Search for a 5-CT alternative. \textit{In vitro} and \textit{in vivo} evaluation of novel pharmacological tools: 3-(1-alkyl-1H-imidazol-5-yl)-1H-indole-5-carboxamides, low-basicity 5-HT\textsubscript{7} receptor agonists\textsuperscript{†}

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Close structural analogues of 5-carboxamidotryptamine (5-CT) based on the newly discovered indole-imidazole scaffold were synthesized and evaluated to search for a 5-HT\textsubscript{7} receptor agonist of higher selectivity. \textit{In vitro} drug-likeness studies and \textit{in vivo} pharmacological evaluation of potent and selective low-basicity 5-HT\textsubscript{7} receptor agonists, previously published \textit{7} (3-(1-ethyl-1H-imidazol-5-yl)-1H-indole-5-carboxamide, AH-494) and \textit{13} (3-(1-methyl-1H-imidazol-5-yl)-1H-indole-5-carboxamide), have supported their usefulness as pharmacological tools. Comprehensive \textit{in vitro} comparison studies between \textit{7}, \textit{13} and the commonly used 5-CT showed their very similar ADMET properties. Compound \textit{7} at 1 mg kg\textsuperscript{-1} reversed MK-801-induced disruption in novel object recognition in mice and alleviated stress-induced hyperthermia (SIH) at high doses. Taking into account both \textit{in vitro} and \textit{in vivo} data, \textit{7} and \textit{13} may be considered as alternatives to 5-CT as pharmacological tools with important additional benefit associated with their low-basicity: high selectivity over 5-HT\textsubscript{1A}.R.

\textbf{Introduction}

The 5-HT\textsubscript{7} receptor (5-HT\textsubscript{7}-R), which was discovered independently by three teams in 1993, is the most recent one to be identified in the human serotonin system.\textsuperscript{1-6} To date, extensive studies on 5-HT\textsubscript{7}-R have indicated its role in CNS development, circadian rhythm, memory and learning, thermoregulation, locomotion, hormonal regulation, hippocampus activity and mood control, as well as the pathophysiology of migraine, pain, depression and epilepsy.\textsuperscript{1-6} Unfortunately, despite its possible therapeutic potential, no selective 5-HT\textsubscript{7}-R drug has yet been introduced to the market. Interestingly, the results obtained from animal models support the possible therapeutic applications of both 5-HT\textsubscript{7}-R agonists and antagonists.\textsuperscript{7} For instance, the symptoms observed in an animal model caused by brain molecular alterations specific for Rett syndrome (RRT) were relieved after seven days of 5-HT\textsubscript{7}-R stimulation.\textsuperscript{7,8} The selective activation of 5-HT\textsubscript{7}-R was also found to be beneficial in an animal model of fragile X syndrome (FXS), which is one of the genetic causes of intellectual disability and autism.\textsuperscript{9} Investigations examining the long-term consequences of 5-HT\textsubscript{7}-R stimulation in adolescent rats have indicated plastic rearrangements within forebrain networks, accounting for long-lasting behavioral changes.\textsuperscript{10} Pharmacological blockade of 5-HT\textsubscript{7}-R produced strong antidepressant-like response more rapidly than the classic antidepressant fluoxetine.\textsuperscript{1-6}

Great efforts have been exerted by several research groups to develop new therapeutics and better molecular probes acting as selective 5-HT\textsubscript{7}-R ligands.\textsuperscript{6} A few 5-HT\textsubscript{7}-R agonists have been used to date as tool compounds (Table 1), including the long-chain diphenylpiperazine, \textit{e.g.}, LP-211 (1), and its low-weight metabolite RA-7 (2),\textsuperscript{11} as well as several small molecules: pyrazoles AS-19 (3),\textsuperscript{12} E-55888 (4)\textsuperscript{13} and 5-carboxamidotryptamine (5-CT) (5)\textsuperscript{14} (Fig. 1). However, due to their poor selectivity (RA-7, 5-CT),\textsuperscript{15} low metabolic stability (LP-211),\textsuperscript{16,17} failure to induce a full functional agonistic...
response of intracellular signaling cascades (AS-19) and low BBB permeability (5-CT and to some extent LP-211), there is still a great demand for a “perfect” selective 5-HT7 agonist, which could serve not only as a 5-HTR tool compound but also as a positron emission tomography (PET) radiotracer. The ability to image serotonin 5-HT-R in a living brain would allow direct assessments of the involvement of this receptor in neuropsychiatric diseases and possible therapies.

The recently reported 5-(1H-indole-3-yl)-1-alkylimidazoles represent the first examples of low-basicity scaffolds exhibiting high affinity and selectivity for 5-HT7R together with agonist function. The hit compound AGH-107 (Fig. 2) exhibited high affinity for and intrinsic activity at the receptor (Ki = 6 nM, EC50 = 19 nM, respectively). Compound 6 was also very selective over 5-HT3R, which is not a common feature of 5-HT7R agonists (Table 1). Moreover, in drug-likeness studies in vitro, 6 was shown to be metabolically stable after incubation with human liver microsomes (Clint = 6.3 ml min⁻¹ kg⁻¹) and exhibited very low toxicity in human embryonic kidney HEK-293 and hepatoma HepG2 cell lines and excellent water solubility. A PK experiment showed a high blood–brain barrier permeation, consistent with its high activity in vivo, where all three tested i.p. doses (0.5, 1 and 5 mg kg⁻¹) reversed MK-801-induced novel object recognition (NOR) impairment in mice. The promising results highlighted the possible use of labeled 6 as a potential radioligand for both in vitro and in vivo studies.

Table 1 Pharmacological in vitro profile of 5-HT7R agonists

<table>
<thead>
<tr>
<th>Ligand/Ki [nM]</th>
<th>5-HT14</th>
<th>5-CT15</th>
<th>AS-19 (ref. 15)</th>
<th>E-55888 (ref. 13)</th>
<th>LP-211 (ref. 11)</th>
<th>RA-7 (ref. 11)</th>
<th>AGH-107 (ref. 20) (6)</th>
<th>AH-494 (ref. 20) (7)</th>
<th>AGH-282 (13)</th>
<th>14</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT14A</td>
<td>3.17</td>
<td>0.3</td>
<td>89.7</td>
<td>700</td>
<td>188</td>
<td>99</td>
<td>1053</td>
<td>123</td>
<td>220</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>5-HT14B</td>
<td>11.55</td>
<td>5012</td>
<td>N.D.</td>
<td>&gt;1000</td>
<td>626</td>
<td>1190</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>5-HT14C</td>
<td>251</td>
<td>20</td>
<td>98.5</td>
<td>N.D.</td>
<td>178</td>
<td>76</td>
<td>&gt;1000d</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>5-HT6</td>
<td>98.41</td>
<td>720</td>
<td>N.D.</td>
<td>&gt;10000</td>
<td>596</td>
<td>1673</td>
<td>&gt;10000</td>
<td>6910</td>
<td>7271</td>
<td>6062</td>
<td></td>
</tr>
<tr>
<td>5-HT7</td>
<td>8.11</td>
<td>0.4</td>
<td>0.6</td>
<td>2.5</td>
<td>0.6</td>
<td>1.4</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>6417</td>
<td>161</td>
</tr>
<tr>
<td>D2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>142</td>
<td>N.D.</td>
<td>4847</td>
<td>&gt;10000</td>
<td>5561</td>
<td>&gt;10000</td>
<td>&gt;10000</td>
</tr>
</tbody>
</table>

*a* Value estimated based on the results of a screening experiment.

During this study, our attention was focused on other potent compounds from that series: the 1H-indole-5-carboxamides AH-494 (7) (Ki = 5 nM, EC50 = 45 nM) and the newly obtained derivative AGH-282 (13) (Ki = 6 nM) (Fig. 2), which are close structural analogues of 5-carboxamidotryptamine (5) (Fig. 1), a molecular probe commonly used in serotonergic system-related research.

Results and discussion

Chemistry

Aldehydes 8–10 were synthesized from the corresponding indoles via Vilsmeier–Haack formylation (Scheme 1). Compounds 11 and 12 which were the intermediates for the synthesis of 7 (AH-494) and 13, respectively, as well as final compounds 14 and 15 were synthesized via van Leusen 3-component imidazole synthesis using the previously described protocol. Compounds 7 and 13 were synthesized by a controlled hydrolysis of 11 and 12, respectively.

In vitro

Radioligand displacement assays of compounds 7 and 13–15 showed, that only compounds with a carbonyl group (either ester or amide) at the 5th position of indole were found to possess significant affinity for the 5-HT7R (Table 1). Compound 15 exhibited only medium binding to 5-HT7R (Ki = 161 nM), while compound 13 was found only slightly less potent than 7 (Ki = 6 and 5 nM, respectively). Compounds 7 and 13 showed selectivity over 5-HT1A in opposition to 5-CT (25-fold and 37-fold vs. no selectivity at all).

The in vitro drug-likeness studies of 7, 13 and the reference 5-HT7R molecular probe 5 included the assessment of ADME-Tox parameters by using recombinant protein and enzymes (Pgp, CYP3A4, CYP2D6, CYP2C9), human (HLMs)
rat (RLMs) and mouse (MLMs) liver microsomes, human embryonic kidney (HEK-293), neuroblastoma (SH-SY5Y) and hepatoma (HepG2) eukaryotic cell cultures and the bacterial strain *Salmonella typhimurium*. The artificial membranes (PAMPA) were used for determination of passive membrane transport of 5 and 7. The assay protocols and culture growth conditions were applied as described previously.\(^2^2,2^3\)

The *in vivo* activity of CNS drugs depends on their penetration through biological membranes.\(^2^4\) The parallel artificial membrane permeability assay (PAMPA) is commonly used in the early discovery and development process for the determination of compound passive transport. The Corning® PAMPA Plate System Gentest\(^\text{TM}\) was used to determine the potential permeability of 5 and 7. The method has been shown to provide good predictability of the membrane permeation and a high degree of correlation to the data obtained from absorption in the Caco-2 cell line.\(^2^5\) The calculated permeability coefficient (Pe) for 5 (Pe = 3.54 × 10\(^{-6}\) cm s\(^{-1}\)) was slightly higher than for 7 (Pe = 2.78 × 10\(^{-6}\) cm s\(^{-1}\)) (Table 2). The results were compared to the references - highly permeable caffeine (Pe = 3.61 × 10\(^{-6}\) cm s\(^{-1}\)) and the low-permeable drug norfloxacin (Pe = 0.95 × 10\(^{-6}\) cm s\(^{-1}\)) and indicated very good passive transport of both 5 and 7 (Table 2).

The potential CNS bioavailability of 5, 7 and 13 was also tested with respect to their influence on glycophorin P (Pgp), the main efflux pump in both intestinal epithelium and the blood–brain barrier (BBB). The luminescence Pgp-Glo\(^\text{TM}\) Assay System (Promega\(^\text{®}\)) based on measurements of the changes in ATP consumed by Pgp\(^2^6\) was used. The difference in the luminescent signal between samples treated with 100 μM of the selective Pgp inhibitor Na\(_3\)VO\(_4\) (100% inhibition observed) and untreated samples represented the basal Pgp ATPase activity, which was considered herein as a negative control. The results were juxtaposed as the % of Pgp basal activity compared to the reference inhibitor (Na\(_3\)VO\(_4\)) and stimulator (verapamil, VL) (Fig. 3). Compounds 5 and 7 showed comparable, statistically significant inhibition effects on Pgp at 100 μM, with a decrease in ATP consumption to ∼26 and ∼19% of the basal activity, respectively (Fig. 3, Table 2). Despite the potentially enhanced risk of drug–drug interactions (DDIs), Pgp inhibition of CNS targeting compound 7 could exert a desirable effect by reducing its efflux from the BBB. No statistically significant influence of 13 on Pgp was also determined.

The metabolic stability of compounds 5, 7 and 13 was evaluated *in silico* using MetaSite 5.1.1 (ref. 27) and *in vitro* using human, rat and mouse models with the respective liver microsomes, according to previously described methods and protocols.\(^2^2,2^3,2^8\)

The *in silico* algorithm predicted, that the most likely metabolic site of 5 was the 2-aminooethyl chain, whereas the metabolic transformations for 7 and 13 were expected at the N-alkyl substituent at the imidazole ring (see Fig. S1 and S2 in ESI†).

The tested compounds were incubated with HLMs, RLMs and MLMs for 120 min at 37 °C in the presence of the NADPH regeneration system to determine their relative *in vitro* metabolic stability. UPLC analysis of the reaction mixtures collected after reaction with HLMs showed that approximately 20% of 5 was converted into two metabolites, M1 and M2 (see Fig. S3A in ESI†). However, due to the ambiguous MS spectrum of metabolite M1, only the M2 quasimolecular ion [M + H]\(^+\) (m/z = 219.11) could be interpreted (see Fig. S4C and D in ESI†) (Table 2). The molecular mass of M2 (+14 units) suggests oxidation to the carbonyl group at the

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**Table 2** ADMET parameters of 5, 7 and 13

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>5</th>
<th>7</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permeability</td>
<td>(10(^{-6}) cm s(^{-1}) ± SD)</td>
<td>3.54 ± 8.2(^a)</td>
<td>2.78 ± 9.2(^a)</td>
<td>N.E.(^d)</td>
</tr>
<tr>
<td>Pgp</td>
<td>(% of basal activity at 50 μM)</td>
<td>26.3 ± 12.0(^b)</td>
<td>19.3 ± 12.3(^a)</td>
<td>60.0 ± 16.0(^b)</td>
</tr>
<tr>
<td>Metabolism with HLMs</td>
<td>(% of metabolized substrate)</td>
<td>26</td>
<td>N.A.(^c)</td>
<td>N.A.(^c)</td>
</tr>
<tr>
<td>Metabolism with RLMs</td>
<td>(% of metabolized substrate)</td>
<td>20</td>
<td>7</td>
<td>N.A.(^c)</td>
</tr>
<tr>
<td>Metabolism with MLMs</td>
<td>(% of metabolized substrate)</td>
<td>2</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>CYP3A4 activity</td>
<td>(% of control ± SD at 10 μM)</td>
<td>95.7 ± 1.4(^a)</td>
<td>2.9 ± 0.8(^a)</td>
<td>29.37 ± 2.2(^a)</td>
</tr>
<tr>
<td>CYP2D6 activity</td>
<td>(% of control ± SD at 10 μM)</td>
<td>108.3 ± 2.6(^a)</td>
<td>43.4 ± 0.8(^a)</td>
<td>55.0 ± 9.7(^b)</td>
</tr>
<tr>
<td>CYP2C9 activity</td>
<td>(% of control ± SD at 25 μM)</td>
<td>78.7 ± 1.6(^a)</td>
<td>104.2 ± 15.8(^d)</td>
<td>37.20 ± 21.1(^a)</td>
</tr>
<tr>
<td>HEK-293 viability</td>
<td>(% of control ± SD at 100 μM)</td>
<td>105.3 ± 7.0(^b)</td>
<td>78.7 ± 16.8(^b)</td>
<td>112.2 ± 5.2(^b)</td>
</tr>
<tr>
<td>SH-SY5Y viability</td>
<td>(% of control ± SD at 100 μM)</td>
<td>91.0 ± 5.7(^b)</td>
<td>106.7 ± 3.5(^b)</td>
<td>88.4 ± 7.1(^b)</td>
</tr>
<tr>
<td>HepG2 viability</td>
<td>(% of control ± SD at 100 μM)</td>
<td>101.3 ± 3.8(^b)</td>
<td>78.7 ± 3.8(^b)</td>
<td>82.2 ± 5.7(^b)</td>
</tr>
<tr>
<td>ATP level in HepG2</td>
<td>(% of control ± SD at 100 μM)</td>
<td>N.E.(^d)</td>
<td>101.6 ± 2.2(^a)</td>
<td>N.E.(^d)</td>
</tr>
<tr>
<td>Ames test</td>
<td>(No. of positive wells ± SD at 10 μM)</td>
<td>4.0 ± 2.9(^a)</td>
<td>5.6 ± 1.2(^a)</td>
<td>12.3 ± 1.5(^a)</td>
</tr>
</tbody>
</table>

\(^a\) Tested in triplicate. \(^b\) Tested in quadruplicate. \(^c\) Not applicable – no metabolism was observed. \(^d\) N.E. – not examined.
2-aminoethyl substituent as a metabolic pathway, which was also predicted in silico (see Fig. S2 in ESI†). UPLC analysis of the reaction mixtures of 7 and 13 after incubation with HLMs indicated their excellent metabolic stability, as no metabolites were present after incubation (see Fig. S3B and C in ESI†) (Table 2). Rodent models (mouse and rat) have shown similarities in the metabolism of 5—only one metabolite with the same retention time (tR = 1.62) was found after incubation with RLMs and MLMs (see Fig. S6A and S9A in ESI†). Comparison of the retention time of the metabolite obtained in rodent models with metabolite M1 (found after incubation with HLMs, tR = 1.61) indicated that it could be the same molecule (see Fig. S4C, S7B and S10B in ESI†). However, as mentioned above, the molecular mass of this metabolite was not determined. The UPLC-MS spectra obtained from the reaction mixtures after incubation of 7 with RLMs and MLMs revealed the presence of the same metabolite with tR ~ 1.60 min. and a molecular mass m/z of ~271 in both models (see Fig. S6B, S8C, S9B and S11C in ESI†). The molecular mass of the metabolite of compound 7 (+16 units) suggested that hydroxylation had occurred, presumably at the N-ethyl substituent, according to the fragmentation analysis and in silico data (see Fig. S1, S2 and S12 in ESI†). Moreover, 7 seemed to be slightly more stable in RLMs than in MLMs with 7% and 14% conversion, respectively (see Fig. S6B and S9B in ESI†) (Table 2). The results obtained for 13 after incubation with RLMs were similar to that for HLMs, as no metabolites were found (see Fig. S3C and S6C in ESI†) (Table 2). The UPLC-MS spectra obtained from the reaction mixture after incubation of 13 with MLMs revealed the presence of one metabolite with molecular mass m/z of ~274 and with 9% conversion of 13 (see Fig. S9C and S13 in ESI†). According to the fragmentation analysis and in silico data, the most probable metabolic pathway of 13 was a double hydroxylation (see Fig. S1, S2 and S14 in ESI†).

The cytochrome P450 isoforms 3A4 (CYP3A4), 2D6 (CYP2D6) and 2C9 (CYP2C9) are responsible for the metabolism of approximately 60–70% of all marketed drugs; the influence on CYP activity may be a source of potential dangerous DDI.29 Luminescence-based CYP3A4, CYP2D6 and CYP2C9 P450-Glo™ assays (Promega®) were conducted to determine their activity at the presence of 5, 7 and 13. The assays were performed according to the manufacturer’s protocols as described previously.22,23 A strong cytochrome inhibition effect of 7 (IC50 = 0.31 μM), which is similar to the reference inhibitor ketoconazole (IC50 = 0.14 μM), was revealed (Fig. 4A) (Table 2). The CYP3A4 inhibition was also observed for 13 (IC50 = 3.53 μM) (Fig. 4A) (Table 2). Surprisingly, no effect on CYP3A4 activity was observed in the presence of 5 (Fig. 4A) (Table 2). An inhibition effects of 7 and 13 on CYP2D6 were also observed (IC50 = 7.47 and 27.60 μM, respectively), whereas slight CYP2D6 induction by 5 was observed at the highest doses (Fig. 4B) (Table 2). The highest inhibition effect on CYP2C9 was observed for 13 (~40% of the enzyme activity) only at the dose 25 μM (Fig. 4C) (Table 2). Thus, the N-substituted imidazole moiety of 7 and 13 is responsible for the interaction with cytochromes, as imidazole is the common molecular feature of many CYP inhibitors.20

In vitro safety profiling is an essential component of the drug discovery process, providing a reliable alternative to animal testing. The cytotoxicity, neurotoxicity, hepatotoxicity and mutagenicity of 5, 7 and 13 were tested using HEK-293, SH-SY5Y, and HepG2 eukaryotic cell lines and the bacterial strain Salmonella typhimurium. The growth conditions and assay protocols have been described previously.22,23 The colorimetric standard MTS assay allowed for the determination of eukaryotic cell viability after 72 h of incubation with 5, 7 or 13. A statistically significant influence on viability was
shown for 7 and 13 in one (HepG2) among the three examined cell lines (Fig. 5). However, the decrease in HepG2 viability to ~80% was observed only for the highest dose of 100 μM (Fig. 5) (Table 2), suggesting a very weak toxicity of 7 and 13 in comparison to the effect of the references DX and CCCP, which decreased the cell viability to ~40% at 1 and 10 μM, respectively (Fig. 5). Potential hepatotoxic effects of 7 were ruled out in additional tests in which the ATP level was measured via luminescence after a short 3 h exposure of the cells to 7. No influence of 7 on cell metabolism and mitochondrial respiration was observed, even at the highest dose (100 μM, Fig. 6).

The mutagenicity potential of 5, 7 and 13 was evaluated using the Ames microplate fluctuation protocol (MPF), as described previously. The *Salmonella typhimurium* strain TA100 was used, which enables the detection of frame shift mutations. The number of revertants observed in the control (growth medium + 1% DMSO) allowed for determination of the medium control baseline (MCB). According to the assay kit manufacturer, a 2-fold increase over the MCB is considered a mutagen alert. The results were compared to the reference mutagen nonyl-4-hydroxyquinoline-N-oxide (NQNO) at 0.5 μM. Mutagenic potential of all compounds was not observed (Fig. 7).

**In vivo**

The *in vivo* evaluation of the biological activity of 7 included a novel object recognition (NOR) and stress-induced hyperthermia (SIH) assays. Compound 7 reversed MK-801-induced...
disruption in the NOR test at relatively low doses (Fig. 8), supporting its potential to improve short-term working memory – however, this effect has also been observed for 5-HT7R antagonists. It is likely that the 5-HT7-R may play a different role in the acquisition and consolidation of recognition memory, which could explain why the administration of both agonists and antagonists can lead to procognitive effects. The reverse dose-dependence can potentially be attributed to the activity of the compound at the 5-HT1A receptor (or other targets), which may influence the effect of 5-HT-R activation at the highest doses. Moreover, the activity of the compound was also evident in the SIH test, which reflects the autonomic response to stress in animals and is one of the most recognized tests for anxiety. However, the activity was observed only at the highest dose (Fig. 9).

**Experimental**

**Chemicals**

The reference compounds used during in vitro study, as follows: caffeine, norfloxacain, doxorubicin, carbonyl cyanide 3-chlorophenylhydrazone, ketoconazole, quinidine, nonyl-4-oxo-1,6-dihydropyridinone-3-oxide were provided by Sigma-Aldrich (St. Louis, MO, USA). The references in Pgp assay: Na3VO4 and ve-roxyquinoline-3-chlorophenylhydrazone, ketoconazole, quinidine, nonyl-4-oxo-1,6-dihydropyridinone-3-oxide were provided by Promega (Madison, WI, USA).

All the chemicals for syntheses were supplied by the companies: Combi-Blocks, Inc. 7949 Silverton Avenue, Suite 915 San Diego, CA 92126 USA, Sigma-Aldrich Sp. z o.o. Poznan, Poland and Chempur, Jana Lortza 70A, 31-940 Piekary Śląskie.

**General procedures for the preparation of compounds 7–15**

**General procedure 1 for the synthesis of Indole-3-carboxaldehydes 8–10.** Aldehydes were prepared as described previously. The Vilsmeier–Haack reagent was generated by the addition of 2 ml of POCl3 over the course of 15 minutes to 8 ml of dry DMF cooled in an ice-salt bath. After the addition was complete, the ice bath was removed and the contents of the flask were left to warm to room temperature over approx. 30 minutes. The substituted indole (21.9 mmol) was dissolved in 10 ml of DMF and added over a period of 15 minutes to the formylating mixture. The stirring was continued for an hour during which the flask contents were heated to 40 °C in a hot water bath. A total of 20 ml of 5 M NaOH was added, and the mixture was diluted with H2O, quickly brought to a boil and left to cool slowly. The crystals were removed by filtration, washed with cold water and vacuum dried. The products thus obtained were in most cases sufficiently pure for the subsequent reactions. The impure aldehydes were recrystallized from ethanol–water mixtures.

**General procedure 2 for the synthesis of 1-alkylimidazoles 11, 12, 14, 15.** Van Leusen reaction was conducted as described previously. Aromatic aldehyde (3 mmol) was mixed with amine (15 mmol) in 20 ml dry methanol. Reaction mixture was left overnight to complete the imine formation although it can be TLC monitored (SiO2/CHCl3). Anhydrous K2CO3 (3 mmol) and TosMIC (tosylmethylisocyanide, 3 mmol) were subsequently added. The mixture was stirred for an additional 8 hours, diluted with 50 ml H2O, and extracted three times with 20 ml ethyl acetate. The combined extracts were washed twice with 20 ml H2O, and once with 20 ml brine, treated with anhydrous magnesium sulfate and evaporated. The final products were purified by trituration under a 2 : 1 hexane : isopropanol mixture.

**General procedure 3 for the synthesis of 3-(1-alkyl-1H-imidazol-5-yl)-1H-indole-5-carboxamides (7 and 13).** Compounds were synthesized from appropriate nitriles (11 and 12, respectively), the cyanide group hydrolysis was accomplished according to a modified procedure outlined by Agarwal et al. A suspension of the nitrile (3.4 mmol) in 1 ml MeOH and 5 ml THF was stirred in an ice-salt bath for 15 minutes until 0 °C was reached. Hydrogen peroxide (30% solution, 5.4 ml) was added dropwise keeping the temperature below 10 °C. Stirring was continued for 15 minutes and sodium hydroxide (20% solution, 5.4 ml) was added dropwise keeping temperature below 10 °C. The mixture was allowed to warm to room temperature and stirred for 24 hours. The product was extracted from the reaction mixture with chloroform–methanol mixture (9 : 1 v/v) and purified by trituration with acetone.

**Analytical methods**

UPLC/MS analysis was performed on Waters TQD spectrometer combined with UPLC Acquity H-Class with PDA eLambda.
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Influence on Pgp activity

The influence of 5, 7 and 13 on glycoprotein P (Pgp) activity was estimated using the luminescent Pgp-Glo™ Assay System (Promega, Madison, WI, USA) as described previously. The reference inhibitor NaN₃ was used in 100 μM concentration and reference stimulator verapamil in 200 μM, according to the manufacturer’s recommendations. Compounds were examined at 100 μM. All compounds were incubated with Pgp membranes for 40 minutes at 37 °C in triplicate. GraphPad Prism™ software (version 5.01, San Diego, CA, USA) was used to calculate statistical significance.

Metabolic stability

Human (HLMs), rat (RLMs) and mouse liver microsomes (MLMs) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The NADPH regeneration system was purchased from Promega® (Madison, WI, USA). All experiments were performed as described before. Each compound was tested in triplicate at the final concentrations similar for all CYP3A4, CYP2D6 and CYP2C9 assays in range from 0.01 to 25 μM. The luminescent signal was measured by using a microplate reader EnSpire PerkinElmer (Waltham, MA, USA).

Safety

Human embryonic kidney HEK-293 cell line (ATCC® CRL-1573™) was kindly donated by Prof. Dr. Christa Müller (Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn). Hepatoma HepG2 (ATCC® HB-8065™) cell line was kindly donated by the Department of Pharmacological Screening, Jagiellonian University Medical College. SH-SY5Y (ATCC® CRL-2266™) cell line was purchased from ATCC. The cell cultures’ growth conditions were applied as described before. The CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) was purchased from Promega (Madison, WI, USA). The absorbance of the samples was measured using a microplate reader EnSpire PerkinElmer, Waltham, MA USA) at 490 nm. The reference compounds CCCP (10 μM) and DX (1 μM) as well as compounds 5, 7 and 13 (0.1–100 μM) were tested in four repetitions.

The CellTiter-Glo Luminescent Cell Viability Assay for ATP-level assessment was purchased from Promega® (Madison, WI, USA). The ATP level was measured as described previously after 3 h of HepG2 cells’ exposure on compound 7 tested at three concentrations (1, 10 and 100 μM), and on the reference compounds CCCP (10 μM) and DX (1 μM). The luminescence signal was measured with a microplate reader EnSpire PerkinElmer, Waltham, MA USA) in luminescence mode. The assay was performed in quadruplicate.

The Salmonella typhimurium TA100 strain with base pair substitution (hisG46 mutation, which target is GGG) was purchased from Xenometrix, Allschwil, Switzerland, and has been used in mutagenicity Ames 384 – well microtiter assay. All experiments were performed as described before.

GraphPad Prism™ software (version 5.01, San Diego, CA, USA) was used to calculate statistical significance in all safety experiments.

Novel object recognition experiment

The method was performed according to Nilsson et al. The animals were trained and tested in a black, plastic, open field (50 × 30 cm, 35 cm high). The open field was in a dark room illuminated only by a 25 W bulb. On the first day (adaptation) the animals were allowed to explore the open field for 10 min. On the next day (training, T1) the
animals were administered the tested drugs, placed in the apparatus and allowed to explore two identical objects (a red, glass cylinder, 6.5 cm in diameter, 4.5 cm high). For the retention trial (T2) conducted one hour later, one of the objects presented in T1 was replaced with a novel object (a transparent glass elongated sphere-like object with an orange cap). The duration of exploration of each object (i.e., sitting in close proximity to the objects or sniffing or touching them) during 5 min time was video recorded and measured separately by a trained observer. All drugs were administered before the training (T1) session. MK-801 (0.3 mg kg⁻¹, i.p.) was given 30 min before the session. Investigated compounds were administered 60 min before MK-801.

The modified stress-induced hyperthermia in singly-housed mice

Each experimental group consisted of eight to ten animals in a modified stress-induced hyperthermia (SIH) test. The animals were housed individually in a 26 × 21 × 14 cm Macelon cage, 24 h before testing. The procedure for the modified stress-induced hyperthermia was adapted from Van der Heyden et al. and based on the procedure introduced by Borsini et al. For this assay, the body temperature was measured for each mouse at T = 0 min (T₁) and T = +15 min (T₂). Albino Swiss mice were placed into a new cage immediately following T₁, with the difference in temperature (T₂ − T₁) used as the measure of stress-induced hyperthermia. The pilot studies of Spooren demonstrated that a T₂ − T₁ interval of 15 min is optimal for assay of SIH. A comparison between T₁ in vehicle-treated mice and those administered with test compound was used to determine whether the agent affects the body temperature alone. The rectal temperature was measured to the nearest 0.1°C by a Physitemp Thermalert Thermometer, TH-5, (Clifton, NJ, USA), with the temperature sensor for mice, type T, copper-constantan thermocouple, BrainTrent Scientific Inc. The lubricated thermistor probe (2 mm diameter) was inserted 20 mm into the rectum. The mouse was held at the base of the tail during this determination and the thermistor probe was left in place until a constant reading was obtained for 15 s. Compound 7 was administered at the doses 1, 5 and 15 mg kg⁻¹ 60 min before test. Neither vehicle nor the tested doses of the compound had any influence on the basal body temperature, which was between 36 and 37 °C, typically for Albino Swiss mice used in our laboratory. The statistical significance was evaluated by one-way ANOVA followed by Dunnett’s post hoc analysis.

The procedures were conducted in accordance with the European Communities Council Directive 2010/63/EU and Polish legislation acts concerning animal experimentation. The experiments were approved by II Local Ethics Committee in Krakow by the Institute of Pharmacology, Polish Academy of Sciences in Krakow (no. 181/2016). All procedures were conducted according to the guidelines of the National Institutes of Health Animal Care and Use Committee.

Conclusions

The recently described 5-(1H-indole-3-yl)-1-alkylimidazoles are the first examples of low-basicty agonists of the 5-HT₇ receptor. Several compounds from this group have been discovered as suitable molecular probe candidates, which could be used for both in vitro and in vivo studies examining the physiology and pathophysiology of the CNS associated with 5-HT₇. Indole-imidazole derivatives 7 (previously published) and 13 are the closest structural analogues of the commonly used molecular probe 5-CT [5]. However, compound 5 exhibits a multi-receptor profile, i.e., high affinity for 5-HT₇-R, 5-HT₁AR, 5-HT₅AR and to some extent also 5-HT₆R receptors. The lack of selectivity as well as low blood-brain barrier permeability are the main disadvantages of 5 as a pharmacological tool. The high affinity of 7 and 13 for 5-HT₇-R as well as high selectivity over the 5-HT₁AR receptor (25-fold and 37-fold, respectively, Table 1) are the main advantages of this compound over 5-CT. In vitro comparison studies between 5 and 13 demonstrated their similarities (Table 2). Compounds 5 and 7 displayed very good ability to diffuse passively across biological membranes and also significantly inhibited Pgp. For compound 13 slight, not statistically significant Pgp inhibition was observed. Excellent metabolic stability of 5, 7, and 13 was also determined, in particular of compound 13, for which no metabolites were observed after incubation with HLMs and RLMs. A significant difference between 5 and 3-(1-alkyl-1H-imidazol-5-yl)-1H-indole-5-carboxamides was observed in the in vitro assays only in terms of the influence on cytochromes P-450 activity. However, inhibition activity of 7 and 13 was expected herein, as had been previously observed for another imidazole-based 5-HT₇-R agonist – compound 6. Finally, no significant toxicity or mutagenicity was found for 5, 7 and 13.

The in vivo activity of 7 was confirmed in pharmacological experiments in mice, providing indirect confirmation of blood-brain barrier permeation. Compound 7 was active in the NOR test in mice at a relatively low dose of 1 mg kg⁻¹, which is in agreement with previous results obtained for 5-CT. Moreover, its activity was also observed in the SIH test at 15 mg kg⁻¹.

We report compounds 7 and 13 as a very promising alternatives to 5-CT with comparable in vitro receptor affinity, very high-water solubility, similar in vitro ADMET properties and a desirable high selectivity over 5-HT₁AR receptor. Compound 7, also confirmed in vivo activity in novel object recognition and the modified stress-induced hyperthermia assays.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The study was partially supported by the grant OPUS 2017/25/B/NZ7/02929 from the Polish National Science Centre.
Notes and references