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Isoquinoline Derivatives Isolated from the Fruit of *Annona muricata* as 5-HTergic 5-HT_{1A} Receptor Agonists in Rats: Unexploited Antidepressive (Lead) Products

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Abstract

The fruit and the leaves of *Annona muricata* (Annonaceae) are used in traditional medicine for their tranquillizing and sedative properties. Extracts of the plant have been shown to inhibit binding of [^{3}H]rauwolscine to 5-HTergic 5-HT_{1A} receptors in calf hippocampus, and three alkaloids, annonaine (**1**), nornuciferine (**2**) and asimilobine (**3**), isolated from the fruit have been shown to have IC50 values of 3 μM, 9 μM and 5 μM, respectively, although in ligand-binding studies it was not possible to determine whether interaction of these ligands with the receptor was agonistic or antagonistic. This paper presents the results of functional assays of the alkaloids.

The inhibition of cAMP accumulation was tested in NIH-3T3 cells stably transfected with the 5-HT_{1A} receptor from man. None of the alkaloids showed antagonistic properties towards the 5-HT_{1A} receptors because in the antagonistic tests no influence on the forskolin-stimulated increase of cAMP level was detected. Full agonistic properties were measured for all three compounds; the inhibition constants (K_i) for **1**, **2** and **3** were < 10 μM. Inhibition of the binding of the radioligand to the 5-HT_{1A} receptor was observed in every ligand-binding assay performed with the alkaloids; the K_i values for **1**, **2** and **3** were in the μM range.

These results imply that the fruit of *Annona muricata* possesses anti-depressive effects, possibly induced by compounds **1**, **2** and **3**, and that in the past potent leads for the development of anti-depressive therapeutics have not been used.

In traditional medicine *Annona muricata* (Annonaceae) has been used for its action on the central nervous system. In Surinam (South America) the fruit and the leaves are claimed to have tranquillizing effects (Hasrat et al 1997) and in Guam and the Virgin Islands the seeds are claimed to have sedative properties (Gupta 1995). This activity might be mediated by the 5-HT_{1A} receptor, because of the influence of this receptor in the brain (Hoyer et al 1994). Extracts of fruit (without seeds), seeds and leaves were investigated for inhibition of the binding of [^{3}H]rauwolscine to 5-HTergic 5-HT_{1A} receptors in calf hippocampus and potent inhibition was shown for at least one extract. Three alkaloids, annonaine (**1**), nornuciferine (**2**) and asimilobine (**3**) isolated from fruit extracts were subsequently shown to have IC50 values of 3 μM, 9 μM and 5 μM, respectively (Hasrat et al 1997). However, in ligand-binding studies it was not possible to determine the mechanism of action of the ligands, i.e., whether their interaction with the receptor was agonistic or antagonistic. Functional in-vitro assays are currently used for determination of the mechanism of action of compounds. In this respect, because of advances in biological techniques, cells transfected with the appropriate receptor(s) have become one of the methods of choice. This rather easy and acceptable method can furnish data which enable distinction whether a ligand is an agonist or an antagonist.

In recent years much information has been collected about the 5-HT_{1A} receptor, including evidence that influencing this receptor in the central nervous system can lead to beneficial effects in certain types of psychiatric disorders, especially depression (Blier & de Montigny 1994; Hoyer et al 1994; Launay et al 1994; Sanders-Bush & Mayer 1995). Moreover, stimulation of the 5-HT_{1A} receptor on 5-HTergic neurons in the limbic system might be the site of action of 5-HT_{1A} ligands. Further, it has been observed that stimulation of the 5-HT_{1A} receptor is negatively coupled to cAMP formation. Thus, in certain circumstances in-vitro, stimulation of cAMP synthesis might be antagonized by 5-HT_{1A} receptor agonists and might not be influenced by 5-HT_{1A} receptor antagonists. In this study the functional assay tests were performed with NIH-3T3 cells stably transfected with 5-HT_{1A} receptors from man.

There is a need for anti-depressive drugs that act more rapidly and shorten the delayed onset of action, because the latency of the effects is at least two weeks for currently available therapeutics. The explanation of this phenomenon is that 5-HTergic neuron activity is restored after being depressed owing to stimulation of somatodendritic 5-HT_{1A} receptors and pre-synaptic 5-$HT_{1B/1D}$ receptors on the neuron, but the continuous presence of agonists desensitizes the receptors (Blier & de Montigny 1994). The use of selective 5-HT_{1A} receptor antagonists at somatodendritic 5-HT_{1A} receptors and selective 5-$HT_{1B/1D}$ receptor antagonists at presynaptic receptors could be a solution to this problem (Blier & de Montigny 1994).

Post-synaptic 5-HT_{1A} receptors which are also involved in depressive disorders have also been established (Blier & de Montigny 1994), but are probably not the dominant factor in the production of an anti-depressive effect with currently available therapeutics.

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Although, no scientific studies have been conducted to examine the 'tranquillizing experience' after use of the fruit of *Annona muricata* in traditional medicine, it is reasonable to assume that effects on the central nervous system are produced rapidly and that the 5-HT_{1A} receptor ligands isolated from the fruit might be responsible for this activity. However, interactions with dopaminergic, adrenergic and other 5-HT receptors might also account for this activity and the rapid onset of action, in addition to inhibition of re-uptake systems of these bioaminergic compounds.

The alkaloids **1**, **2** and **3** from the fruit of *Annona muricata* are isoquinoline derivatives; more specifically they belong to the aporphine alkaloids. The isoquinolines, the largest single group of plant alkaloids (Harborne & Baxter 1993), can be divided into approximately twenty categories with different activity. Among these the central nervous effects of morphine and derivatives are the best known. Other isoquinoline alkaloids have antimicrobial, anthelminthic, cardiovascular, gastrointestinal, oxytocic, spasmolytic, insecticidal, tumour inhibiting, cytotoxic, anti-inflammatory and neuromuscular-blocking effects (Harborne & Baxter 1993). Only a few of these effects have been related to interaction with specific receptors. The effects of morphine, which result from interactions with encephalin, endorphin and dynorphin receptors, have been thoroughly described. It is obvious, however, that all the effects of isoquinoline derivatives are unlikely to be produced by action at a single type of receptor. Many of these interactions might result in changes of intracellular messengers such as cAMP.

Materials and Methods

Test medium

NIH-3T3 cells stably transfected with 5-HT_{1A} receptors from man were used for 2–3 months (up to passage number 30).

Vehicle

Cells were cultured in M505 medium containing 10% cosmic calf serum. Test compounds (agonists, antagonists, reference compounds) were dissolved in an appropriate vehicle and diluted with M505 culture medium containing 500 μM 3-isobutyl-1-methylxanthine to a concentration ten times higher than the final concentration in the assay. The final DMSO concentration in the assay of 1% is tolerated.

Reagents

M505: Dulbecco's Modified Eagle Medium and Ham's Nutrient Mixture in a 1 : 1 ratio supplemented with: $NaHCO_3$ (2500 mg L^{-1}), Na-pyruvate (55 mg L^{-1}), ethanolamine (20 μM), Na selenite (25 μM), L-glutamine (350 mg L^{-1}), Na penicillin (62·5 mg L^{-1}), streptomycin (62·5 mg L^{-1}), β-mercaptoethanol (2·1 μL L^{-1}); Cosmic calf serum (Hyclone, A-2169-L); trypsin-EDTA solution (Sigma, T-5775); trypan blue (Sigma, T-6146) 1 mg mL^{-1} in 0·9% NaCl (sterile; stored at 4°C); poly-L-lysine (Sigma, P-5899) stock solution 5 mg in 50 mL sterile water (stored at −20°C); sterile water (Milli-Q grade); 3-isobutyl-1-methylxanthine (Sigma, I-5879; MW 222·3) stock solution: 10^{-2} M in DMSO (stored at −20°C); ethanol 100%; cAMP standard solution; cAMP assay buffer (Innogenetics); anti-cAMP antibody-coated test tubes (Innogenetics); $[^{125}I]$cAMP tracer (Innogenetics).

The alkaloids **1**, **2** and **3**, were isolated from fresh and frozen fruits of *Annona muricata* as described elsewhere (Hasrat et al 1997).

Assay

The functional assay has been described elsewhere (Hasrat et al 1997). It is based on inhibition of the accumulation of cAMP in NIH-3T3 cells stably transfected with 5-HT_{1A} receptors from man. The test is used to evaluate the ability of compounds to act as agonists or antagonists at the 5-HT_{1A} receptor. Briefly, cells are seeded on poly-L-lysine-coated plates (5×10^4 cells mL^{-1} per well), grown on M505 culture medium with 10% cosmic calf serum for 1 day, and washed with serum-free medium (2 × 1 mL). The cells are then pre-warmed to 37°C and pre-incubated for 30 min with 900 μL M505 + 0·5 mM 3-isobutyl-1-methylxanthine (agonistic test), or pre-incubated for 30 min with 800 μL M505 + 0·5 mM 3-isobutyl-1-methylxanthine + 100 μL 10-fold-concentrated compound (antagonistic test), or incubated for 30 min with 100 μL compound added (10 × concentrated (agonistic test); forskolin (final concentration 1 μM) is added with the compound), or incubated for 30 min with 100 μL 5-HT added (antagonistic test). In each experiment the reaction is stopped by removal of 500 μL extracellular medium and 100 μL of this is used for determination of cAMP.

Determination of cAMP

cAMP concentrations were determined by radioimmunoassay. A calibration curve was used to convert counts min^{-1} into cAMP concentration. A typical calibration curve is shown in Fig. 1.

Evaluation of results for agonistic activity

Concentration–response curves were generated by plotting the concentration of the test compound against the concentration of cAMP. The data were analysed by non-linear regression (GraphPad Prism, sigmoidal curve (variable slope)). Duplicate or triplicate values were considered individually. The analysis yields a pEC50 value, defined as the negative logarithm of the concentration of test compound that induces half-maximum inhibition of forskolin-induced cAMP accumulation for that particular compound, and the Hill-slope factor. To calculate

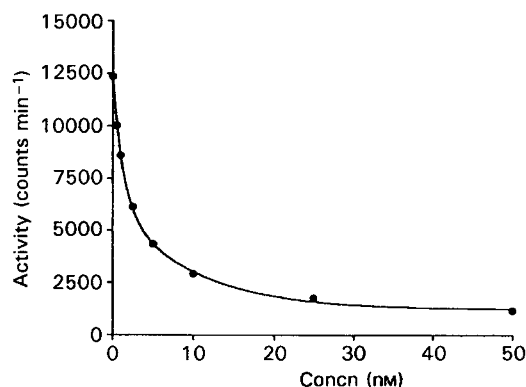

FIG. 1. A typical calibration curve used to convert counts min^{-1} measured for the samples to cAMP concentration in functional assays with NIH-3T3 cells stably transfected with the 5-HT_{1A} receptor from man.

the intrinsic efficacy of a test compound, the mean maximum effect was determined for the test compound and for a reference agonist. The intrinsic efficacy, α, was calculated from the formula:

$$\alpha = (\text{MaxF} - \text{MaxT})/(\text{MaxF} - \text{MaxR}) \quad (1)$$

where MaxF, MaxR and MaxT are, respectively, the mean concentrations of cAMP after stimulation with 1 μM forskolin, with 10^{-5} M 5-HT and with the highest concentration of test compound. In all experiments basal cAMP concentration and maximum stimulation by 1 μM forskolin were recorded as controls.

Evaluation of results for antagonistic activity

Concentration–response curves were generated by plotting the concentration of the test compound against the concentration of cAMP. The data were analysed as described for the evaluation of agonistic activity. The analysis yields a pIC50 value, defined as the negative logarithm of the concentration of test compound that reduces the effect of 300 nM 5-HT by 50% of the maximum reduction. The maximum reduction by the test compound, expressed as percentage of the maximum inhibition by 5-HT, was calculated from the formula:

$$\text{Maximum reduction (\%)} = \{(\text{MaxT} - \text{MinR})\}/(\text{MaxF} - \text{MinR})\} \times 100 \quad (2)$$

where MaxT is the mean concentration of cAMP after stimulation with the highest concentration of test compound in the presence of 300 nM 5-HT and 1 μM forskolin, MaxF is the mean concentration of cAMP after stimulation with 1 μM forskolin, and MinR is the mean concentration of cAMP after stimulation with 300 nM 5-HT in the presence of 1 μM forskolin.

Results

Fig. 2 shows the results obtained from experiment 1, i.e. measurement of the agonistic activity of compound **2**, nornuciferine, and extracts of the leaves and seeds of *Annona muricata*. 5-HT$_{1A}$ receptor-agonistic activity with weak affinity was measured for the extracts tested; activity with moderate affinity (< 10 μM) was observed for nornuciferine.

Fig. 3 shows the results obtained from experiment 2, i.e. measurement of the agonistic activity of compounds **1**, **2** and **3**. All three alkaloids have agonistic properties and completely reduced forskolin-stimulated cAMP accumulation. The affinity of nornuciferine obtained in this experiment was lower than obtained in experiment 1. Chemical analysis of the samples containing **1** and **2** revealed that the concentrations of the compounds were actually 5–10 times lower in the second experiment, so the affinity was, therefore, many times higher. The intrinsic efficacy and the Hill-slope calculated from the results are listed in Table 1.

Fig. 4 shows the results obtained from experiment 3, i.e. measurement of the antagonistic activity of compounds **1**, **2** and **3**. Except for the highest concentrations used, in this experiment the compounds did not induce accumulation of cAMP. The increase of extracellular cAMP concentration observed with the highest concentrations of the compounds was probably a result of the death of all the cells which was observed at this concentration.

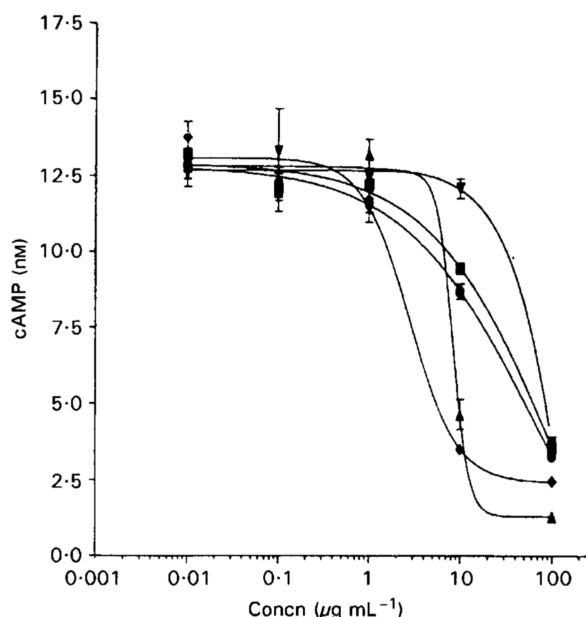

FIG. 2. Effects of nornuciferine (◆) and fractions from extracts of the leaves and seeds of *Annona muricata* on forskolin-induced cAMP formation in NIH-3T3 cells stably transfected with the 5-HT$_{1A}$ receptor from man. Inhibiting effects on the binding of [^{3}H]rauwolscine to 5-HT$_{1A}$ receptors in calf hippocampus were: the EC50 of **2** was < 10 μM; the EC50 values of the fractions of the leaf extracts, AML-1 (■), AML-2 (▼) and AML-3 (▲) were 120, 170 and 8·4 mg mL^{-1}, respectively; the EC50 of the fraction from the seed extract (●, AMS-1) was 68 mg mL^{-1} (Hasrat et al 1997).

Discussion

The results of the functional assay tests on NIH-3T3 cells stably transfected with 5-HT$_{1A}$ receptors from man have clearly demonstrated the 5-HT$_{1A}$ receptor agonistic properties of the alkaloids **1**, **2** and **3** isolated from the fruit of *Annona muricata*. The activities of these compounds might explain the effects on the central nervous system when this plant is used in traditional medicine. Because, as a result of their action in the

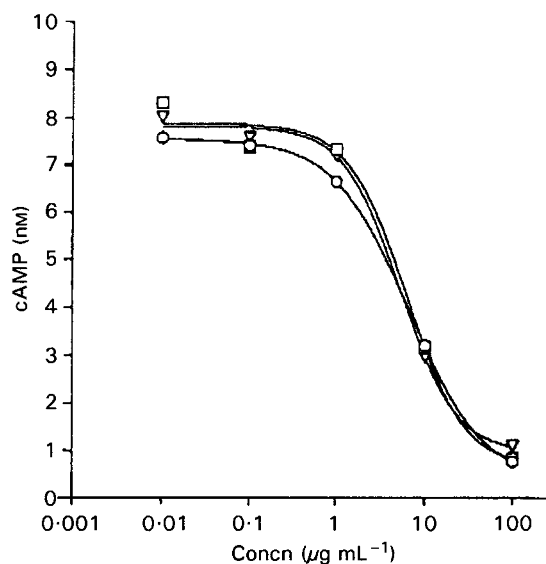

FIG. 3. Effect of annonaine (○), nornuciferine (□) and asimilobine (▽) on forskolin-induced cAMP formation in NIH-3T3 cells stably transfected with the 5-HT$_{1A}$ receptor from man.

Table 1. EC50 values, intrinsic efficacy, and Hill slope of annonaine, nornuciferine and asimilobine after functional assay tests for 5-HT_{1A} receptor agonistic activity on NIH-3T3 cells stably transfected with the 5-HT_{1A} receptor from man.

Compound	EC50	Intrinsic efficacy	Hill slope
Annonaine (**1**)	< 10 μM	1·0	1·0
Nornuciferine (**2**)	< 10 μM	1·0	1·3
Asimilobine (**3**)	20 μM	1·0	1·4

The data were analysed by non-linear regression (GraphPad Prism, sigmoidal curve (variable slope)).

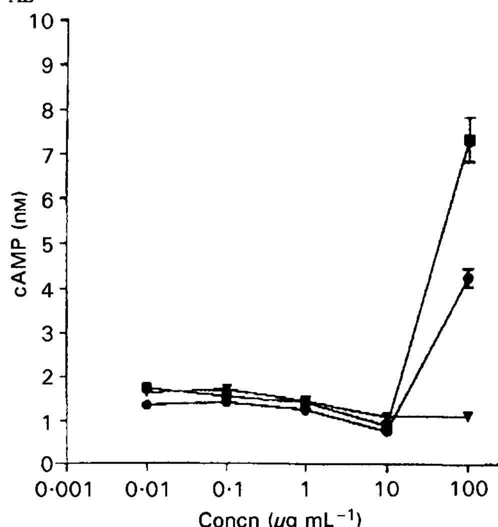

FIG. 4. Effect of annonaine (●), nornuciferine (■) and asimilobine (▼) on 5-HT (300 nM)-mediated inhibition of forskolin-induced cAMP formation in NIH-3T3 cells stably transfected with the 5-HT_{1A} receptor from man.

brain, 5-HT_{1A} receptor agonists have anti-depressive effects, the tranquillizing effects claimed in traditional medicine are a subject of debate. In addition, compounds **1**, **2** and **3** might not be receptor-specific, for example, interactions with non-5-HT_{1A} receptors might potentiate the effects on the 5-HT_{1A} receptor.

Besides morphine and its derivatives, aporphines and other isoquinoline alkaloids such as apoglazionine, bulbocapnine, isocorydine and xylopine have been implicated as producing sedative or tranquillizing effects, or both (Harborne & Baxter 1993; Sanders-Bush & Mayer 1995). Adrenergic-blocking properties have been reported for isocorydine and xylopinine and interactions of aporphines and benzylisoquinolines with dopaminergic receptors have been recorded (Bradbury et al 1983). Inhibition of platelet aggregation has been demonstrated for isoquinoline derivatives (Chen et al 1995a, b), which provides evidence for interactions of these compounds with 5-HT_2 receptors. Interaction of annonaine with the dopamine re-uptake pump has also been reported (Protais et al 1995). Endogenous aporphines have been discovered, and might be implicated in the neurochemical mechanisms underlying addictive drinking of alcohol (Rommelspacher et al 1991; Wodarz et al 1996). Some isoquinoline derivatives have been shown to be weakly to moderately potent acetylcholinesterase and butylcholinesterase inhibitors (Ulrichová et al 1983a, b). All these data indicate that natural isoquinoline derivatives, in particular aporphines such as **1**, **2** and **3** might induce the observed effects by interaction with different systems.

Results obtained with compounds in-vivo do not always reflect results obtained in-vitro; for example, the weak central anti-dopaminergic and peripheral anti-adrenergic effects of sertindole observed in-vivo contrast with the high affinity of this compound for 5-HT_{2A}, 5-HT_{2C}, dopaminergic D_2 and α-adrenergic receptors in-vitro (Andersen et al 1996). It is possible that for this compound the in-vivo accessibility of some of the receptors plays an important role. Although **1**, **2** and **3** showed moderate affinity for the 5-HT_{1A} receptor in-vitro, it is possible that the effects observed in traditional medicine might arise from the greater accessibility of these receptors in-vivo.

To conclude, compounds **1**, **2** and **3** are presumably non-selective 5-HT_{1A} receptor agonists, but interaction with other receptors might contribute to the rapid onset of the central effects of these substances. Although compounds have been developed that have highly selective affinity for one of the receptors involved in mental disorders, none is currently applied in clinical practice. This is because of the many other processes that can be affected by the use of drugs with very high affinity for one type of receptor, especially when the ligand is an agonist. Cellular regulatory mechanisms are affected by this kind of compound, leading to delayed onset of tachyphylaxis and often unexpected side-effects. In many therapeutic areas the development of high-affinity partial agonists has solved some of these problems (Ijzerman et al 1996), although examples of partial agonists in clinical practice are scarce. It is not clear why drugs that influence different receptors involved in mental disorders are not attractive enough for therapeutic use. Pharmacologically it might be argued that a drug which interacts with different receptors resulting in the same effect will act synergistically to its own effect. Thus, even if the compound has moderate affinity (μM concentrations) it might produce sufficient effect as a result of synergism that occurs. Although not yet demonstrated, compounds **1**, **2** and **3** might interfere with different receptors, leading to clinically interesting effects. In this study annonaine, compound **2**, is an agonist at the 5-HT_{1A} receptor, but also has inhibitory activity on dopamine re-uptake, with the same affinity for the receptor involved (Protais et al 1995).

The detection and investigation of natural bioactive compounds are not of interest to pharmaceutical companies, especially for compounds already isolated, as long as the problem of the patentee remains to be solved. This is, in general, a drawback to research on natural substances, and one which must be changed as soon as possible. New international rules must be formulated to ensure protection of the scientific knowledge gathered by pharmaceutical companies or governmental organizations, even for compounds already isolated. This will not only promote research on natural bioactive compounds, but will help to create funds for developing countries to investigate traditional medicine.

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