Full Length Research Paper

Inhibitory effect of betulinic acid and 3β-acetoxybetulinic acid on rat platelet aggregation

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Accepted 21 November, 2013

Platelet aggregation is one of the major causes of cardiovascular diseases. Our search for bioactive molecules from nature, led to the isolation of betulinic acid (BA) and structural modification of BA to 3β-acetoxybetulinic acid (BAA). Both inhibited blood platelet aggregation induced by thrombin, adenosine diphosphate (ADP) and epinephrine. BAA showed an enhance inhibition of platelet aggregation, in the thrombin-induced platelet aggregation (54.5±0.01 at 1 mg/ml, 63.5±0.17 at 3 mg/ml and 73.5±0.15 at 10 mg/ml; IC50 0.81 mg/ml) which was observed to be significantly (p<0.05) similar to that of the standard aspirin (65.4±0.07 at 1 mg/ml, 72.1±0.03 at 3 mg/ml and 76.5±1.22 at 10 mg/ml; IC50 0.33 mg/ml). The results clearly shows that functional group modification of BA to give BAA led to enhanced activity, hence BAA provides a better option as lead in the search for anti-platelet aggregation agents from nature.

Key words: Anti-platelet aggregation, aggregation inducer, Betulinic acid, acetoxybetulinic acid, Melaleuca bracteata.

INTRODUCTION

Platelets are cells in the blood that help in the formation of clot. They play an important role in the hemostasis and in pathophysiological processes such as thrombosis (Shattil et al., 1998; Stouffer and Smyth, 2003). Intravascular thrombosis is central to the development of a wide variety of cardiovascular diseases (Grenache et al., 2003; Huo and Ley, 2004). Platelet over aggregation is usually the main cause of internal blood clot formation and if not checked, can be fatal leading to atherothrombotic diseases such as strokes and heart attack (Valko et al., 2005). Thrombin, adenosine diphosphate (ADP), epinephrine, arachidonic acid, collagen and other risk factors such as free radicals, inflammation, stress and hypercholesterolemia significantly contribute to platelet dysfunction (Ambrosio et al., 1997; Davi and Patrono, 2007; Bakdash and Williams, 2008; Verhamme and Hoylaaert, 2009). The activation of platelet by thrombin is mediated through two protease activating receptors PAR-1 and PAR-4, in the activation of platelets; these receptors work cooperatively (Fabre and Gurney, 2010). ADP acts through G-protein coupled receptors P2Y1 and P2Y12, they activates phospholipase C and thus resulting in the elevation of intracellular calcium concentration (Davi and Patrono, 2007). Epinephrine is a weak platelet agonist and exerts its effect on human platelets through α2-adrenergic receptors (A2AR) and potentiates the aggregation potency of other inducers (Choi, 2002).

Antiplatelet drugs help stop blood clot formation and this significantly contributes to the management of pathogenesis of cardiovascular diseases. Among the many drugs used in the management of the condition is aspirin,
but these drugs are not without side effect, fuelling the search for more effective drugs from natural origin that will help in overcoming the challenges of toxicity and other undesirable side-effects.

The triterpene betulinic acid (BA) is one of the major components of *Malaleuca bracteata*, a plant native to Australia. The essential oil from the leaves of the plant has antiseptic, germicidal and insecticidal properties (Cribb and Cribb, 1981; Yatagai, 1997). The leaves and their volatile oils are locally used in West Africa in the treatment of skin infection (Oliver, 1960; Irvine, 1961; Howes, 1974; Goldstein et al., 1990; Belousova and Denisova 1992). BA possesses diverse biological functions, which includes, but not limited to anticancer (Amico et al., 2006; Huang et al., 2007), HIV-1 maturation inhibition (Fukoja et al., 1994) and antibacterial (Chandramu et al., 2003). The present study reports our findings on blood platelet aggregation (BPA) inhibition of BA and the effect of change in the functionality of BA on BPA.

**MATERIALS AND METHODS**

**Plant**

*Melaleuca bracteata* was supplied by Prof. F. O. Shode of the School of Chemistry, University of KwaZulu-Natal, Durban, South Africa. The plant was identified and a voucher specimen (Glow 001) was prepared and deposited in the Herbarium, School of Biological Sciences, University of KwaZulu-Natal, Durban, South Africa.

**Extraction and isolation of BA from *M. bracteata***

The pulverized plant material (2.5 kg) was extracted by cold maceration in dichloromethane (5 L×2) for 48 h, filtered and concentrated under reduced pressure at 40°C using rotary evaporator and allowed to dry under room temperature. The dried residue was defatted with n-hexane to yield a solid mass (9.8 g). The solid mass (5 g) was subjected to column chromatography using ethyl acetate:hexane (8:2) as solvent of elution to give a white amorphous powder which was identified as betulinic acid (BA). The structure was confirmed by nuclear magnetic resonance (NMR) spectra (Figure 1) and comparison with literature values (Mahato and Kundu, 1994).

![Figure 1. C-NMR, DEPT 90 and DEPT 135 spectrum of BA.](image-url)
Preparation of 3β-acetoxy betulinic acid (BAA)

BA (2 g) in a round bottom flask was added acetic anhydride (5 ml) and pyridine (5 ml), the mixture was then refluxed for 2 h. Then stirred at room temperature for 24 h, after which water (10 ml) was added and stirring continued for another 30 min. The mixture was then filtered under suction and washed thoroughly with 10% hydrochloric acid to give a white powder. NMR analysis and comparison with literature (Mahato and Kundu, 1994) confirmed the structure of BAA (Figure 2).

Animals

Ethical clearance for the use of animals in this study was obtained from the research animal ethics committee of the University of Zululand, South Africa. Adult rats (Sprague-Dawley) of either sex were collected from the animal house in the Department of Biochemistry and Microbiology, University of Zululand, South Africa. The animals were maintained under standard conditions and had free access to standard pellet feed and drinking water, for a minimum of 7 days before use.

Preparation of blood platelets

The blood platelet was prepared according to the method described by Tomita et al. (1983). Briefly, blood was surgically collected from the heart immediately after the rat has been rendered unconscious. The blood was transferred into a centrifuge tube and mixed (5:1 v/v) with an anticoagulant, acid-dextrose-anticoagulant (0.085 M trisodium citrate, 0.065 M citric acid, 2% dextrose). The platelets were obtained by series of centrifugation at 1200 rpm for 15 min and at 2200 rpm for 3 min consecutively, the supernatant was collected and discarded and the sediment (platelets) was re-suspended in 5 ml washing buffer (pH 6.5). This was centrifuged again at 300 rpm for 15 min after which the supernatant was discarded and the platelets were finally suspended in a (pH 7.4; containing 0.14 M NaCl, 15 mM Tris-HCl, 5 mM glucose buffer). The platelet was diluted in the re-suspending buffer (1:10), and the resulting solution was mixed with calcium chloride (0.4 ml; 10 µl CaCl2).

Anti-platelet aggregation study

The method of Mekhfi et al. (2004) was adopted with some modifications. Various concentrations (1, 3 and 10 mg/ml) of the test compounds were made, by separately dissolving the compounds in dimethyl sulfoxide (DMSO) and making up the volume with a buffer (pH 7.4) solution. The antiplatelet aggregation activity of the compounds were separately tested on thrombin (5 U/ml), ADP (5 mM) and epinephrine (10 mM) induced platelet aggregation; the platelets (150 µl) were incubated in a 96-well microtitre plate at 37°C for 5 min, with 50 µl of different concentrations of the compounds (1, 3, and 10 mg/ml) and an aggregation inducer (20 µl)
was introduced to the mixtures. Aggregation was determined with the Biotek plate reader using Gen5 software by following change in absorbance at 415 nm for 20 min at 30 s intervals. DMSO (1%) and aggregation inducer were used as negative control; aspirin was used as positive control.

The inhibitory effects of the compounds on each agonist were calculated as:

\[
\text{Inhibition (\%)} = \left\{ 1 - \left( \frac{A_t}{A_c} \right) \right\} \times 100
\]

The inhibitory concentration providing 50% inhibition (IC\(_{50}\)) was determined using statistical package (Origin 6.1), where At is the absorbance of the mixture containing the compounds and Ac is absorbance of the control without the test compounds.

**RESULTS**

The results of the NMR analysis, \(^{13}\)C, DEPT-90 and DEPT-135 for both BA and BAA are shown in Figures 1 and 2, respectively, were in agreement with those reported by Mahato and Kundu (1994). The results presented in Table 1 showed the inhibitory concentration providing 50% inhibition (IC\(_{50}\)) of the test compounds and standard, on platelet aggregation for all the three agonist used (thrombin, ADP and epinephrine). While the results of the percentage inhibition of platelet aggregation of the various concentration of the test compounds on each of the agonist are shown in Figures 3, 4 and 5.

**DISCUSSION**

Platelet aggregation is one of the major causes of
The results of our investigation showed that anti-platelet aggregation inhibitions of BA and BAA against thrombin, ADP and epinephrine-induced platelet aggregation (Figures 3 to 5) showed a dose dependent increase in percentage inhibition, with increase in concentration. BA and BAA were more potent in the thrombin-induced platelet aggregation with IC_{50} of 1.99 and 0.81 mg/ml, respectively, which were comparable to that of aspirin (0.33 mg/ml). BAA showed more enhance...
activity in all the three agonist used (IC50: thrombin, 0.81 mg/ml; ADP, 1.39 mg/ml; epinephrine, 1.81 mg/ml), as compared to BA (IC50: thrombin, 1.99 mg/ml; ADP, 4.20 mg/ml; epinephrine, 4.63 mg/ml) the parent nucleus. The observed activity was attributed to the modification of the 3\'-hydroxyl (-OH) of BA to 3\'-acetyl (OCOCH3) functional group. The overall result of the anti-platelet aggregation studies reveals that both compounds, BA and BAA inhibit platelet aggregation at a significant difference (p<0.05) between the different concentrations used in all the three agonist. Our findings are in agreement with Tzakos et al. (2012) who reported the potency of betulinic acid to inhibit human platelet aggregation. The results of this study showed that the isolated (BA) and modified (BAA) compounds are good candidates in the search for anti-platelet aggregation agents from nature.

Conclusion

The results showed that structural modification of the isolated compound (BA) to mimic the functionality of aspirin, led to enhanced platelet aggregation inhibition, induced by thrombin, ADP and epinephrine. The findings suggest that BA and BAA may be considered as lead candidates, in the search for anti-platelet aggregation agents from nature that may offer a better alternative to aspirin.

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