REGULAR ARTICLE

Gingerols and Related Analogues Inhibit Arachidonic Acid-Induced Human Platelet Serotonin Release and Aggregation

Karen L.K. Koo, Alaina J. Ammit, Van H. Tran, Colin C. Duke and Basil D. Roufogalis
Faculty of Pharmacy, University of Sydney, Sydney, NSW 2006, Australia
(Received 19 July 2000 by Editor J. S. Bennett; revised/accepted 18 June 2001)

Abstract

Gingerols, the active components of ginger (the rhizome of Zingiber officinale, Roscoe), represent a potential new class of platelet activation inhibitors. In this study, we examined the ability of a series of synthetic gingerols and related phenyl-alkanol analogues (G1–G7) to inhibit human platelet activation, compared to aspirin, by measuring their effects on arachidonic acid (AA)-induced platelet serotonin release and aggregation in vitro. The IC50 for inhibition of AA-induced (at EC50 = 0.75 mM) serotonin release by aspirin was 23.4 ± 3.6 μM. Gingerols and related analogues (G1–G7) inhibited the AA-induced platelet release reaction in a similar dose range as aspirin, with IC50 values between 45.3 and 82.6 μM. G1–G7 were also effective inhibitors of AA-induced human platelet aggregation. Maximum inhibitory (ICmax) values of 10.5 ± 3.9 and 10.4 ± 3.2 μM for G3 and G4, respectively, were ~2-fold greater than aspirin (ICmax = 6.0 ± 1.0 μM). The remaining gingerols and related analogues maximally inhibited AA-induced platelet aggregation at ~20–25 μM. The mechanism underlying inhibition of the AA-induced platelet release reaction and aggregation by G1–G7 may be via an effect on cyclooxygenase (COX) activity in platelets because representative gingerols and related analogues (G3–G6) potently inhibited COX activity in rat basophilic leukemia (RBL-2H3) cells. These results provide a basis for the design of more potent synthetic gingerol analogues, with similar potencies to aspirin, as platelet activation inhibitors with potential value in cardiovascular disease. © 2001 Elsevier Science Ltd. All rights reserved.

Key Words: Platelets; Aspirin; Gingerols; Serotonin release; Platelet aggregation; COX

Antiplatelet therapy has been widely used in preventing ischaemic heart diseases. Aspirin has been the mainstay of this antiplatelet therapy for several decades. In an overview of randomised trials of antiplatelet therapy reported by the Anti-Platelet Trialists’ Collaboration [1], it is stated that antiplatelet therapy (mainly aspirin) reduces the risk of nonfatal stroke, nonfatal myocardial infarction and vascular death by 25–32% in patients with high-risk factors. However, there are reports of adverse effects of aspirin, mainly associated with gastric ulcers and gastrointestinal bleeding [2]. Thus, the use of aspirin may be confined to elderly patients whose benefits are likely to outweigh any increased risk of gastrointestinal ulcers or
bleeding [3]. Even though low-dose aspirin (75–150 mg) is used to minimise the incidence of adverse effects, longitudinal studies show that 75 mg daily dose can still cause a small but significant increase in gastrointestinal bleeding [4]. Thus, research and development of new antiplatelet drugs with a more favourable adverse effect profile are required.

Ginger (the rhizome of Zingiber officinale, Roscoe) is one of the most widely used species of the ginger family (Zingiberaceae) and is a common condiment for various foods and beverages. It has been used extensively in traditional oriental medicine for alleviating symptoms of the common cold, rheumatism and digestive disorders [5] and has been reported to reduce inflammation and inhibit platelet activation [6]. Unlike nonsteroidal anti-inflammatory drugs, oral administration of ginger extracts in albino rats was shown to be cytoprotective and can inhibit gastric mucosal damage induced by nonsteroidal anti-inflammatory drugs or alcohol [7]. Moreover, in a study on ethanol-induced gastric lesions in rats [6]-gingerol inhibited gastric lesions by 54.5% [8]. Thus, gingerols and related analogues may offer an alternative antiplatelet therapy for the elderly and patients with gastric ulcers.

In in vitro studies, aqueous ginger extracts have been shown to inhibit platelet aggregation induced by arachidonic acid (AA), adenosine diphosphate and collagen in a dose-dependent manner in human blood [9]. The main principles in ginger are the series of pungent oleoresin constituents known as gingerols, with [6]-gingerol being the major gingerol component. Ginger also contains shogaol homologues that are formed by dehydration of corresponding gingerols during storage or thermal processing. Other constituents include giderdiols, paradols and zingerone [6,10,11]. The gingerols in ginger extract may be the major active principles that inhibit platelet activation as it has been reported that [6]-gingerol, isolated from ginger, inhibits secondary platelet activation and adenosine triphosphate release from platelets in human platelet-rich plasma (PRP). This inhibition is reversible and is mainly due to inhibition of AA metabolism and cyclooxygenase (COX) activity [12,13].

We have synthetically prepared a series of gingerols and related phenylalkanol analogues (G1–G7) that vary in terms of hydrophobicity,
alkyl chain length and side chain substitution (Fig. 1). In this series, the previously examined [6]-gingerol [12,13] was designated G3. We examined the ability of these compounds to inhibit human platelet activation, compared to aspirin, by measuring their effects on AA-induced platelet serotonin release and aggregation in vitro. To examine whether the underlying mechanism may be via an effect on COX activity, we used rat basophilic leukemia 2H3 cells (RBL-2H3), a well-characterised cell type for investigating AA metabolism by COX [14–16]. While nucleated cells, such as RBL-2H3, may contain two types of COX enzymes, that is, constitutive COX-1 and inducible COX-2, only COX-1 is present in anucleate platelets [23,24]. However, studies performed in RBL-2H3 cells allowed us to compare the inhibitory effect of representative gingerol and related analogues series (G3–G6) on COX enzyme activity, although the individual contribution of each COX isoform in RBL-2H3 cells was not examined in this study.

1. Materials and Methods

1.1. Materials

Gingerols and related analogues were prepared synthetically in our laboratory. The structures of these compounds are shown in Fig. 1. All synthetic gingerols and related analogues were racemic. G6 was racemic and a mixture of diastereoisomers. 5-Hydroxy[G-3H]tryptamine creatinine sulfate ([3H]serotonin) (specific activity 17.8 Ci/mmol; concentration 1 mCi/ml) was purchased from Amersham Life Science (Arlington Heights, IL, USA). RBL-2H3 cells were provided by Dr. Russell I. Ludowyke (Centre for Immunology, St. Vincent’s Hospital, Sydney, Australia). RPMI 1640 and penicillin–streptomycin antibiotic solution were purchased from Gibco Life Technologies (Victoria, Australia). Foetal calf serum was purchased from Commonwealth Serum Laboratories (Victoria, Australia). PGD2-MOX enzyme immunoassay (EIA) kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Unless otherwise specified, all chemicals used in this study were purchased from the Sigma Chemical (St. Louis, MO, USA).

1.2. Preparation of Human PRP

Human venous blood was collected from 15 healthy volunteers (between 21 and 55 years old) using 3.8% trisodium citrate dihydrate (9:1 v/v) as the anticoagulant. Volunteers had taken no medication for at least 2 weeks before blood collection. Approval for experiments with human blood was provided by the Human Ethics Committee of the University of Sydney. PRP was prepared by centrifuging the citrated venous blood at 290 g for 12 min. Platelet-poor plasma (PPP) was obtained by further centrifugation at 1200 g for 10 min.

1.3. Bioassay of Human Platelet Serotonin Release Induced by AA

Platelet activation was assessed by measuring the release of [3H]serotonin from platelet granules in human PRP, using a method modified from previous publications [17–19]. Briefly, platelets were labelled by incubating [3H]serotonin with PRP (28 pmol/ml PRP) at 37 °C for 20 min (with mixing). To induce the platelet release reaction, a range of concentrations of AA (0.25–1.5 mM, dissolved in 100 mM sodium carbonate and diluted in 150 mM NaCl) was added to [3H]serotonin-labelled PRP and the mixture was incubated at 37 °C for 10 min (with mixing). To ensure that the increase in [3H]serotonin release was directly attributed to the effects of AA, the baseline release throughout the incubation period was also measured. To stop the release reaction in each incubation mixture, ice-cold Na2–EDTA–formaldehyde solution (132 mM NaCl, 0.5% EDTA and 0.74% formaldehyde) was added. To measure the total amount of [3H]serotonin in the labelled PRP, 20% Triton X-100 and Na2–EDTA–formaldehyde solution (132 mM NaCl, 0.5% EDTA and 0.74% formaldehyde) was added. To measure the total amount of [3H]serotonin in the labelled PRP, 20% Triton X-100 and Na2–EDTA–formaldehyde were added to lyse the platelets. All the incubation mixtures and Triton X-100 treated PRP were then centrifuged at 2450 g for 3 min. Radioactivity of each supernatant (50 μl) was measured using Tri-Carb 1900CA Liquid Scintillation Analyser (Canberra Packard, Mt. Waverley, Victoria, Australia) and the percentage release of [3H]serotonin was...
calculated as follows:


\[ \frac{[^3]H_{AA} -[^3]H_{control}}{[^3]H_{total} -[^3]H_{control}} \times 100\% \]


1.4. Inhibition of AA-Induced Serotonin Release by Gingerols and Related Analogues, Compared to Aspirin

Dose–response curves for aspirin (diluted in 150 mM NaCl) and the gingerol series (dissolved in 0.3% DMSO and diluted in 150 mM NaCl) were established by adding a range of concentrations of aspirin (10–100 μM) or G1–G7 (20–200 μM) to[^3]H]serotonin-labelled PRP at 37 °C for 3 min. The release reaction was then initiated with AA at a concentration that induced 50%[^3]H]serotonin release (EC_{50}). Percentage inhibition of[^3]H]serotonin release was calculated and dose–response curves of aspirin, gingerols and related analogues were established. To examine whether gingerols and related analogues induced nonspecific effects on the platelet release reaction, initial control experiments were performed where[^3]H]serotonin-labelled PRP were incubated for 10 min with high concentrations (200 μM) of gingerols and related analogues (G1–G7). To calculate IC_{50} values, the dose–response curves for aspirin and gingerol analogues were fitted using the MicroMath Scientist program (Version 2.02; Salt Lake City, UT) by utilizing a sigmoid E_{max} model as follows:

\[ E = \frac{(E_{max} \cdot C^\gamma)}{(IC_{50} + C^\gamma)} \]

where E: percentage inhibition of platelet activation; E_{max}: percentage maximum inhibition of platelet activation; IC_{50}: concentration required to inhibit platelet activation by 50%; C: concentration of gingerols and their related analogues or aspirin; and \( \gamma \): gamma value for modelling using the sigmoid E_{max} model.

1.5. Measurement of Human Platelet Aggregation by Turbidometric Aggregometry

To confirm the effectiveness of gingerols and related analogues as inhibitors of platelet activation, turbidometric aggregometry was examined using the Chrono-Log Lumi-Aggregometer (Model 460, Dual sample; Havertown, PA, USA) according to the manufacturer's instructions. Briefly, an aliquot of PRP was incubated at 37 °C for 2 min (with mixing) to allow for temperature equilibration. Baselines were adjusted using PPP. To induce aggregation, a range of concentrations of AA (0.5–1.5 mM) was added and the mixture was incubated at 37 °C for up to 5 min (with mixing). Platelet aggregation was detected as an increase in light transmission. To ensure that the increase in platelet aggregation was directly attributed to the effects of AA, the baseline release throughout the incubation period was also measured.

1.6. Inhibition of AA-Induced Platelet Aggregation by Gingerols and Related Analogues, Compared to Aspirin

Dose–response curves for aspirin and the gingerol series were established by adding a range of concentrations of aspirin (2.5–100 μM) or G1–G7 (5–100 μM) to temperature-equilibrated PRP at 37 °C for 3 min. Platelet aggregation was then initiated with AA at a concentration that induced a 50% change in light transmission (EC_{50}). The IC_{max} concentration was defined as the minimal concentration that totally inhibited platelet aggregation induced by the AA EC_{50}. To examine whether gingerols and related analogues had nonspecific effects on platelet aggregation, initial control experiments were performed where PRP was incubated with high concentrations (100 μM) of gingerols and related analogues (G1–G7).

1.7. Estimation of Hydrophobicity of Gingerols and Related Analogues

Hydrophobicity of gingerols and related analogues was estimated using three-dimensional molecular models constructed using the WindowsChem Molecular Modelling Pro (Version 2.11; Fairfield, CA, USA) and were expressed as log P values.
1.8. RBL-2H3 Cell Culture and COX Activity Assay

RBL-2H3 cells were cultured in RPMI 1640 containing 10% foetal calf serum and 0.5% penicillin-streptomycin antibiotic as described previously [20]. Confluent cells were harvested and subsequently seeded on 24-well plates at $1 \times 10^6$ cells/ml at 37 °C for 3 h. Cells were washed twice with incubation buffer (5 mM HEPES, 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl$_2$, 1 mM CaCl$_2$ and 55 mM glucose), then incubated with either G3–G6 (0.1–100 μM) or vehicle control for 5 min at 37 °C (with mixing). In addition, the effect of indomethacin (0.1–10 μM), a nonsteroidal anti-inflammatory agent known to inhibit COX enzyme activity, was used as a positive control [21]. AA (12.5 μM) was subsequently added, and the plate incubated for a further 10 min. The effects of indomethacin, and representative gingerols and related analogues (G3–G6) on COX enzyme activity, were assessed by measuring PGD$_2$, a product of AA metabolism by COX enzymes [14–16]. Because PGD$_2$ is relatively unstable, cell supernatants underwent methoxime derivatization of PGD$_2$ prior to quantification by EIA, according to the manufacturer's instructions (Cayman Chemicals). Data were fitted to the sigmoid $E_{max}$ model using the MicroMath Scientist program (see above) to determine the IC$_{50}$ values of the test compounds.

2. Results

2.1. Inhibition of AA-Induced Human Platelet Serotonin Release by Gingerols and Related Analogues

Using a quantitative bioassay for measuring human platelet activation in vitro, based on the release of [3H]serotonin from platelet granules in PRP, we found that AA rapidly induced the platelet granule release reaction within a 10-min incubation period (results not shown) in a dose-dependent manner (Fig. 2). The increase in [3H]serotonin release was directly attributed to the effects of AA because baseline release was stable throughout the incubation period (<5% [3H]serotonin release). The concentration of AA that induced 50% [3H]serotonin release was 0.75 mM (Fig. 2). At this concentration, the bioassay was reproducible since the interbioassay coefficient of variation was 9.7% ($n = 15$). In addition, the intrabioassay coefficient of variation was examined to monitor changes in sensitivity of PRP to AA within 3 h after blood collection. This was conducted by examining the dose–response curve of AA-induced platelet activation at various time points after blood collection using the same batch of [3H]serotonin-labelled PRP. The AA dose–response curves established using PRP collected at various times within 3 h of blood collection were similar, resulting in an acceptable intrabioassay coefficient of variation of 4.5% at 0.75 mM AA (data not shown).

Due to the hydrophobic nature of gingerols and their related analogues, dissolution in 0.3% DMSO (in 150 mM NaCl) was necessary. At this concentration, DMSO does not nonspecifically inhibit AA-induced serotonin release (data not shown). To exclude the possibility that any of the gingerols and related analogues induced nonspecific effects on the platelet release reaction, initial control experiments were performed whereby [3H]serotonin-labelled platelets in PRP were incubated for 10 min with high concentrations (200 μM) of gingerols and related analogues (G1–G7) and the baseline release of [3H]serotonin detected. None of the compounds G1–G7 caused any appreciable effect on platelet release reaction (data not shown). Fig. 3 shows the dose–response curves for the

Fig. 2. Dose–response curve of AA-induced serotonin release from human platelets. Human PRP was labelled with [3H]serotonin at 37 °C for 20 min followed by addition of AA at specified concentrations and incubation for a further 10 min. The assay was stopped by addition of ice-cold Na$_2$–EDTA–formaldehyde solution. Supernatants were sampled for the measurement of the amount of [3H]serotonin release by liquid scintillation counting. Platelets were subsequently lysed with 20% Triton X-100 and Na$_2$–EDTA–formaldehyde solution to obtain the total [3H]serotonin count. Percentage release of [3H]serotonin was calculated according to the equation described in Materials and Methods. The data are presented as the mean ± S.E.M. of 15 dose–response curves.
inhibition of the platelet release reaction (induced by 0.75 mM AA) by gingerols and their related analogues compared to aspirin. [3H]Serotonin-labelled PRP were incubated with either (●) aspirin, (■) G1, (▲) G2, (◆) G3, (○) G4, (□) G5, (△) G6 or (○) G7 at specified concentrations at 37 °C for 3 min prior to addition of AA (0.75 mM). The amount of [3H]serotonin release was determined as described in the legend of Fig. 2. The data are presented as the mean ± S.E.M. of three independent determinations performed in duplicate.

To confirm the effectiveness of gingerols and related analogues as inhibitors of platelet activation, turbidometric aggregometry was also examined. To induce aggregation, a range of concentrations of AA (0.5–1.5 mM) was added to the aggregometer, and platelet aggregation detected as an increase in light transmission (%). The concentration of AA that induced a 50% change in light transmission was 0.75 mM (data not shown). When aspirin (2.5–100 μM) was added to the aggregometer cuvette prior to the addition of AA at 0.75 mM, 6.0 ± 1.0 μM aspirin totally inhibited AA-induced platelet aggregation. Fig. 4A shows a representative aggregometry trace, where 5 μM aspirin total inhibited platelet aggregation induced by 0.75 mM AA. However, at 2.5 μM, total inhibition was lost (Fig. 4A). G1–G7 were also effective inhibitors of AA-induced human platelet aggregation (Table 1). With IC_max values of 10.5 ± 3.9 and 10.4 ± 3.2 μM, G3 and G4 were ~1.8-fold less potent than aspirin (IC_max = 6.0 ± 1.0 μM). The remaining gingerols and related analogues inhibited AA-induced platelet aggregation at ~20–25 μM (Table 1). Fig. 4B

Table 1. Log P, IC_{50} and IC_{max} values for inhibition of AA-induced platelet release reaction and aggregation by aspirin, gingerols and related analogues (G1–G7)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log P</th>
<th>IC_{50} (μM) against AA-induced platelet release reaction</th>
<th>IC_{max} (μM) against AA-induced platelet aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>1.19</td>
<td>23.4 ± 3.6</td>
<td>6.0 ± 1.0</td>
</tr>
<tr>
<td>G1</td>
<td>5.33</td>
<td>45.1 ± 1.1</td>
<td>20 ± 3.2</td>
</tr>
<tr>
<td>G2</td>
<td>6.39</td>
<td>71.4 ± 8.2</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>G3</td>
<td>1.85</td>
<td>73.7 ± 1.2</td>
<td>10 ± 3.9</td>
</tr>
<tr>
<td>G4</td>
<td>2.91</td>
<td>62.7 ± 8.3</td>
<td>10 ± 3.2</td>
</tr>
<tr>
<td>G5</td>
<td>5.46</td>
<td>56.9 ± 4.8</td>
<td>25 ± 0.0</td>
</tr>
<tr>
<td>G6</td>
<td>3.25</td>
<td>80.9 ± 5.1</td>
<td>25 ± 0.0</td>
</tr>
<tr>
<td>G7</td>
<td>3.90</td>
<td>67.9 ± 3.1</td>
<td>25 ± 0.0</td>
</tr>
</tbody>
</table>

Log P values of aspirin, gingerols and related analogues were calculated using the WindowChem Molecular Modelling Pro, as described in Materials and Methods. Results for inhibition of AA-induced platelet release reaction represent the mean ± S.E.M. of three independent determinations performed in duplicate. IC_{50} values were calculated using a sigmoidal E_{max} model as described in Materials and Methods. Results for inhibition of AA-induced platelet aggregation represent the mean ± S.E.M. of up to six independent determinations. The IC_{max} concentration was defined as the minimal concentration that totally inhibited platelet aggregation induced by the AA EC_{50}.
shows the aggregometry traces for G3 as a representative of the gingerol series. At concentrations as high as 100 μM, gingerols and related analogues did not nonspecifically affect platelet aggregation (data not shown).

2.3. Relationship Between the Hydrophobicity of Gingerols and Related Analogues and Inhibition of AA-Induced Platelet Activation

Table 1 compares the hydrophobicity of aspirin, gingerols and their related analogues (expressed as log \( P \) values) with their IC\(_{50}\) and IC\(_{\text{max}}\) values against the AA-induced platelet release reaction and aggregation, respectively. The potency of gingerols and their related analogues against AA-induced platelet serotonin release appeared to follow a bimodal relationship with log \( P \) values. G1 and G5 had the highest potencies in the gingerol series, with IC\(_{50}\) values of 45.1 ± 1.1 and 56.9 ± 4.8 μM, respectively. These data would suggest that the log \( P \) optima for inhibition of the platelet release reaction would be in the range of 5.3–5.5, as shown graphically in Fig. 5A. In contrast, the least hydrophobic compounds studied, G3 (log \( P = 1.85 \)) and G4 (log \( P = 2.91 \)), were the most potent inhib-
itors of AA-induced platelet aggregation (IC\textsubscript{max} values of 10.5 ± 3.9 and 10.4 ± 3.2 μM, respectively) (Fig. 5A). These results suggest that the hydrophobicity optima for inhibiting platelet aggregation differ from those required for the inhibition of platelet release reaction.

2.4. Inhibition of COX Enzyme Activity in RBL-2H3 Cells by Representative Gingerols and their Related Analogues

To assess the effects of representative gingerols and their related analogues (G3–G6) on COX enzyme activity, we measured PGD\textsubscript{2}, a product of AA metabolism by COX enzymes, secreted from intact RBL-2H3 cells. Cells were incubated with increasing concentrations of G3–G6 (0.1–100 μM) and their effect on PGD\textsubscript{2} secretion (induced by 12.5 μM AA) measured by EIA. Indomethacin (0.1–10 μM) was used as positive control. As shown in Fig. 6, G5 inhibited AA-induced PGD\textsubscript{2} secretion in a dose-dependent manner. Inhibition curves were also obtained for G3, G4, and G6 (data not shown) and were used to fit to the sigmoid \( E_{\text{max}} \) model in order to obtain comparative IC\textsubscript{50} values as shown in Table 2. G4–G6 potently inhibited COX in intact RBL-2H3 cells. As shown in Table 2, the IC\textsubscript{50}

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC\textsubscript{50} (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3</td>
<td>50</td>
</tr>
<tr>
<td>G4</td>
<td>1.54</td>
</tr>
<tr>
<td>G5</td>
<td>1.22</td>
</tr>
<tr>
<td>G6</td>
<td>3.3</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Inhibition curves for indomethacin and G3–G6 against AA-induced COX enzyme activity, in intact RBL-2H3 cells, were obtained as described in Materials and Methods. The data were fitted to the sigmoid \( E_{\text{max}} \) model to determine IC\textsubscript{50} values as described in Materials and Methods. The data presented are the mean of two independent determinations performed in duplicate.
values for G4–G6 ranged from 1.22 to 3.33 μM and were two- to four-fold less potent than indomethacin (IC_{50} = 0.76 μM). In contrast, G3 was less effective against AA-induced COX activity, with an IC_{50} value of 50 μM.

3. Discussion

In this study, we examined the ability of a series of synthetic gingerols and related analogues to inhibit human platelet activation, compared to aspirin, by measuring their effects on AA-induced platelet serotonin release and aggregation in vitro. The study revealed that gingerols and related analogues were approximately two- to threefold less potent than aspirin against the platelet release reaction initiated by AA, and two- to four-fold less potent than aspirin at inhibiting AA-induced platelet aggregation. Using RBL-2H3 cells, we found that representative gingerols and selected analogues (G3–G6) inhibited COX activity, assessed by measuring PGD_{2}, a product of AA metabolism by COX. These results suggest that inhibition of COX activity by gingerols and related analogues may be the underlying mechanism for their effect on AA-induced platelet activation.

Platelets normally exist in a resting state despite exposure to various physiological activators and inhibitors. Activators perturb the resting state of platelets by inducing platelet activation, whilst inhibitors keep platelets dormant during circulation. Four well-defined stages of platelet activation are recognised in vitro: (A) platelet shape change; (B) platelet adhesion and primary platelet aggregation; (C) platelet granule release reaction and/or AA liberation; and (D) secondary platelet aggregation. There is a wide range of platelet activators, including proteins (thrombin, collagen), nucleotides (adenosine diphosphate), amines (serotonin) and lipids (AA, thromboxane, prostaglandins (PGs) and platelet-activating factor). However, AA plays a central role, since activation of platelets by most activators is accompanied by liberation of AA from membrane phospholipids. AA is rapidly converted to PGs and then to thromboxane A_{2} by COX and thromboxane synthase, respectively [22]. The oxygenated metabolites of AA, especially PGG_{2}, PGH_{2} and thromboxane A_{2}, are potent platelet activators. In this way, AA plays a key role in the initiation and perpetuation of platelet activation. Hence, the development of well-tolerated antiplatelet therapies that inhibit platelet activation, mediated via AA-dependent pathways, is of the utmost importance.

In this study, we show that gingerols and related analogues can inhibit both the platelet granule serotonin release reaction, as well as platelet aggregation, induced by AA in human PRP. To examine whether underlying mechanism may be via an effect on COX enzyme activity, we used RBL-2H3 cells, a well-characterised cell type for investigating AA metabolism by COX [14–16]. Although both constitutive COX-1 and inducible COX-2 are present in RBL-2H3, and only COX-1 is present in anucleate platelets [23,24], studies performed in RBL-2H3 cells allowed us to compare the inhibitory effect of representative gingerol and related analogues series (G3–G6) on COX enzyme activity. We found that these compounds potently inhibited COX enzyme activity in RBL-2H3 cells with the following rank order of potency (IC_{50} μM): G5 (1.2 μM) > G4 (1.5 μM) > G6 (3.3 μM) > G3 (50 μM).

Interestingly, the potency of gingerols and related analogues against COX enzyme activity in RBL-2H3 cells appeared to depend on hydrophobicity (log P). G3 ([6]-gingerol [12,13]), the least hydrophobic compound studied (log P = 1.85), was the least effective at inhibiting COX activity (IC_{50} = 50 μM). G4, a compound that differed from G3 by having a greater alkyl chain length (Fig. 1), was more hydrophobic (log P = 2.91) and also more effective at inhibiting COX activity (IC_{50} = 1.54 μM). G5 (log P = 5.46), the most hydrophobic compound studied, had the greatest inhibitory effect on COX activity (IC_{50} = 1.2 μM). Similar observations were made from the inhibition of platelet release reaction induced by AA by the gingerols and related analogues, as shown in Table 1 and Fig. 5A, where the potencies of gingerols and related analogues against AA-induced platelet release reaction appeared to follow a bimodal relationship with hydrophobicity, where log P optima for inhibition of the platelet release reaction would be in the
range of 5.3–5.5. In contrast, the least hydrophobic compounds studied, G3 (log $P = 1.85$) and G4 (log $P = 2.91$) were the most potent inhibitors of AA-induced platelet aggregation (Fig. 5B). Taken together, these results suggest that the hydrophobicity optima for inhibiting platelet aggregation, as compared to platelet release reaction and/or AA liberation, differ. While greater hydrophobicity may be necessary for the analogue to be transported through platelet cell membrane in order to inhibit the platelet granule release reaction and/or AA liberation (after COX activity), less hydrophobic compounds may be able to inhibit primary platelet aggregation (independent of COX activity) in a more efficient manner. Although additional experiments are needed to identify the precise events responsible for these effects on platelet activation, these results highlight the need for the examination of hydrophobicity as part of the rational redesign of new gingerol analogues. For example, it may be important to ensure that the hydrophobicity of the analogues should not be so high that the molecule will reside mostly in the platelet cell membrane or that it will interact non-specifically with other plasma proteins, such as albumin. An optimal balance between COX inhibition and platelet aggregation may be reached through judicial choice of structures for further drug development.

This research project is the first stage in the development of synthetic gingerol analogues as potential platelet activation inhibitors with widespread clinical application. Although further studies are required to design synthetic analogues with greater potencies than aspirin, these results can provide important leads that can be used to aid rational drug design. These gingerols and related analogues could prove to be potentially effective as well-tolerated platelet activation inhibitors with a more favourable therapeutic profile in cardiovascular disease. They may also possess favourable platelet activation inhibition as an adjunct to their possible use as anti-inflammatory agents.

We express our thanks to all the volunteers for blood donation. We are also grateful to Dr. Andrew J. McLachlan, Faculty of Pharmacy, University of Sydney, for helping us with the modelling of dose–response curves; Dr. Russell I. Ludowyke, Centre for Immunology, St. Vincent’s Hospital, Sydney, for providing RBL-2H3 cells; and Prof. Joel S. Bennett and Gaston Vilaire, Hematology–Oncology Division, University of Pennsylvania, for their aggregometry expertise.

References


