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Lauryl Gallate Inhibits the Activity of Protein Tyrosine Kinase c-Src Purified from Human Platelets

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The inhibitory effect of gallic acid (3,4,5-trihydroxybenzoic acid), and its ester derivatives methyl, propyl, octyl and lauryl has been tested on the tyrosine kinase activity of affinity purified c-Src from human platelets, using the artificial substrate Poly (Glu.Na, Tyr) 4:1. When tested as inhibitor of the autophosphorylation of the enzyme and the phosphorylation of the protein tyrosine phosphatase SHP-1 by c-Src, lauryl gallate was found to be a more potent inhibitor than other widely used protein tyrosine kinase (PTK) inhibitors such as genistein and herbimycin A. However, lauryl gallate did not inhibit the activity of the serine threonine kinases protein kinase A (PKA) and casein kinase II (CKII) from rat brain.

Keywords: PTK c-Src; PTP SHP-1; Inhibitors of PTKs

Abbreviations: PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; SHP-1, Src homology region 2 [SH2]-domain phosphatase 1; LG, lauryl gallate; PKA, protein kinase A; CKII, casein kinase II; GST, glutathione S-transferase; GT 4:1, Poly (Glu.Na, Tyr) 4:1; TBS, tris buffered saline (0.01 M tris–HCl pH 7.4; 0.15 M NaCl); SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; *p*NPP, *p*-nitrophenyl phosphate; OPD, *o*-phenylenediamine; IgG, immunoglobulin; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide

INTRODUCTION

Protein tyrosine kinases (PTKs) are enzymes that phosphorylate specific tyrosine residues in a wide variety of proteins. They are common mediators for transmitting mitogenic signals, and regulate numerous cellular processes.[1] c-Src was the first member described from the family Src of PTKs,[2] and the cellular homologue of the transforming protein of the Rous sarcoma virus, v-Src. c-Src is present in many cells and is involved in the signal transduction pathways activated by the platelet-derived growth factor (PDGF),[3] the colony stimulating factor-1 (CSF-1),[4] and the epidermal growth factor (EGF)[5] receptors. Other functions, such as cell adhesion

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and migration[6] have also been postulated to be regulated by this kinase.

A potential role for certain PTKs in tumourigenesis is evident from their ability to transform normal cells to a neoplasic phenotype when expressed in a mutated unregulated form, or expressed in an abnormally high level. Enhanced PTK activity of c-Src, has been implicated in several human cancers: neuroblastoma,[7] colon,[8] bladder[9] and oesophagus[10] carcinomas, as well as in breast cancer.[11] An increase in c-Src protein expression has also been reported in breast,[12] and pancreatic cancer patients.[13] The prevalence of activated c-Src in cancer indicates that this protein may play a significant role in the progression of many cancers, and thus, is a likely target for drug discovery efforts. The recent use of c-Src inhibitors in leukaemia cells[14] supports the validity of this hypothesis. A wide variety of compounds such as genistein,[15] herbimycin A,[16] and tyrphostins[17] have frequently been used as PTK inhibitors, and a number of pyrido[2,3-*d*]pyrimidines have been characterized as selective inhibitors of c-Src ($IC_{50} < 10\,nM$).[18] In a previous work, we have investigated the effect of different monophenolic, diphenolic and triphenolic compounds on a partially purified PTK activity from human spleen, and found that gallic acid (3,4,5-trihydroxybenzoic acid) and its ester derivatives methyl, propyl, octyl and lauryl gallate, behaved as very good inhibitors of this activity.[19] We have also shown that lauryl gallate inhibits total tyrosine phosphorylation in Wehi 231 cells upon pervanadate treatment or IgM stimulation at lower concentrations than herbimycin A or genistein.[20] Here, we demonstrate that these compounds, and particularly lauryl gallate, are also strong inhibitors of c-Src activity purified from human platelets, showing an inhibitory potency superior to other widely used inhibitors of PTKs. However, lauryl gallate failed to inhibit the serine threonine kinases protein kinase A (PKA) and casein kinase II (CKII).

MATERIALS AND METHODS

Antibodies, Materials and Chemicals

Antiphosphotyrosine monoclonal antibody 1G2 (ATCC SD 990, USA), Monoclonal antibody 327 anti c-Src[21] and peroxidase-labelled goat antibody against mouse IgG were from BioRad.

Expression vector for recombinant protein tyrosine phosphatase SHP-1 (Src homology region 2 [SH2]-domain phosphatase 1) was pKG- SHP-1, (kindly provided by Dr R.J. Matthews, University of Wales College of Medicine).[22]

MicroELISA polystyrene plates (Nunc, The Netherlands, Cat. Ref. 449852), Poly (Glu.Na, Tyr) 4:1, gallic acid, methyl gallate, genistein and herbimycin A were from SIGMA (USA). Propyl, octyl and lauryl gallate were from Fluka (Buchs, Switzerland). [γ-^{32}P]ATP (sp act: 3000 Ci/mmol) was from DuPont NEN (Boston, MA). Other chemicals were of the highest quality available and were purchased from Sigma and Merck.

Purification of c-Src from Human Platelets

PTK c-Src was obtained from platelet membranes prepared from 1 day outdated platelet concentrates, by affinity chromatography on Monoclonal Antibody 327 bound to Sepharose, according to the published procedures.[23] About 0.2 mg of purified PTK c-Src, with purity above 85% as judged by SDS-PAGE, were obtained from 10 units of platelet concentrates.

Expression of Recombinant SHP-1

A 1.5 Kb *Eco* R1-*Hind* III fragment of mouse cDNA, encoding amino acid residues 1–595 of SHP-1, was inserted into expression vector pGEX-KG. Expression of GST fusion proteins was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside as previously described.[22] The GST-SHP-1 fusion protein was cleaved with thrombin, in a glutathione

Sepharose 4B column (0.5 × 2 cm) (Pharmacia Biotech) incubated with 50 units of thrombin in 1 ml of cleavage buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 2.5 mM $CaCl_2$), for 1 h at room temperature. Following incubation, the column was eluted with TBS, releasing a mixture of SHP-1 and thrombin, while GST remained bound to the column. In order to separate SHP-1 and thrombin, the eluted sample was loaded onto a Sephacryl S-300 gel filtration equilibrated with TBS. Fractions with significant PTP activity (assays for activity with *p*NPP were performed as described[24]) were pooled and concentrated to about 3 mg/ml. Glycerol was then added to a final concentration of 50% and the enzyme was stored frozen at −40°C.

Preparation of Brain Fractions

Adult Sprague–Dawley rats were used to prepare brain extracts as described previously.[25] Briefly, animals were killed by decapitation and their brains were immediately homogenized 1:5 in buffer A (20 mM Tris–HCl pH 7.5, 5 mM magnesium acetate, 100 mM NH_4Cl, 10 mM 2-mercaptoethanol, 10 mM EGTA, 1 mM PMSF and 200 mM sucrose). Cell debris was removed by centrifugation at 27,000*g* for 30 min and the supernatant was loaded on a 1.8 M sucrose cushion (in buffer A) and centrifuged at 100,000*g* for 18 h. The supernatant (fraction S) was made up to 0.02% (w/v) in sodium azide and kept at 4°C.

Phosphorylation Assays

Tyrosine kinase activity of c-Src against the artificial substrate Poly (Glu.Na, Tyr) 4:1 (GT 4:1), was measured by means of an ELISA developed in our laboratory[26] with minor modifications. Plates were coated overnight with GT 4:1 (0.01 mg/ml) and incubated with a blocking solution containing 1% BSA. About 6.75 ng of c-Src were incubated in the presence of the different inhibitors, in phosphorylation buffer (50 mM Hepes, 10 mM $MgCl_2$, 300 μM ATP) 30 min at 37°C. Phosphorylated GT 4:1 was then probed with anti-phosphotyrosine monoclonal antibody 1G2 (50 ng/ml) and goat anti-mouse IgG antibody labelled with peroxidase (1/2000). OPD was used as substrate and the absorbance was read at 492 nm in a multichannel electrophotometer (Multiskan Titertek Flow).

The autophosphorylation of c-Src was followed by the incorporation of P^{32} from [γ-^{32}P]ATP, SDS-PAGE and autoradiography. About 80 ng of c-Src was incubated in the presence of lauryl gallate, genistein or herbimycin A and [γ-^{32}P]ATP (5 μl of a solution 100 μCi/ml in a final volume of 50 μl), in phosphorylation buffer (50 mM Hepes, 25 mM $MgCl_2$). After incubation for 30 min at room temperature, the reaction was stopped by addition of 50 μl of SDS-PAGE sample buffer, samples were then boiled and run on a 12% SDS-PAGE. After electrotransference to an Immobilon-P membrane (Millipore), P^{32} labelled proteins were visualized by autoradiography.

The phosphorylation of SHP-1 by c-Src was followed in the same way as the autophosphorylation of c-Src, 400 ng of SHP-1 previously inactivated by heating 10 min at 50°C, was used as substrate of c-Src in each reaction.

Protein kinase assay to measure kinase activity of PKA and CKII, were performed as described[25] with minor modifications. Reaction mixtures in a final volume of 50 μl contained: 20 mM Tris–HCl pH 7.5, NaF 5 mM, potassium acetate 50 mM and magnesium acetate 5 mM. Histone IIs (50 μg) and fosvitine (2 mg/ml) were used as exogenous substrates for PKA and CKII, respectively. c-AMP (5 μM) and heparin (5 μg/ml) were used to measure specific activity of PKA and CKII, respectively, and 10 μg of protein from fraction S were used as kinase source. After 2 min preincubation at 30°C, the reaction was started by addition of [γ-^{32}P]ATP (100 μM, 25 μCi) and maintained for 30 min after which the reaction was stopped by spotting the samples on a

TABLE I Inhibition of PTK c-Src from human platelets by gallic acid derivatives

Compounds	Percentage inhibition at 500 μM	I_{50} (μM)
Gallic acid		136
Methyl gallate		46
Propyl gallate		48
Octyl gallate*		4
Lauryl gallate*		2
Genistein*	23	

The substrate GT 4:1 was used at 0.01 mg/ml in these assays. All compounds were dissolved in water except (*), which were dissolved in dimethyl sulfoxide (DMSO) and assayed in the presence of 10% DMSO in the assay mix due to solubility problems. Results are representative of three independent experiments.

Whatman P-81 (histone) or ET-31 (fosvitine) filter paper followed by immersion in either 0.5% orthophosphoric acid (P-81) or 10% trichloroacetic acid (ET-31). The strips were washed twice more with either 0.5% orthophosphoric acid (P-81) or 5% trichloroacetic acid (ET-31). Filters were counted and protein kinase activity was expressed as pmoles of ^{32}P incorporated/10 min/mg protein.

RESULTS AND DISCUSSION

Gallic Acid and Derivatives Inhibit the Kinase Activity of c-Src Using GT 4:1 as Substrate

Previous work on the effect of mono, di or triphenolic compounds on the PTK activities present in membranes or cytosolic extracts of human spleen, allowed us to detect that gallic acid and its alkyl esters behaved as potent inhibitors for these enzymes.[19] As shown in Table I, these compounds also inhibit the phosphorylation of the artificial substrate GT 4:1 by affinity purified PTK c-Src from human platelets, showing a 50% inhibition in a range between 2 and 136 μM. The more hydrophobic compounds (octyl and lauryl gallate) showed the highest inhibitory potency ($I_{50} = 4$ and 2 μM, respectively). We have also shown that lauryl gallate inhibits total tyrosine phosphorylation in Wehi 231 cells,[20] and proliferation of human peripheral blood lymphocytes and tumour cell lines.[27] Therefore, all subsequent studies were performed with that compound. Genistein produced a 23% inhibition when used at 500 μM. Herbimycin A did not show inhibitory activity when used at 50 μM.

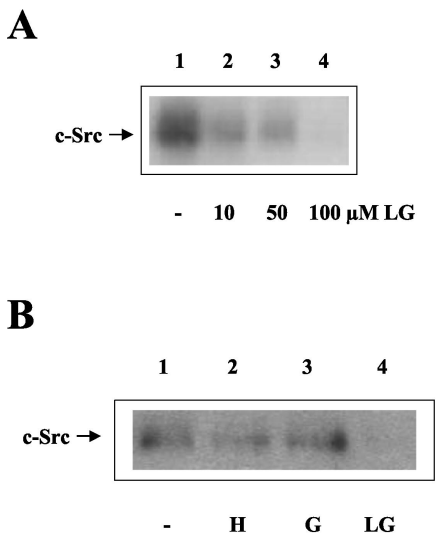

FIGURE 1 Effect of lauryl gallate on the autophosphorylation of c-Src, and comparison with herbimycin A and genistein. (A) Lane 1: Autophosphorylation of PTK c-Src in the presence of 10% DMSO. Lanes 2, 3 and 4: Autophosphorylation in the presence of 10, 50 and 100 μM lauryl gallate. (B) Lane 1: Autophosphorylation of PTK c-Src in the presence of 10% DMSO. Lanes 2, 3 and 4: Effect of herbimycin A (H), genistein (G) or lauryl gallate (LG) when added at 100 μM in the assay. Autophosphorylation conditions and detection of the phosphorylated proteins were performed as described under methods.

Lauryl Gallate Inhibits the Autophosphorylation of c-Src from Human Platelets

In order to investigate if this compound could also inhibit the activity of PTK c-Src towards more physiological substrates, we studied the effect of lauryl gallate on the autophosphorylation of c-Src from human platelets *in vitro*. Figure 1ashows that the inhibition of the enzyme autophosphorylation is already detectable when used at 10 μM, and is complete when used at

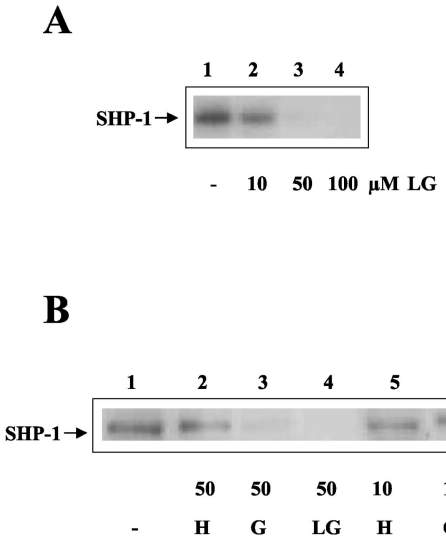

FIGURE 2 Effect of lauryl gallate on the phosphorylation of PTP SHP-1 by c-Src, and comparison with herbimycin A and genistein. (A) Lane 1: Phosphorylation of recombinant PTP SHP-1 by purified PTK c-Src in the presence of 10% DMSO. Lanes 2, 3 and 4: phosphorylation of PTP SHP-1 in the presence of 10, 50 and 100 μM lauryl gallate, respectively. (B) Lane 1: Phosphorylation of PTP SHP-1 in the presence of 10% DMSO. Lanes 2, 3 and 4: Effect of herbimycin A, genistein or lauryl gallate added at 50 μM. Lanes 5, 6 and 7: Effect of the same inhibitors added at 10 μM. Phosphorylation conditions and detection of the phosphorylated proteins were performed as described under methods.

100 μM. On the other hand, genistein or herbimycin A, when used at the same concentration (100 μM) as lauryl gallate, behaved as poor inhibitors of c-Src autophosphorylation (Fig. 1b).

Lauryl Gallate Inhibits the Phosphorylation of PTP SHP-1 by PTK c-Src from Human Platelets

There are several pieces of evidence concerning the mutual regulation of PTP SHP-1 phosphorylation and c-Src. First, SHP-1 has been found associated to c-Src in human platelets upon thrombin stimulation.[28] On the other hand, it has also been shown that the activity of PTK c-Src can be positively regulated by PTP SHP-1,[29] in contrast with the negative regulation observed on a wide spectrum of signalling effectors such

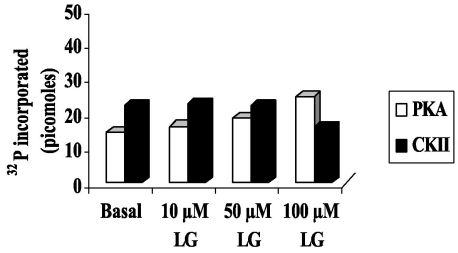

FIGURE 3 Effect of lauryl gallate on protein kinase A (PKA) and casein kinase II (CKII) activities. Supernatant fraction from rat brain was used as source of kinase activities, and the effect of increasing concentrations of lauryl gallate on phosphorylation of exogenous substrates was measured as described under methods. Basal activity was measured in the presence of 10% DMSO.

as ZAP-70 and JAK 2 tyrosine kinases.[30,31] These results prompted us to investigate if lauryl gallate could also inhibit the phosphorylation by c-Src of recombinant PTP SHP-1. Figure 2ashows the effect of different concentrations of lauryl gallate when SHP-1 is used as a substrate for c-Src *in vitro*. The inhibitory effect can be observed when this compound is used at 10 μM, but when used at concentrations up to 50 μM, the inhibition of phosphorylation is complete. The inhibitory potency of lauryl gallate is superior to the inhibitory potency shown by herbimycin A or genistein when are used at 50 or 10 μM, as shown in Fig. 2b.

Lauryl Gallate Does Not Inhibit the Activity of the Serine Threonine Kinases PKA, and CKII

Another point of interest was to investigate if lauryl gallate specifically inhibits PTKs activity, or could also inhibit the activity of serine threonine kinases such as PKA or CKII. Figure 3shows that lauryl gallate failed to cause inhibition of these two enzymes when used at the same concentration as in the studies performed for tyrosine phosphorylation. This specificity of lauryl gallate in the inhibition of PTK activity confers additional advantages to this compound in comparison with broader spectrum inhibitors that could affect the activity

of serine threonine kinases involved in cell regulation.

In summary, we have demonstrated that gallic acid and its derivatives are good inhibitors of the kinase activity of c-Src purified from human platelets. The fact that lauryl gallate inhibits proliferation of several tumoural cell lines,[27] and that gallic acid provides protection against carcinogenesis induced by a variety of tumour promoters,[32] could justify a study on long chain alkyl gallates as potential antitumoral drugs. Their simple chemical structures, very low commercial cost, their use as common antioxidant food additives, i.e. E-310 (propyl gallate); E-311 (octyl gallate); E-312 (lauryl gallate) and the fact that the latter does not show inhibitory properties on serine threonine kinases such as PKA or PKCII, are also important aspects to consider.

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