 cells/0.5 mL).

5.4.4. Preparation of nuclear extracts. Nuclear extracts were obtained as described previously.[44] Briefly, cells (5×10^6 cells/mL) were incubated in ice-cold lyses buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiotreitol, and 0.5 mM PMSF). After incubation (15 min), NP-40 was added (0.5%, v/v) and centrifuged (1810*g*; 5 min at 4 °C). Nuclear pellets were suspended in ice-cold buffer C (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 μg/mL pepstatin, 1 μg/mL leupeptin, and 20% (v/v) glycerol and 1 mM EDTA, EGTA, PMSF, and dithiotreitol) and incubated for 30 min. Nuclear proteins were collected in the supernatant after centrifugation (12,000*g*; 10 min at 4 °C). The total protein content was determined by the Bradford method.[45]

5.4.5. Immunoblotting analysis. Nuclear lysates were denatured in a buffer (50 mM Tris–HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue) and heated in a bath of boiling water for 3 min. Samples (30 μg total protein) were run in 12% SDS–PAGE and the proteins were transferred to PVDF membranes. Rainbow markers were run in parallel. The PVDF membranes were blocked with Tween–TBS (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.1% Tween-20, 5% milk) and incubated overnight with rabbit polyclonal anti-NFκB antibody (1:1000 dilution with Tween–TBS). The membranes were then washed with Tween–TBS, followed by incubation (1 h) with anti-rabbit IgG antibody biotin-conjugated (1:1000) with streptavidin-conjugated horseradish peroxidase (1:1000). Immunoreactive proteins were visualized by 3,3′-diaminobenzidine staining. The bands were quantified by densitometry, using Scion Image Software (Scion Co, Frederick, Maryland, USA).

5.4.6. Statistical analysis. The results were presented as means ± SD of triplicates from three-independent experiments. Statistical significance was assessed by ANOVA followed by Bonferroni's *t*-test, and $p < 0.05$ was taken as statistically significant.

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