

Activity of *Mitragyna speciosa* ("Kratom") Alkaloids at Serotonin ReceptorsFrancisco León,<sup>○</sup> Samuel Obeng,<sup>○</sup> Marco Mottinelli, Yiming Chen, Tamara I. King, Erin C. Berthold, Shyam H. Kamble, Luis F. Restrepo, Avi Patel, Lea R. Gamez-Jimenez, Carolina Lopera-Londoño, Takato Hiranita, Abhisheak Sharma, Aidan J. Hampson, Clinton E. Canal, Lance R. McMahon,\* and Christopher R. McCurdy\*Cite This: *J. Med. Chem.* 2021, 64, 13510–13523

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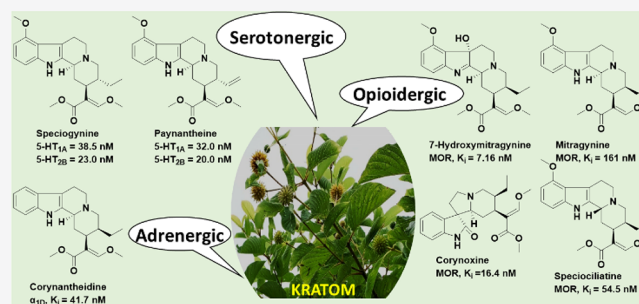


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**ABSTRACT:** Kratom alkaloids have mostly been evaluated for their opioid activity but less at other targets that could contribute to their physiological effects. Here, we investigated the in vitro and in vivo activity of kratom alkaloids at serotonin receptors (5-HT<sub>1A</sub>R and 5-HT<sub>2B</sub>R), unlike the principal kratom alkaloid mitragynine. Both alkaloids produced antinociceptive properties in rats via an opioid receptor-independent mechanism, and neither activated 5-HT<sub>2B</sub>R in vitro. Paynantheine, speciogynine, and mitragynine induced lower lip retraction and antinociception in rats, effects blocked by a selective 5-HT<sub>1A</sub>R antagonist. In vitro functional assays revealed that the in vivo 5-HT<sub>1A</sub>R agonistic effects may be due to the metabolites 9-O-desmethylspeciogynine and 9-O-desmethylpaynantheine and not the parent compounds. Both metabolites did not activate 5-HT<sub>2B</sub>R, suggesting low inherent risk of causing valvulopathy. The 5-HT<sub>1A</sub>R agonism by kratom alkaloids may contribute to the mood-enhancing effects associated with kratom use.



## INTRODUCTION

Kratom, *Mitragyna speciosa* (Korth.), is a widely used medicinal plant from Southeast Asia.<sup>1</sup> Kratom's leaves are chewed or boiled to prepare beverages that are consumed to treat diverse maladies, including fever, diarrhea, and cough.<sup>2</sup> Kratom has been used also to enhance productivity and energy, combat anxiety, and manage opioid dependence.<sup>2</sup> The consumption of kratom preparations appears to be well-tolerated by individuals in Southeast Asia including former and existing opioid users.<sup>3</sup> In the United States (US), kratom use has proliferated in the past decade and is used typically in the form of capsules, powdered leaves consumed either dry or as a tea, in energy drinks, and in elixirs.<sup>2</sup> A recent estimate of use in the US showed that around 3.3 million people consume kratom.<sup>4</sup> In the US, kratom is used as a recreational product often to treat chronic pain, opioid use disorder symptoms, and as a remedy for anxiety and depression.<sup>5</sup> These purported therapeutic effects of kratom products suggest potential clinical value, though no kratom-derived products have been approved for use by the US Food and Drug Administration (FDA).<sup>6–8</sup> Currently, kratom is not federally regulated, although its possession is banned in some US jurisdictions and states. The US Drug Enforcement Administration lists kratom as a drug of concern.<sup>9</sup>

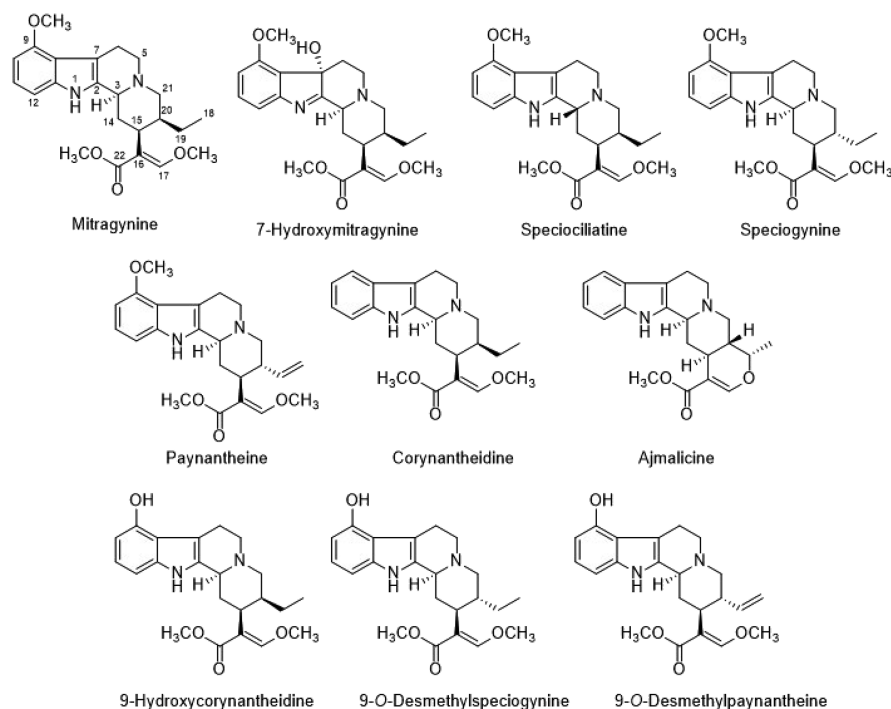
To date, about 45 different indole and oxindole alkaloids isolated from kratom leaves have been reported, including mitragynine, paynantheine, speciogynine, and speciociliatine.<sup>1,2,7</sup> However, the major kratom alkaloids and the estimates of their relative abundance vary due to different growing conditions, origin of the kratom plant, and other factors that have not been fully elucidated.<sup>2,6–10</sup> 7-Hydroxymitragynine represents <2% of the alkaloids but has a much greater binding affinity at  $\mu$ -opioid receptors (MORs) than mitragynine, which comprises 20–60% of the total alkaloid content.<sup>7,11</sup>

Functional assays at human  $\mu$ -,  $\kappa$ -, and  $\delta$ -opioid receptors (hMORs, hKORs, and hDORs, respectively) using a bioluminescence resonance energy transfer (BRET) G protein activation assay demonstrated that mitragynine is not only less potent but also a lower-efficacy hMOR agonist ( $EC_{50}$  = 339 nM;  $E_{max}$  = 34%) than 7-hydroxymitragynine ( $EC_{50}$  = 34 nM;

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**Figure 1.** Chemical structures of the selected isolated kratom alkaloids and metabolites.

**Table 1.** Percent Displacement of Radioligands from Human 5-HTRs by 100 nM and 10  $\mu$ M Concentrations of Kratom Alkaloids<sup>a</sup>

kratom alkaloid	binding site					
	5-HT <sub>1A</sub>	5-HT <sub>1B</sub>	5-HT <sub>2A</sub>	5-HT <sub>2B</sub>	5-HT <sub>2C</sub>	5-HT <sub>3</sub>
mitragynine						
100 nM	9.7	−0.2	−0.9	23.0	−8.2	−9.1
10,000 nM	<b>76.7</b>	8.2	49.9	<b>90.7</b>	14.7	−12.7
7-hydroxymitragynine						
100 nM	3.1	−5.6	−0.2	−1.4	−18.3	8.2
10,000 nM	1.2	−0.8	3.9	12.4	−13.9	19.5
speciociliatine						
100 nM	−4.8	3.8	1.5	−3.4	0.4	11.1
10,000 nM	33.3	16.8	<b>68.1</b>	47.1	14.3	0.7
speciogynine						
100 nM	<b>58.9</b>	2.6	2.8	70.9	−18.6	11.3
10,000 nM	<b>98.7</b>	30.4	<b>82.7</b>	<b>102.2</b>	<b>77.9</b>	1.5
paynantheine						
100 nM	<b>69.4</b>	8.9	16.8	<b>53.8</b>	12.7	−9.8
10,000 nM	<b>98.7</b>	19.5	<b>92.8</b>	<b>101.5</b>	<b>89.3</b>	−0.8
9-hydroxycorynantheidine						
100 nM	0.2	3.0	−6.5	5.2	−10.4	−1.3
10,000 nM	29.6	−2.1	28.4	<b>71.3</b>	12.5	7.3
corynantheidine						
100 nM	2.5	−4.2	−6.8	14.8	2.5	6.2
10,000 nM	<b>77.3</b>	10.7	28.7	<b>76.3</b>	3.8	6.8
ajmalicine						
100 nM	33.2	21.6	5.1	6.2	−12.5	−2.5
10,000 nM	<b>95.2</b>	<b>98.3</b>	36.5	<b>89.3</b>	−5.3	1.8

<sup>a</sup>Bold fonts indicate  $\geq 50\%$  displacement of radioligands from the respective 5-HTR. Each experiment was conducted in duplicate. [<sup>3</sup>H]8-OH-DPAT, [<sup>125</sup>I]CYP (+ 30  $\mu$ M isoproterenol), and [<sup>3</sup>H]imipramine were used to label 5-HT<sub>1A</sub>Rs, 5-HT<sub>1B</sub>Rs, and the 5-HT transporter, respectively. [<sup>125</sup>I]( $\pm$ )DOI was used to label 5-HT<sub>2A</sub>(h), 5-HT<sub>2B</sub>(h), and 5-HT<sub>2C</sub>(h) receptors.

$E_{\max}$  = 47%), whereas speciociliatine, speciogynine, and paynantheine are low-potency, competitive antagonists at hMOR; none of the compounds show appreciable activity at

hKORs or hDORs.<sup>1</sup> However, the physiological effects of kratom use likely reflect the summed actions of multiple alkaloids at a range of receptor targets, which include MOR,  $\alpha$ -

**Table 2.**  $K_i$  Values (Mean  $\pm$  SEM in nM) of Mitragynine, Speciogynine, and Paynantheine at Human 5-HT<sub>1A</sub>, 5-HT<sub>7A</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, and 5-HT<sub>2C</sub>R Subtypes<sup>i</sup>

kratom alkaloid	binding site				
	5-HT <sub>1A</sub>	5-HT <sub>7A</sub>	5-HT <sub>2A</sub>	5-HT <sub>2B</sub>	5-HT <sub>2C</sub>
mitragynine	5880 $\pm$ 828 <sup>a</sup>	>10,000 <sup>f</sup>	5010 $\pm$ 1150 <sup>g</sup>	1260 $\pm$ 138 <sup>g</sup>	>10,000 <sup>g</sup>
speciogynine	38.5 $\pm$ 3.9 <sup>b</sup>	1600 $\pm$ 82 <sup>f</sup>	1320 $\pm$ 365 <sup>g</sup>	23.0 $\pm$ 5.7 <sup>h</sup>	5430 $\pm$ 922 <sup>g</sup>
paynantheine	95.5 $\pm$ 34.7 <sup>a</sup>			108 $\pm$ 39.5 <sup>g</sup>	
	32.0 $\pm$ 3.5 <sup>b</sup>	870 $\pm$ 72 <sup>f</sup>	815 $\pm$ 192 <sup>g</sup>	20.0 $\pm$ 2.8 <sup>h</sup>	1770 $\pm$ 417 <sup>g</sup>
	71.8 $\pm$ 13.2 <sup>a</sup>			83.1 $\pm$ 32.9 <sup>g</sup>	
9-O-desmethylspeciogynine (gambirine)	294.1 $\pm$ 189.1 <sup>a</sup>	ND	ND	508.1 $\pm$ 96.0 <sup>g</sup>	ND
9-O-desmethylpaynantheine (gambireine)	97.7 $\pm$ 44.2 <sup>a</sup>	ND	ND	335.8 $\pm$ 83.3 <sup>g</sup>	ND
ipsapirone	0.240 <sup>c</sup>	ND	ND	ND	ND
	49.7 $\pm$ 10.5 <sup>d</sup>				
8-OH-DPAT	0.75 $\pm$ 0.03 <sup>b</sup>	ND	ND	ND	ND
DOI	ND	ND	6.80 $\pm$ 1.00 <sup>g</sup>	2.20 $\pm$ 0.30 <sup>h</sup>	ND
WAY 100635	0.90 <sup>e</sup>	ND	ND	ND	ND
5-HT	4.50 $\pm$ 2.70 <sup>a</sup>	2.10 $\pm$ 0.50 <sup>f</sup>	17.2 $\pm$ 12.5 <sup>g</sup>	10.8 $\pm$ 2.60 <sup>g</sup>	ND
SB 206553	ND	ND	ND	ND	2.80 $\pm$ 0.30 <sup>g</sup>

<sup>a</sup>[<sup>3</sup>H]8-OH-DPAT binding to human 5-HT<sub>1A</sub>Rs expressed in CHO cells in the present study. <sup>b</sup>[<sup>3</sup>H]8-OH-DPAT binding to human 5-HT<sub>1A</sub>Rs expressed in HEK cells in the present study. <sup>c</sup>[<sup>3</sup>H]8-OH-DPAT binding to human 5-HT<sub>1A</sub>Rs expressed in COS-7 cells. <sup>d</sup>[<sup>3</sup>H]5-CT binding to human 5-HT<sub>1A</sub>Rs expressed in CHO cells in the present study. <sup>e</sup>[<sup>3</sup>H]8-OH-DPAT binding to human 5-HT<sub>1A</sub>Rs expressed in CHO cells. <sup>f</sup>[<sup>3</sup>H]5-CT binding to human 5-HT<sub>7A</sub>Rs expressed in HEK cells in the present study. <sup>g</sup>[<sup>3</sup>H]LSD binding to human 5-HT<sub>2A</sub>Rs, 5-HT<sub>2B</sub>Rs, or 5-HT<sub>2C</sub>Rs expressed in HEK cells in the present study. <sup>h</sup>[<sup>125</sup>I]DOI binding to human 5-HT<sub>2B</sub>Rs expressed in CHO cells in the present study. <sup>i</sup>Two (Eurofins) to three (Mercer University) independent experiments were conducted to calculate  $K_i$  values. In every experiment, compounds were tested at each concentration in duplicate (Eurofins) or triplicate (Mercer University). ND: not determined.

adrenergic, muscarinic, dopaminergic, and serotonergic receptors.<sup>8</sup> The affinity of some kratom alkaloids for adrenergic  $\alpha$  receptor subtypes has been reported previously.<sup>12</sup> Corynantheidine has moderate nanomolar affinity for adrenergic  $\alpha_{1D}$  receptors ( $K_i = 41$  nM), while mitragynine has micromolar affinity for adrenergic  $\alpha_{1A}$ ,  $\alpha_{1B}$ ,  $\alpha_{1C}$ ,  $\alpha_{2A}$ ,  $\alpha_{2B}$ , and  $\alpha_{2C}$  receptor subtypes.<sup>12</sup> A recent report, principally using in silico methodology,<sup>13</sup> extended those findings to predict (with some experimental corroboration) the affinities of kratom alkaloids at adrenergic ( $\alpha_{2A}$ ,  $\alpha_{2B}$ , and  $\alpha_{2C}$ ) receptors and serotonergic 5-HT<sub>1A</sub>Rs and 5-HT<sub>2A</sub>Rs.<sup>12</sup> The data suggested that speciogynine, mitragynine, and ajmalicine have affinity for the human 5-HT<sub>1A</sub> receptor orthosteric binding site ( $K_i$  values = 0.54, 5.8, and 0.42  $\mu$ M, respectively).<sup>13</sup> Contribution of descending serotonergic systems to the antinociceptive effects of mitragynine in mice has also been suggested.<sup>14</sup>

The current study reports experimentally obtained affinities for the major kratom alkaloids, mitragynine, speciociliatine, speciogynine, and paynantheine and minor available ones, 7-hydroxymitragynine, 9-hydroxycorynantheidine, corynantheidine, and ajmalicine (Figure 1) at serotonin receptor subtypes 5-HT<sub>1A</sub>, 5-HT<sub>1B</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>3</sub>, and 5-HT<sub>7</sub>.<sup>15</sup> Based on their relevant affinities, we also evaluated the in vitro 5-HT<sub>1A</sub> and 5-HT<sub>2B</sub> receptor functional activity of select kratom alkaloids. Finally, we evaluated the in vivo activity of select kratom alkaloids at 5-HT<sub>1A</sub> receptors by testing their effects on lower lip retraction and on antinociception using a hotplate assay<sup>16</sup> in rats pretreated with a vehicle or the selective 5-HT<sub>1A</sub>R antagonist WAY 100635.

The independent pharmacokinetic profiles of speciogynine and paynantheine were evaluated following a single dose (i.v. administration) to rats. These pharmacokinetic parameters were compared to those of mitragynine to understand their

corresponding clearance and overall contributions to the pharmacological activity of kratom products.

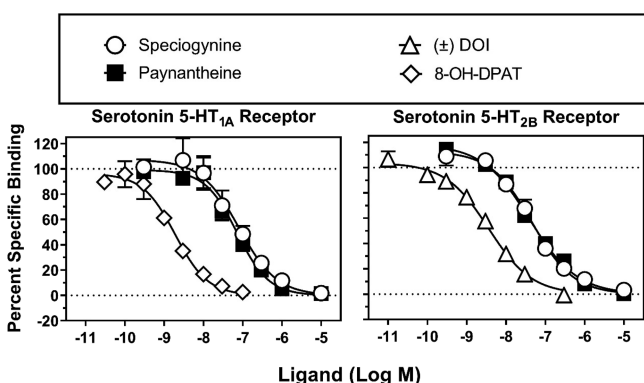
## RESULTS AND DISCUSSION

Affinity screens of select kratom alkaloids at recombinant human 5-HTRs expressed in human embryonic kidney 293 (HEK-293) cell membranes were determined by radioligand competition binding using 100 nM and 10  $\mu$ M concentrations (Table 1). Radioligand probe “displacement” values of 50% or greater at 100 nM were considered significant “hits”, and  $K_i$  values of hits were subsequently determined.  $K_i$  values of mitragynine at the 5-HT receptors were also determined, regardless of affinity screen results, as mitragynine is the most prevalent alkaloid in kratom and is often extracted from the plant and used in scientific studies independent from the other kratom alkaloids.

At 100 nM, only speciogynine and paynantheine displaced a significant percentage (58.9 and 69.4%, respectively) of [<sup>3</sup>H]8-OH-DPAT [(±)-8-hydroxy-2-(dipropylamino)tetralin] bound to 5-HT<sub>1A</sub>Rs (Table 1), while 10  $\mu$ M concentrations of mitragynine, speciogynine, paynantheine, corynantheidine, and ajmalicine displaced 76.7, 98.7, 98.7, 77.3, and 95.2% (respectively) of [<sup>3</sup>H]8-OH-DPAT bound to 5-HT<sub>1A</sub>Rs. As ligand affinities at 5-HT<sub>1A</sub>Rs and 5-HT<sub>7</sub>Rs often correlate,<sup>17</sup> we also determined  $K_i$  values of speciogynine and paynantheine at agonist-labeled, [<sup>3</sup>H]5-CT [3-(2-aminoethyl-1,2-*t*<sub>2</sub>)-1*H*-indole-5-carboxamide], human 5-HT<sub>7A</sub> receptors expressed in HEK-293 cells (Table 4). Alkaloid binding to recombinant 5-HT<sub>1B</sub>Rs was assessed using [<sup>125</sup>I]CYP [4-2-(hydroxy-3-(*tert*-butylamino)propoxy)-3-iodo-1*H*-indole-2-carbonitrile]; no significant radioligand displacement was observed for the kratom alkaloids tested, with the exception of ajmalicine at 10  $\mu$ M (Table 1). Similar to what we observed at 5-HT<sub>1A</sub>Rs, 100 nM concentrations of speciogynine and paynantheine displaced, respectively, 70.9 and 53.8% of [<sup>125</sup>I]2,5-dimethoxy-4-iodoamphetamine ([<sup>125</sup>I]DOI) binding to recombinant, human 5-

HT<sub>2B</sub> receptors expressed in Chinese hamster ovary K1 (CHO-K1) cells. Other alkaloids including mitragynine, 9-hydroxycorynantheidine, corynantheidine, and ajmalicine displaced  $\geq 50\%$  of [<sup>125</sup>I]DOI from 5-HT<sub>2B</sub>Rs only at 10  $\mu\text{M}$  concentrations. As ligand affinities at 5-HT<sub>2B</sub>Rs, 5-HT<sub>2A</sub>Rs, and 5-HT<sub>2C</sub>Rs often correlate,<sup>18</sup> we determined  $K_i$  values of speciogynine and paynantheine at agonist-labeled, [<sup>3</sup>H]LSD [(6aR,9R)-N,N-diethyl-7-(methyl-*t*<sub>3</sub>)-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide], human 5-HT<sub>2A</sub>Rs, and 5-HT<sub>2C</sub>Rs expressed in HEK-293 cells (Table 2). None of the alkaloids bound to 5-HT<sub>3</sub>Rs at concentrations of 10  $\mu\text{M}$  (Table 1).

$K_i$  values of mitragynine, speciogynine, and paynantheine at human 5-HT<sub>1A</sub>, 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, 5-HT<sub>2C</sub>, and 5-HT<sub>7</sub> receptors are shown in Table 2, along with  $K_i$  values of positive controls assessed in parallel. Speciogynine and paynantheine exhibited moderate ( $\sim 100$  nM or less) affinity at 5-HT<sub>1A</sub> and 5-HT<sub>2B</sub> receptors (Figure 2 and supplementary



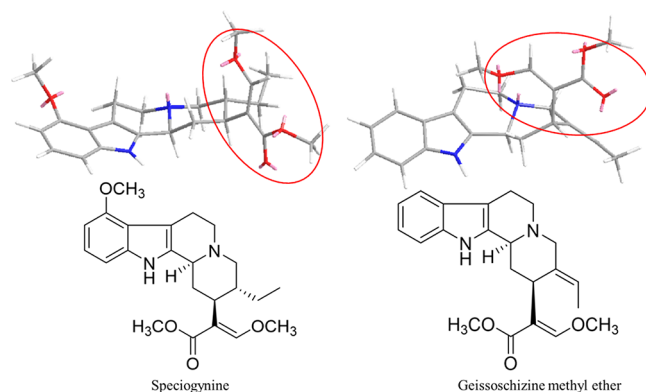
**Figure 2.** Radioligand competition binding at human 5-HT<sub>1A</sub>Rs and 5-HT<sub>2B</sub>Rs. Ordinates: percent specific binding of the radioligand to receptors expressed in cell membranes (described in the Experimental Section). Abscissae: concentrations of test ligands (log scale). The left panel shows percent specific binding of [<sup>3</sup>H]8-OH-DPAT at 5-HT<sub>1A</sub>Rs. The right panel shows percent specific binding of [<sup>125</sup>I]DOI at 5-HT<sub>2B</sub>Rs. These experiments were conducted in duplicate and repeated twice ( $N = 2$ ), and the data shown were obtained by Eurofins. We conducted additional affinity tests to validate these results, and the binding curves are shown in the Supporting Information (Figure S21).

Figure S21 show full concentration–response curves). The next highest affinities were observed for paynantheine at 5-HT<sub>7</sub>Rs and 5-HT<sub>2A</sub>Rs, though they were relatively low, i.e., 870 and 815 nM, respectively.  $K_i$  values of mitragynine, speciogynine, and paynantheine determined at each of the

other 5-HTRs were  $>1.2$   $\mu\text{M}$  (Table 2). The affinity of speciogynine for 5-HT<sub>1A</sub>Rs was  $>10$ -fold higher than previously reported (540 nM; the species from which the 5-HT<sub>1A</sub>R was derived was unspecified).<sup>13</sup>

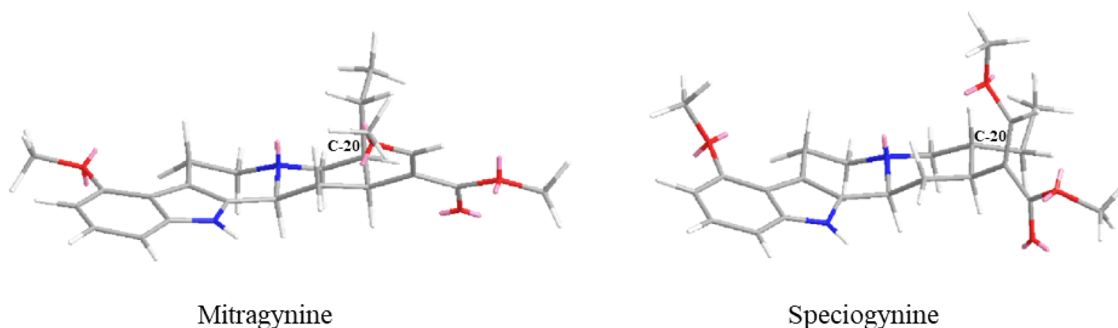
Structurally, speciogynine and mitragynine are diastereomers with different configurations at C-20, meanwhile paynantheine has the same configuration as speciogynine (speciogynine is 3S, 15S, 20R; paynantheine is 3S, 15S, 20R; mitragynine is 3S, 15S, 20S). This primary configurational difference at the C-20 position makes the  $\beta$ -methoxyacrylate group in speciogynine and paynantheine perpendicular to the molecule's plane, whereas the substituent at C-20 in mitragynine is planar (see Figure 3).

It is noteworthy here that a structurally related natural product, geissoschizine methyl ether, produced by the plant *Uncaria rhynchopilla* (yokukansan), is a Japanese traditional medicine used to treat irritability and insomnia in children and disorders such as psychosis, dementia, and anxiety. Geissoschizine methyl ether is a potent antagonist at 5-HT<sub>1A</sub> receptors<sup>19,21,22</sup> and exhibits a similar free  $\beta$ -methoxyacrylate group to that of speciogynine, the motif that significantly increases the affinity of speciogynine at 5-HT<sub>1A</sub>Rs relative to mitragynine (Figure 4).



**Figure 4.** Two- and three-dimensional chemical structures for speciogynine and geissoschizine methyl ether.

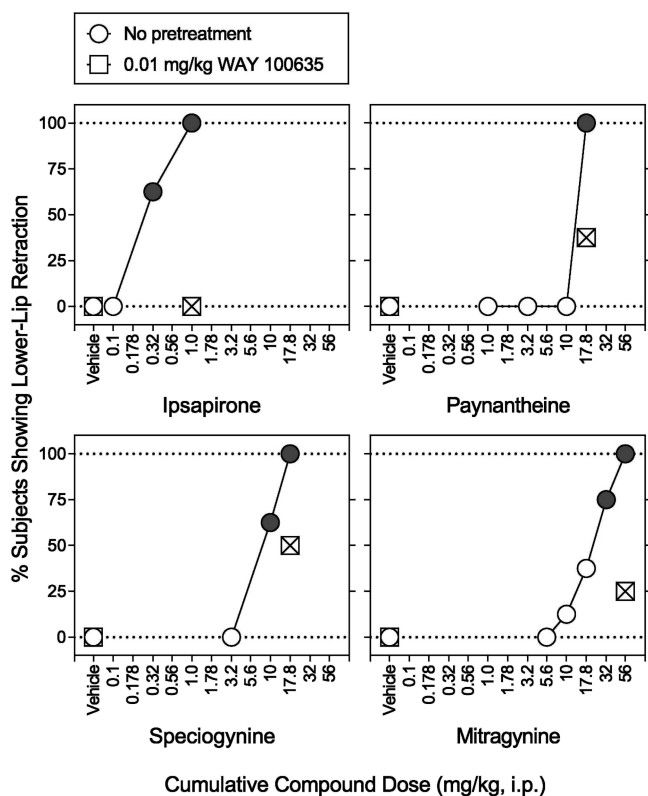
As kratom reportedly can produce anxiolytic effects—which can be elicited by 5-HT<sub>1A</sub>R activation—we evaluated the in vivo 5-HT<sub>1A</sub>R activity of mitragynine, speciogynine, and paynantheine using the lower-lip retraction assay in rats, a behavior elicited by 5-HT<sub>1A</sub>R activation and blocked by 5-HT<sub>1A</sub>R antagonists.<sup>23</sup> Ipsapirone, a 5-HT<sub>1A</sub>R agonist, and the selective 5-HT<sub>1A</sub>R antagonist WAY 100635 were used to



**Figure 3.** Three-dimensional structure comparison of mitragynine and speciogynine (most stable conformation, Chemdraw3D).



confirm in vivo 5-HT<sub>1A</sub>R-dependent effects in the assay. Intraperitoneal (i.p.) administration of ipsapirone dose-dependently increased the percent of animals showing lower lip retraction (100% at 1.0 mg/kg) (Figure 5, top left panel,



**Figure 5.** Effects of i.p. administration of various compounds alone and in combination with the 5-HT<sub>1A</sub>R antagonist WAY 100635 on expression of lower lip retraction in rats. Abscissae: vehicle and cumulative compound dose in mg/kg (i.p., log scale). Ordinates: percentage of subjects showing lower lip retraction. Each point represents the mean  $\pm$  SEM ( $N = 8$ , four subjects per sex). No lower lip retraction was observed during each baseline measurement. Following the baseline measurement, a vehicle was administered i.p. 60 min prior to the next measurement (open circles). Following the second measurement, each lowest dose of test compounds was administered. Cumulative doses of each test compound were administered every 15 min prior to each measurement. Note that ipsapirone, paynantheine, speciogynine, and mitragynine produced 100% lower lip retraction. Each gray symbol indicates a significant difference from the respective vehicle. Using separate groups of rats, sensitivity of respective expression of lower lip retraction to WAY 100635 (0.01 mg/kg) was assessed. Following the baseline measurement, WAY 100635 (0.01 mg/kg) and then one of the highest doses of test compounds were administered i.p. 60 and 15 min, respectively, prior to measurement (squares). Behaviorally toxic effects were observed in a pilot observational study of higher doses. WAY 100635 significantly antagonized lower lip retraction produced by each test compound. Each square with cross hatch indicates a significant difference from the respective highest dose of test compounds. Details for statistical analyses are shown in Table 3 and Tables S2 and S3.

circles). Details for statistical analyses are shown in Tables S2 and S3. The ED<sub>50</sub> value (95% confidence intervals) of ipsapirone to produce lower lip retraction was 0.431 (0.261–0.585) mg/kg (Table 3), and there was no sex difference in ED<sub>50</sub> values (data not shown). Administration of 0.01 mg/kg WAY 100635 (i.p.) completely eliminated the lower lip

**Table 3.** ED<sub>50</sub> Values (95% Confidence Intervals) for the Induction of Lower Lip Retraction and Antinociceptive Effects of Test Compounds as Shown in Figures 5 and 8<sup>a</sup>

test compound	ED <sub>50</sub> (95% confidence intervals) in mg/kg	
	lower lip retraction (i.p.)	hotplate (i.v.)
ipsapirone	0.431 (0.261–0.585)	3.41 (1.80–5.22)
ipsapirone in the presence of WAY 100635	not assessed	7.68 (5.72–9.59)
paynantheine	12.7 (11.3–14.4)	5.45 (4.51–6.48)
paynantheine in the presence of WAY 100635	not testable	not testable
speciogynine	9.10 (6.97–11.0)	5.92 (4.13–8.35)
speciogynine in the presence of WAY 100635	not testable	11.6 (9.88–13.5)
mitragynine	26.8 (21.2–32.9)	not testable

<sup>a</sup>The sample sizes are described in each figure legend. Each value represents data collected from males and females combined.

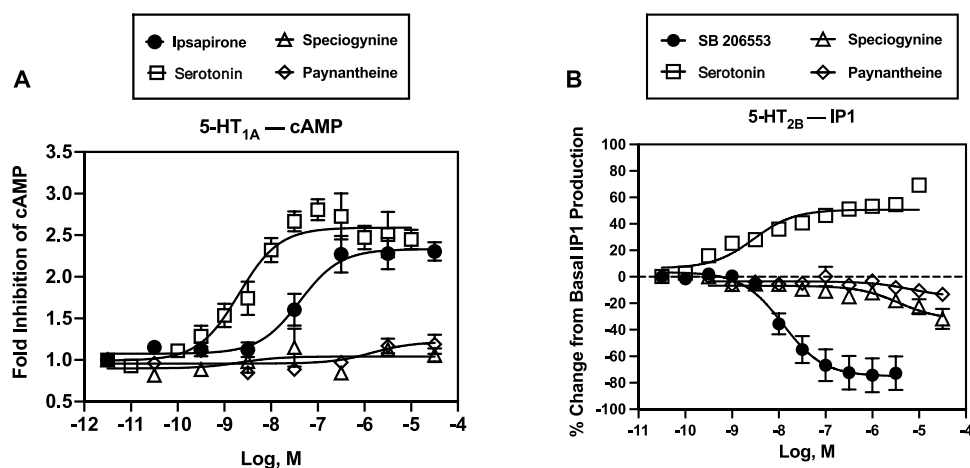
retraction produced by 1.0 mg/kg dose of ipsapirone (Figure 5, top left panel, squares with cross hatch; Table S3), confirming the involvement of 5-HT<sub>1A</sub>R in this assay. There was no significant interaction of sex with ipsapirone dose  $\times$  WAY 100635 (Table S3).

Like ipsapirone, paynantheine (17.8 mg/kg i.p.) produced lower lip retraction in 100% of animals tested (Figure 5, circles; Table S2). In the presence of 0.01 mg/kg WAY 100635, the 17.8 mg/kg dose of paynantheine resulted in 37.5% subjects showing lower lip retraction (two out of four females and one out of four males) (Figure 5, top right panel, squares with cross hatch; Table S3). These data suggest that paynantheine functions, in part, as a 5-HT<sub>1A</sub>R agonist in vivo. There was no significant interaction of sex with paynantheine dose or paynantheine dose  $\times$  WAY 100635 (Tables S2 and S3).

Similar to paynantheine, speciogynine (i.p.) at 17.8 mg/kg elicited lower lip retraction in 100% of animals tested (Figure 5, lower left panel, circles; Table S2). In the presence of 0.01 mg/kg WAY 100635, the 17.8 mg/kg dose of speciogynine resulted in half of subjects (i.e., 50%) showing lower lip retraction (Figure 5, lower left panel, squares with cross hatch; Table S3). Thus, speciogynine, like paynantheine, showed effects in vivo suggestive of 5-HT<sub>1A</sub>R activation. There was no significant interaction of sex with the speciogynine dose or speciogynine dose  $\times$  WAY 100635 (Tables S2 and S3).

Mitragynine (i.p.) at 56 mg/kg elicited lower lip retraction in 100% of animals tested (Figure 5, lower right panel, circles; Table S2). In the presence of 0.01 mg/kg WAY 100635, the 56 mg/kg dose of mitragynine elicited lower lip retraction in 25% of animals tested (two out of four females and zero out of four males) (Figure 5, lower right panel, squares with cross hatch; Table S3). These data suggest mitragynine likely behaves as a 5-HT<sub>1A</sub>R agonist in vivo, though with substantially lower potency than speciogynine and paynantheine, paralleling mitragynine's much lower affinity at 5-HT<sub>1A</sub>R. There was no significant interaction of sex with the mitragynine dose or mitragynine dose  $\times$  WAY 100635 (Tables S2 and S3).

We also evaluated paynantheine's and speciogynine's in vitro functional activity at human 5-HT<sub>1A</sub>R in a luminescent cAMP assay. Neither alkaloid activated or inactivated 5-HT<sub>1A</sub>R; 5-



**Figure 6.** In vitro function of speciogynine and paynantheine at human 5-HT<sub>1A</sub>Rs (A) and 5-HT<sub>2B</sub>Rs (B). Abscissae: concentrations of test compounds (molar, log scale). Ordinates: (A) serotonin and ipsapirone activated 5-HT<sub>1A</sub>Rs as measured by inhibition of forskolin-stimulated production of cAMP, whereas speciogynine and paynantheine showed no significant functional effects. (B) Serotonin activated 5-HT<sub>2B</sub>Rs as measured by increased production of inositol phosphate 1 (IP1), whereas SB 206553 inactivated 5-HT<sub>2B</sub>Rs; speciogynine and paynantheine showed no significant functional effects. Each symbol represents the mean  $\pm$  SEM from at least three independent determinations.

HT and ipsapirone were tested in parallel as positive controls (Figure 6). The discrepancy observed between in vivo and in vitro results led us to hypothesize that paynantheine and speciogynine are metabolized to alkaloids that are 5-HT<sub>1A</sub>R agonists. As shown in Figure 7 and Table 4, 9-*O*-desmethylpaynantheine (gambireine) and 9-*O*-desmethylspeciogynine (gambirine), a metabolite of paynantheine and speciogynine, respectively, behaved as fully efficacious 5-HT<sub>1A</sub>R agonists. Also shown in Figure 7 and Tables 2 and 4, affinities and potencies of both metabolites at 5-HT<sub>1A</sub>Rs were similar, though lower, than those of the parent compounds. These data provide, for the first time, evidence that metabolites of kratom alkaloids, in addition to 7-hydroxymitragynine, may have in vivo pharmacological activity.

Chronic 5-HT<sub>2B</sub>R activation has been linked with valvular heart disease,<sup>24,25</sup> and so the binding of speciogynine, paynantheine, and their 9-*O*-desmethyl metabolites to 5-HT<sub>2B</sub>R raised the question of a potential cardiovascular risk to chronic kratom users. To evaluate this concern, we examined the functional activity of these alkaloids using a 5-HT<sub>2B</sub>R-phosphoinositide hydrolysis assay with 5-HT and SB 206553 as the positive and inverse agonist controls (respectively, Figure 6B). Speciogynine and paynantheine showed no agonist activity at human 5-HT<sub>2B</sub>Rs, while 9-*O*-desmethylpaynantheine and 9-*O*-desmethylspeciogynine metabolites clearly demonstrated an inverse partial agonist action (Figure 7D). These data suggest that speciogynine, paynantheine, and their 9-*O*-desmethyl metabolites are unlikely to induce the 5-HT<sub>2B</sub>R-mediated cell proliferation linked to cardiac valvulopathy.

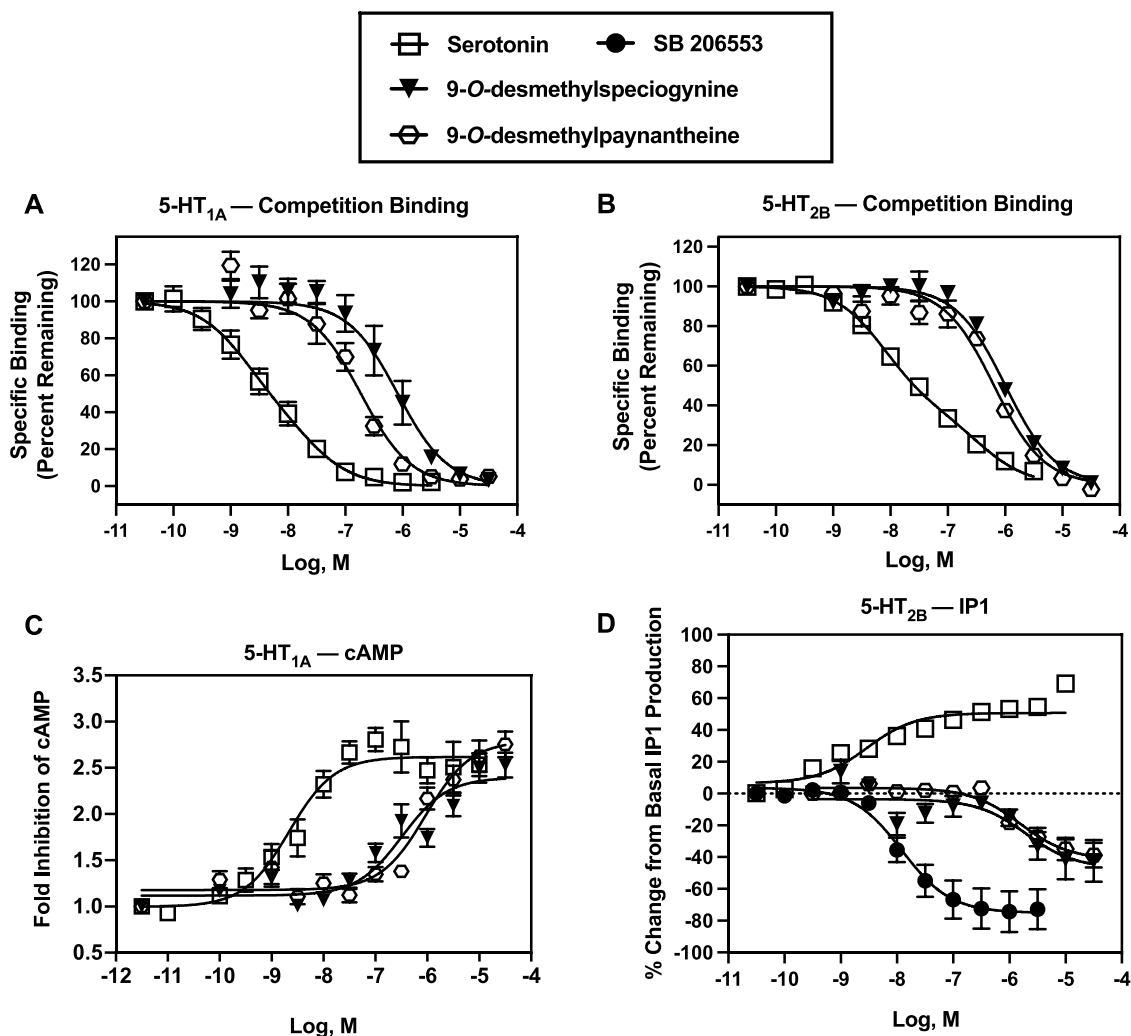
Using the hotplate assay, cumulative mitragynine doses administered 5 min apart (up to 10 mg/kg, i.v.) did not produce antinociception, and 32 mg/kg i.v. mitragynine was lethal in our previous study.<sup>12</sup> The lack of antinociceptive effects of mitragynine when cumulatively administered i.v. in a shorter (5 min) interval in the previous study<sup>12</sup> may reflect less conversion of mitragynine to an active metabolite. Indeed, the CYP-450 3A metabolite 7-hydroxymitragynine was 108-fold more potent than the parent compound mitragynine in producing morphine-like discriminative-stimulus effects in rats.<sup>11,15,26,27</sup>

In contrast to i.v. mitragynine,<sup>12</sup> a 10 mg/kg dose of i.v. ipsapirone produced 87.4% (8.78%) of the percentage of maximum possible effect in the hotplate assay; the antinociception dose-effect function of ipsapirone was shifted to the right 2.3-fold by WAY 100635 (0.01 mg/kg, i.p.) (Figure 8, squares; Table S4). Details for statistical analyses are shown in Table S4. The ED<sub>50</sub> value of i.v. ipsapirone to produce antinociception was 3.41 (1.80–5.22) mg/kg (Table 3). Thus, the present results suggest that the antinociceptive effects of ipsapirone are 5-HT<sub>1A</sub>R mediated.

As with i.v. ipsapirone, a 10 mg/kg dose of i.v. paynantheine produced antinociceptive effects that were sensitive to WAY 100635 (0.01 mg/kg) (Figure 8, squares; Table S4). The ED<sub>50</sub> value of i.v. paynantheine to produce antinociception was 5.45 (4.51–6.48) mg/kg (Table 3). Similar to i.v. ipsapirone and i.v. paynantheine, i.v. speciogynine produced antinociception in a manner sensitive to WAY 100635 (Figure 8, squares; Table S4). The ED<sub>50</sub> value of i.v. speciogynine to produce antinociception was 5.92 (4.13–8.35) mg/kg (Table 3). Collectively, the present results suggest that the apparent antinociceptive effects of paynantheine and speciogynine may be mediated by stimulation of 5-HT<sub>1A</sub>Rs.

The WAY 100635-reversible lower lip retraction illustrates involvement of 5-HT<sub>1A</sub>R in the in vivo effects of ipsapirone, paynantheine, speciogynine, and mitragynine. Though K<sub>i</sub> values at 5-HT<sub>1A</sub>R did not significantly correlate with the ED<sub>50</sub> values for lower-lip retraction or antinociception, the correlation approached significance ( $P = 0.0508$ , Figure 9); this may be due to the low sample size ( $n = 4$ ) chosen in the experimental design. Given the apparently similar affinity of corynantheidine and ajmalicine at 5-HT<sub>1A</sub>Rs to the alkaloids evaluated here (Table 1), future in vitro 5-HT<sub>1A</sub>R and in vivo lower lip retraction studies with these two alkaloids may also be warranted.

In vivo pharmacokinetic studies were performed to assess the systemic exposure, distribution, and elimination of i.v. paynantheine and speciogynine (Figure 10 and Table 5). Single-dose pharmacokinetic studies of paynantheine (2.5 mg/kg,  $n = 4$ ) and speciogynine (1.25 mg/kg,  $n = 3$ ) were performed in male Sprague Dawley rats. Blood samples (100  $\mu$ L) were collected at pre-dose and 0.08, 0.25, 0.5, 0.75, 1, 2, 4,



**Figure 7.** In vitro binding and function at human 5-HT<sub>1A</sub>Rs (A, C) and 5-HT<sub>2B</sub>Rs (B, D). Abscissae: concentrations of test compounds (molar, log scale). Ordinates: (A) 5-HT<sub>1A</sub>R competition binding with [<sup>3</sup>H]8-OH-DPAT. (B) 5-HT<sub>2B</sub>R competition binding with [<sup>3</sup>H]LSD. Note that serotonin data from both 5-HT<sub>1A</sub>R [<sup>3</sup>H]8-OH-DPAT and 5-HT<sub>2B</sub>R [<sup>3</sup>H]LSD competition binding fit best to a two-site model, relative to a one-site model (*P* values: <0.05). *K<sub>i</sub>* values for serotonin at 5-HT<sub>1A</sub>Rs were 0.41 nM (high affinity) and 6.7 nM (low affinity), and *K<sub>i</sub>* values for serotonin at 5-HT<sub>2B</sub>Rs were 5.0 nM (high affinity) and 230 nM (low affinity); *R*<sup>2</sup> values were 0.726 and 0.924, respectively. The *K<sub>i</sub>* results for serotonin at 5-HT<sub>1A</sub> and 5-HT<sub>2B</sub>Rs reported in Table 2 of the manuscript are from one-site analyses, wherein *R*<sup>2</sup> values were 0.715 and 0.895, respectively. (C) Serotonin, 9-O-desmethypaynantheine, and 9-O-desmethyspeciogynine activated 5-HT<sub>1A</sub>Rs as measured by inhibition of forskolin-stimulated production of cAMP. (D) Serotonin activated 5-HT<sub>2B</sub>Rs as measured by increased production of IP1, whereas SB 206553, 9-O-desmethypaynantheine, and 9-O-desmethyspeciogynine inactivated 5-HT<sub>2B</sub>Rs. Table 4 shows the pIC/EC<sub>50</sub> and *E*/*I*<sub>max</sub> values. Each data point represents the mean (±SEM) of at least three independent experiments. In each experiment, serotonin and SB 206553 were tested in duplicate while the kratom alkaloids were tested in triplicate.

8, 12, 18, and 24 h post-dose. The plasma concentration–time profiles for each alkaloid are shown in Figure 10, and pharmacokinetic parameters are shown in Table 5.

The concentration–time profiles of paynantheine and speciogynine showed biexponential decay, which is typical of i.v. administered drugs. Like mitragynine, both paynantheine and speciogynine exhibited a volume of distribution (*V<sub>d</sub>*) greater than the rat blood volume (0.085 L/kg),<sup>29</sup> indicating extensive tissue distribution. Both paynantheine and speciogynine were cleared from systemic circulation more rapidly than mitragynine so that when exposure was normalized for dose (AUC<sub>inf</sub>/dose), mitragynine exposure was 3.6-fold higher than paynantheine and 1.7-fold higher than speciogynine. It is worth noting that i.p. administration was used in the in vivo studies where paynantheine and speciogynine exhibited 5-HT<sub>1A</sub>R-mediated effects, an administration route that is subject to first-

pass hepatic metabolism (unlike i.v. administration) and so may well produce higher metabolite levels. Further pharmacokinetic studies examining 9-desmethylmetabolite formation following i.p. and p.o. administration of paynantheine and speciogynine are warranted given our discovery that these metabolites are more efficacious than the parent molecules as 5-HT<sub>1A</sub>R agonists.

## CONCLUSIONS

Speciogynine and paynantheine comprise approximately 15% of the total alkaloid content of kratom.<sup>2,7</sup> This study demonstrates that these alkaloids have a high binding affinity for 5-HT<sub>1A</sub>R and 5-HT<sub>2B</sub>Rs (relative to mitragynine, the predominant alkaloid in kratom) but do not activate canonical G-protein mediated signal transduction through these receptors. The results from 5-HT<sub>1A</sub>R functional assays were



**Table 4. Potencies ( $EC_{50}$  or  $IC_{50} \pm SEM$  in nM) and Efficacies ( $E_{max}$  or  $I_{max} \pm SEM$ ) of Test Compounds at Human  $5-HT_{1A}$  and  $5-HT_{2B}$ Rs<sup>a</sup>**

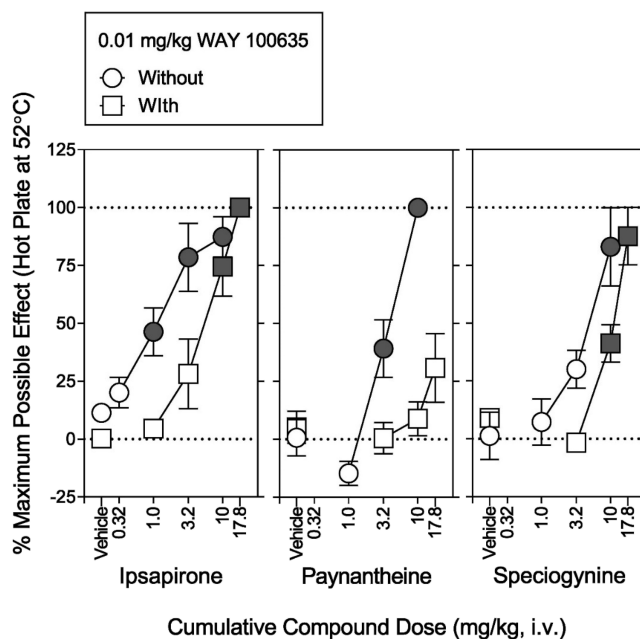
compounds	$5-HT_{1A}$		$5-HT_{2B}$	
	IC/ $EC_{50}$	$E/I_{max}$	IC/ $EC_{50}$	$E/I_{max}$
speciogygine	N.A.	N.A.	2016 (1888)	24.8 (6.9)
paynantheine	N.A.	N.A.	N.A.	N.A.
9-O-desmethylspeciogygine (gambirine)	837.6 (253.9)	99 (0.08)	1472 (731.6)	45 (14.3)
9-O-desmethylpaynantheine (gambireine)	865.4 (126.4)	100 (0.08)	2339 (499.7)	38 (10.3)
mitragynine	N.A.	N.A.	N.T.	N.T.
serotonin	5.194 (2.5)	100	31.31 (20.3)	100
ipsapirone	67.10 (44.7)	82 (0.1)	N.T.	N.T.
SB 206553	N.T.	N.T.	15.33 (4.89)	100

<sup>a</sup> $E_{max}$  at  $5-HT_{1A}$ Rs is the percent maximum serotonin, agonist, response;  $I_{max}$  at  $5-HT_{2B}$ Rs is the percent maximum SB 206553, inverse agonist, response. N.T. = not tested. N.A. = no activation or inactivation up to 30  $\mu M$ .

surprising as we also found that speciogygine and paynantheine exert in vivo pharmacological effects consistent with  $5-HT_{1A}$ R activation (effects blocked in the presence of a selective  $5-HT_{1A}$ R antagonist). These apparently paradoxical findings are perhaps reconcilable through our discovery that paynantheine and speciogygine 9-O-desmethyl metabolites also bind to  $5-HT_{1A}$ Rs and  $5-HT_{2B}$ Rs and are efficacious at stimulating  $5-HT_{1A}$ Rs and inhibiting  $5-HT_{2B}$ Rs. As neither parent nor metabolites were agonists at  $5-HT_{2B}$ Rs, they are unlikely to induce cardiac valvulopathy. Our results suggest that just as mitragynine's metabolism to 7-hydroxymitragynine results in greater activity at  $\mu$ -opioid receptors,<sup>11</sup> paynantheine's and speciogygine's activity at  $5-HT_{1A}$ Rs and  $5-HT_{2B}$ Rs may be principally mediated via their metabolites, 9-O-desmethylspeciogygine and 9-O-desmethylpaynantheine. Future studies may clarify whether the serotonergic activity of these metabolites contributes to the analgesic, anxiolytic, or antidepressant effects reported by kratom users.<sup>30,31</sup>

## EXPERIMENTAL SECTION

**General Chemistry.** The compounds were available in our alkaloid library and were isolated and structurally elucidated through <sup>1</sup>H and <sup>13</sup>C NMR using a Bruker model Avance NEO 600 NMR spectrometer operating at 600 MHz in <sup>1</sup>H and 151 MHz in <sup>13</sup>C (Bruker Co, Billerica, MA, USA). High-resolution mass spectrometry HRMS and purity ( $\geq 98\%$ ) were determined using an Agilent 1290 Infinity series ultraperformance liquid chromatography (UPLC) system equipped with a photodiode array detector and quadrupole-time-of-flight (QTOF) Agilent 6540 mass spectrometer (Agilent Co, Santa Clara, CA, USA). The data was acquired by MassHunter data acquisition software (version B.09.00). The samples at a 10  $\mu M$  concentration were injected onto a Waters Acquity UPLC BEH C18 column (1.7  $\mu m$ , 2.1  $\times$  100 mm). The gradient elution was performed using a mobile phase of 10 mM ammonium acetate (Sigma Aldrich Co., St. Louis, MO) buffer pH 3.5 (solvent A) and acetonitrile (solvent B, Sigma Aldrich Co.) at a flow rate of 0.5 mL/min. The gradient was started at 10% B and maintained till 1 min, then linearly increased to 90% B till 3 min, maintained at 90% B till 3.5 min, then decreased to 10% B from 3.5 to 4 min, and maintained at 10% B till 5 min. The column oven and autosampler temperatures were maintained at 45 and 4  $^{\circ}C$ , respectively. The HPLC purity of samples was determined using the UV chromatogram extracted at a

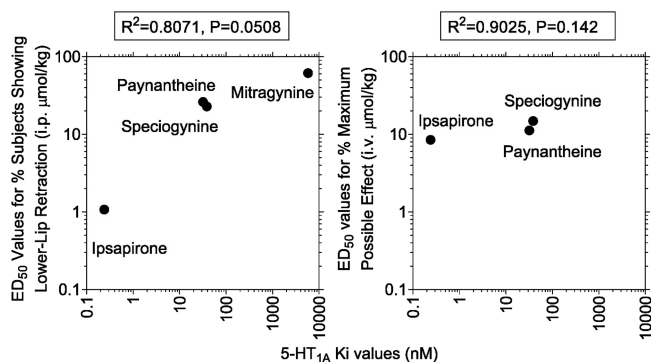


**Figure 8.** Effects of i.v. injection of ipsapirone, paynantheine, and speciogygine on hotplate latency in catheterized rats. The catheterized rats differ from rats in Figure 5. Abscissae: vehicle, cumulative compound dose in mg/kg (i.v., log scale). Ordinates: percentage of maximum possible effects. The hotplate was maintained at  $52 \pm 0.1$   $^{\circ}C$ . Each symbol represents the mean  $\pm$  SEM ( $N = 6$ , three subjects per sex). In baseline values across six groups (ipsapirone alone and in combination with WAY 100635, paynantheine alone and in combination with WAY 100635, and speciogygine alone and in combination with WAY 100635), there was no significant difference of the treatment group ( $F_{5,24} = 1.44$ ;  $P = 0.245$ ), sex ( $F_{1,24} = 3.16$ ;  $P = 0.088$ ), and interaction of the two ( $F_{5,24} = 1.54$ ;  $P = 0.215$ ). Following the baseline measurement, the vehicle was administered i.p. 60 min prior to the next measurement (open squares). Following the second measurement, cumulative doses were sequentially administered. Cumulative doses of each test compound were administered every 5 min prior to each measurement. Note that ipsapirone, paynantheine, and speciogygine significantly increased the maximum possible effect. Each gray symbol indicates a significant difference from the respective vehicle. Using separate groups of rats, sensitivity of respective antinociceptive effects to WAY 100635 (0.01 mg/kg) was assessed. Following the baseline measurement, WAY 100635 (0.01 mg/kg) was administered i.p. 60 min prior to measurement (squares). WAY 100635 shifted to the right dose-effect functions of each test compound. Paynantheine (32 mg/kg) in the presence of WAY 100635 caused lethality in all rats tested. Details for statistical analyses are shown in Table 3 and Table S4.

254 nm wavelength. The high-resolution mass spectrometry chromatograms for each compound were extracted as base peak chromatograms at respective molecular ( $M + H$ )<sup>+</sup> ion peaks. The isolation of mitragynine, speciociliatine, speciogygine, paynantheine, corynantheidine, and ajmalicine were conducted using a procedure described previously.<sup>32</sup> 7-Hydroxymitragynine and 9-hydroxycorynantheidine were obtained by semi-synthesis from mitragynine using published methods.<sup>12</sup> The detailed characterization and purity determination of corynantheidine, 9-hydroxycorynantheidine, mitragynine, 7-hydroxymitragynine, and speciociliatine have been reported previously.<sup>12</sup> The detailed characterization of ajmalicine, 9-O-desmethylpaynantheine, 9-O-desmethylspeciogygine, paynantheine, and speciogygine are shown below; the <sup>1</sup>H and <sup>13</sup>C NMR spectra, HR-MS spectra, and UPLC-UV chromatograms are shown in the Figures S1–S20 (Supporting information).

*Methyl(4S,4aR,13bS,14aS)-4-methyl-4a,5,7,8,13,13b,14,14a-octahydro-4H-indolo[2,3-a]pyrano[3,4-g]quinolizine-1-carboxylate*





**Figure 9.** Correlations between in vitro 5-HT<sub>1A</sub>R affinity ( $K_i$  values) and potency to produce lower lip retraction and antinociception. Ordinates: (left panel) ED<sub>50</sub> value in  $\mu\text{mol/kg}$  (i.p.) to produce lower lip retraction; (right panel) ED<sub>50</sub> value in  $\mu\text{mol/kg}$  (i.v.) to produce antinociception. Abscissae:  $K_i$  values at 5-HT<sub>1A</sub>Rs in nM. (Left panel) The  $R^2$  value was 0.807 ( $P = 0.0508$ ). (Lower panels) The  $R^2$  values were 0.9025 ( $P = 0.142$ ).

(Ajmalicine). <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>):  $\delta$  9.20 (s, 1H), 7.54 (d,  $J = 1.8$  Hz, 1H), 7.48–7.43 (m, 1H), 7.34 (d,  $J = 8.0$  Hz, 1H), 7.12 (ddd,  $J = 8.2, 7.1, 1.1$  Hz, 1H), 7.09–7.03 (m, 1H), 4.43 (dd,  $J = 6.7, 4.0$  Hz, 1H), 3.73 (s, 3H), 3.38 (dq,  $J = 11.3, 2.3$  Hz, 1H), 3.24 (dt,  $J = 12.6, 3.1$  Hz, 1H), 3.10 (dd,  $J = 11.2, 5.9$  Hz, 1H), 3.05–2.98 (m, 1H), 2.95 (dd,  $J = 10.7, 3.0$  Hz, 1H), 2.79–2.73 (m, 1H), 2.67 (td,  $J = 11.2, 4.4$  Hz, 1H), 2.43–2.35 (m, 1H), 2.22 (t,  $J = 11.0$  Hz, 1H), 2.16–2.09 (m, 1H), 1.28 (q,  $J = 11.8$  Hz, 1H), 1.18 (d,  $J = 6.6$  Hz, 3H). <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>):  $\delta$  167.86, 155.07, 136.30, 134.35, 127.01, 121.14, 119.00, 117.88, 111.01, 107.18, 106.42, 73.71, 60.26, 56.65, 53.16, 51.00, 40.58, 32.37, 30.44, 21.57, 14.86. NMR data align with a previous report.<sup>33</sup> HRMS (ESI<sup>+</sup>) calculated for C<sub>21</sub>H<sub>25</sub>N<sub>2</sub>O<sub>3</sub><sup>+</sup> [M + H]<sup>+</sup>: 353.1860, found: 353.1859. HPLC purity: >99%.

Methyl(E)-3-methoxy-2-((2S,3R,12bS)-8-methoxy-3-vinyl-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-a]quinolizin-2-yl)acrylate (Paynantheine). <sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>):  $\delta$  8.00 (s, 1H), 7.35 (s, 1H), 7.01 (t,  $J = 7.9$  Hz, 1H), 6.89 (d,  $J = 8.0$  Hz, 1H), 6.47 (d,  $J = 7.7$  Hz, 1H), 5.68–5.53 (m, 1H), 5.02 (ddd,  $J = 17.2, 2.1, 0.9$  Hz, 1H), 4.97 (dd,  $J = 10.2, 2.0$  Hz, 1H), 3.88 (s, 3H), 3.76 (s, 3H), 3.71 (s, 3H), 3.31–3.24 (m, 1H), 3.24–3.14 (m, 1H), 3.12–2.99 (m, 4H), 2.78 (td,  $J = 11.7, 3.8$  Hz, 1H), 2.60 (td,  $J = 11.2, 4.4$  Hz, 1H), 2.35–2.25 (m, 1H), 2.11 (d,  $J = 12.1$  Hz, 1H), 2.02–1.93 (m, 1H). <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>):  $\delta$  168.98, 159.94, 154.50, 139.43, 137.42, 133.00, 121.85, 117.53, 115.49, 111.59, 107.71, 104.35, 99.70, 61.59,

**Table 5.** Pharmacokinetic Parameters of Speciogynine, Paynantheine, and Mitragynine Following a Single i.v. Administration in Male Rats<sup>a</sup>

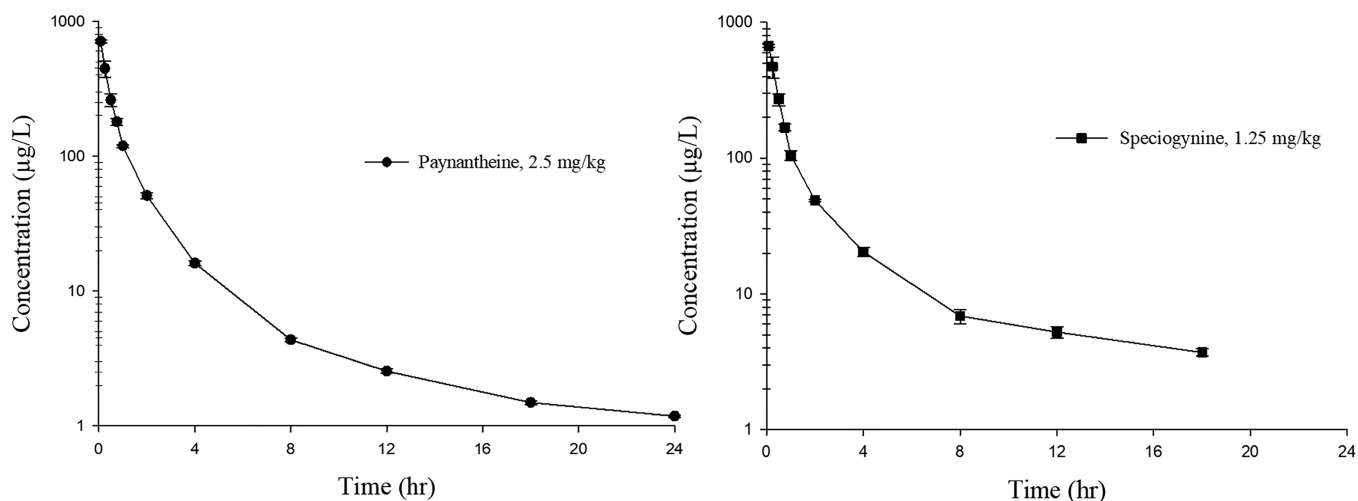
parameters	paynantheine	speciogynine	mitragynine <sup>28</sup>
	2.5 mg/kg (n = 4)	1.25 mg/kg (n = 3)	5 mg/kg (n = 6)
CL (L/h/kg)	5.3 ± 0.21	3.2 ± 0.01	1.3 ± 0.1
V <sub>z</sub> (L/kg)	71.9 ± 6.5	30.1 ± 8.4	8.0 ± 1.5
AUC <sub>inf</sub> /dose (h· $\mu\text{g}$ /L/mg/kg)	235.2 ± 8.9	495.1 ± 2.7	849.8 ± 112.8
k <sub>e</sub> (1/h)	0.08 ± 0.01	0.13 ± 0.05	0.12 ± 0.04

<sup>a</sup>Values are mean ± SEM. Abbreviation: CL, clearance; V<sub>z</sub>, apparent volume of distribution; AUC<sub>inf</sub>, total exposure extrapolated to infinity; k<sub>e</sub>, elimination rate constant.

61.37, 60.09, 55.31, 53.14, 51.36, 42.79, 38.82, 33.37, 23.78. NMR data align with a previous report.<sup>34</sup> HRMS (ESI<sup>+</sup>) calculated for C<sub>23</sub>H<sub>29</sub>N<sub>2</sub>O<sub>4</sub><sup>+</sup> [M + H]<sup>+</sup>: 397.2122, found: 397.2130. HPLC purity: >96%.

Methyl(E)-2-((2S,3R,12bS)-3-ethyl-8-methoxy-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-a]quinolizin-2-yl)-3-methoxyacrylate (Speciogynine). <sup>1</sup>H NMR (strong line broadening for all ring protons) (600 MHz, CDCl<sub>3</sub>):  $\delta$  7.88 (s br, 1H), 7.38 (s br, 1H), 7.00 (t,  $J = 7.9$  Hz, 1H), 6.90 (d,  $J = 8.0$  Hz, 1H), 6.52–6.42 (m, 1H), 3.88 (s, 5H), 3.82–3.67 (m, 5H), 3.32–2.98 (m, 5H), 2.73–2.53 (m, 2H), 2.34–2.26 (m, 1H), 2.13–1.88 (m, 3H), 1.44 (s, 1H), 0.97–0.76 (m, 3H). <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) (incomplete due to broadening of signals):  $\delta$  160.07, 154.60, 137.46, 133.22, 122.00, 117.65, 107.83, 104.35, 99.85, 61.76, 58.54, 55.43, 51.58, 38.82, 24.51, 23.83, 11.35. For an explanation of the line broadening in the demethoxy (corynantheine) series, see ref.<sup>35</sup> HRMS (ESI<sup>+</sup>) calculated for C<sub>23</sub>H<sub>31</sub>N<sub>2</sub>O<sub>4</sub><sup>+</sup> [M + H]<sup>+</sup>: 399.2278, found: 399.2286. HPLC purity: >98%.

Methyl(E)-2-((2S,3R,12bS)-8-hydroxy-3-vinyl-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-a]quinolizin-2-yl)-3-methoxyacrylate (Gambireine, 9-O-desmethylpaynantheine). AlCl<sub>3</sub> (594 mg, 4.389 mmol) was added to a stirring solution of paynantheine (580 mg, 1.463 mmol) in anhydrous DCM (25 mL) previously cooled to 0 °C under an argon atmosphere. Ethanethiol (2.13 mL, 29.26 mmol) was slowly introduced dropwise, and the mixture was allowed to reach room temperature and then stirred at room temperature for 2 h. The mixture was quenched with sat. aq. NaHCO<sub>3</sub> and then partitioned between water and DCM. The aqueous layer was extracted with DCM (2×), and the combined organic layers were dried with



**Figure 10.** Mean plasma concentration-time profile of (left) i.v. paynantheine and (right) i.v. speciogynine in male rats. Each symbol represents mean ± SEM ( $n = 4$  paynantheine,  $n = 3$  speciogynine).

Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated to give a yellow solid (510 mg). The crude compound was purified by flash chromatography (0–5% MeOH in DCM) to give the product as a pale yellow solid (350 mg, 71%) spectroscopically equivalent to previously reported examples.<sup>36</sup>

<sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>): δ 7.72 (s, 1H), 7.33 (s, 1H), 6.90 (t, *J* = 7.8 Hz, 1H), 6.83 (dd, *J* = 8.2, 0.7 Hz, 1H), 6.38 (dd, *J* = 7.5, 0.7 Hz, 1H), 5.62–5.53 (m, 1H), 5.24 (broad s, 1H), 5.03–4.92 (m, 2H), 3.75 (s, 3H), 3.69 (s, 3H), 3.31–3.25 (m, 1H), 3.23 (dd, *J* = 15.2, 8.4, 2.6 Hz, 1H), 3.11–3.00 (m, 3H), 2.98 (ddt, *J* = 15.5, 4.3, 1.7 Hz, 1H), 2.76 (td, *J* = 11.7, 3.8 Hz, 1H), 2.60 (td, *J* = 11.3, 4.4 Hz, 1H), 2.34–2.25 (m, 1H), 2.15–2.04 (m, 1H), 1.94 (d, *J* = 12.4 Hz, 1H). <sup>13</sup>C NMR (151 MHz, CDCl<sub>3</sub>): δ 168.86, 159.95, 150.10, 139.33, 137.98, 133.27, 122.04, 116.63, 115.55, 111.43, 106.90, 104.40, 103.86, 61.59, 61.29, 59.98, 52.99, 51.36, 42.79, 38.59, 33.35, 23.48. HRMS (ESI<sup>+</sup>) calculated for C<sub>22</sub>H<sub>27</sub>N<sub>2</sub>O<sub>4</sub><sup>+</sup> [*M* + *H*]<sup>+</sup>: 383.1965, found: 383.1976. HPLC purity: >99%.

*Methyl(E)-2-((2S,3R,12bS)-3-ethyl-8-hydroxy-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizin-2-yl)-3-methoxyacrylate (Gam-birine, 9-O-desmethylspeciogyne)*. AlCl<sub>3</sub> (510 mg, 3.765 mmol) was added to a stirring solution of speciogyne (500 mg, 1.255 mmol) in anhydrous DCM (25 mL) previously cooled to 0 °C under an argon atmosphere. Ethanethiol (1.82 mL, 25.10 mmol) was slowly introduced dropwise, and the mixture was allowed to reach room temperature and then stirred at room temperature for 2 h. The mixture was quenched with sat. aq. NaHCO<sub>3</sub> and then partitioned between water and DCM. The aqueous layer was extracted with DCM (2×), and the combined organic layers were dried with Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated to give a yellow solid (470 mg). The crude compound was purified by flash chromatography (0–10% MeOH in DCM) to give the product as a pale yellow solid (360 mg, 75%) spectroscopically equivalent to previously reported examples.<sup>37</sup>

<sup>1</sup>H NMR (600 MHz, MeOD): δ 7.49 (s, 1H), 6.80 (dd, *J* = 8.1, 7.0 Hz, 2H), 6.78 (dd, *J* = 8.1, 1.3 Hz, 2H), 6.32 (dd, *J* = 7.1, 1.3 Hz, 1H), 3.97–3.77 (m, 3H), 3.77–3.60 (m, 3H), 3.28 (broad s, 1H), 3.27–3.18 (m, 1H), 3.13 (dd, *J* = 11.4, 3.9 Hz, 1H), 3.08 (dd, *J* = 11.6, 5.8 Hz, 1H), 3.03 (ddt, *J* = 15.9, 4.7, 1.6 Hz, 1H), 2.70 (broad s, 1H), 2.62 (td, *J* = 11.6, 4.6 Hz, 1H), 2.34 (tdd, *J* = 11.3, 9.4, 3.6 Hz, 1H), 2.23–1.99 (m, 3H), 1.45 (broad s, 1H), 1.09 (broad s, 1H), 0.90 (t, *J* = 7.5 Hz, 3H). <sup>13</sup>C NMR (151 MHz, MeOD): δ 169.73, 160.41, 151.15, 138.69, 132.16, 121.20, 116.59, 111.23, 109.68, 106.10, 102.85, 102.83, 60.82, 60.48, 53.37, 50.56, 49.86, 38.15, 38.01, 33.19, 24.08, 22.96, 9.59. HRMS (ESI<sup>+</sup>) calculated for C<sub>22</sub>H<sub>29</sub>N<sub>2</sub>O<sub>4</sub><sup>+</sup> [*M* + *H*]<sup>+</sup>: 385.2122, found: 385.2136. HPLC purity: >99%.

**In Vitro Radioligand Competition Binding.** The kratom alkaloids were tested at Eurofins Cerep (Celle l'Evescault, France) and at Mercer University College of Pharmacy (Atlanta, GA) for their in vitro receptor binding affinities. 5-HT hydrochloride (Alfa Aesar, Haverhill, MA), SB 206553 hydrochloride (Tocris, Minneapolis, MN), (±)-DOI hydrochloride (Sigma-Aldrich, St. Louis, MO), M100907 hydrochloride (a gift from Dr. Kevin Murnane, synthesized by Dr. Kenner Rice), and mianserin hydrochloride (Alfa Aesar, Haverhill, MA) were used as controls and/or for nonspecific binding in tests conducted at Mercer University. Briefly, each cell membrane homogenate was incubated with a radioligand in the absence or presence of test compounds in a buffer. Nonspecific binding was determined in the presence of a specific agonist or antagonist at the target receptor. Following incubation, each sample was filtered rapidly under vacuum through glass fiber filters presoaked in a buffer and rinsed several times with an ice-cold buffer using a cell harvester. The filters were soaked with scintillation fluid, and counts per minute were detected using photodetectors. The experimental conditions are summarized in Table S1. The IC<sub>50</sub> values (concentration causing a half-maximal inhibition of control specific binding) from the displacement data were computed using a nonlinear, least squares regression analysis (Prism; GraphPad Software, Inc., San Diego, CA) and then converted to K<sub>i</sub> values using the Cheng–Prusoff equation.<sup>38</sup>

**In Vitro 5-HT<sub>1A</sub> Receptor cAMP Signaling.** cAMP measurements were performed with a GloSensor cAMP Assay (Promega Corporation, Madison, WI) according to the manufacturer's protocol. CHO cells were transfected with 7–11 μg of human 5-HT<sub>1A</sub>R cDNA

and 1 μg of 22F Glosensor plasmid (Promega) using a TransIT-CHO reagent (Mirus Bio, Madison WI), based on established methods.<sup>39</sup> Twenty-four hours after transfection, cells were plated in media containing 10% dialyzed FBS and DMEM in white, opaque 96-well plates (PerkinElmer, Waltham, MA) at a density of 150,000 cells/100 μL and incubated at 37 °C, 5% CO<sub>2</sub> overnight. The next day, media were removed from plates, and cells were incubated with 90 μL of assay buffer (1× HBSS +20 mM HEPES) + 1% Glosensor substrate for 2 h in the dark at room temperature. Cells were then treated with compounds made in assay buffer for 30 min in the dark at room temperature. Then, cells were stimulated with 10 μM (final concentration) forskolin for 10 min in the dark at room temperature. Luminescence was measured with a Microbeta<sup>2</sup> Microplate Counter (PerkinElmer). Data were analyzed as normalized means (± SEM) from at least three independent determinations, with controls and the kratom alkaloids tested at each concentration in triplicate.

**5-HT<sub>2B</sub> Receptor Phosphoinositide Hydrolysis.** The activity of kratom alkaloids at human 5-HT<sub>2B</sub> receptors was measured using the IP-One homogeneous time-resolved fluorescence (HTRF) assay (Cisbio, Bedford, MA). The assay was performed per the manufacturer's protocol with optimization. Transfection of HEK-293 cells with 7–11 μg of human 5-HT<sub>2B</sub> receptor cDNA using the TransIT 2020 reagent (Mirus) was performed as previously described.<sup>39</sup> Forty-eight hours after transfection, cells were serum-starved for 90 min in DMEM and plated at 25,000 cells per well in 384-well plates. Cells were treated with compounds and incubated at 37 °C, 5% CO<sub>2</sub> for 90 min. The reaction was terminated with lysis buffer containing d2-labeled IP1 and anti-IP1-Cryptate. HTRF-FRET was measured by a Mithras LB 940 Multimode Microplate Reader (Berthold Technologies, Bad Wildbad, Germany). Data were analyzed as normalized means (± SEM) from at least three independent determinations, with controls and the kratom alkaloids tested at each concentration in triplicate.

**In Vivo Functional Studies.** Salt and enantiomeric forms of the compounds used in the present study were as follows: ipsapirone (Tocris), mitragynine hydrochloride, paynantheine oxalate, speciogyne free base, and WAY 100635 maleate (Sigma Aldrich Co.). Doses were expressed as the weight of the salt or free base form as listed above. Compounds were dissolved in a vehicle consisting of sterile water (HyClone Water for Injection, 1 L, Fisher Scientific, Waltham, MA) containing 5% Tween80 (polyoxyethylenesorbitanmonooleate, Fisher Scientific) and 5% propylene glycol (Sigma Aldrich Co.). Compounds and vehicle were administered i.p. or i.v. Each solution was filtered with a 0.2 μm pore size syringe filter (Millex-LG, 0.20 μm, SLLG025SS, Sigma Aldrich Co.) prior to injections. The injection volumes were 1.0–5.0 mL/kg of the subject body weight. The dose ranges and pretreatment times were chosen based on our preliminary data and published methods.<sup>12,27,40</sup>

**Animals.** Adult, naïve female and male (*N* = 42 rats per sex) Sprague Dawley rats (Envigo, Indianapolis, IN), age 10 weeks and weighing approximately 200 and 310 g for females and males, respectively, were singly acclimated in ventilated cages for at least 3 days to a temperature- (21.9 °C ± 1.9 °C) and humidity-controlled (53% ± 14%) vivarium with a 12 h light/dark cycle (lights on at 07:00 h) during which food (2918 Teklad global 18% protein rodent diets, Envigo) and reverse osmosis water were available in the home cage at all times. Among the 82 rats, 36 rats (18 rats per sex) were pre-implanted with a catheter into a right external jugular vein prior to entering our facility. After at least 3 days of acclimation, experiments commenced. These studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments were conducted in the light cycle (08:00–17:00 h) using drug naïve subjects.

**Lower Lip Retraction Following Intraperitoneal Administration.** The sample size of each experimental group per treatment was eight (four subjects per sex). The temperature, humidity, and light/dark

cycle in the experimental room were equivalent to those in the vivarium. Prior to commencement of each experiment, the body weight of each subject was measured. Each experimental session started with a baseline measurement for lower lip retraction (observation of visible lower incisors)<sup>23</sup> in each subject (see below for more details). Vehicle and cumulative doses of a test compound were administered i.p. at intervals of 15 min to assess the effects of compounds alone. Following the baseline measurements, the vehicle or WAY 100635 (0.01 mg/kg) were administered followed by cumulative doses (every 15 min) or a single dose of a test compound i.p. at 60 and 15 min, respectively, prior to a measurement of lower lip retraction. The assessment of effects of a test compound was continued until 100% subjects showed lower lip retraction. Lower lip retraction was assessed by trained experimenters who were blind to treatment conditions. The expression of lower lip retraction was assessed by observing each subject for 5 s before or every 15 min after an injection.

**Hotplate.** The whole size of the hotplate analgesia meter that included a hard-black anodized aluminum plate was 33.1 cm long, 28.8 cm wide, and 9.9 cm high and a built-in digital thermometer (1440 Analgesia Hotplate with RS-232 Port and Software, Columbus Instruments, Columbus, OH). A clear acrylic cage (26.7 cm long, 26.7 cm wide, and 31.0 cm high) was fitted over the square plate to confine an animal on the plate (total height: 40.9 cm). The size of the heated surface was 25.4 cm long, 25.4 cm wide, and 1.9 cm high; the plate surface temperature could be set between 30 and 79.9 °C with an accuracy of  $\pm 0.1$  °C. To minimize biotransformation at the time of testing, tests were conducted following i.v. administration. The antinociceptive effects of ipsapirone, paynantheine, and speciogynine alone and in combination with WAY 100635 were tested. Following the baseline measurements, vehicle or WAY 100635 (0.01 mg/kg) was administered i.v. at 60 and 15 min, respectively, followed by cumulative doses (every 5 min) of a test compound. Across six treatment groups in the present study (three subjects per sex), the baseline hotplate latencies [mean (SEM) in seconds] were as follows: ipsapirone alone [11.0 (2.05)] and in combination with WAY 100635 [7.98 (1.18)], paynantheine alone [16.2 (1.69)] and in combination with WAY 100635 [10.3 (1.31)], and speciogynine alone [16.8 (2.48)] and in combination with WAY 100635 [8.72 (1.17)]. For the baseline values, two-way ANOVA indicated no significant main effect on the treatment group ( $F_{5,24} = 1.44$ ;  $P = 0.245$ ), sex ( $F_{1,24} = 3.16$ ;  $P = 0.088$ ) and interaction of the two ( $F_{5,24} = 1.54$ ;  $P = 0.215$ ). The assessment of effects of a test compound was continued until the hotplate latency reached 60 s to avoid tissue damage. Hotplate latency was assessed by trained experimenters who were blind to treatment conditions.

**Data Analysis. Lower Lip Retraction.** Percent subjects showing lower lip retraction were compared with the vehicle or WAY 100635. Across eight treatment groups, lower lip retraction was never observed at the baseline (i.e., in the absence of the drug).

**Hotplate (i.p.).** The hotplate latencies were normalized to the percentage of the MPE using the following formula: % MPE =  $100 \times (\text{post-injection latency} - \text{pre-injection baseline latency}) / (\text{maximum latency } 60 \text{ s} - \text{pre-injection baseline latency})$ . All data per treatment group in each figure above were presented as a mean ( $\pm$  an SEM). Using the SigmaPlot software (ver. 14.0; Systat Software Inc., San Jose, CA) and GraphPad Prism software (ver. 8; GraphPad Software, San Diego, CA), a two- and three-way (repeated-measures) ANOVA, with post hoc Bonferroni *t* tests, was used (factors were the compound dose and sex for two-way ANOVA and test compound treatment, the presence or absence of WAY 100635, and sex for three-way ANOVA). Outcomes were considered significant at  $P < 0.05$ . The ED<sub>50</sub> values (95% confidence intervals) for hotplate latency were calculated by linear regression<sup>41</sup> of the dose-effect functions using the GraphPad Prism software. To compare potency, potency ratios and corresponding 95% CIs were calculated.<sup>42</sup>

**Correlational analysis.** Fisher's exact test (two tails) was used to assess the association between in vivo activity (lower lip retraction and antinociceptive effects of test compounds) and receptor affinity

(i.e.  $K_i$  values) for serotonin 5-HT<sub>1A</sub> receptors using GraphPad Prism software. The  $R^2$  and  $P$  values are shown.

**Pharmacokinetic of Speciogynine and Paynantheine in Rats.** These procedures were approved by the University of Florida IACUC. As with the in vivo functional studies, adult, naïve male Sprague Dawley rats weighing  $250 \pm 25$  g were housed individually in ventilated cages at the University of Florida vivarium with ad libitum access to food and water. During the pharmacokinetic tests, animals were placed inside a CulexNxT automated blood sampling system (BASi Co., West Lafayette, IN). A 1.5 mg/mL paynantheine freebase equivalent formulation was prepared by weighing an accurate amount of paynantheine oxalate and dissolving it in normal saline with 1% (v/v) Tween 80 (Sigma Aldrich Co.). A 2.5 mg/mL speciogynine formulation was prepared by weighing an appropriate amount of speciogynine freebase and dissolving it in normal saline with 2% (v/v) Tween 80 and 2% (v/v) Tween 20 (Sigma Aldrich Co.). Both solutions were filtered through a 0.2  $\mu\text{m}$  syringe filter (Millex, Sigma Aldrich Co.) prior to use. Two short, sensitive, and robust bioanalytical methods were developed and partially validated according to FDA guidelines in terms of accuracy, precision, recovery, selectivity, dilution integrity, carryover, linearity, and sensitivity. The methods used a mobile phase of purified water acidified with 0.1% formic acid and either acetonitrile or acetonitrile acidified with 0.1% formic acid. All solvents were of LC-MS grade. Formic acid, acetonitrile, and water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Blank rat plasma was purchased from VWR International (Suwanee, GA, USA). Sodium heparin-coated blood collection vials and sterile blood collection tubing were obtained from BASi Co. (West Lafayette, IN, USA). Both methods used a gradient elution, had a linearity range of 3–500 ng/mL, and a low sample volume of 20–25  $\mu\text{L}$ . Verapamil hydrochloride (Sigma Aldrich Co., St. Louis, MO, USA) served as the internal standard (IS) for paynantheine, while yohimbine hydrochloride (Selleckchem, Houston, TX, USA) served as the IS for speciogynine. Chromatographic separation was achieved using a Waters Acquity UPLC BEH C18 column (1.7  $\mu\text{m}$ , 2.1  $\times$  50 mm) with a VanGuard pre-column of the same chemistry (Waters Co, Milford, MA), and retention times for speciogynine and paynantheine were 1.0 and 2.1 min, respectively. A Waters Acquity I-class chromatograph (Waters Co, Milford, MA) was coupled to a Xevo-TQS Micro triple-quadrupole mass spectrometer (Waters Co) in positive electrospray ionization mode using multiple reaction monitoring. Following the quantification of the samples in TargetLynx (Waters Co), plasma concentration versus time functions were constructed in SigmaPlot 12.0 (Systat Software, Inc.). The data were further subjected to a noncompartmental analysis using a linear trapezoidal method in Phoenix (Certara USA, Inc., Princeton, NJ).

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00726>.

Experimental procedures, statistical analysis of in vivo data, additional binding data at 5HT<sub>1A</sub> and 5HT<sub>2B</sub> receptors, and spectroscopic data for the kratom alkaloids and metabolites (PDF)  
Molecular formula strings (CSV)

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## Author Contributions

The manuscript was written through contributions from all authors. All authors have given approval to the final version of the manuscript. A.J.D. was substantially involved in his role as Scientific Officer of UG3 DA048353 (e.g. study designs, weekly discussion over two years, and manuscript review). A.J.D. had no substantial involvement in other cited grants.

## Notes

The authors declare no competing financial interest.

The views and opinions expressed in this manuscript are those of the authors only and do not necessarily represent the views, official policy, or position of the U.S. Department of Health and Human Services or any of its affiliated institutions or agencies.

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## ABBREVIATIONS USED

(8-OH-DPAT), ( $\pm$ )-8-Hydroxy-2-(dipropylamino)tetralin; (9-O-desmethylpantaneine), gambireine; (9-O-desmethylspeociognine), gambirine; (AAALAC), Association for Assessment and Accreditation of Laboratory Animal Care; (ANOVA), analysis of variance; (CHO), Chinese hamster ovary; (DOI), 2,5-dimethoxy-4-iodoamphetamine; (DOR),  $\delta$ -opioid receptor; (HEK), human embryonic kidney; (IACUC), Institutional Animal Care and Use Committee; (i.p.), intraperitoneally; (IS), internal standard; (i.v.), intravenous; (KOR),  $\kappa$ -opioid receptor; (MOR),  $\mu$ -opioid receptor; (MPE), maximum possible effects; (5-HT), serotonin; (SEM), standard of error of mean

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