J. Biochem. **139**, 495–502 (2006)
doi:10.1093/jb/mvj050

Tannic Acid, a Potent Inhibitor of Epidermal Growth Factor Receptor Tyrosine Kinase

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Received November 4, 2005; accepted January 5, 2006

Increasing evidence supports the hypothesis that tannic acid, a plant polyphenol, exerts anticarcinogenic activity in chemically induced cancers. In the present study, tannic acid was found to strongly inhibit tyrosine kinase activity of epidermal growth factor receptor (EGFr) *in vitro* (IC_{50} = 323 nM). In contrast, the inhibition by tannic acid of $p60^{c\text{-}src}$ tyrosine kinase (IC_{50} = 14 μM) and insulin receptor tyrosine kinase (IC_{50} = 5 μM) was much weaker. The inhibition of EGFr tyrosine kinase by tannic acid was competitive with respect to ATP and non-competitive with respect to peptide substrate. In cultured cells, growth factor–induced tyrosine phosphorylation of growth factor receptors, including EGFr, platelet-derived growth factor receptor, and basic fibroblast growth factor receptor, was inhibited by tannic acid. No inhibition of insulin-induced tyrosine phosphorylation of insulin receptor and insulin-receptor substrate-1 was observed. EGF-stimulated growth of HepG2 cells was inhibited in the presence of tannic acid. The inhibition of serine/threonine-specific protein kinases, including cAMP-dependent protein kinase, protein kinase C and mitogen-activated protein kinase, by tannic acid was only detected at relatively high concentration, IC_{50} being 3, 325 and 142 μM respectively. The molecular modeling study suggested that tannic acid could be docked into the ATP binding pockets of either EGFr or insulin receptor. These results demonstrate that tannic acid is an *in vitro* potent inhibitor of EGFr tyrosine kinase.

Key words: EGF receptor, inhibitor, molecular modeling, tyrosine kinase.

Protein tyrosine kinases play an important role in the regulation of cell differentiation and proliferation. Ligand-activated growth factor receptor tyrosine kinases catalyze the receptor autophosphorylation and the phosphorylation of their cellular substrates at tyrosine residues to carry out signal transduction in the cells (*1*). In human cancers, such as breast and liver cancers, some growth factor receptors with tyrosine kinase activity are known to be overexpressed (*2–4*). In recent years, a number of promising new anticancer drugs have been developed which target intracellular pathways or extracellular molecules. One group of clinically effective compounds among these drugs functions as tyrosine kinase inhibitors. The inhibitors of protein tyrosine kinases represent an important new class of therapeutic agents for the treatment of cancers in which tyrosine kinases are overexpressed (*5*, *6*). Some growth factor receptor tyrosine kinase inhibitors, such as the EGFr tyrosine kinase inhibitor ZD1839, the c-kit tyrosine kinase inhibitor STI571, the vascular endothelial growth factor receptor (VEGFr) tyrosine kinase inhibitor PTK787/ZK222584 and multitarget tyrosine kinase inhibitors ZD6474 and SU6668, have been reported (*7–11*).

Some plant polyphenols, such as genistein and quercetin, were reported to be tyrosine kinase inhibitors (*12*, *13*). We previously reported that other plant polyphenols, including butein, marein and phloretin, also had ability to inhibit protein tyrosine kinases (*14*, *15*). Tannic acid, an ester of one glucose molecule with three gallic acid molecules, is also a plant polyphenol (*16*). The structures of tannic acid and gallic acid are shown in Fig. 1. In this study, the specificity and kinetics of tannic acid–induced inhibition of tyrosine-specific protein kinases, such as EGFr, $p60^{c\text{-}src}$ and insulin receptor (IR), and serine/threonine-specific protein kinases, such as Protein kinase C (PKC), cAMP-dependent protein kinase (PKA) and mitogen-activated protein kinase (MAPK), were determined *in vitro*. The inhibitory effect of tannic acid on growth factor–induced tyrosine phosphorylation of growth factor receptors, including EGFr, basic fibroblast growth factor receptor (bFGFr), platelet-derived growth factor receptor (PDGFr) and IR, in cultured cells was detected by ECL Western blotting assay system. We also docked tannic acid into the ATP binding pockets of EGFr and IR to release the possible interaction of tannic acid with EGFr and IR by the computational method.

MATERIALS AND METHODS

Assay of EGFr, $p60^{c\text{-}src}$ and IR Tyrosine Kinase Activities—The activities of EGFr and $p60^{c\text{-}src}$ tyrosine kinases were determined by the method previously described by Yang *et al.* (*19*, *20*) with some modification.

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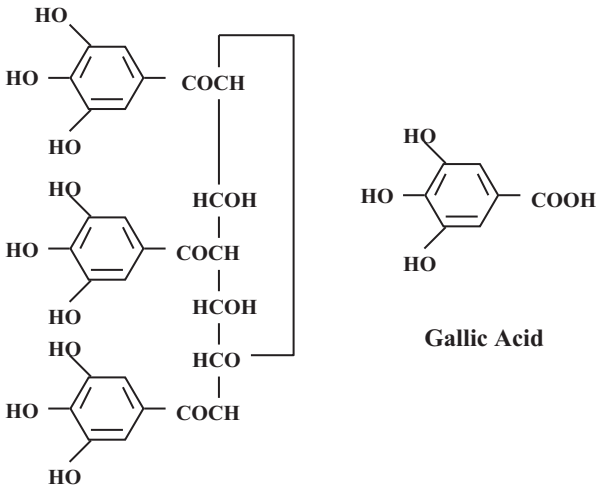

Fig. 1. **Molecular structures of tannic acid and gallic acid.**

Five microliters of diluted EGFr solution (Sigma Chemicals, MO, USA) with 500 ng/ml EGF in 50 mM Tris-HCl buffer, pH 7.5, or 5 μl of diluted $p60^{c\text{-}src}$ solution (Calbiochem-Novabiochem Corporation, CA, USA) in 50 mM Tris-HCl buffer, pH 7.5, containing 20 mM $MgCl_2$, was mixed with 10 μl of 4 mg/ml poly (Glu, Tyr) 4:1 (Sigma Chemicals, MO, USA) for EGFr tyrosine kinase activity assay or 10 μl of 0.5 μg/ml Raytide (Calbiochem-Novabiochem Corporation, CA, USA) for $p60^{c\text{-}src}$ tyrosine kinase activity assay. After the addition of 5 μl of different concentrations of tannic acid in water (Extrasynthese, Genay, France), 5 μl of 0.5 mM ATP containing 20–25 μCi/ml [γ-^{32}P]ATP (Amersham Pharmacia Biotech, Backinghanshire, UK) was added to start the reaction. After the reaction mixtures were incubated at 30°C for 60 min, then the reaction was stopped by adding 20 μl of 10% H_3PO_4, and 20 μl of each reaction solution was spotted onto a piece of phosphocellulose disc (ø 2 cm). Discs were washed 3 times with 1% H_3PO_4 at room temperature for 10 min, then peptide-incorporated ^{32}P on the discs was counted in a scintillation counter.

The inhibition of IR tyrosine kinase by tannic acid was evaluated by ELISA method. One hundred microliters of 2 mg/ml poly (Glu, Tyr) 4:1 in PBS was coated on a 96-well plate at 4°C overnight. After washing four times with Tris-buffered saline (TBS)/Tween 20 containing 20 mM Tris-HCl buffer, pH 8.0, 0.15 M NaCl, and 0.2% Tween 20, 40 μl of IR kinase buffer containing 20 mM Tris-HCl buffer, pH 7.6, 10 mM MnAc, and 25 μM ATP with various concentrations of tannic acid was added into the plate. The IR tyrosine kinase–catalysed reaction was started by the addition of 10 μl of diluted IR solution (Sigma Chemicals, MO, USA). After incubation at 25°C for 30 min, the reaction was stopped by washing four times with TBS/Tween 20. The non-specific binding sites were blocked by incubation with 100 μl of 3% BSA in TBS/Tween 20 at room temperature for 1 h. After washing twice with TBS/Tween 20, 100 μl of recombinant RC20 anti–phosphotyrosine peroxidase conjugate (Affiniti, Nottinghamshire, UK) (1:1,000) in TBS/Tween 20 was added. The plate was incubated at room temperature for 1 h, then washed four times with TBS/Tween 20 and incubated with 100 μl of TMB-ELISA (Gibco, Gaithersburg, MD, USA) in dark with gentle agitation at room temperature for 30 min. Then 25 μl of 0.5 M H_2SO_4 was added to stop the reaction, and the plate was read in a microreader at 450 nm.

Assay of PKC, PKA and MAPK Activities—To detect the inhibition of PKC and PKA by tannic acid, 10 μl of diluted PKC or PKA solution (Calbiochem-Novabiochem Corporation, CA, USA) in 50 mM Tris-HCl buffer, pH 7.5, with various concentrations of tannic acid was mixed with 5 μl of lipid preparation containing 100 μM phorbol 12-myristate 13-acetate, 2.8 mg/ml phosphatidyl serine and Triton X-100 mixed micelles in 50 mM Tris-HCl buffer, pH 7.5, for PKC assay or 5 μl of 40 μM cAMP in 50 mM Tris-HCl buffer, pH 7.5, for PKA assay. MAPK solution (Sigma Chemicals, MO, USA) was diluted with MAPK kinase buffer containing 20 mM Tris-HCl buffer, pH 7.6, 20 mM $MgCl_2$ and different concentrations of tannic acid. The volume of this solution for each reaction was 15 μl. The reactions were started by adding 10 μl of PKC substrate solution containing 250 μM acetylated myelin basic protein (*4–14*) (Gibco BRL, MD, USA), 100 μM ATP, 5 mM $CaCl_2$, 10 mM $MgCl_2$, 50 mM Tris-HCl buffer, pH 7.5, and 20–25 μCi/ml [γ-^{32}P] ATP, 10 μl of PKA substrate solution containing 200 μM Kemptide (Gibco BRL, MD, USA), 400 μM ATP, 40 mM $MgCl_2$, 1 mg/ml BSA, 50 mM Tris-HCl buffer, pH 7.5, and 20–25 μCi/ml [γ-^{32}P]ATP or 10 μl of MAPK substrate solution containing 2.5 mg/ml MBP (Sigma Chemicals, MO, USA), 125 μM ATP, and 20–25 μCi/ml [γ-^{32}P]ATP in MAPK kinase buffer. The reaction mixtures were incubated at 30°C for 5 min, then peptide-incorporated ^{32}P was measured by the method described above.

Analysis of Growth Factor–Induced Tyrosine Phosphorylation of Growth Factor Receptors—Confluent Swiss 3T3 mouse fibroblasts (ATCC, Rockville, MD, USA) in a 6-well plate were incubated in serum-free Dulbecco's minimum essential medium (DMEM) at 37°C for 48 h. After preincubating with different concentrations of tannic acid in fresh serum-free medium for 1 h, the cells were incubated with 5 ng/ml EGF, 20 ng/ml bFGF, or 20 ng/ml PDGF (Sigma Chemicals, MO, USA) at 37°C for 5 min. Then 200 μl of boiling Laemmli sample buffer was added to stop the reaction and make whole-cell extracts, and 20 μl of each extract was subjected to 7.5% SDS-PAGE (*17*) and electrophoretically transferred to nitrocellulose membrane. After blocking with 1% BSA, the blot was incubated with recombinant RC20 anti–phosphotyrosine peroxidase conjugate (1:2,000) at room temperature for 1 h. The signals of phosphotyrosine-containing proteins were identified by ECL Western blotting assay system (Amersham Pharmacia Biotech, Backinghanshire, UK) according to the instructions of producer. HepG2 cells (ATCC, Rockville, MD, USA) were used to detect the effect of tannic acid on insulin-induced tyrosine phosphorylation of IR and insulin receptor substrate-1 (IRS-1). Confluent HepG2 cells in a 6-well plate were preincubated with serum-free DMEM containing 20 mM glucose for 48 h. After preincubating with different concentrations of tannic acid in fresh serum-free DMEM at 37°C for 1 h, the cells were incubated with 0.2 IU/ml

insulin at 37°C for 5 min. Tyrosine phosphorylation of IR and IRS-1 was also analyzed using ECL Western blotting as described above.

Analysis of Cell Growth—HepG2 cells in 100 μl of DMEM containing 10% fetal calf serum (FCS) were transferred to a 96-well plate (2×10^3 cells/well) and allowed to attach overnight. The cells in fresh medium were incubated with or without 1 ng/ml EGF and/or 80 μM tannic acid for 24 h. Cell number was counted by the 3 (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) method as described by Hansen *et al.* (*18*). Briefly, 25 μl of 5 mg/ml MTT stock solution was added to each well and the plate was incubated at 37°C for 2 h. Then 100 μl of extraction buffer [20% sodium dodecyl sulfate (SDS), 50% *N,N*-dimethyl formamide (DMF), pH 4.7] was added and incubation at 37°C was continued overnight. Then the optical densities (OD) at 570 nm were measured using a microreader, employing the mixture of 100 μl of culture medium, 25 μl of MTT stock solution, and 100 μl of extraction buffer as the blank.

Protein Modeling and Small Molecular Preparation—The 3D model of EGFr tyrosine kinase domain was constructed by homology modeling as described by Yang *et al.* (*15*). The 3D structure of tannic acid was obtained from Molecular Database of the Combined Chemical Dictionary. Atom charge was calculated using Gaussian 94 (Gaussian, Pittsburgh, PA, USA). No further optimisation was performed on this structure.

Molecular Docking—Tannic acid was docked into the EGFr tyrosine kinase domain using the standard molecular docking procedure (*19*). Briefly, the molecular surface of EGFr was calculated by DMS program in MidasPlus (*20*). Based on the site point information acquired, spheres on the EGFr surface were determined and grouped into clusters using program SPHGEN (*21*). Clusters around the ATP-binding pocket, which is located in the cleft between the C-terminal and N-terminal lobes, were chosen for the docking study. The steric and electrostatic environment of the ATP-binding pocket in EGFr was evaluated by GRID (*22*, *23*). Tannic acid, the EGFr tyrosine kinase inhibitor, was docked into this site using DOCK (*19*). During the matching process, the inhibitor was regarded as flexible and subjected to energy minimization. The conformations with the lowest potential energy were selected and considered to represent the global minimal status of inhibitor in EGFr. The inhibitor with the optimum orientation was merged into EGFr. The complex acquired was subjected to an energy minimization for 3,000 iterations using Powell method in SYBYL 6.5.

IR tyrosine kinase ligated with its peptide substrate and ATP analogue (PDB entry:1ir3, resolution 1.9 Å) (*24*) as template to study the interaction of tannic acid with IR. Tannic acid was docked into the IR ATP-binding pocket using the standard molecular docking procedure (*19*).

RESULTS

Effect of Tannic Acid on Protein Kinase Activities—The inhibitory effects of tannic acid and gallic acid at different concentrations on the activities of tyrosine-specific protein kinases, including EGFr, $p60^{c\text{-}src}$ and IR, were determined. As shown in Fig. 2A, tannic acid strongly inhibited EGFr-catalyzed tyrosine phosphorylation of exogenous substrate, poly (Glu, Tyr) 4:1, IC_{50} being 323 nM. Compared with EGFr tyrosine kinase, the activity of $p60^{c\text{-}src}$ and IR tyrosine kinases was inhibited by tannic acid at relatively high concentration, IC_{50} being 14 μM and 5 μM respectively.

The inhibition of serine/threonine-specific protein kinases, including PKC, PKA and MAPK, by tannic acid at different concentrations was tested. Tannic acid, as shown in Fig. 2B, inhibited PKA activity at relatively high concentration, IC_{50} being 3 μM. The inhibition of PKC and MAPK by tannic acid could be only measured at very high concentration, IC_{50} reaching 325 and 142 μM respectively.

Compared with tannic acid, gallic acid, the monomer of tannic acid, was able to inhibit EGFr and $p60^{c\text{-}src}$ tyrosine kinase activities at relatively high concentration, IC_{50} being 3 and 21 μM respectively, as shown in Fig. 2C. Gallic acid at concentration of up to 100 μM inhibited only about 25% of PKA activity. At high concentration, gallic acid was also able to inhibit PKC activity, IC_{50} reaching 70 μM.

Effect of Tannic Acid on the Kinetics of EGFr Tyrosine Kinase Activity—By the Lineweaver-burk plot analysis, tannic acid showed a non-competitive inhibition toward poly (Glu, Tyr) 4:1 as V_{max} was decreased and K_m was unchanged (Fig. 3A), while, as shown in Figure 3B, tannic acid was a competitive inhibitor toward ATP because V_{max} was unchanged and K_m was increased.

Inhibition of Growth Factor–Induced Tyrosine Phosphorylation of Growth Factor Receptors—After Swiss 3T3 mouse fibroblasts were incubated with various concentrations of tannic acid followed by 5 ng/ml EGF, 20 ng/ml bFGF, or 20 ng/ml PDGF, and the tyrosine phosphorylation of their receptors was detected by ECL Western blotting assay using recombinant anti–phosphotyrosine peroxidase conjugate. HepG2 cells were used to detect the effect of tannic acid on insulin-induced IR and IRS-1 tyrosine phosphorylation. As shown in Fig. 4, the growth factor–induced tyrosine phosphorylation of EGFr, bFGFr, and PDGFr was inhibited by tannic acid. The inhibition was enhanced by increasing the concentrations of tannic acid in culture medium. Insulin-induced tyrosine phosphorylation of both IR and IRS-I was not inhibited in cultured HepG2 cells.

Inhibition of EGF-Stimulated Cell Growth by Tannic Acid—After HepG2 cells were transferred to a 96-well plate (2×10^3 cells/well) and incubated with or without 1 ng/ml EGF and/or 80 μM tannic acid for 24 h, then incubated with MTT, and the number of cells was determined in a microreader. As shown in Fig. 5, the cells incubated with EGF grew faster than those incubated in the absence of EGF ($p < 0.01$), but cells incubated with EGF plus tannic acid grew more slowly than those incubated with EGF in the absence of tannic acid ($p < 0.01$), demonstrating that tannic acid inhibited EGF-stimulated cell growth.

Binding Models of Tannic Acid—The inhibition of EGFr tyrosine kinase by tannic acid was ATP-competitive as shown in Fig. 3. In order to study the possible mechanism of the inhibition of tyrosine kinases by tannic acid, tannic acid was docked into the ATP-binding pockets of EGFr and IR. The possible interaction of tannic acid with EGFr and IR is shown in Fig. 6. The images were produced using Conic module in MidasPlus (*25*).

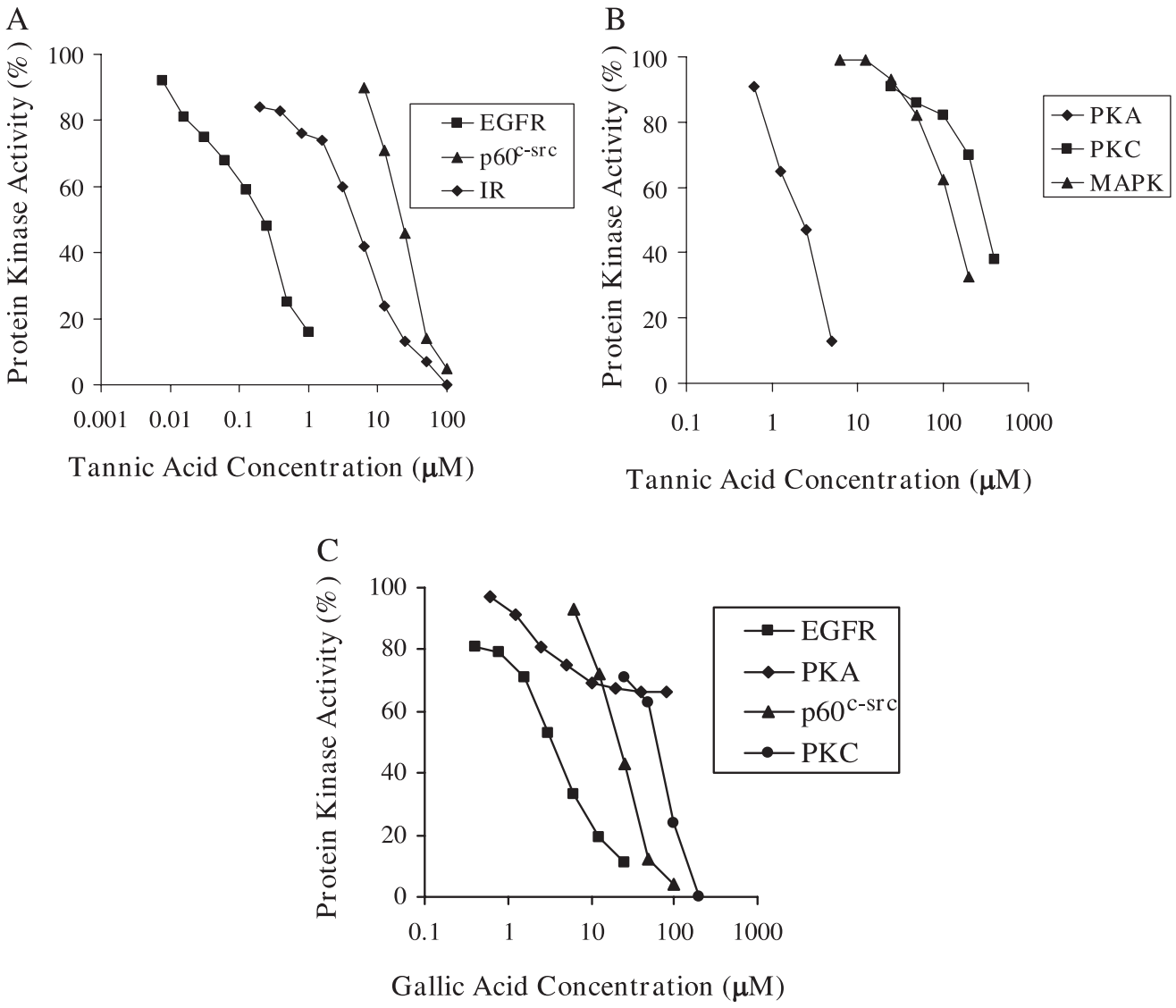

Fig. 2. **Inhibition of protein kinases by tannic acid and gallic acid.** The effect of tannic acid concentration on the activities of tyrosine-specific protein kinases (A), including EGFr (squares), $p60^{c\text{-}src}$ (triangles) and IR (diamonds) tyrosine kinases, and serine/threonine-specific protein kinases (B), including PKA (diamonds), PKC (squares) and MAPK (triangles), and the effect of gallic acid on the activities of protein kinases (C), including EGFr tyrosine kinase (squares), $p60^{c\text{-}src}$ tyrosine kinase (triangles), PKA (diamonds), and PKC (circles), was measured as described in "MATERIALS AND METHODS."

The binding orientations of tannic acid on EGFr (Fig. 6A) are: (i) C-ring C in tannic acid is located at the N-terminal lobe of EGFr, (ii) C-rings B and D are both located at the C-terminal lobe of EGFr, (iii) C-ring A is located at the entrance of the binding pocket, and (iv) C-rings A, B and D each form a hydrogen bond with EGFr, and C-ring C forms two such hydrogen bonds. Hydrogen bonding and hydrophobic interaction may both play important roles in stabilizing the inhibitor-protein binding. The hydrogen bonds between EGFr and tannic acid are formed between: (i) the side-chain amino hydrogen of Lys720 and the hydroxyl oxygen of tannic acid at C5 in C-ring A (2.712 Å), (ii) the backbone amide nitrogen of Gly832 and the hydroxyl oxygen of tannic acid at C4 in C-ring B (2.786 Å), (iii) the backbone carbonyl oxygen of Met768 and the hydroxyl oxygen of tannic acid at C3 in C-ring D (2.799 Å), (iv) the backbone amide nitrogen of Thr765 and the hydroxyl oxygen of tannic acid at C4 in C-ring C (2.676 Å), and (v) the backbone amide nitrogen of Tyr702 and the hydroxyl oxygen of tannic acid at C5 in C-ring C (3.237 Å). Two amino acids (Val701 and Cys772) contribute to the hydrophobic interaction between tannic acid and EGFr.

The binding orientations of tannic acid on IR (Fig. 6B) are: (i) C-ring C in tannic acid is located at the N-terminal lobe of IR, (ii) C-rings B and D are both located at the C-terminal lobe of IR, and (iii) C-ring A is located at the center of the binding pocket. Hydrogen bonding and hydrophobic interaction may both play important roles in the stabilization of the inhibitor-protein binding. There are five hydrogen bonds involved in the binding of IR with tannic acid. They are formed between: (i) the backbone

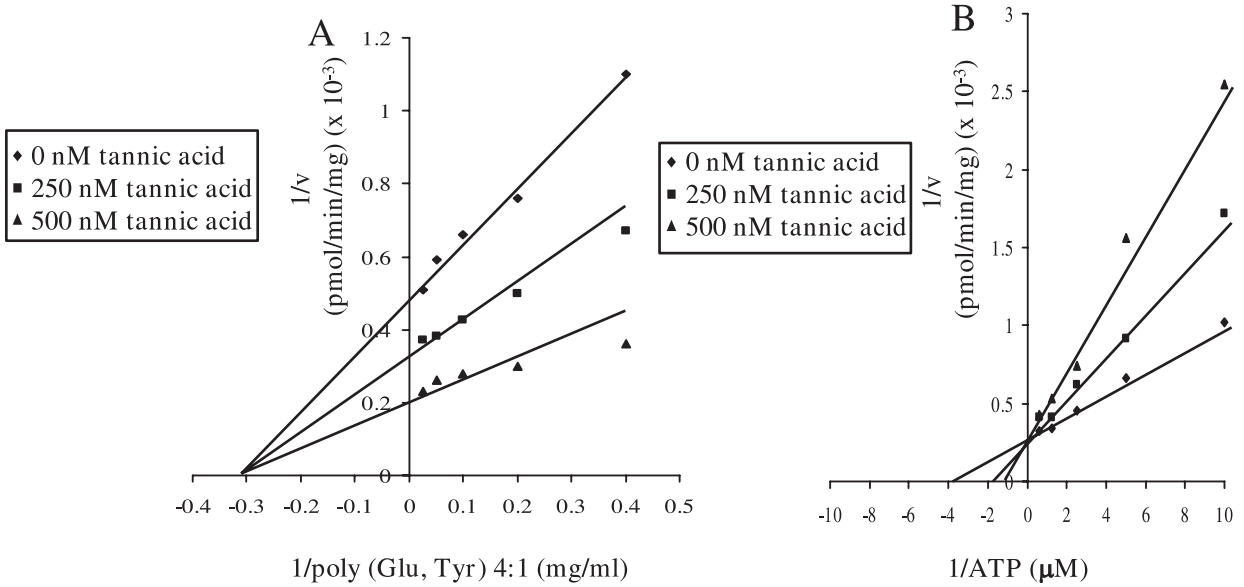

Fig. 3. **Inhibitory kinetics of EGFr tyrosine kinase by tannic acid.** Experiments were carried out with various concentrations of poly (Glu, Tyr) 4:1 or ATP as described in "MATERIALS AND METHODS." Tannic acid showed non-competitive inhibition with respect to poly (Glu, Tyr) 4:1 (V_{max} decreased and K_m unchanged) (Fig. 3A) and competitive inhibition with respect to ATP (V_{max} unchanged and K_m increased) (Fig. 3B).

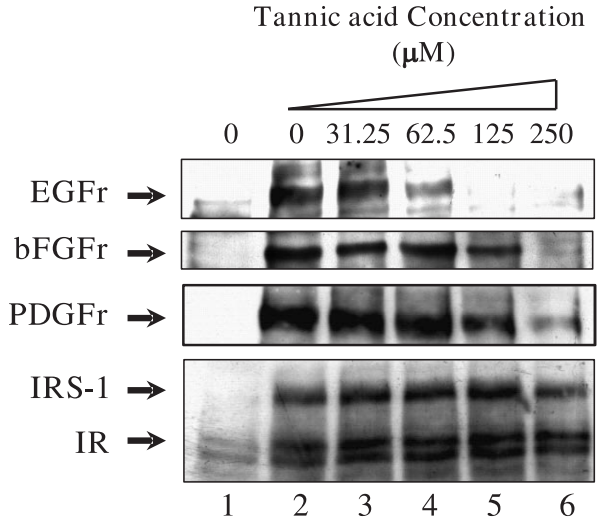

Fig. 4. **Inhibitory effect of tannic acid on growth factor–induced tyrosine phosphorylation of growth factor receptors.** Swiss 3T3 mouse fibroblasts in a 6-well plate were preincubated with various concentrations of tannic acid at 37°C for 1 h then incubated with or without 5 ng/ml EGF, 20 ng/ml bFGF, and 20 ng/ml PDGF at 37°C for 5 min. Then tyrosine phosphorylation of growth factor receptors was analyzed by ECL Western blotting assay using recombinant RC20 anti–phosphotyrosine peroxidase conjugate. HepG2 cells were used to detect the effect of tannic acid on insulin-induced tyrosine phosphorylation of IR and IRS-1. Confluent cells in a 6-well plate were cultured with serum-free DMEM containing 20 mM glucose for 48 h, preincubated with different concentrations of tannic acid at 37°C for 1 h and stimulated with 0.2 IU/ml insulin at 37°C for 5 min before Western blotting analysis. A positive control experiment in which cells were treated with growth factors but without tannic acid indicated phosphorylation of growth factor receptors in the absence of tannic acid (lane 2). Lane 1 shows negative controls without treatment of tannic acid and growth factors. Lanes 3 to 6 show cells incubated with increasing concentration of tannic acid.

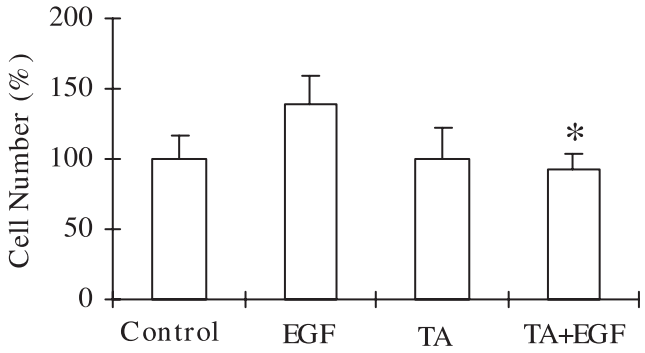

Fig. 5. **Inhibition of EGF-induced cell growth by tannic acid.** HepG2 cells in a 96-well plate (2×10^3 cells/well) were incubated with or without 1 ng/ml EGF and/or 80 μM tannic acid for 24 h then cell number was counted described in "MATERIALS AND METHODS." When the cells were incubated with EGF, they grew faster than control cells incubated in the absebce of EGF and tannic acid ($*p < 0.01$). When the cells were cultured in the presence of tannic acid, EGF-stimulated cell growth was inhibited ($**p < 0.01$).

amide nitrogen of Asp1150 and the oxygen atom linking C-rings A and B in tannic acid (2.7269 Å), (ii). the backbone amide nitrogen of Gly1082 and the hydroxyl oxygen of tannic acid at C3 in C-ring D (2.7390 Å); (iii) the backbone carbonyl oxygen of Met1079 and the hydroxyl oxygen of tannic acid at C3 in C-ring D (2.7495 Å), (iv) the backbone amide nitrogen of Val1074 and the hydroxyl oxygen of tannic acid at C4 in C-ring C (3.0175 Å), and (v) the backbone carbonyl oxygen of Lys1030 and the hydroxyl oxygen of tannic acid at C3 in C-ring C (2.6319 Å). Three amino acids (Met1076, Leu1002 and Gly1152) contribute to the hydrophobic interaction between tannic acid and IR.

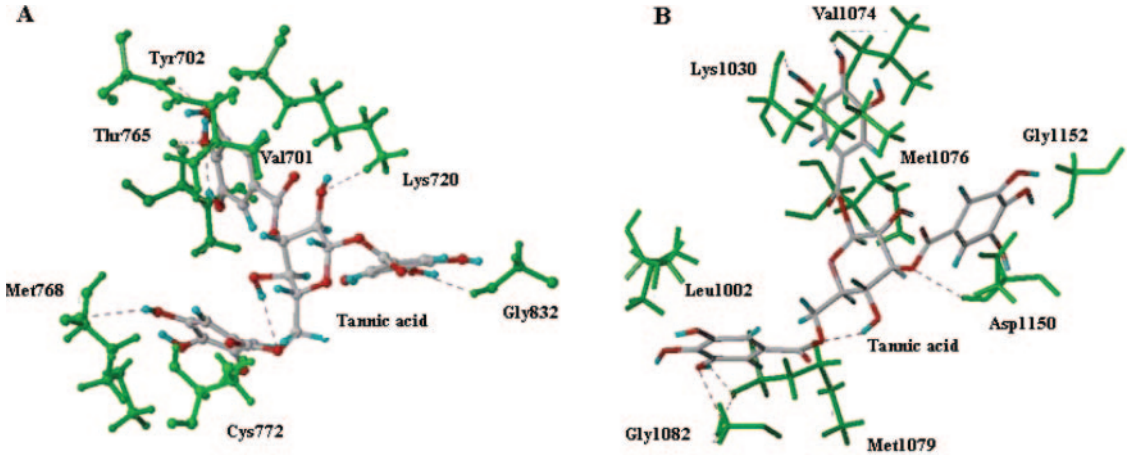

Fig. 6. **Binding of tannic acid with the ATP binding pockets of EGFr and IR.** The hydrogen bonds between tyrosine kinase inhibitor and amino acids in the ATP binding pockets of EGFr (Fig. 6A) and IR (Fig. 6B) are shown by dotted lines.

DISCUSSION

Tyrosine kinases are known to play an important role in the control of cell proliferation and differentiation and to be overexpressed in certain cancer tissues. Finding of specific and potent tyrosine kinase inhibitors appears to be very necessary for the study of signal transduction and the discovery of new anticancer drugs. Thousands of natural products and synthetic chemicals have been screened to discover potential tyrosine kinase inhibitors. Among them, plant polyphenols such as genistein, quercetin, butein, marein, and phloretin have been found to inhibit tyrosine kinase activity (*12–15*). Of coumarin and its derivatives, only daphnetin was shown to inhibit the activities of protein kinases, including tyrosine-specific and serine/threonine-specific protein kinases, *in vitro* (*26*). In addition, genistein was used to investigate the relationship between EGFr tyrosine kinase activity and EGF-induced internalization and degradation of EGFr. It was found that EGF-induced internalization and degradation of EGFr depended on EGFr tyrosine kinase activity (*27*).

Tannic acid is not a unique plant polyphenol that has been used in analyzing potential inhibition of tyrosine kinase activity, but we found that it was the most potent EGFr tyrosine kinase inhibitor among plant polyphenols. In the present work, tannic acid was found to strongly inhibit EGFr tyrosine kinase activity with 323 nM IC_{50} *in vitro*. The inhibitory effect of tannic acid on the activity of other protein kinases, including $p60^{c\text{-}src}$ tyrosine kinase, IR tyrosine kinases, PKA, PKC and MAPK, was much less than that on EGFr tyrosine kinase, IC_{50} reaching 14, 5, 3, 325 and 142 μM respectively. Compared with tannic acid, gallic acid, the monomer of tannic acid, was found to inhibit EGFr at relatively high concentration, IC_{50} being 3 μM.

EGFr catalyzes not only tyrosine phosphorylation of its substrates *in vivo* and *in vitro* but also autophosphorylation of tyrosine residues at its C-terminal tail (*1*). In this study, tannic acid was found to inhibit EGF, bFGF and PDGF-induced autophosphorylation of their receptors in Swiss 3T3 mouse fibroblasts. The inhibition was enhanced by increasing tannic acid concentrations in culture medium. The inhibition of EGF-induced EGFr autophosphorylation was also found by the preincubation of HepG2 cells with butein or A431 cells with genistein (*14*, *12*). The inhibition of insulin-induced IR autophosphorylation by tannic acid was not detected. This inhibition was also not observed when using quercetin (*13*). These results imply that the mechanism involved in the inhibition of IR tyrosine kinase by plant polyphenols may be different from other receptor tyrosine kinases. The molecular modeling study in this work shows that the binding of tannic acid with EGFr is different from that with IR. Although the interaction of tannic acid with EGFr and IR tyrosine kinase domains is caused by hydrogen bonding and hydrophobic interaction, amino acids of EGFr and IR and groups of tannic acid involved in the interaction are different.

In the current study, the concentration of tannic acid for the inhibition of EGF-induced EGFr autophosphorylation in cultured Swiss 3T3 mouse fibroblasts was much higher than that for the inhibition of EGFr tyrosine kinase activity in cell-free experiments. This phenomenon was also observed in the previous studies in which butein and PD153035 were used to inhibit EGFr tyrosine kinase activity (*14*, *7*).

Although tannic acid is known to exert cancer-preventative activity (*28–30*), the molecular mechanisms responsible for this activity remain unclear. Studies have suggested that tannic acid–induced apoptosis (*31–33*) may play a role. Reactive oxygen species (ROS) react with biological molecules and destroy the structure of cells, and eventually cause free radical-induced disease, such as cancer. Previous studies showed that tannic acid had potent antioxidant and free radical scavenging activity (*34*, *35*), suggesting that the anticarcinogenic activity of tannic acid may be associated with its antioxidant activity. In the present work, it was found that tannic acid was a tyrosine kinase inhibitor, suggesting that one of the mechanisms of the anticarcinogenic activity of tannic acid may involve the inhibition of protein kinase activity in cancer cells.

Molecular docking by the computational method is a useful tool for studying the interaction of specific ligands with their receptors. It explores the possible orientation of a ligand bound to its target protein. The binding site is characterized by the sphere created by the program

SPHGEN and mapped by GRID to evaluate its steric and electrostatic environment. The ligand is then put into the binding site by DOCK to match the sphere. The affinity between the ligand function groups and the spatial point in the binding site is examined. By searching for the favourable interactions, it is possible to speculate how the ligand may co-ordinate with the binding site.

Based on the evidence that the inhibition of EGFr tyrosine kinase activity by butein was competitive with respect to ATP, we previously used homology modeling to build the 3D structure of the EGFr tyrosine kinase domain and docked this inhibitor into the ATP-binding site of EGFr tyrosine kinase domain (*15*). In the present work, the inhibition of EGFr tyrosine kinase by tannic acid was also found to be competitive with respect to ATP, so a similar system was used to explore the possible interaction of tannic acid with the EGFr ATP-binding site. At the same time, tannic acid was also docked into the IR ATP-binding domain. Five hydrogen bonds were found to contribute to the interaction of EGFr or IR with tannic acid. Two amino acids (Val701 and Cys772) in EGFr and three amino acids (Met1076, Leu1002 and Gly1152) in IR which contribute to the hydrophobic interaction of tannic acid with EGFr and IR ATP binding domains appear to be very important in the inhibition of EGFr and IR tyrosine kinases by tannic acid. A more exact binding model may be established when the X-ray crystal structure of EGFr is elucidated.

This work was supported by grants from the National Medical Research Council of Singapore (NMRC/0137/1995 and NMRC/0498/2000). The authors thank Biomedical Research Council, Agency for Science, Technology and Research, Singapore for funding support (BMRC 03/1/22/18/229) to WN Chen.

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