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Serotonin transporter protein (SERT) and P-glycoprotein (P-gp) binding activity of montanine and coccinine from three species of *Haemanthus* L. (Amaryllidaceae)☆

G.I. Stafford [a], C. Birer [b], B. Brodin [c], S. Brøgger Christensen [b], A.H. Eriksson [d,1], A.K. Jäger [b], N. Rønsted [a,*]

[a] Natural History Museum of Denmark, University of Copenhagen, Sølvgade 83, DK-1307 Copenhagen K, Denmark
[b] Natural Products Research, Department of Drug Design and Pharmacology, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark
[c] Drug Transporters in ADME, Department of Pharmacy, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark
[d] Bioneer: FARMA, Department of Pharmacy, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark

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ABSTRACT

The alkaloid rich extracts from an acid/base extraction of bulb material of *Haemanthus coccineus* L., *H. montanus* Baker and *H. sanguineus* Jacq. revealed that two montanine type Amaryllidaceae alkaloids, montanine (1) and coccinine (2) were the major alkaloid constituents. Together these two alkaloids constituted 88, 91 and 98% of the total alkaloid extract from each species respectively. GC–MS analysis revealed that *H. coccineus* and *H. sanguineus* had a relative abundance of coccinine (74 and 91% respectively) to montanine (14 and 7% respectively); whereas *H. montanus* had 20% coccinine and 71% montanine. The three extracts and two isolated alkaloids were evaluated for binding to the serotonin transporter protein (SERT) in vitro. Affinity to SERT was highest in *H. coccineus* (IC_{50} = 2.0 ± 1.1 μg/ml) followed by *H. montanus* (IC_{50} = 6.8 ± 1.0 μg/ml) and *H. sanguineus* (IC_{50} = 28.7 ± 1.1 μg/ml). Montanine (IC_{50} = 121.3 ± 3.6 μM or 36.56 ± 1.14 μg/ml; K_i = 66.01 μM) was more active than coccinine (IC_{50} = 196.3 ± 3.8 μM or 59.15 ± 1.08 μg/ml; K_i = 106.8 μM), both of which were less active than the total alkaloid extracts of each species investigated. The possible synergistic effects of two coccinine/montanine mixtures (80:20 and 20:80) were investigated, however the mixtures gave similar activities as the pure compounds and did not show any increase in activity or activity similar to the total alkaloid extracts. Thus the considerably higher activity observed in the total alkaloid extracts is not correlated to the relative proportions of coccinine and montanine in the extracts and thus are likely to be due to more potent unidentified minor constituents. Both alkaloids exhibited low binding affinity to P-glycoprotein (P-gp) as demonstrated by low inhibition of calcein-AM efflux in the MDCK-MDR1 cell line. This indicates that P-gp efflux will not be limiting for blood–brain-barrier passage of the alkaloids.

1. Introduction

Depression is generally considered to be associated with reduction of monoamine neurotransmitters in the brain. Several antidepressants exert their effect by selective inhibition of serotonin reuptake by binding to the serotonin transporter protein (SERT) (Stahl, 1998). In a screening of plants used for depression-like disorders in traditional medicine in South Africa, an ethanolic extract of leaves and bulbs of *Boophone disticha* (L.f.) Herb. (Amaryllidaceae) has shown affinity to SERT in both in vitro (Nielsen et al., 2004) and in vivo (Pedersen et al., 2008). Four active alkaloids have been isolated from the extract; buphanamine, buphanidrine, buphanisine and distichamine, with IC_{50}-values of 55 μM, 62 μM, 199 μM and 65 μM respectively, in the SERT-binding assay. The alkaloids also showed activity in a functional assay, buphanidrine and distichamine being the most active with IC_{50}-values of 513 μM and 646 μM, respectively (Sandager et al., 2005; Neergaard et al., 2009).

The Amaryllidaceae alkaloids include more than 500 identified compounds, which have been divided into eighteen structural types based on their hypothetical biosynthetic pathways (Jin, 2009). Structurally, buphanamine and buphanadrine belong to the crinine-type and have the benzo-1,3-dioxole moiety in common with the clinically used SSRI, paroxetine, which could explain their affinity to the SERT. A detailed screening of alkaloids isolated from Amaryllidaceae (Elgorashi et al., 2006; Neergaard et al., 2009), suggested that Amaryllidaceae

Abbreviations: AChE, acetylcholinesterase; BBB, blood–brain-barrier; BCRP, breast cancer resistance protein; CNS, central nervous system; P-gp, P-glycoprotein; SERT, serotonin transporter protein; SSRI, selective serotonin reuptake inhibitor.

* Corresponding author at: Natural History Museum of Denmark, Sølvgade 83, Opg. S., DK-1307 Copenhagen K, Denmark. Tel.: +45 33 32 22 48.
E-mail address: nronsted@snm.ku.dk (N. Rønsted).
[1] Current address: DMPK & Safety, LEO Pharma A/S, Industriparken 55, DK-2750 Ballerup, Denmark.

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alkaloids with affinity to SERT primarily are of the crinine type (Jin, 2009). In a recent study on the Southern African Amaryllidaceae tribe Haemantheae (Bay-Smidt et al., 2011), we found that SERT activity appears to be pronounced and restricted to the genus *Haemanthus* within this tribe. Alkaloid rich extracts of three of the eight *Haemanthus* species tested had IC_{50} < 10 μg/ml. Two of the most active extracts contained primarily montanine type alkaloids, which have previously not been tested for SERT affinity. Several of the closely related white-flowered *Haemanthus* (Bay-Smidt et al., 2011), which are found in the summer rainfall regions of southern Africa, are used in African traditional medicine (Crouch et al., 2005). These include the bulbs and roots of *H. albiflos* Jacq., which are used by the Xhosa in infusions or poultices to promote the healing of broken bones, (Crouch et al., 2005; Dold and Cocks, 2000). *Haemanthus* species are not reported to be used in African traditional medicine to treat mental illnesses, although several closely related Amaryllidaceae genera are (Sobiecki, 2002; Stafford et al., 2008), such as *Scadoxus puniceus* (L.) Friis & Nordal (syn = *Haemanthus kalbreyeri* Baker), *Ammocharis coranica* (Ker-Gawl.) Herb, *Boophone disticha* (L.f.) Herb., *B. haemanthoides* F.M.Leight. and *Pancratium tenuifolium* Hochst. ex A.Rich.

The first study to isolate alkaloids from *Haemanthus* found 10 alkaloids from several species (Wildman and Kaufman, 1955). Coccinine ((2β)-2-*O*-methylpancracine) was found to be the principle component in *H. coccineus* and montanine ((2α)-2-*O*-methylpancracine) the only alkaloid detected in *H. montanus*.

In a study by da Silva et al. (2006), where montanine was isolated from the bulbs of the South American *Hippeastrum vittatum* (L'Hér.) Herb., montanine showed a LD_{50} of 64.7 mg/kg and 67.6 mg/kg for male and female mice, respectively. When given i.p., montanine dose-dependently decreased sodium pentobarbital-induced sleep, protected against pentylenetetrazole-provoked convulsions, increased the number of entries and the time spent in the open arms of an elevated plus maze and augmented the time spent struggling during a forced swimming test. When given immediately after inhibitory avoidance training, montanine did not affect avoidance memory retention in rats. The reported in vivo activity of montanine, particularly in the forced swimming test, supports the possibility that montanine and its beta 2-*O*-methyl-isomer, coccinine (Fig. 1), may serve as leads for SERT binding compounds. Montanine has also been shown to significantly inhibit acetylcholinesterase (AChE) (Pagliosa et al., 2010). An activity utilized in treatment of neurological disorders and neurodegenerative diseases related to the levels of acetylcholine. For this reason galanthamine, an important alkaloid isolated from several Amaryllidaceae, is approved for the pharmacological treatment of Alzheimer's disease.

It has however been estimated that 98% of CNS drug candidates do not enter the brain (Pardridge, 2007). The passage by passive diffusion of compounds with optimal physicochemical properties may be hindered by the activity of efflux transporters, amongst which P-glycoprotein (P-gp; MDR1 gene product) plays a major role. Our understanding of the substrate-specificity of P-gp is still rather limited (Li et al., 2007). In an evaluation of the interaction of nine potentially CNS-active Amaryllidaceae alkaloids of the crinine, lycorine and galanthamine types with P-gp (Eriksson et al., 2012), structurally similar compounds such as crinine and epibuphanisine showed very different P-gp interaction emphasizing the difficulty in predicting P-gp interaction.

In the present study we investigated affinity of extracts of three *Haemanthus* species, *H. coccineus* L., *H. montanus* Baker and *H. sanguineus* Jacq., and major alkaloid constituents, coccinine and montanine, to the SERT protein. The pure alkaloids were also evaluated with for their interaction with P-gp using the calcein-AM assay. *Haemanthus coccineus* L. (Synonyms: *H. concolor* Herb., *H. moschatus* Jacq., *H. splendens* Dinter, *H. tigrinus* Jacq.) is a perennial geophyte which occurs at an altitude of 15–1200 m in the winter rainfall region of southern Africa from Namibia through the Northern Cape and Western Cape to Eastern Cape of South Africa. *Haemanthus montanus* Baker (synonyms: *H. amarylloides* in sense of Baker, not of Jacq., misapplied name) a perennial geophyte which occurs in the summer rainfall regions of Botswana and Northern West, Griqualand, Mpumalanga, Free State, KwaZulu-Natal, Eastern Cape provinces of South Africa. *Haemanthus sanguineus* Jacq. (synonyms: *H. incarnatus* Burch. ex Herb., *H. rotundifolius* Ker Gawl.) is a perennial geophyte found only in the Western and Eastern Cape of South Africa.

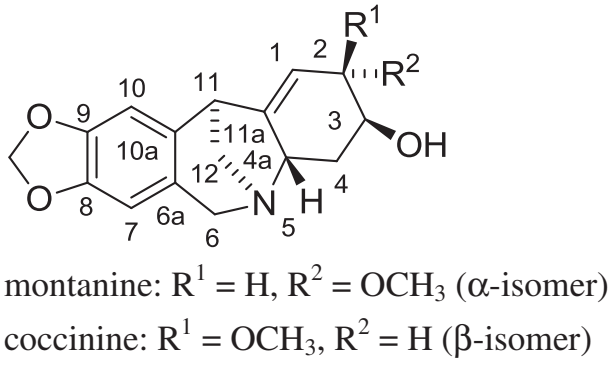

Fig. 1. Montanine and coccinine.

2. Materials and methods

2.1. Plant material

Three species of *Haemanthus* L. (Amaryllidaceae) were obtained from a specialist nursery (www.rareplants.co.uk) for this study. The plant material was identified by NR and GIS and vouchers (Table 1) are deposited at the Herbarium, Botanical Garden and Museum, University of Copenhagen (**C**). Plant material was cultivated in a greenhouse and harvested in February 2011.

2.2. Alkaloid extraction for initial screening

Alkaloids were extracted from 300 mg dried bulb scales. The pulverized plant material was macerated in 400 μl methanol for 5 min. Three milliliters 0.1% H_2SO_4 was added and the material was extracted for 45 min under ultra-sonification followed by shaking for 2 h. The sample was centrifuged for 10 min at 4000 rpm and the supernatant taken off. Extraction was repeated twice more, but the sample was left on the shaker overnight during the second extraction. The three extracts were pooled, centrifuged for 10 min at 4000 rpm, and retained on an ion-exchange SPE (solid phase extraction) column (Isolute SCX, 500 mg, 3 ml, Mikrolab, Denmark). The column was preconditioned with 2 ml methanol and 2 ml 0.1% H_2SO_4. After addition of the alkaloid extract, the column was washed with 2 ml 0.1% H_2SO_4 and 4 ml methanol. The alkaloid extracts were then with 6 ml 25% NH_4OH in methanol, pH 11–12, concentrated under vacuum until dryness and re-dissolved to a concentration of 5 mg/ml in methanol.

2.3. Isolation of montanine and coccinine

The same extraction protocol that was used for the initial screening was performed on 11.97 g dried *Haemanthus coccineus* (fw 71.21 g) which yielded 275 mg extract (23 mg/g dried bulb), 12.00 g dried *H. montanus* (fw 59.53 g) yielded 496 mg extract (41 mg/g dried bulb) and 12.00 g dried *H. sanguineus* yielded 443 mg extract (37 mg/g dried bulb). Two hundred milligrams of the *H. coccineus* and *H. montanus* extracts were suspended in methanol and separated by column chromatography on a silica gel column (170 ml Merck 9385 silica gel, height 80 cm, diameter 2 cm). An eluent system consisting of 80:20 chloroform:methanol was used and 150 fractions collected. The fractions were pooled by TLC profile into 2 fractions for each column.