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Drug Annotation

Discovery and Preclinical Characterization of Usmarapride (SUVN-D4010): A Potent, Selective 5-HT$_4$ Receptor Partial Agonist for the Treatment of Cognitive Deficits Associated with Alzheimer's Disease

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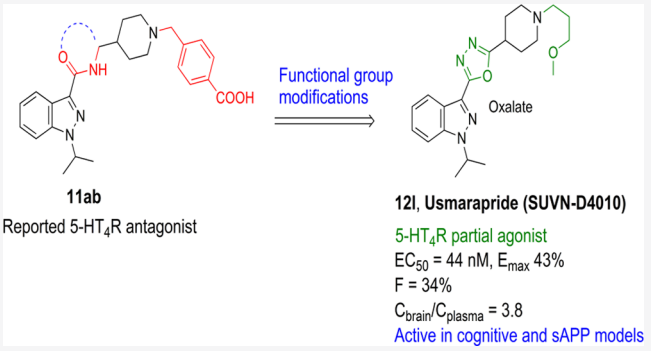
ABSTRACT: A series of oxadiazole derivatives were synthesized and evaluated as 5-hydroxytryptamine-4 receptor (5-HT$_4$R) partial agonists for the treatment of cognitive deficits associated with Alzheimer's disease. Starting from a reported 5-HT$_4$R antagonist, a systematic structure−activity relationship was conducted, which led to the discovery of potent and selective 5-HT$_4$R partial agonist 1-isopropyl-3-{5-[1-(3-methoxypropyl) piperidin-4-yl]-[1,3,4]-oxadiazol-2-yl}-1H-indazole oxalate (Usmarapride, **12l**). It showed balanced physicochemical−pharmacokinetic properties with robust nonclinical efficacy in cognition models. It also showed disease-modifying potential, as it increased neuroprotective soluble amyloid precursor protein alpha levels, and dose-dependent target engagement and correlation of efficacy with oral exposures. Phase 1 clinical studies have been completed and projected efficacious concentration was achieved without any major safety concerns. Phase 2 enabling long-term safety studies have been completed with no concerns for further development.



INTRODUCTION

Alzheimer's disease (AD) is a debilitating progressive neurodegenerative disorder, affecting mostly the aging population. AD is characterized by the presence of Aβ plaques and pathologic tau deposits with clinical symptoms like confusion, memory loss, and dementia. Dysfunction of acetylcholine containing neurons in the brain contributes substantially to the symptoms. The current pharmacotherapy provides only symptomatic relief through augmentation of cholinergic function with several undesired side effects such as nausea, vomiting, and bradycardia. Moreover, no disease-modifying therapy is available to the patients to date. Thus, there is a need to explore new therapies through different modes of action. 5-Hydroxytryptamine-4 receptor (5-HT$_4$R) is one of such targets which have attracted the attention of scientific community.[1] The 5-HT$_4$R belongs to a superfamily of seven transmembrane G-protein coupled receptors (GPCRs) and is coupled to a G-protein containing Gαs subunit.[2] The 5-HT$_4$R is reported to have a potential role in many central nervous system (CNS) disorders such as AD[3,4] and peripherally mediated disorders such as irritable bowel syndrome[5] and gastroparesis.[6] The 5-HT$_4$ receptor is present at a high density in brain regions like the hippocampus, amygdala, and cerebral cortex, suggesting the possible involvement of this receptor in cognitive processes.[7] The 5-HT$_4$R agonists modulate amyloid precursor protein (APP)-derived peptides, amyloid-beta (Aβ), and soluble amyloid precursor protein alpha (sAPPα).[8] The sAPPα is a soluble nonamyloidogenic protein, and it is reported to have a potent neuroprotective role against the neurotoxic effects of glutamate and β-amyloid.[9] Human 5-HT$_4$R isoforms are positively coupled to adenylyl cyclase production. Thus, activation of this G-protein coupled receptor activates adenylyl cyclase, consequently increasing the production of cyclic adenosine monophosphate (cAMP).

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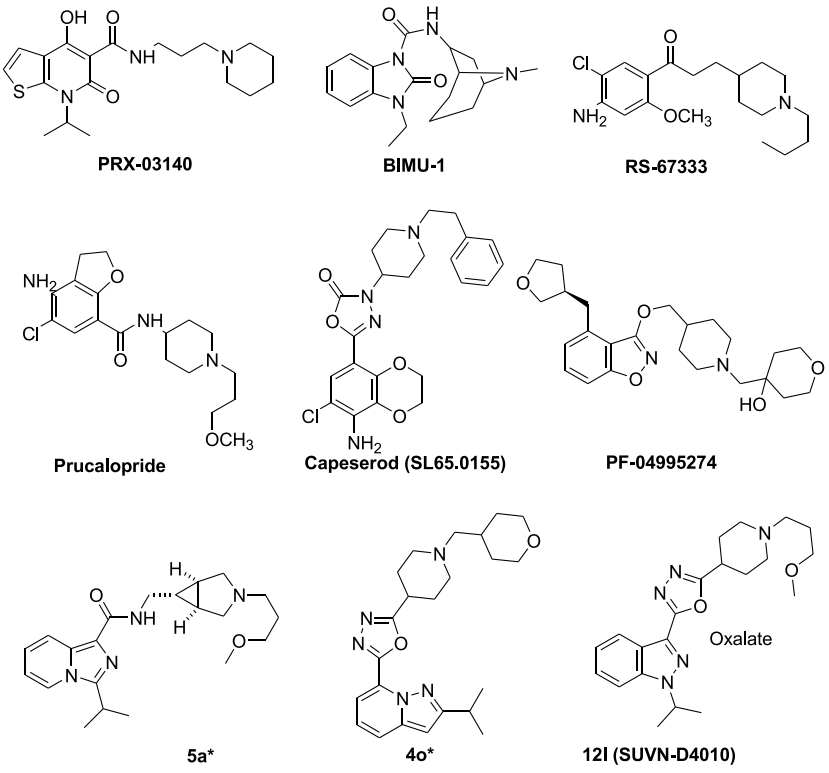

Figure 1. Reported 5-HT$_4$R ligands along with the current series of compounds.

The increased cAMP, in turn, increases sAPPα release. The stimulatory effect of 5-HT$_4$R on sAPPα release is mimicked by forskolin, a direct activator of adenylyl cyclase, and 8-bromo-cAMP (membrane-permeant cAMP analog), suggesting the involvement of adenylyl cyclase and cAMP in modulating sAPPα by 5-HT$_4$R.[9] The 5-HT$_4$R also increases the neuronal acetylcholine (ACh) levels in the brain.[10] However, prolonged exposure of GPCRs to a full agonist can result in receptor desensitization, thereby limiting the compound's therapeutic usefulness. Partial agonists have shown to minimize the potential for desensitization while retaining the desired activity.[11] Thus, 5-HT$_4$R partial agonists may be of use for both disease modifying and symptomatic treatment of cognitive disorders associated with AD.[12] Several structurally diverse heteroaromatic derivatives[13−18] as 5-HT$_4$R agonists/partial agonists/antagonists have been explored for CNS and peripheral indications. The 5-HT$_4$R partial agonist PRX-03140 showed significant improvement in cognition assessed by the Alzheimer's Disease Assessment Scale-Cognition (ADAS-Cog) score in a phase 2a study for AD.[19,20] Apart from this compound, several other 5-HT$_4$R compounds such as BIMU-1,[21] RS-67333,[22] prucalopride,[11] capeserod (SL65.0155),[23] and PF-04995274[13] are in development (Figure 1).

In our continuous efforts of discovering novel small molecules for the potential treatment of AD with a diverse mechanism of actions,[24−27] the 5-HT$_4$R agonists attracted our attention as they have a dual mechanism of action in treating AD[10] and availability of positive proof-of-concept phase 2 clinical results of PRX-03140. Most of the reported 5-HT$_4$R ligands which have been explored for both CNS and peripheral indications belong to benzamide, imidazole, imidazopyridine, pyrazolopyridine, 2-oxoquinoline, and indazole derivatives. Previous work from our group disclosed a series of imidazo-[1,5-*a*]pyridine[24] and pyrazolo[1,5-*a*]pyridine derivatives[27] represented by compounds **5a** and **4o** (Figure 1), respectively, as 5-HT$_4$R partial agonists with procognitive effects in animal models. While selected compounds from these series are at various stages of development, we focused on earlier reported indazole derivatives[28] represented by compound **11ab** (Figure 2). Compound **11ab** was reported as a 5-HT$_4$R antagonist, and its structure can also be envisaged from 5-HT$_4$R partial agonist **5a**[24] based on the structural similarity (Figure 2). We aimed to identify a structurally diverse 5-HT$_4$R partial agonist as a lead compound with an adequate brain penetration, acceptable pharmacokinetics, robust nonclinical efficacy, and sufficient safety margin for the treatment of AD and associated disorders. Selective 5-HT$_4$R partial agonists are reported to have the potential to modulate the centrally located 5-HT$_4$ receptors while showing minimal effects on the gastrointestinal system through peripheral 5-HT$_4$R. Activation of peripheral 5-HT$_4$R is implicated in modulating gastric intestinal motility and cardiac function. The structural modifications were done keeping in mind the 5-HT$_4$R pharmacophore requirements such as an aromatic moiety, a coplanar carbonyl group, or its bioisosteres like oxadiazole, and a voluminous substituent in the basic amino framework of the molecule.[29,30] The basic amino framework part of reported indazole derivative **11ab**[28] was modified appropriately by implementing earlier understandings.[24] The well-optimized[24,27] *N*-isopropyl group on indazole nitrogen was kept constant throughout the structure−activity relationship (SAR) (Figure 2). Modifications in the basic amino framework led to chemical scaffolds showing desired partial agonistic activity at 5-HT$_4$R. The successive modifications led to 1-isopropylindazole-carboxamide derivatives

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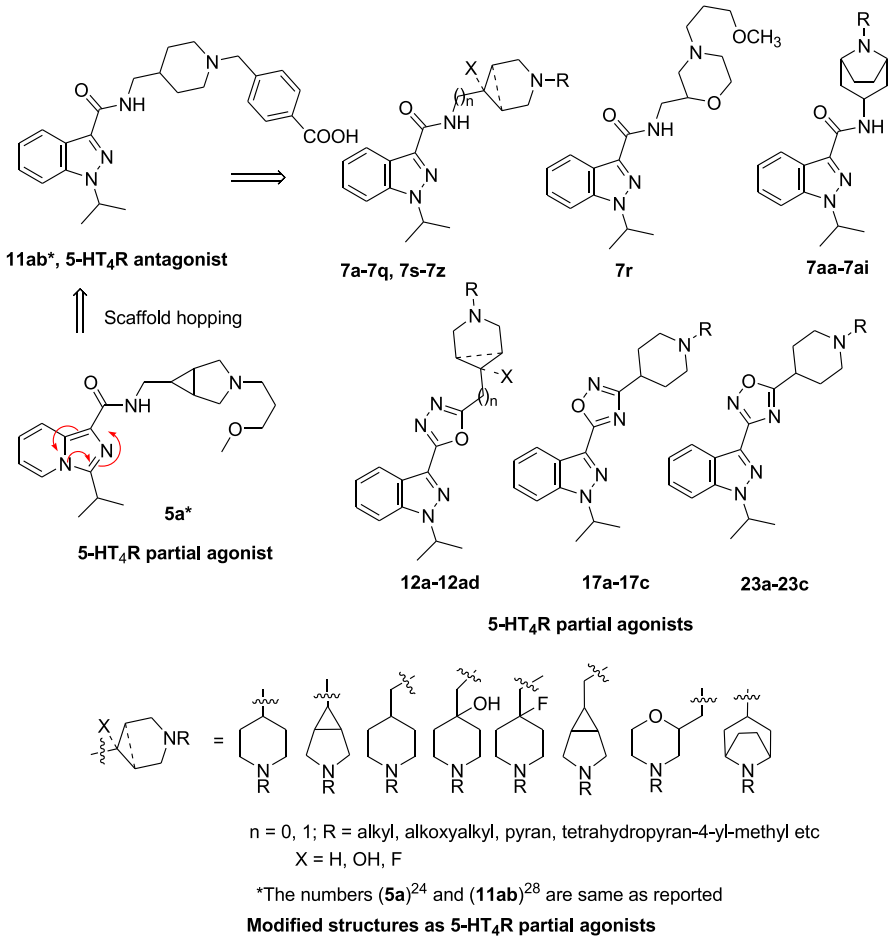
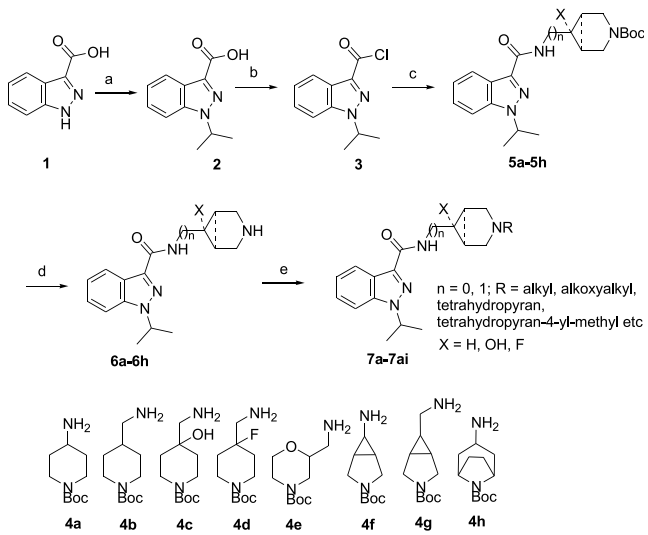


Figure 2. Genesis of ligands.

(**7a**−**7ai**), 1-isopropylindazole-1,3,4-oxadiazole derivatives (**12a**−**12ad**), and 1-isopropylindazole-1,2,4-oxadiazole derivatives (**17a**−**17c** and **23a**−**23c**). The SAR covering *in vitro* binding affinity, hERG affinity, metabolic stability, CYP inhibition, pharmacokinetics, receptor occupancy, and *in vivo* efficacy resulted in clinical candidate 1-isopropyl-3-{5-[1-(3-methoxypropyl) piperidin-4-yl]-[1,3,4]oxadiazol-2-yl}-1H-indazole oxalate (**12l**, Usmarapride). Compound **12l** has completed phase 1 clinical studies and achieved projected efficacious concentrations within the tested doses. Phase 2 enabling nonclinical safety studies for compound **12l** in rats and dogs were completed with no concerns for further development.

Chemistry. The general synthetic strategy used for the preparation of the present series of compounds is summarized in Schemes 1−4. The starting material indazole-3-carboxylic acid was sourced from a commercial vendor, and other building blocks were synthesized as per the earlier reported[24,27,31,32] protocols. Synthesis of compounds **7** commenced (Scheme 1) with indazole-3-carboxylic acid **1** which on N-isopropylation reaction with 2-iodopropane in the presence of NaO^{t}Bu afforded *N*-isopropylindazole-3-carboxylic acid **2** as a single isomer. The carboxylic acid **2** was then converted to acid chloride **3** by treating it with thionyl chloride. The acid chloride **3** was then reacted with different N-Boc protected cyclic amines **4** in the presence of K_2CO_3 to afford N-Boc protected carboxamide derivatives **5**. Deprotection of Boc in compounds **5** in the presence of trifluoroacetic acid followed by basification with $NaHCO_3$ yielded compounds **6**. The N-alkylation of compounds **6** by

Scheme 1. Synthesis of Compounds 7a−7ai[a]



[a]Reagents and conditions: (a) NaO^{t}Bu, 2-iodopropane, DMF, r.t., 16.0 h, 60%. (b) $SOCl_2$, 1,2-dichloroethane, 0 °C-reflux, 1.0 h, 100%. (c) K_2CO_3, Boc protected amine **4a**−**4h**, DCM, 0 °C-r.t., 16.0 h. (d) TFA, DCM, 0 °C-r.t., 16.0 h, H_2O, $NaHCO_3$. (e) K_2CO_3, acetonitrile, R−X, r.t.-reflux, 7.0−16.0 h.

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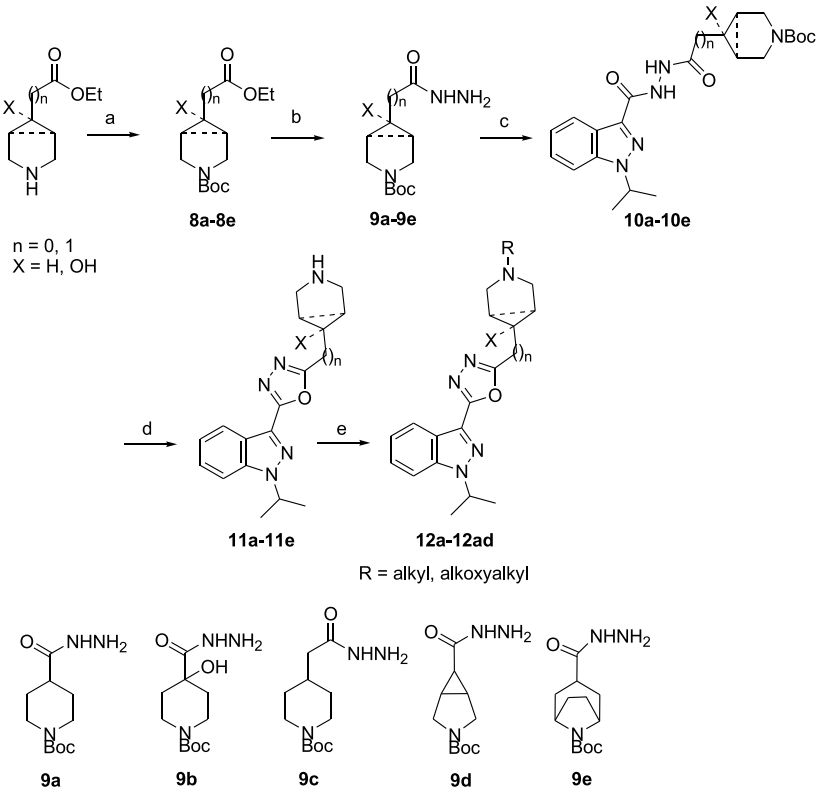
Scheme 2. Synthesis of Compounds 12a−12ad[a]



[a]Reagents and conditions: (a) Boc_2O, Et_3N, DMAP, DCM, 0 °C-r.t., 16 h. (b) Hydrazine-hydrate, MeOH, r.t.-reflux, 10.0 h. (c) (i) DCM, acid chlroride 3, 0 °C-r.t., 4.0 h; (ii) H_2O, $NaHCO_3$, 2 h. (d) $SOCl_2$, 1,2-dichloroethane, r.t.-reflux, 6.0−10.0 h. (e) K_2CO_3, acetonitrile, R−X, r.t-reflux, 7.0−16.0 h.

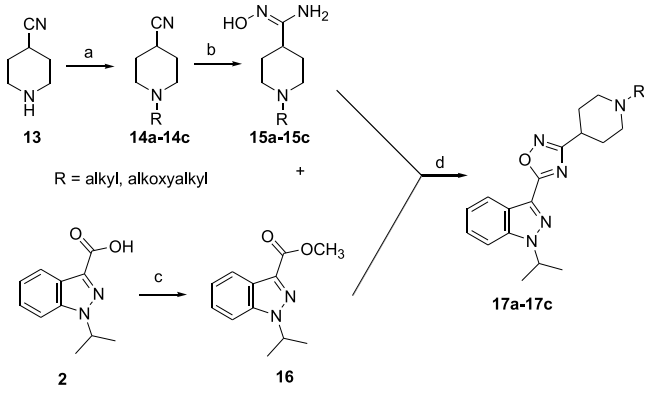
reacting with alkyl halides yielded N-alkylated carboxamide derivatives **7**.

The 1,3,4-oxadiazole derivatives **12** were prepared as mentioned in Scheme 2. The hydrazides **9** were prepared from the corresponding esters **8** by refluxing with hydrazine in methanol. The esters **8** were either sourced commercially or prepared by reported procedures. The hydrazides **9** were then reacted with acid chloride **3** in DCM to afford compounds **10** after basification with $NaHCO_3$. Cyclization of compounds **10** with thionyl chloride in 1,2-dichloroethane at reflux temperature afforded compounds **11** with concomitant Boc deprotection. The N-alkylation of compounds **11** with appropriate alkyl halides in the presence of K_2CO_3 afforded 1,3,4-oxadiazole derivatives **12**.

The 1,2,4-oxadiazoles **17** were prepared as mentioned in Scheme 3. Commercially available 4-cyanopiperidine **13** was alkylated using a standard N-alkylation procedure to obtain compounds **14**. Compounds **14** on treatment with hydroxylamine in ethanol in the presence of K_2CO_3 afforded compounds **15**. The *N*-isopropylindazole-3-carboxylic acid **2** was converted to its methyl ester **16** by reacting with methanol in the presence of dry HCl. The desired 1,2,4-oxadiazole derivatives **17** were obtained upon treatment of compounds **15** with ester **16** in DMF in the presence of NaH.

1,2,4-Oxadiazoles **23** were prepared as mentioned in Scheme 4. The *N*-isopropyl-indazole-3-carboxylic acid **2** was converted to *N*-isopropyl-indazole-3-carboxamide **18** with Boc anhydride and NH_4HCO_3. The carboxamide derivative **18** was subjected to dehydration using TFAA and triethylamine to afford nitrile **19**. The commercially available ethyl isonipecotate **21** was

Scheme 3. Synthesis of Compounds 17a−17c[a]



[a]Reagents and conditions: (a) K_2CO_3, R−X, acetonitrile, r.t-reflux, 7.0−16.0 h. (b) NH_2-OH·HCl, K_2CO_3, EtOH, r.t.-reflux, 16.0 h. (c) $SOCl_2$, CH_3OH, 0 °C-r.t., 6.0 h. (d) NaH, DMF, 120 °C 0.5 h.

alkylated ith appropriate alkyl halides using a standard N-alkylation procedure to obtain compounds **22a−22c**. The desired 1,2,4-oxadiazole derivatives **23a−23c** were obtained upon treatment of compounds **22** with compound **20** in DMF in the presence of NaH.

RESULTS AND DISCUSSION

The newly synthesized compounds were screened in the cell-based luciferase reporter gene assay that measures the levels of cAMP inside cells upon activation or inhibition of the receptor, the results of which are shown in Table 1. Among the first

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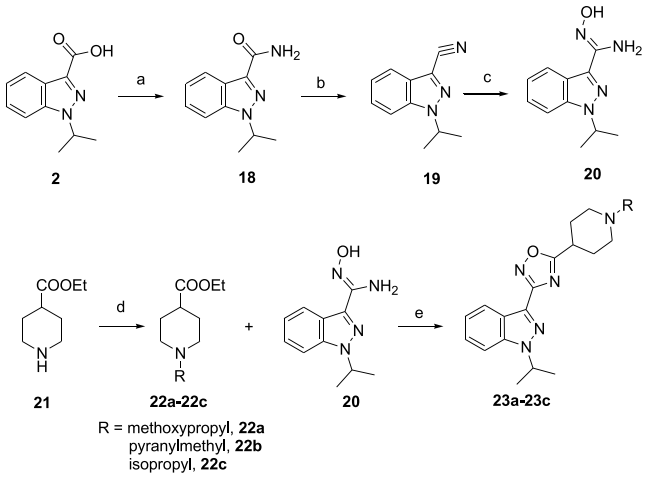
Scheme 4. Synthesis of Compounds 23a−23c[a]



[a]Reagents and conditions: (a) Boc_2O, pyridine, NH_4HCO_3, acetonitrile, r.t., 16.0 h, 83%. (b) TFAA, Et_3N, THF, 0 °C-r.t., 2.0 h, 86%. (c) NH_2-OH·HCl, K_2CO_3, EtOH, r.t.-reflux, 16.0 h, 100%. (d) K_2CO_3, R−X, acetonitrile, r.t-reflux, 7.0−16.0 h. (e) NaH, DMF, 120 °C, 16.0 h.

prepared 1-isopropylindazole-carboxamide derivatives, **7a**−**7c**, consisting of aminopiperidine **4a**, 1-hydroxypropyl derivative **7a** and 1-fluoroethyl derivative **7c** showed potent *in vitro* efficacies (EC_{50}) of 6.4 and 5.8 nM with intrinsic activity (E_{max}) values of 60 and 71%, respectively. Compound **7b** with a 1-hydroxyethyl group showed a modest EC_{50} of 38 nM and E_{max} of 39%. The observed partial agonist activity was in contrast with the antagonist activity reported[28] for structurally similar types of indazole derivatives (**11ab**)[28] with bigger alkyl groups on the piperidine ring (Figure 2). Encouraged by these findings, additional analogs (**7d**−**7g**) with branched alkyl groups with fluoro, hydroxy, or methoxy group substitutions were synthesized and evaluated. These compounds were 5−7-fold less potent as compared to normal alkyl group derivatives (**7a**−**7c**). Compounds with cyclic ethers such as pyran, methylpyranyl groups (**7h**−**7k**) showed 2−3-fold more potency than the branched alkyl-substituted compounds (**7d**−**7g**) but were 2−4-fold less potent than the alkyl-substituted derivatives (**7a** and **7c**). Overall, the straight alkoxyalkyl or fluoroalkyl-substituted piperidine derivatives were more potent than other branched alkyl or oxy-cyclic-substituted piperidine derivatives. In the next change in the compounds, a methylene linker was introduced between piperidine and indazolyl-3-carboxamide moiety. The methyl-piperidine derivatives **7l** and **7n** consisting of aminopiperidine **4b** showed very weak activity with higher EC_{50} and lower E_{max} (∼10%) values, indicating the linker was not tolerated. Introducing hydroxy and fluoro groups at the fourth position of the piperidine ring (aminopiperidine **4c** and **4d**) yielded compounds **7o**−**7q**. Though this modification resulted in compounds with potent EC_{50} values (<50 nM), the E_{max} was on the lower side as compared to **7a** and **7c**. The methylpiperidine was then replaced with morpholine methyl-amine (**4e**) derivative **7r** with an EC_{50} value of 3906 nM. Introducing the bicyclic 3-aza-bicyclo[3.1.0]hexane ring in place of piperidine resulted in less potent derivatives **7s**−**7u**. Bringing the methylene linker in 3-aza-bicyclo[3.1.0]hexane group (**4g**) dramatically increased the potency of the synthesized compounds (**7v**−**7z**) with EC_{50} values <12 nM, albeit with weak agonist activity (E_{max} < 20%). The bicyclic 3-aza-bicyclo[3.1.0]hexane group was replaced with 8-aza-bicyclo[3.2.1]octan (**4h**) which resulted in potent compounds (**7aa**−**7ai**) with EC_{50} values ranging from 3 nM to 62 nM and the agonist activity ranging from partial (E_{max}, 64%) to full agonist (E_{max}, 98%). Noteworthy in these compounds is barring branched *N*-alkyl derivatives (**7aa**, **7ad**, **7ae**, and **7ai**), all other compounds showed <10 nM EC_{50} values with >50% agonist activity. Compound **7ac** showing a EC_{50} value of 3 nM with 66% E_{max} value stands out to be the most potent partial agonist from the indazole 3-carboxamide derivatives synthesized.

Bioisosteres have been explored in the past to improve potency, enhance selectivity, and optimize pharmacokinetic (PK) profiles or alter toxicophores in addition to being a source of novel intellectual property.[33] To have compounds with improved potency and favorable pharmacokinetics, the symmetrical 1,3,4-oxadiazole and asymmetrical 1,2,4-oxadiazole derivatives were envisaged[34] as carboxamide bioisosteres (Table 2). Compound **12a** without any alkyl substitution on piperidine nitrogen showed very weak activity with EC_{50} of 1507 nM, however; it retained partial agonist activity showing an E_{max} value of 43% (Table 2). The alkyl-substituted derivatives **12b**−**12i** showed EC_{50} values between 23 nM and 199 nM with moderate E_{max} values ranging from 28% to 56%. Among the lot, the isopropyl derivative **12b** and phenethyl derivative **12d** showed potent EC_{50} values of 23 nM and 24 nM, respectively. The fluoroethyl derivative **12j** showed very weak activity with an EC_{50} value of 1814 nM, as against its carboxamide counterpart **7c** which showed a potent EC_{50} value of 5.8 nM. The other heteroalkyl derivatives **12k**−**12t** showed moderate EC_{50} values ranging between 22 nM and 172 nM. These compounds showed a weak agonist activity ranging from (E_{max}, 17%) to moderate (E_{max}, 53%) partial agonist activity. The heteroalicyclic-substituted derivatives **12o** and **12p** showed EC_{50} values of 79 nM and 84 nM, respectively, whereas compound **12u** having a methylpyranyl group with junction hydroxy showed a weak EC_{50} value of 210 nM. The higher EC_{50} value for compound **12u** is in sharp contrast to the corresponding carboxamide derivative **7j** with the same substitution that showed a potent EC_{50} value of 15 nM. Compound **12v** showed very weak *in vitro* potency with an EC_{50} value of 675 nM with a hydroxy group on the junction of piperidine. The introduction of an additional methylene group between 1,3,4-oxadiazole and piperidine resulted in compounds **12w**−**12y** with weak *in vitro* potency. The bicyclic 3-aza-bicyclo[3.1.0]hexane 1,3,4-oxadiazole derivatives **12z**−**12ab** turned out to be inactive. The similar carboxamide derivative **7v** was potent albeit with weak partial agonist activity as compared to inactive 1,3,4-oxadiazole derivative **12ab**. The tropane derivatives **12ac** and **12ad** showed weak potency with an EC_{50} value of 695 nM and 1060 nM respectively.

The 1,2,4-oxadiazole derivatives were made to compare their activity with that of 1,3,4-oxadiazole counterparts. The 1,2,4-oxadiazole derivative **17a** having a methoxypropyl group on the piperidine ring which is at the 5-position of oxadiazole showed an EC_{50} value of 156 nM which is ∼3-fold less than that of 1,3,4-oxadiazole derivative **12l**. However, the other synthesized derivatives **17b** and **17c** showed 2−4-fold more potency than their corresponding 1,3,4-oxadiazole derivatives**12p** and **12b** respectively. On the other hand, 1,2,4-oxadiazole derivative **23a** with an EC_{50} value of 21 nM showed

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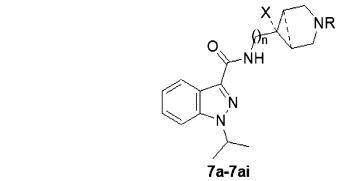
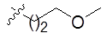
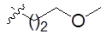
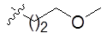
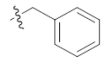
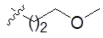
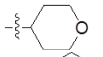
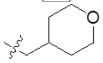
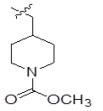
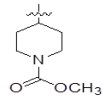
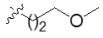
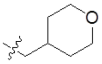
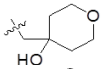
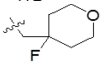
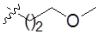
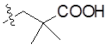
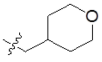
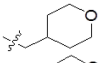
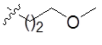
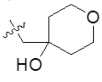
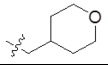
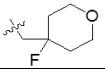
Table 1. 5-HT$_4$R Functional Activity of Indazole Carboxamide Derivatives 7a−7ai[a]



Compd.	Amino-piperidine	R	EC$_{50}$ (nM)	E$_{max}$ (%)	Compd.	Amino-piperidine	R	EC$_{50}$ (nM)	E$_{max}$ (%)
PRX-03140	NA	NA	58 ± 7.2	48 ± 5.9	**7q**	4d		49.4 ± 4.1	12 ± 3.5
RS-67333	NA	NA	2 ± 0.8	47 ± 13.4	**7r**	4e		3906 ± 595.4	5 ± 2.8
PF-04995274	NA	NA	1.3 ± 0.6	18 ± 9.2	**7s**	4f		>10000	NA
7a	4a		6.4 ± 1.0	60 ± 2.8	**7t**	4f		318 ± 25.5	16 ± 4.2
7b	4a		38 ± 4.2	39 ± 5.7	**7u**	4f		343 ± 39.6	30 ± 7.1
7c	4a		5.8 ± 1.9	71 ± 4.2	**7v**	4g		3.8 ± 1.1	18 ± 2.8
7d	4a		29 ± 2.7	51 ± 7.1	**7w**	4g		5 ± 0.6	16 ± 3.9
7e	4a		48 ± 8.6	57 ± 5.7	**7x**	4g		12.4 ± 2.1	19 ± 2.5
7f	4a		48 ± 5.7	63 ± 11.3	**7y**	4g		4.3 ± 0.4	14 ± 4.4
7g	4a		45 ± 5.8	42 ± 1.4	**7z**	4g		3.7 ± 0.3	9 ± 5.3
7h	4a		10.5 ± 1.3	69 ± 3.5	**7aa**	4h		18.5 ± 1.8	72 ± 3.2
7i	4a		14 ± 4.2	48 ± 2.8	**7ab**	4h		7.3 ± 1.0	64 ± 1.6
7j	4a		15 ± 1.6	48 ± 4.2	**7ac**	4h		3 ± 0.4	66 ± 5.1
7k	4a		32 ± 5.7	43 ± 8.5	**7ad**	4h		14.8 ± 2.3	67 ± 2.3
7l	4b		291 ± 31.0	8 ± 4.2	**7ae**	4h		62 ± 15.6	98 ± 1.4
7m	4b		10.4 ± 0.7	11 ± 2.8	**7af**	4h		5.2 ± 0.8	70 ± 2.1
7n	4b		>10000	NA	**7ag**	4h		5.8 ± 1.7	67 ± 1.8
7o	4c		21.6 ± 2.1	34 ± 4.2	**7ah**	4h		4.2 ± 1.0	89 ± 5.3
7p	4c		25 ± 1.3	22 ± 2.8	**7ai**	4h		23 ± 2.8	82 ± 5.5

[a]Data represent mean ± SD. Functional activity determined in CHO cells stably expressing the human 5HT$_{4E}$ isoform and a reporter gene was used. EC$_{50}$ values were determined from average of two independent experiments tested in duplicate. The in-house data are consistent with

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Table 1. continued

reported values of PRX-03140 (17−52 nM/30−61%), PF-04995274 (0.26 nM/7%), and prucalopride (26 nM/52%) (Supporting Information); NA, not applicable.

2-fold more potency than 1,3,4-oxadiazole derivative **12l**. The other synthesized derivatives **23b** and **23c** from this series showed a weaker potency with EC_{50} values of 106 and 297 nM, respectively.

Several *in vitro* potent compounds which represent structural diversity during the SAR were further evaluated for their rat and human liver microsomal metabolic stability, metabolizing enzymes cytochrome P450 (CYP) 3A4 and 2D6 inhibition, time-dependent inhibition (TDI) for CYP 3A4 enzyme, and the human ether-a-go-go related gene (hERG) inhibition and *in vivo* rat pharmacokinetics, bioavailability, and brain penetration (Table 3).

A compound's metabolic stability is an important parameter to consider during the early stages of SAR. The metabolic stability of the compound is its susceptibility to biotransformation, which helps in predicting the compound's PK properties which in turn helps in designing compounds with favorable PK properties. The metabolic stability results are usually reported as measures of intrinsic clearance, from which secondary PK parameters such as bioavailability and half-life can be calculated if the data on the volume of distribution and fraction absorbed are available. Since these parameters are very important in defining the PK and toxicological profile of the compound, the researchers need to give more emphasis on the metabolic stability during SAR optimization. The metabolic stability of the compounds reported here both in rat and human liver microsomes was found to be low (∼5%) to high (>70%). The carboxamide derivatives showed suboptimal ADME properties, as given in Table 3. The carboxamides **7c** with fluoroethyl group **7f**, **7ad** with a branched hydroxy-alkyl group, and **7ag**, **7ah** with a pyranyl-methyl group on piperidine nitrogen were extensively metabolized in rat liver microsomes; whereas, in human liver microsomes, the metabolism was low to moderate. The high metabolism of these compounds in rat liver microsomes is very well reflected in their poor *in vivo* exposures and bioavailability in rats for the tested analogs **7c**, **7f**, and **7ah**. Low to moderate microsomal metabolism observed with the select carboxamide derivatives such as **7d**, **7v**, **7w**, and **7x** is well correlated with modest oral exposure and bioavailability in rats. Compound **7o**, however, was the exception, as its good metabolic stability both in rat and human microsomes did not translate into good *in vivo* exposures and bioavailability in rats.

The 1,3,4-oxadiazole derivatives **12h**, **12l**, and **12p** showed good microsomal stability both in rats and humans that translated into good oral exposures, good bioavailability, and good brain exposures in rats. The 1,2,4-oxadiazole derivatives **17c** and **23a** showed poor rat microsomal metabolic stability. Rat PK data for **17c** indicated poor oral exposures and bioavailability, but that was not the case for **23a**. The 1,2,4-oxadiazole derivative **23a** showed moderate oral exposures and bioavailability. Good brain penetration property is essential for the compounds intended to treat brain disorders. Most of the compounds such as **7d**, **12d**, **12f**, **12h**, **12l**, **12p**, and **23a** showed very good brain penetration properties. Selected compounds were further evaluated for their CYP and hERG inhibiting properties. Cytochrome P450 (P450) is a family of enzymes that play a major role in the metabolism of drugs. Evaluation of the compound's potential to inhibit a specific P450 enzyme is important, as coadministration of compounds may result in one or both inhibiting the other's metabolism. This may affect the compound's plasma exposures and potentially lead to adverse drug−drug interactions (DDI) or toxicity. The CYP enzyme inhibition liability of the selected compounds is remote, as most of them showed IC_{50} values of >45 μM. The TDI of CYP enzyme refers to a change in compound's potency during an *in vitro* incubation or *in vivo* dosing period. Potential mechanisms include the formation of inhibitory metabolites and mechanism-based inhibition. TDI is of particular concern as it is irreversible, and typically *de novo* synthesis of the enzyme is required to restore the activity. The consequences of TDI can be drug withdrawal or serious restrictions of use. Gratifyingly, the tested compounds either showed minimal or no TDI against CYP3A4 enzyme, therefore discharging them from the potential for DDI liability in a clinical setup. There is a literature precedent that disclosed the cardiovascular adverse events associated with 5-HT_4R agonists. For instance, Cisapride, a 5-HT_4R agonist, was withdrawn from the market due to the risk of fatal cardiac arrhythmias.[35−37] The hERG affinity may have detrimental effects on cardiac safety which prompted us to identify 5-HT_4R agonists having minimal hERG liability. Compounds **7d** and **12k** showed hERG IC_{50} values >10 μM, compounds **12p** and **12l** showed IC_{50} values of 8.5 μM and 3.0 μM respectively, and compound **12h** showed an IC_{50} of 2.4 μM. Compounds **7d**, **12h**, **12k**, **12l**, and **12p**, based on their moderate to good metabolic stability, good *in vivo* exposures in rats, and modest affinity for hERG channel, were evaluated in the object recognition test (ORT), long-term memory deficit assay. The ORT[38,39] is regarded as a spontaneous delayed nonmatching-to-sample (DNMS) test. The test is based on spontaneous behavior. The main assumption of this test is that access to novelty (object or environment) can elicit approach behaviors in animals. The "unconditioned preference" of animals for novelty has been used in the ORT to study memory functions, assessing the ability of animals to recognize a novel object in a familiar environment. ORT does not involve reference memory components, thus it can be considered as a "pure" recognition memory test and a valid task to assess working memory. Additionally, ORT does not involve positive or negative reinforces (food or electric shocks), and this makes it comparable to memory tests currently used in the clinic. These advantages make the ORT efficacy model, quick, and simple.Thus, the ORT model has been widely used for assessing procognitive effects in preclinical models. In this set of experiments, the rats were treated with compounds **7d**, **12h**, **12k**, **12l**, and **12p** which also included reported 5-HT_4R partial agonist RS-67333[22] as a positive control (Figure 3). The reference compound, RS-67333, evaluated at 0.3, 1.0, and 3.0 mg/kg i.p. showed statistically significant activity at 0.3 and 1.0 mg/kg, and the effects faded at 3.0 mg/kg dose, which is in line with literature reports. Compound **7d** showed activity only at 10 mg/kg p.o. dose. Compound **12h** showed activity at all tested doses; however, procognitive activity reduced as the dose increased. Compound **12k** did not show activity at any of the tested doses. Compound **12l** showed activity at 1 and 3

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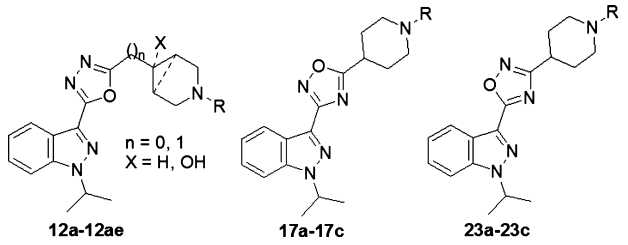
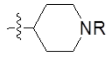
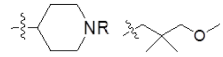
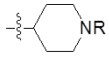
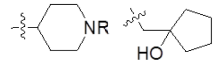
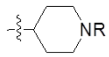
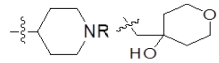
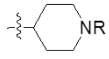
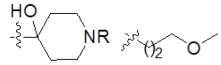
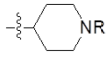
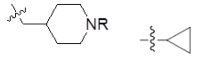
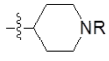
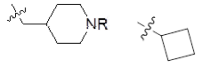
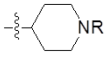
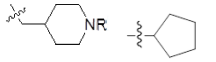
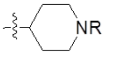
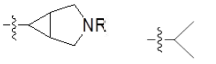
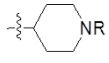
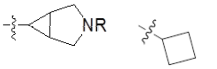
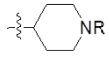
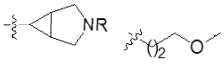
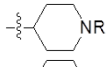
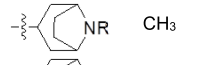
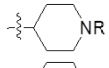
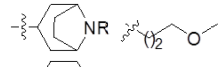
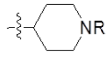
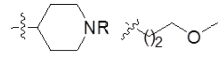
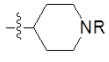
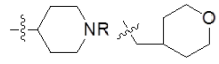
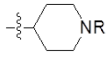
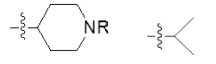
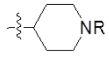
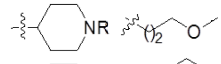
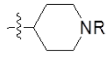
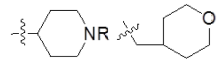
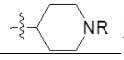
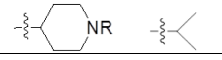
Table 2. 5-HT$_4$R Functional Activity of Indazole Carboxamide Derivatives 12a−12ad, 17a−17c, 23a−23c[a]



Compd.	Azacycle	R	EC$_{50}$ (nM)	E$_{max}$ (%)	Compd.	Azacycle	R	EC$_{50}$ (nM)	E$_{max}$ (%)
RS-67333	NA	NA	2 ± 0.8	47 ± 13.4	RS-67333	NA	NA	2 ± 0.8	47 ± 13.4
12a		H	1507 ± 134	43 ± 2.8	12s			59 ± 4.2	28 ± 4.6
12b			23 ± 2.7	56 ± 2.1	12t			22 ± 1.4	22 ± 8.1
12c			116 ± 17	28.4 ± 10.5	12u			210 ± 17.3	32 ± 3.3
12d			24 ± 5.1	42 ± 5.7	12v			675 ± 19.2	49 ± 4.8
12e			169 ± 16.6	41 ± 9.9	12w			5541 ± 1754	26 ± 10.2
12f			66 ± 15.6	54 ± 5.7	12x			255 ± 12.7	27 ± 6
12g			199 ± 26.9	42 ± 3.4	12y			989 ± 42.4	33 ± 9.6
12h			127 ± 23.3	41 ± 4.7	12z			>10000	NA
12i			72 ± 5.2	28 ± 10.1	12aa			>10000	NA
12j			1814 ± 129	36 ± 16.3	12ab			>10000	NA
12k			158 ± 17.5	26 ± 11.3	12ac		CH$_3$	695 ± 24.0	49 ± 5.3
12l			44 ± 3.5	43 ± 5.8	12ad			1060 ± 63.6	79 ± 4.2
12m			172 ± 13.1	26 ± 2.8	17a			156 ± 25.5	47 ± 2.8
12n			143 ± 9.0	41 ± 8.8	17b			19.9 ± 6.4	38 ± 4.6
12o			79 ± 5.8	53 ± 6.5	17c			8.6 ± 1.1	53 ± 2.8
12p			84 ± 4.7	51 ± 12.8	23a			23 ± 2.5	49 ± 8.8
12q			151 ± 17.3	29 ± 13.3	23b			106.7 ± 10	55 ± 4.7
12r			51 ± 4.7	17 ± 8.6	23c			297.9 ± 13.8	67 ± 4.0

[a]Data represent mean ± SD. Functional activity determined in CHO cells stably expressing the human 5HT$_{4E}$ isoform, and a reporter gene was used. EC$_{50}$ values were determined from average of two independent experiments tested in duplicate. The in-house data are consistent with reported values of PRX-03140 (17−52 nM/30−61%), PF-04995274 (0.26 nM/7%), and prucalopride (26 nM/52%) (Supporting Information); NA, not applicable.

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Table 3. Metabolic Stability, CYP and hERG Inhibition, Pharmacokinetics, Brain Penetration of Selected 5-HT$_4$R Compounds[a]

compd	rat liver microsomes % metabolized @ 30 min	human liver microsomes % metabolized @ 30 min	hERG IC_{50} (μM)	hERG % inhibition (%)	CYP3A4 inhibition IC_{50} (μM)	CYP2D6 inhibition IC_{50} (μM)	CYP 3A4 TDI (at 30 min) % inhibition	%*F*	C_{max} (ng/mL)	AUC (ng·hr/mL)	$t_{1/2}$ (h)	V_{dss} (L/kg)	CL (mL/min/kg)	C_{brain}/C_{plasma} @ 1 h
PRX-03140	NA	NA	1.3	92	NA	NA	NA	2 ± 0	17 ± 5	22 ± 0	1.5 ± 0.3	7.6 ± 0.5	62 ± 10	1.6 ± 0.4
7c	87.5 ± 0.4	45.8 ± 0.2	NA	NA	NA	NA	NA	3 ± 2	31 ± 18	26 ± 13	0.8 ± 0	2.8 ± 0.3	59 ± 12	3.2 ± 1.8
7d	30.5 ± 4.4	16.7 ± 0.6	>10	26	>45	>45	0	22 ± 2	177 ± 36	269 ± 30	2.3 ± 1.3	7.4 ± 0.8	32 ± 8	3.3 ± 0.3
7f	90.9 ± 0.2	4.6 ± 0.1	NA	NA	>45	>45	N.A	2 ± 2	36 ± 50	16 ± 17	2.3 ± 0.9	5.7 ± 0.9	70 ± 4	4.0 ± 0.8
7o	15.6 ± 11.1	20.2 ± 3.1	1.5	88	N.A	N.A	N.A	26 ± 13	99 ± 83	242 ± 118	1.2 ± 0.2	5.5 ± 0.9	53 ± 3	0.4 ± 0.1
7v	36.6 ± 0.7	17.9 ± 1.8	NA	NA	36.4	>45	7.9 ± 5.7	20 ± 3	69 ± 11	102 ± 18	0.9 ± 0.1	4.9 ± 0.4	95 ± 4	1.7 ± 0.2
7w	44.8 ± 3.6	45.1 ± 4.1	NA	NA	20.4	>45	12.5 ± 1.7	21 ± 1	64 ± 6	110 ± 6	1.7 ± 0.1	7.6 ± 0.6	92 ± 2	2.1 ± 0.0
7x	42.7 ± 12.5	27.4 ± 0.3	NA	NA	9.1	>45	>45	17 ± 1	65 ± 6	144 ± 7	1.3 ± 0.5	6.6 ± 1.2	62 ± 19	2.4 ± 0.3
7ad	98.4 ± 0.2	26.9 ± 1.4	NA	NA	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
7ag	74.8 ± 3.1	21.4 ± 6.2	NA	NA	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A	N.A
7ah	62.9 ± 3.8	31.7 ± 4.3	NA	NA	26.7	>45	0	2.2 ± 1	12 ± 8	10 ± 6	1.3 ± 0.3	6.8 ± 0.9	106 ± 2	5.0 ± 0.7
12b	35.7 ± 2.9	15.3 ± 1.9	NA	NA	>45	>45	0	9 ± 4	91 ± 40	133 ± 61	1.9 ± 0.4	10.0 ± 1.0	105 ± 6	3.6 ± 0.6
12d	98.5 ± 0.2	76.0 ± 2.7	NA	NA	11.2	13.9	0	17 ± 5	151 ± 3	520 ± 3	4.0 ± 0.2	4.0 ± 1.0	28 ± 4	1.3 ± 0.1
12e	55.1 ± 1.4	62.5 ± 1.4	NA	NA	>45	>45	0	2.6 ± 1.8	14 ± 8.0	16 ± 11	2.3 ± 0.8	4.9 ± 0.7	81 ± 13	0.0 ± 0.0
12f	83.8 ± 1.4	43.6 ± 1.8	NA	NA	>45	36	5.9 ± 0.7	19 ± 8	179 ± 80	394 ± 174	1.7 ± 0.5	8.0 ± 1.0	78 ± 14	4.7 ± 0.7
12h	64.9 ± 4.3	64.8 ± 0.5	2.4	79	>45	37	0	22 ± 3	248 ± 43	392 ± 103	1.7 ± 0.3	10.0 ± 3.0	96 ± 20	4.0 ± 0.6
12k	79.2 ± 0.4	53.8 ± 2.1	>10	36	>45	>45	0	19 ± 3	43 ± 11	74 ± 12	1.2 ± 0.1	12.7 ± 1.1	126 ± 17	0.8 ± 0.1
12l	25.1 ± 1.0	26.2 ± 0.8	3.0	82	9.1	>45	0	34 ± 13	360 ± 46	709 ± 120	1.7 ± 0.1	8.0 ± 1.0	76 ± 18	3.8 ± 0.2
12p	43.0 ± 0.4	33.6 ± 0.6	8.5	58	>45	>45	0	66 ± 18	587 ± 340	1323 ± 364	1.4 ± 0.2	8.5 ± 1.4	82 ± 10	3.1 ± 0.6
12s	81.5 ± 0.3	74.1 ± 2.9	1.4	91	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
12t	62.1 ± 1.6	36.4 ± 3.0	1.1	88	>45	2.8	NA	NA	NA	NA	NA	NA	NA	NA
17a	89.0 ± 1.5	29.3 ± 12.3	2.8	75	>45	>45	NA	NA	NA	NA	NA	NA	NA	NA
17b	64.1 ± 3.1	40.4 ± 0.8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
17c	73.0 ± 3.8	44.4 ± 3.8	NA	NA	NA	NA	NA	7 ± 3	12 ± 4	31 ± 13	1.8 ± 0.3	19.5 ± 7.7	124 ± 38	7.3 ± 0.5
23a	95.4 ± 0.7	56.49 ± 2.3	0.95	88	>45	37.7	NA	21 ± 7	157 ± 22	303 ± 100	1.5 ± 0.1	4.6 ± 1.6	35 ± 10	6.4 ± 1.2
23b	52.5 ± 1.6	32.0 ± 9.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
23c	46.7 ± 2.5	40.9 ± 0.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

[a]Data represent mean ± SD. RLM, rat liver microsomes; HLM, human liver microsomes; hERG, human ether-a-go-go related gene; CYP, cytochrome P450; TDI, time-dependent inhibition. Doses used for PK experiments: 3.0 mg/kg for p.o. dosing, 1.0 mg/kg for i.v. dosing. %*F*, oral bioavailability; C_{max}, maximum systemic concentration; AUC, area under the curve; $t_{1/2}$, elimination half-life; V_{dss}, volume of distribution; CL, intravenous clearance; C_{brain}/C_{plasma}, brain to plasma concentration ratio; NA, not available.

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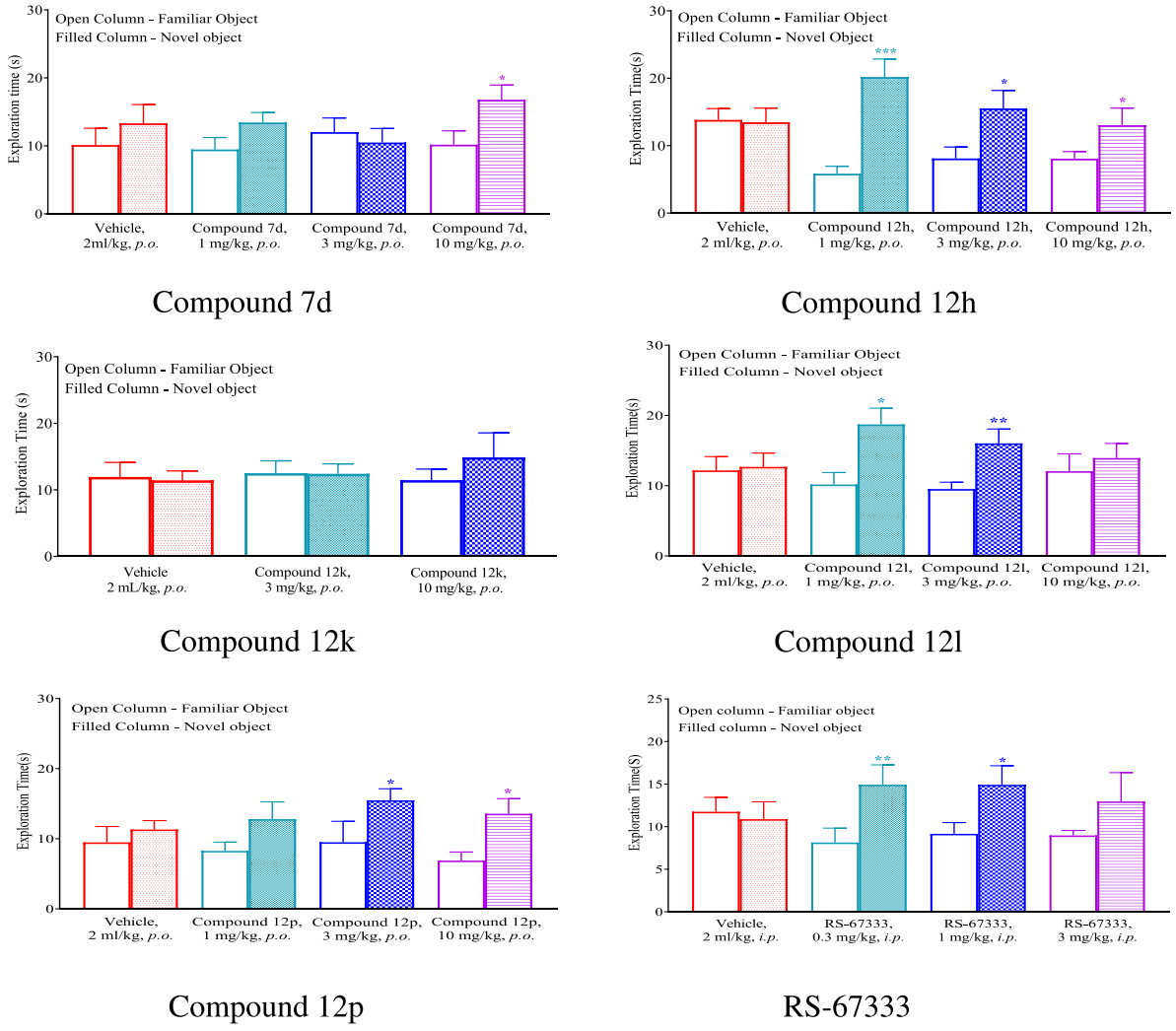

Figure 3. Effect of compounds **7d**, **12h**, **12k**, **12l**, **12p**, and RS-67333 on attenuation of long-term memory deficits in ORT. Data represent mean ± SEM of exploration time in seconds; (N = 7−8); *p < 0.05, **p < 0.01, ***p < 0.001. Paired−t-test comparison of novel vs familiar object.

mg/kg p.o. doses. Compound **12p** showed activity at 3 and 10 mg/kg p.o. doses. Compound **12k** did not show activity probably due to its poor brain penetration and lower plasma exposures. The difference in the efficacy for the other tested compounds could be due to differences in the free fractions which could have led to a difference in receptor occupancy thereby a difference in the ORT.

Based on efficacy observed in ORT assay and good PK properties, compounds **12h** and **12l** were shortlisted for further evaluation. Compounds **12h** and **12l** were evaluated in another cognitive assay, the radial arm maze task (RAM).[40] The RAM is a popular test of working memory in rodents. RAM exploits the natural tendency of rodents to explore, learn, and remember different locations of food reinforcement. RAM in rodents requires rats to explore a series of visually identical arms arranged around a central start location. Each arm will be baited with a reward, and the rat is required to remember which arms it has visited. A rat returning to a previously visited arm is recorded as an error, and one can infer how the rat is using various cues based on the pattern of navigation and errors. In this assay, compound **12h** at tested doses of 1, 3, and 10 mg/kg p.o. did not reverse the scopolamine-induced cognitive deficits; whereas, compound **12l** significantly reversed the scopolamine-induced amnesia at the tested doses of 1, 3, and 10 mg/kg p.o. (Figure 4).

In order to see correlation between dose and efficacy, compound **12l** was evaluated in ORT at doses 0.03, 0.1, 0.3, 1.0, 3.0, and 10.0 mg/kg p.o. In this study, compound **12l** did not show activity at 0.03 and 0.1 mg/kg, however did show significant efficacy from 0.3 to 3.0 mg/kg. The efficacy of compound **12l** in ORT assay at doses of 0.1−10 mg/kg, p.o. along with the recognition index is depicted in Figure 5A,B (exploration time and recognition index, respectively). Compound **12l** showed a statistically significant effect at 3.0 mg/kg on both exploration time and recognition index.

An inverted U-shaped dose−response curve has been observed with procognitive compounds and approved procognitive drugs.[41] The exact reason for such a phenomenon is not quite well understood. One school of thought is that an optimal level of acetylcholine is required for cognition. An increase in acetylcholine levels beyond optimal levels did not show improvement in cognition. One reason could be that moderate arousal is beneficial to cognition; whereas too much activation leads to cognitive impairment.[42] Another reason could be due to an off-target activity at higher doses which may make the rat uncomfortable. It is believed that in case there is

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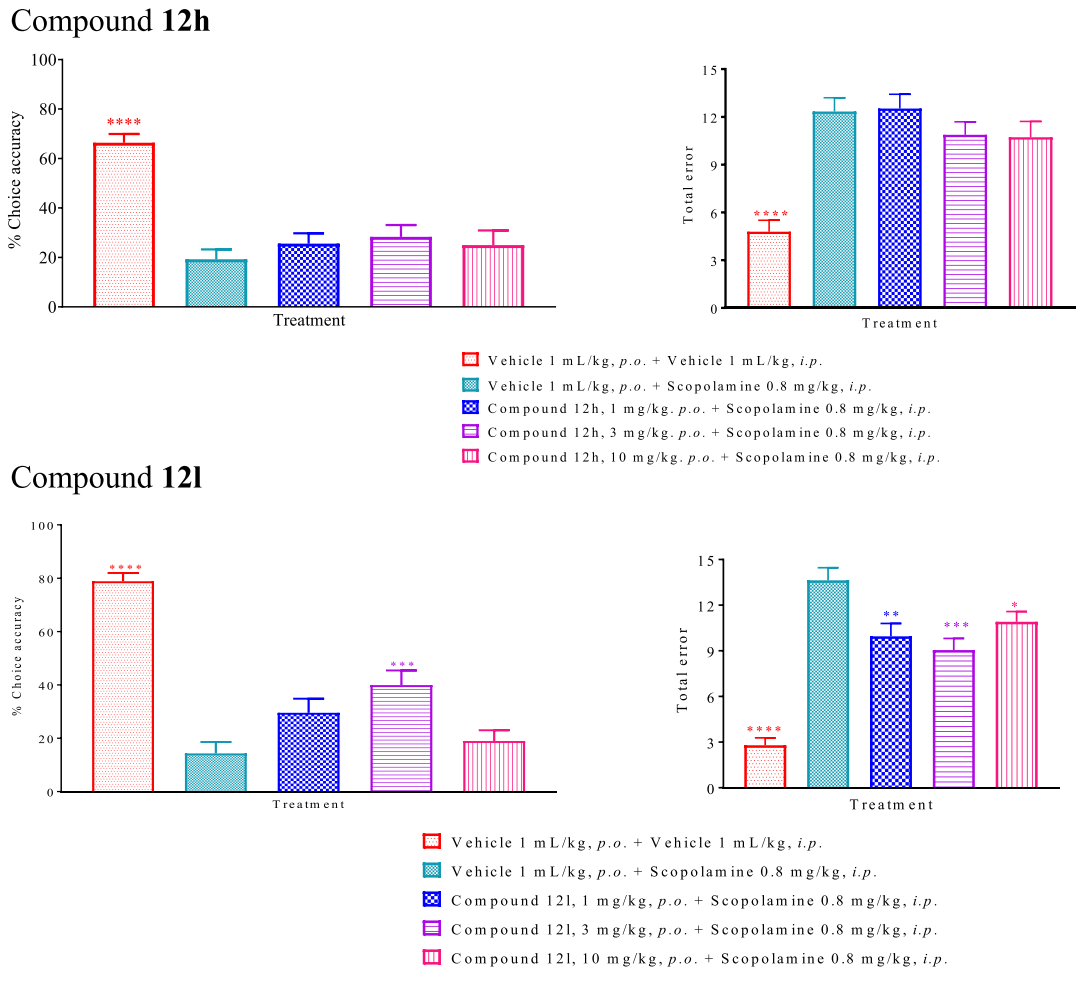

Figure 4. Effect of compounds **12h** and **12l** in RAM. Data represent mean ± SEM of % choice accuracy or total error compared to vehicle ($F_{4,103}$ = 19.54 for choice accuracy and $F_{4,103}$ = 14.81 for total error for compound **12h** and $F_{4,105}$ = 36.16 for choice accuracy and $F_{4,105}$ = 32.88 for total error for compound **12l**); n = 7−8; *p < 0.05, **p < 0.01, ***p < 0.001,****p < 0.0001 vs scopolamine (ANOVA followed by Dunnett's test).

an activation of presynaptic autoreceptors, it may result in reducing the efficacy at higher doses.[41] This could be the reason that **12l** did not show efficacy at 10 mg/kg dose.

To show the relationship between compound **12l** efficacy and its target engagement at 5-HT_4R, it was evaluated for 5-HT_4 receptor occupancy (R.O.).[43] Gratifyingly, compound **12l** showed significant R.O. at tested doses ranging from 0.1 mg/kg to 30 mg/kg. The absolute ED_{50} value was found to be 2.75 mg/kg, p.o. Brain EC_{50} was found to be 184.8 ng/g, and plasma EC_{50} was found to be 59.3 ng/mL (Figure 6). The observed R.O. suggests that the *in vivo* efficacy for this compound may be mediated through 5-HT_4R. The 5-HT_4R partial agonist PF-04995274 showed dose-dependent R.O. with ED_{50} value of 0.033 mg/kg, s.c. which was comparable with the reported value (0.01 mg/kg, s.c.).[44] Another 5-HT_4R partial agonist RS-67333 showed an ED_{50} value of 15.15 mg/kg, p.o. which was also in close agreement with the reported value.[11,43] The *in vivo* efficacy doses of compound **12l** were very well correlated with observed R.O. data. The effective doses (0.3, 1.0, and 3 mg/kg, p.o.) of **12l** showed R.O. from 27% to 60% (Figure 6).

The 5-HT_4R agonists can shift the equilibrium of amyloid precursor protein (APP) processing from an amyloidogenic to nonamyloidogenic form, showing disease-modifying potential.[45] The APP processing shifts equilibrium from an amyloidogenic to nonamyloidogenic form by generating sAPPα which is reported to provide neuroprotective effects.[9,46,47] The 5-HT_4R-mediated regulation of sAPPα secretion has been demonstrated *in vitro* using primary neuronal cultures and recombinant cells.[48] It is reported that the endogenous ligand serotonin (5-HT) enhances the level of secreted sAPPα in a time and dose-dependent manner in Chinese hamster ovary (CHO) cells stably expressing the h5-HT_{4E} receptor isoform.[9] It is also reported that the 5-HT_4R agonists increase sAPPα levels in the cortex and hippocampus of male C57BL/6j mice and the effects were blocked with selective 5-HT_4R antagonist GR-113808.[46] In agreement with the published results, activation of the 5-HT_{4E} receptor isoform dose dependently increased expression levels of nonamyloidogenic sAPPα in tsa201 cells transiently transfected with the 5-HT_{4E} receptor and APP695. At 10 μM concentration, 5-HT, RS-67333, and compound **12l** increased sAPPα secretion by 2.9 and 1.5 and 1.8 fold, respectively. The increase in sAPPα secretion by 5-HT and compound **12l** was completely blocked by GR-113808 (Figure 7), suggesting compound **12l** has disease-modifying potential and the activity is arising from the selective activation of the 5-HT_4 receptor. RS-67333 was not subjected to blockade with GR-113808.

The protein binding experiment was done in rat, dog, and human liver microsomes to ascertain the free fraction available

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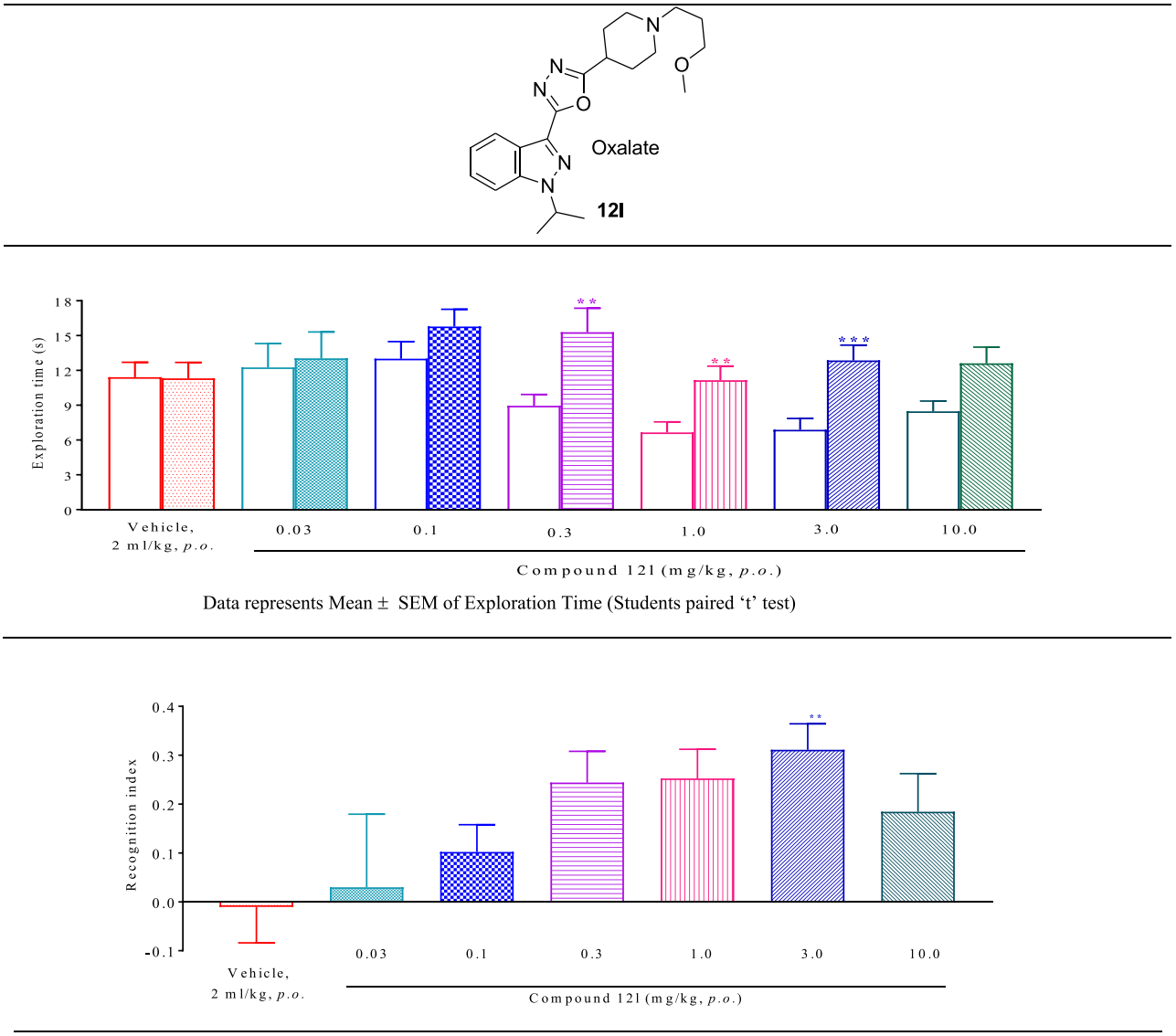

Figure 5. (A) Attenuation of long-term memory deficits in ORT by compound **12l**. Data represent (mean ± SEM) of exploration time compared to vehicle (n = 8−11/group). $^{**}p < 0.01$, $^{***}p < 0.001$ (Paired−t-test comparison of novel vs familiar object). (B) Effect of compound **12l** on attenuation of long-term memory deficits in ORT assay. Data represent mean ± SEM of discrimination index. $^{**}P < 0.01$ vs vehicle group ($H_{7,74}$ = 14.12; Kruskal−Wallis test followed by Dunn's post hoc).

for **12l**. Compound **12l** showed 11.3, 9.2, and 7.3% free fraction in rat dog and human plasma, respectively. The free fraction in rat brain homogenate for **12l** was 5.9% (Table 4).

Compound **12l** showed comparable potencies at tested 5-HT$_4$R isoforms (5-HT$_{4A}$, 5-HT$_{4D}$, and 5-HT$_{4E}$) with EC$_{50}$ values ranging from 32 to 114 nM. Compound **12l** showed excellent selectivity against 5-HT receptors (5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_3$, 5-HT$_6$, 5-HT$_7$), adrenergic receptors (Adα_{1B} and Adα_{2C}), adenosine receptor A$_{2A}$, cannabinoid receptors (CB$_1$ and CB$_2$), dopamine (D$_1$, D$_{2S}$, D$_3$, and D$_5$), histamine (H$_1$ and H$_3$), muscarinic receptors (M$_1$, M$_2$, M$_3$, M$_4$, and M$_5$), and serotonin transporter (SERT). Compound **12l** did not show any significant binding up to the highest tested concentration of 10 μM toward GPCRs, ion channels, and transporters (see Supporting Information). Compound **12l** did not show any agonist activity at the 5-HT$_{2B}$ receptor when evaluated in rat fundus assay[49] (See Supporting Information). Compound **12l** did not show any activity at GPCRs or ion channels other than 5-HT$_4$R, which are implicated in cognition. Compound **12l** is a nonhygroscopic and crystalline oxalate salt with excellent water solubility (17.1 mg/mL). The solubility of **12l** in buffer solutions from pH 2 to 7.0 is about 20−75 mg/mL at 37 °C. The highest possible dose of **12l** is soluble in 250 mL in the entire pH range. Compound **12l** demonstrated high permeability in Caco-2 experiments at a tested concentration (10 μM) with an apparent permeability coefficient (P_{app}) of 38 × 10^{-6} cm·s^{-1}. The high solubility and high permeability suggest that **12l** belongs to class I of the Biopharmaceutics Classification System (BCS). Compound **12l** showed an acid dissociation constant (pK_a) value of 8.4 (Supporting Information).

Pharmacokinetics of compound **12l** was assessed in male beagle dogs (n = 3) at 1 mg/kg, p.o., and 1 mg/kg, i.v., doses. Compound **12l** showed rapid oral absorption (T_{max} in the range of 0.5−0.75 h) with oral exposures (AUC$_{0-24h}$ = 711 ± 369 ng·h/mL) and showed longer terminal i.v. half-life (6.0 ± 1.5 h). The moderate intravenous clearance (18 ± 6 mL/min/kg) and volume of distribution (5.1 ± 0.6 L/kg) indicate a

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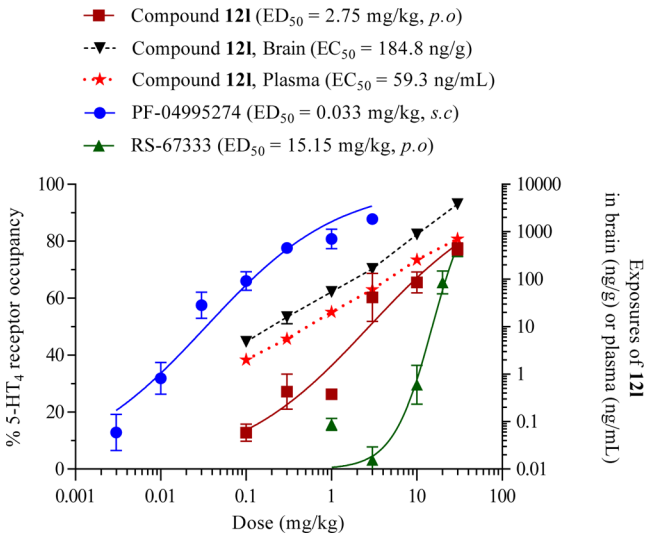

Figure 6. 5-HT$_4$R occupancy of compound **12l** and correlation with plasma and brain concentrations. Data shown are the mean ± SEM (n = 4−6 animals).

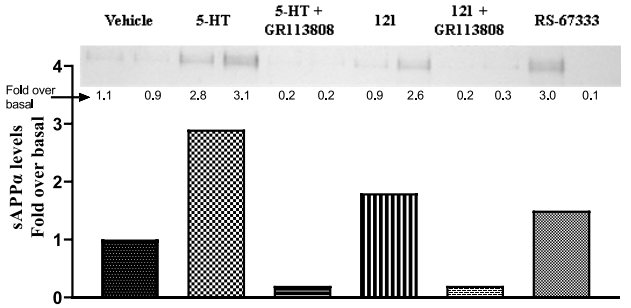

Figure 7. Effect of compound **5-HT**, **12l**, and RS-67333 on sAPPα secretion in the absence or presence of selective 5-HT$_4$R antagonist GR-113808 by Western blot. Data represent mean of duplicates of fold over basal of sAPPα levels. Compound **5-HT** and 5-HT$_4$ receptor partial agonist compound **12l** and RS-67333 evoked stimulation of sAPPα secretion *in vitro* were monitored using tsa201 cells transiently transfected with human 5-HT$_{4E}$ receptor and human APP695.

Table 4. *In Vitro* Plasma Protein Binding for Compound 12l[a]

	%unbound		%bound		
species	replicate 1	replicate 2	replicate 1	replicate 2	%bound[a]
		Plasma Binding			
human	6.4	8.2	93.6	91.8	92.7
beagle dog	8.3	10.0	91.7	90.0	90.8
Wistar rat	11.8	10.9	88.2	89.1	88.7
		Brain Homogenate Binding			
Wistar rat	5.5	6.2	94.5	93.8	94.1

[a]Data represent mean, and experiment was done in duplicates.

higher tissue distribution. The observed absolute oral bioavailability (%F) was 72 ± 11%. Compound **12l** was evaluated for cardiovascular toxicity using dog telemetry assay as per ICH 7A guidelines. In this study, compound **12l** was administered orally to freely moving conscious male dogs. Gratifyingly, it did not show any adverse effect on QT interval prolongation up to the tested dose of 24 mg/kg p.o.

Compound **12l** was found to be nonmutagenic in all four strains of *Salmonella typhimurium* TA1537, TA1535, TA98, and TA100 and *Escherichia coli* WP2 uvrA both in the absence and presence of metabolic activation. It was found to be nonclastogenic in *in vitro* chromosomal aberration tests in human peripheral blood lymphocytes and in *in vivo* micronucleus assay done on mice bone marrow. These studies were conducted per the standard protocols and in line with regulatory guidance documents (OECD 471 and 474 and ICH S2R1). The CYP enzyme inhibition, CYP3A4 TDI assay, CYP induction, and transporter assays were performed as described earlier.[50] Compound **12l** was not an inhibitor of P450 enzymes with IC$_{50}$ values >45 μM against major P450 isoforms. It did not show any TDI liability at CYP3A4 or CYP induction liability at CYP1A2, CYP2B6, and CYP3A4. Compound **12l** was neither an inhibitor (IC$_{50}$ > 100 μM) nor substrate of OAT1, OAT3, OCT1, OCT2, OATP1B1, OATP1B3 uptake transporters. The likelihood of drug−drug interaction potential of compound **12l** as an inhibitor/inducer is remote.

Compound **12l** as a clinical candidate has completed regulatory safety assessment studies in rats and dogs with excellent safety margins which supported its progression to first in human phase 1 clinical studies including pharmacokinetics, safety, and tolerability under US IND (ClinicalTrials.gov, identifiers: NCT02575482 and NCT03031574).[51] Compound **12l** showed a favorable safety and PK profile in a single ascending dose in healthy male and multiple ascending doses for 14 days in healthy male subjects. Gender, age, and food did not have any significant effect on the pharmacokinetics. It was well-tolerated in humans with adequate plasma exposures and a favorable half-life for once-a-day dosing. Phase 2 enabling long-term animal safety evaluation to support repeat dosing has also been completed. Compound **12l** will be evaluated in phase 2 proof of concept studies in patients with cognitive deficits associated with AD in due course of time.

CONCLUSIONS

In summary, indazole-3-carboxamide, indazole 1,2,4-oxadiazole, and indazole 1,3,4-oxadiazole derivatives have been synthesized and tested for their *in vitro* affinity at 5-HT$_4$R. The focused structure−activity relationships for *in vitro* affinity at 5-HT$_4$R combined with microsomal metabolic stability, PK evaluation, efficacy in cognitive models, target engagement, and safety assessments resulted in a safe, potent, and selective 5-HT$_4$R clinical candidate (**12l**, Usmarapride). Compound **12l** showed high selectivity against ganglionic 5-HT$_3$R and other closely related receptors and good pharmacokinetics in rats, dogs, and healthy humans. It also showed promising *in vivo* efficacy in animal models of cognition. Additionally, compound **12l** increased neuroprotective sAPPα levels and may possess disease-modifying potential. Further, **12l** showed a dose-dependent receptor occupancy which correlates well with efficacy doses in tested cognition models. When evaluated in safety models, **12l** did not show any cardiotoxicity in dog telemetry studies. Based on its robust efficacy in cognition models and a wide margin of safety in preclinical species, it was evaluated in healthy human subjects in phase 1 clinical trials for tolerability and PK properties. Compound **12l** was found to be well tolerated and showed good pharmacokinetics. Further evaluation of compound **12l** for its efficacy in cognitive deficits associated with AD in a phase 2 proof-of-concept study is being planned.

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EXPERIMENTAL SECTION

Unless stated otherwise, all reagents, commercially available building blocks, and solvents were purchased from common commercial suppliers and were used without further purification. Synthesized compounds were purified wherever required by passing through 100−200 mesh silica gel using appropriate solvent systems. ^{1}H NMR spectra were recorded at 400 MHz and ^{13}C NMR at 100 MHz on a Bruker NMR spectrometer instrument (Fallanden, Switzerland). All ^{1}HNMR shifts are reported in δ units (ppm) relative to the signals for chloroform (7.27 ppm), DMSO (2.50 ppm), and MeOH (3.31 ppm). All coupling constants (J values) are reported in hertz (Hz). NMR abbreviations are as follows: bs, broadened singlet; s, singlet; d, doublet; t,triplet; q, quartet; m, multiplet and dd, doublet of doublets. Thin-layer chromatography (TLC) was performed on Merck silica gel 60 F_{254} plates. Electrospray ionization mass spectra were recorded on API 4000 triple quadrupole instrument (MDS-SCIEX, Concord, Ontario, Canada). Infrared spectra were recorded on KBr disc and in solid state using PerkinElmer model 1600 FT-IR spectrophotometer (PerkinElmer, Norwalk, CT, USA). Elemental analyses were carried out in an "Elementar" GmbH, Vario microcube instrument, and the analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values. Column chromatography was performed using 100−200 mesh silica gel. The purity of all final compounds was established by HPLC (Agilant, Model-1100 series). All final compounds' HPLC purity was found to be ≥95%. DSC was recorded on Waters DSC Q100 instrument. Melting points of synthesized compounds were determined using an Electro Derman open capillary apparatus and are uncorrected. The reference standards PRX-03140, RS-67333, PF-04995274, GR-113808, prucalopride, and others were synthesized and characterized in-house using respective reported procedures. The purity of all final compounds was determined using HPLC methods unless stated otherwise.

General Procedure 1 (Boc Protection). To the solution of amine/amide (1.0 mmol) in DCM (2.0 mL) stirred at room temperature (r.t.), Boc anhydride (1.1 mmol), DMAP (0.05 mmol), and triethylamine (1.5 mmol) were added. The reaction mixture was stirred for 16 h at r.t. Upon completion of reaction, it was diluted with EtOAc, washed with water and brine, and dried over anhydrous Na_2SO_4. Volatiles were removed under reduced pressure to obtain the crude product, which was triturated with hexanes to obtain titled compound.

General Procedure 2 (Boc Deprotection). To a Boc protected compound (1.0 mmol) in DCM (4.0 mL) under nitrogen at 0 °C, TFA (5.0 mmol) was added. The reaction was gradually warmed to r.t. and stirred for 16 h. The mixture was concentrated under vacuum to obtain a crude product, which was basified with aqueous $NaHCO_3$ solution. The reaction mass was diluted with DCM (10 mL), and the two layers were separated. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum which afforded amino compound.

General Procedure 3 (N-Alkylation). To the stirred solution of amino compound (1.0 mmol) in acetonitrile (4.0 mL) at r.t., K_2CO_3 (1.5 mmol) and alkyl halide (1.05 mmol) were added. The reaction mixture was gradually heated to reflux (82−85 °C) for 7−16 h. After completion of the reaction, volatiles were removed under reduced pressure. The crude mass was diluted with DCM and water and allowed to stir for 15 min, then two layers were separated. The organic layer was washed with brine,and dried over anhydrous Na_2SO_4, and volatiles were removed under pressure to get gummy mass of N-alkylated compound.

General Procedure 4 (Acid Chloride and Amine Coupling Reaction). To a stirred solution of 1-isopropyl-1H-indazole-3-carbonyl chloride (**3**, 1.0 mmol) in DCM (2.0 mL) under nitrogen atmosphere at 0 °C, triethylamine (1.5 mmol) and a solution of amine (**4**, 1.02 mmol) in DCM (2.0 mL) were added. The reaction mixture was gradually warmed to r.t. and stirred for 16 h. After completion of the reaction, the reaction mixture was diluted with water and DCM. The separated organic layer was washed with brine and dried over anhydrous Na_2SO_4, and the solvent was evaporated under vacuum to obtain crude carboxamide derivative, which was purified by silica gel column chromatography with appropriate solvent system.

General Procedure 5 (Hydrazide Formation). To a stirred solution of ester **8a−8f** (1.0 mmol) in methanol (2.0 mL) at r.t., hydrazine hydrate (80% w/v, 3.0 mmol) was added. The reaction mixture was heated to reflux for 4 h. An additional amount of hydrazine (80% w/v, 3.0 mmol) was added, and the reaction was heated until (6 h) all of the ester was consumed. Solvent methanol was evaporated under reduced pressure, and the residue was dissolved in DCM. The two layers were separated, the organic layer was dried over anhydrous Na_2SO_4, and the solvent was evaporated under vacuum to obtain hydrazide derivative (**9a−9f**), which was used in the next reaction without further purification.

General Procedure 6 (Acid Chloride-Hydrazide Coupling). To a stirred solution of hydrazide **9a−9f** (1.0 mmol) in DCM (2.0 mL) cooled at 0 °C, a solution of 1-isopropyl-1H-indazole-3-carbonyl chloride **3** (1.1 mmol) in DCM (2.0 mL) was added over a period of 5 min. The reaction was gradually warmed to r.t. and was stirred for 2 h. The progress of the reaction was monitored by TLC, which showed an absence of hydrazide. The reaction mixture was then diluted with water, and the two layers were separated. The aqueous layer was washed with EtOAc, cooled to 0 °C, and cautiously basified to pH 7.6 with 10% aqueous $NaHCO_3$ solution. The basified aqueous layer was then extracted with DCM. The combined organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The residue was then cooled to r.t. and diluted with hexane. The slurry, thus obtained, was filtered at r.t. under nitrogen atmosphere, and the wet product cake was washed with hexanes to obtain respective compounds **10a−10f** in quantitative yields.

General Procedure 7 (1,3,4-Oxadiazole Formation). To a stirred solution of compounds **10a−10f** (1.0 mmol) in 1,2-dichloroethane (4.0 mL) under nitrogen atmosphere at r.t., thionyl chloride (2.0 mmol) was added over a period of 15 min. The reaction temperature was then gradually raised, and the reaction mass was refluxed for 6−10 h until all starting material was consumed. The excess thionyl chloride and solvent 1,2-dichloroethane were distilled off under reduced pressure. The reaction mass was cooled to r.t. and diluted with water and solvent ether. The resulting mass was stirred for 15 min, and the two layers were separated. The pH of the aqueous layer was adjusted to 9−10 by adding an aqueous NaOH (2.5 N). The basified aqueous layer was then extracted multiple times with DCM. The combined organic layer was washed with cold (5−10 °C) aqueous NaOH (0.6 N) and dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure to afford 1,3,4-oxadiazole derivatives **11a−11f**.

General Procedure 8 (Salt formation). To the stirred solution of N-alkylated carboxamide derivative/oxadiazole derivative (1.0 mmol) in isopropanol (8.0 mL), respective acid (1.0 mmol) was added. The reaction mass was stirred for 1 h before the volatiles were evaporated under reduced pressure. The crude salt was triturated with copious amounts of ether and ethyl acetate to obtain the respective salts. The salts were recrystallized from isopropanol or mixture of solvents wherever required to obtain the desired purity.

General Procedure 9 (Reductive Amination). To a stirred mixture of carboxamide derivatives (1.0 mmol), aldehyde/ketone (1.5 mmol), and acetic acid (0.1 mL) in DCM (4.0 mL) cooled at 0 °C, sodium triacetoxyborohydride (2.0 mmol) was added. The reaction mass was gradually warmed to r.t and stirred for 16 h. The reaction mass was diluted with saturated aqueous $NaHCO_3$ solution and extracted with DCM. The combined organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. The crude product was purified by silica gel column chromatography to obtain the N-alkylated derivatives.

*1-Isopropyl-lH-indazole-3-carboxylic Acid (**2**).* To the stirred DMF (50.0 mL) under nitrogen atmosphere at r.t., sodium *tert*-butoxide (6.0 g, 62.43 mmol) was added over a period of 15 min. The reaction mixture was stirred for 10 min after which it was cooled to 0 to 5 °C. A solution of indazole-3-carboxylic acid (4.0 g, 24.67 mmol) in DMF (50 mL) was added slowly into the reactor over a period of 45 min, maintaining the reaction mass temperature between 0 to 5

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°C. The cooling was removed, and the reaction temperature was gradually raised to r.t. over a period of 30 min. After stirring at this temperature for 1 h, the reaction mixture was cooled to 0 °C, and isopropyl iodide (37.18 mmol) was added over a period of 30 min. The cooling was removed, and the reaction temperature was warmed to r.t. After 16 h of stirring, the HPLC analysis of the reaction mixture revealed l0% of indazole-7-carboxylicacid remaining. The reaction mass was quenched cautiously by adding cold water and extracted with EtOAc. The separated aqueous layer was acidified to pH 4.0 with aqueous HCl solution (6 N). The acidified reaction mass was then extracted with EtOAc. The combined organic layer was washed with water, brine, and dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure to obtain a crude mass. The obtained crude mass was diluted with DCM (28.0 L) and stirred for 15 min. The solids precipitated (unreacted indazole-7-carboxylic acid) were filtered, and the filter bed was washed once with DCM. The combined filtrate was dried over anhydrous Na_2SO_4 and distilled under reduced pressure which yielded a crude mass. The crude mass was triturated with ether, and the solids were filtered to obtain the wet cake which was dried further under reduced pressure to obtain the title compound (3.0 g) in 60% yield. HPLC purity: 99.86%. ^{1}H NMR (400 MHz, $CDCl_3$): 8.27 (d, J = 8.1 Hz, l H), 7.55 (d, J = 8.4 Hz, lH), 7.46 (t, J = 7.6 Hz, lH), 7.34 (t, J = 7.4 Hz, lH), 5.01−4.95 (m, lH), 1.68 (d, J = 6.6 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.6, 139.9, 133.7, 126.6, 124.1, 123.4, 122.3, 109.8, 51.8, 22.0; DSC: (RAMP 5 °C/min): 163.0 °C; mass (m/z): 205.1 (M + H)$^+$.

*1-Isopropyl-1H-indazole-3-carbonyl Chloride (**3**).* To a stirred solution of 1-isopropyl-lH-indazole-3-carboxylic acid **2** (1.0 g, 4.90 mmol) in 1,2-dichloroethane (19.6 mL) cooled at 0 °C under nitrogen atmosphere, thionyl chloride (0.42 mL, 5.88 mmol) was added. The reaction mixture was gradually raised to r.t. and then refluxed for 1 h. After completion of the reaction, volatiles were evaporated which yielded title compound **3** (1.0 g) in quantitative yield. ^{1}H NMR ($CDCl_3$): δ 8.21 (d, J = 8.4 Hz, l H), 7.57 (d, J = 8.4 Hz, l H), 7.49 (t, J = 6.8 Hz, l H), 7.41 (t, J = 7.6 Hz, l H), 5.01−4.96 (m, l H), 1.68 (d, J = 6.6 Hz, 6H); mass (m/z): 223.1, 225.1 (M + H)$^+$.

*4-[(1-Isopropyl-1H-indazole-3-carbonyl)-amino]-piperidine-1-carboxylic Acid tert-Butyl Ester (**5a**).* By following general procedure 4, 1-isopropyl-1H-indazole-3-carbonyl chloride **3** (500 mg, 2.25 mmol) was reacted with 4-amino-piperidine-1-carboxylic acid *tert*-butyl ester **4a** (450.0 mg, 2.25 mmol) to obtain title compound **5a** (825.8 mg) in 95.1% yield with HPLC purity 97.0%. ^{1}H NMR ($CDCl_3$): δ 8.37 (d, J = 8.0 Hz, 1H), 7.48 (d, J = 8.4 Hz, 2H), 7.39 (t, J = 8.4 Hz, 1H), 7.18 (t, J = 7.6 Hz, 1H), 4.90−4.84 (m, 1H), 4.13 (bs, 3H), 2.75 (t, J = 12.0 Hz, 2H), 1.88 (d, J = 17.6 Hz, 4H), 1.64 (d, J = 6.8 Hz, 6H), 1.46 (s, 9H); mass (m/z): 387.2 (M + H)$^+$.

*4-{[(1-Isopropyl-1H-indazole-3-carbonyl)-amino]-methyl}-piperidine-1-carboxylic Acid tert-Butyl Ester (**5b**).* By following general procedure 4, 1-isopropyl-1H-indazole-3-carbonyl chloride **3** (200 mg, 0.9 mmol) was reacted with 4-aminomethylpiperidine-1-carboxylic acid *tert*-butyl ester **4b** (192.6 mg, 0.9 mmol) to obtain title compound **5b** (324.0 mg) in 90.1% yield with HPLC purity 95.0%. ^{1}H NMR ($CDCl_3$): δ 8.39 (d, J = 8.0 Hz, 1H), 7.46 (d, J = 8.4 Hz, 2H), 7.39 (t, J = 8.4 Hz, 1H), 7.26 (t, J = 7.6 Hz, 1H), 4.90−4.84 (m, 1H), 4.13 (bs, 3H), 3.49 (bs, 2H), 2.71 (t, J = 12.0 Hz, 2H), 1.84 (d, J = 17.6 Hz, 4H), 1.61 (d, J = 6.8 Hz, 6H), 1.45 (s, 9H); mass (m/z): 401.2 (M + H)$^+$.

*4-Hydroxy-4-{[(1-isopropyl-1H-indazole-3-carbonyl)-amino]-methyl}-piperidine-1-carboxylic Acid tert-Butyl Ester (**5c**).* By following general procedure 4, 1-isopropyl-1H-indazole-3-carbonyl chloride **3** (1.0 g, 4.50 mmol) was reacted with 4-aminomethyl-4-hydroxy-piperidine-1-carboxylic acid *tert*-butyl ester **4c** (1.03 g, 4.50 mmol) to obtain title compound **5c** (1.7 g) in 95.0% yield with HPLC purity 97.0%. ^{1}H NMR ($CDCl_3$): δ 8.37 (d, J = 8.1 Hz, 1H), 7.44 (d, J = 8.3 Hz, 2H), 7.38 (t, J = 8.4 Hz, 1H), 7.21 (t, J = 7.6 Hz, 1H), 4.20 (bs, 3H), 3.54 (bs, 2H), 2.73 (t, J = 12.0 Hz, 2H), 1.81 (d, J = 17.6 Hz, 4H), 1.64 (d, J = 6.8 Hz, 6H), 1.46 (s, 9H); mass (m/z): 417.2 (M + H)$^+$.

*4-Fluoro-4-{[(1-isopropyl-1H-indazole-3-carbonyl)-amino]-methyl}-piperidine-1-carboxylic Acid tert-Butyl Ester (**5d**).* By following general procedure 4, 1-isopropyl-1H-indazole-3-carbonyl chloride **3** (500 mg, 2.25 mmol) was reacted with 4-aminomethyl-4-fluoro-piperidine-1-carboxylic acid *tert*-butyl ester **4d** (522.5 mg, 2.25 mmol) to obtain title compound **5d** (904.2 mg) in 96.1% yield with HPLC purity 94.0%. ^{1}H NMR ($CDCl_3$): δ 8.42 (d, J = 8.0 Hz, 1H), 7.42 (d, J = 8.4 Hz, 2H), 7.21 (t, J = 8.4 Hz, 1H), 7.14 (t, J = 7.6 Hz, 1H), 4.25 (bs, 3H), 3.49 (bs, 2H), 2.71 (t, J = 12.0 Hz, 2H), 1.84 (d, J = 17.6 Hz, 4H), 1.64 (d, J = 6.8 Hz, 6H), 1.46 (s, 9H); mass (m/z): 419.2 (M + H)$^+$.

*6-{[(1-Isopropyl-1H-indazole-3-carbonyl)-amino]-methyl}-[1,3]-oxazinane-3-carboxylic Acid tert-Butyl Ester (**5e**).* By following general procedure 4, 1-isopropyl-1H-indazole-3-carbonyl chloride **3** (750.0 mg, 3.37 mmol) was reacted with 2-aminomethyl-morpholine-4-carboxylic acid *tert*-butyl ester **4e** (729.7 mg, 3.37 mmol) to obtain title compound **5e** (1.2 g) in 90.1% yield with HPLC purity 90.0%. ^{1}H NMR (DMSO-d_6): δ 8.15 (d, J = 7.6 Hz, 2H), 7.76 (d, J = 8.4 Hz, 1H), 7.43 (t, J = 7.6 Hz, 1H), 7.22 (t, J = 7.6 Hz, 1H), 5.07−5.04 (m, 1H), 3.80−3.44 (m, 3H), 2.65−2.62 (m, 2H), 2.37−2.29 (m, 2H), 1.53 (d, J = 6.8 Hz, 6H), 1.44 (s, 9H), 1.22 (m, 2H); mass (m/z): 403.2 (M + H)$^+$.

*6-[(1-Isopropyl-1H-indazole-3-carbonyl)-amino]-3-aza-bicyclo-[3.1.0]hexane-3-carboxylic Acid tert-Butyl Ester (**5f**).* By following general procedure 4, 1-isopropyl-1H-indazole-3-carbonyl chloride **3** (1.0 g, 4.50 mmol) was reacted with 6-amino-3-aza-bicyclo[3.1.0]-hexane-3-carboxylic acid *tert*-butyl ester **4f** (892.3 mg, 4.50 mmol) to obtain title compound **5f** (1.5 g) in 90.1% yield with HPLC purity 95.3%. ^{1}H NMR (DMSO-d_6): δ 8.15 (d, J = 8.0 Hz, 1H), 7.95 (d, J = 8.4 Hz, 1H), 7.77 (d, J = 8.8 Hz, 1H), 7.43 (t, J = 7.6 Hz, 1H), 7.25 (t, J = 7.6 Hz, 1H), 5.07−5.04 (m, 1H), 2.49−2.42 (m 1H), 2.20 (t, J = 11.2 Hz, 2H), 1.68−1.63 (m, 2H), 1.54 (d, J = 6.4 Hz, 6H), 1.44 (s, 9H), 1.23 (m, 2H); mass (m/z): 385.2 (M + H)$^+$.

*6-{[(1-Isopropyl-1H-indazole-3-carbonyl)-amino]-methyl}-3-aza-bicyclo[3.1.0]hexane-3-carboxylic Acid tert-Butyl Ester (**5g**).* By following general procedure 4, 1-isopropyl-1H-indazole-3-carbonyl chloride **3** (500 mg, 2.25 mmol) was reacted with 6-aminomethyl-3-aza-bicyclo[3.1.0]hexane-3-carboxylic acid *tert*-butyl ester **4g** (477.4 mg, 2.25 mmol) to obtain title compound **5g** (806.7 mg) in 90.1% yield with HPLC purity 93.4%. ^{1}H NMR (DMSO-d_6): δ 8.15 (d, J = 8.0 Hz, 1H), 7.95 (d, J = 8.4 Hz, 1H), 7.77 (d, J = 8.8 Hz, 1H), 7.43 (t, J = 7.6 Hz, 1H), 7.25 (t, J = 7.6 Hz, 1H), 5.07−5.04 (m, 1H), 2.91 (d, J = 11.6 Hz, 2H), 2.71 (d, J = 12.0 Hz, 2H), 2.49−2.42 (m, 1H), 2.20 (t, J = 11.2 Hz, 2H), 1.68−1.63 (m, 2H), 1.53 (d, J = 6.4 Hz, 6H), 1.44 (s, 9H); mass (m/z): 399.1 (M + H)$^+$.

*3-[(1-Isopropyl-1H-indazole-3-carbonyl)-amino]-8-aza-bicyclo-[3.2.1]octane-8-carboxylic Acid tert-Butyl Ester (**5h**).* By following general procedure 4, 1-isopropyl-1H-indazole-3-carbonyl chloride **3** (500 mg, 2.25 mmol) was reacted with 3-amino-8-aza-bicyclo[3.2.1]-octane-8-carboxylic acid *tert*-butyl ester **4h** (509.6 mg, 2.25 mmol) to obtain title compound **5h** (750.7 mg) in 80.8% yield with HPLC purity 92.4%. ^{1}H NMR ($CDCl_3$): δ 8.37 (d, J = 8.4 Hz, 1H), 7.52 (d, J = 6.0 Hz, 1H), 7.43 (d, J = 8.4 Hz, 1H), 7.41 (t, J = 6.8 Hz, 1H), 4.91−4.86 (m, 1H), 4.35−4.34 (m, 1H), 3.65 (bs, 1H), 2.85−2.75 (m, 1H), 2.72−2.65 (m, 1H), 2.41−2.25 (m, 2H), 2.21−2.08 (m, 2H), 2.05−1.9 (m, 2H), 1.83−1.80 (m, 2H), 1.62 (d, J = 6.4 Hz, 6H), 1.44 (s, 9H); mass (m/z): 413.4 (M + H)$^+$.

*1-Isopropyl-1H-indazole-3-carboxylic Acid Piperidin-4-ylamide (**6a**).* Following the procedure of Boc deprotection as mentioned in general procedure 2, compound 4-[(1-isopropyl-1H-indazole-3-carbonyl)-amino]-piperidine-1-carboxylic acid *tert*-butyl ester **5a** (825.8 mg, 2.13 mmol) was converted to title compound **6a** (580.7 mg) in 95.1% yield with HPLC purity 91.4%. ^{1}H NMR ($CDCl_3$): δ 8.39 (d, J = 8.0 Hz, 1H), 7.45 (d, J = 8.4 Hz, 2H), 7.39 (t, J = 8.4 Hz, 1H), 7.28 (t, J = 7.6 Hz, 1H), 5.01- 4.97 (m, 1H), 4.13 (bs, 2H), 2.97 (t, J = 12.0 Hz, 3H), 2.05 (m, 2H), 1.92 (d, J = 17.6 Hz, 4H), 1.61 (d, J = 6.8 Hz, 6H) ; mass (m/z): 287.4 (M + H)$^+$.

*1-Isopropyl-1H-indazole-3-carboxylic Acid (Piperidin-4-ylmethyl)-amide (**6b**).* Following the procedure of Boc deprotection as mentioned in general procedure 2, compound 4-{[(1-isopropyl-1H-

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indazole-3-carbonyl)-amino]-methyl}-piperidine-1-carboxylic acid *tert*-butyl ester **5b** (260.0 mg, 0.65 mmol) was converted to title compound **6b** (190.0 mg) in 97.4% yield with HPLC purity 95.21%. ^{1}H NMR ($CDCl_3$): δ 8.39 (d, J = 8.0 Hz, 1H), 7.45 (d, J = 8.4 Hz, 2H), 7.39 (t, J = 8.4 Hz, 1H), 7.28 (t, J = 7.6 Hz, 1H), 5.01- 4.97 (m, 1H), 4.13 (bs, 3H), 2.97 (t, J = 12.0 Hz, 3H), 2.05 (m, 1H), 1.92 (d, J = 17.6 Hz, 4H), 1.61 (d, J = 6.8 Hz, 6H); mass (m/z): 301.4 $(M + H)^+$.

*1-Isopropyl-1H-indazole-3-carboxylic Acid (4-Hydroxy-piperidin-4-ylmethyl)-amide (**6c**).* Following the procedure of Boc deprotection as mentioned in general procedure 2, compound 4-hydroxy-4-{[(1-isopropyl-1*H*-indazole-3-carbonyl)-amino]-methyl}-piperidine-1-carboxylic acid *tert*-butyl ester **5c** (1.7 g, 4.08 mmol) was converted to title compound **6c** (1.1 g) in 98.0% yield with HPLC purity 97.15%. ^{1}H NMR ($CDCl_3$): δ 8.37 (d, J = 8.1 Hz, 1H), 7.44 (d, J = 8.3 Hz, 2H), 7.38 (t, J = 8.4 Hz, 1H), 7.21 (t, J = 7.6 Hz, 1H), 4.21 (bs, 3H), 3.54 (bs, 2H), 2.73 (t, J = 12.0 Hz, 2H), 1.81 (d, J = 17.6 Hz, 4H), 1.64 (d, J = 6.8 Hz, 6H); mass (m/z): 317.4 $(M + H)^+$.

*1-Isopropyl-1H-indazole-3-carboxylic Acid (4-Fluoro-piperidin-4-ylmethyl)-amide (**6d**).* Following the procedure of Boc deprotection as mentioned in general procedure 2, compound 4-fluoro-4-{[(1-isopropyl-1*H*-indazole-3-carbonyl)-amino]-methyl}-piperidine-1-carboxylic acid *tert*-butyl ester **5d** (1.32 g, 3.15 mmol) was converted to title compound **6d** (0.97 g) in 97.7% yield with HPLC purity 96.2%. ^{1}H NMR ($CDCl_3$): δ 8.36 (d, J = 8.2 Hz, 1H), 7.45 (d, J = 8.1 Hz, 2H), 7.38 (t, J = 8.4 Hz, 1H), 7.21 (t, J = 7.6 Hz, 1H), 4.26 (bs, 3H), 3.55 (bs, 2H), 2.73 (t, J = 12.0 Hz, 2H), 1.81 (d, J = 17.6 Hz, 4H), 1.64 (d, J = 6.8 Hz, 6H); mass (m/z): 319.2 $(M + H)^+$.

*1-Isopropyl-1H-indazole-3-carboxylic Acid (Morpholin-2-yl-methyl)-amide (**6e**).* Following the procedure of Boc deprotection as mentioned in general procedure 2, compound 2-{[(1-isopropyl-1*H*-indazole-3-carbonyl)-amino]-methyl}-morpholine-4-carboxylic acid *tert*-butyl ester **5e** (1.2 g, 2.98 mmol) was converted to title compound **6e** (827.4 g) in 93.4% yield with HPLC purity 94.15%. ^{1}H NMR (DMSO-d_6): δ 8.15 (d, J = 7.6 Hz, 2H), 7.76 (d, J = 8.4 Hz, 1H), 7.43 (t, J = 7.6 Hz, 1H), 7.22 (t, J = 7.6 Hz, 1H), 5.07−5.04 (m, 1H), 3.80−3.44 (m, 3H), 2.71 (t, J = 12.0 Hz, 2H), 2.65−2.62 (m, 2H), 2.37−2.29 (m, 2H), 1.53 (d, J = 6.8 Hz, 6H); mass (m/z): 303.1 $(M + H)^+$.

*1-Isopropyl-1H-indazole-3-carboxylic Acid (3-Aza-bicyclo[3.1.0]-hex-6-yl)-amide (**6f**).* Following the procedure of Boc deprotection as mentioned in general procedure 2, compound 6-[(1-isopropyl-1*H*-indazole-3-carbonyl)-amino]-3-aza-bicyclo[3.1.0]hexane-3-carboxylic acid *tert*-butyl ester **5f** (1.5 g, 3.90 mmol) was converted to title compound (998.0 mg) **6f** in 90.0% yield with HPLC purity 94.1%. ^{1}H NMR (DMSO-d_6): δ 8.15 (d, J = 8.0 Hz, 1H), 7.95 (d, J = 8.4 Hz, 1H), 7.77 (d, J = 8.8 Hz, 1H), 7.43 (t, J = 7.6 Hz, 1H), 7.25 (t, J = 7.6 Hz, 1H), 5.07−5.04 (m, 1H), 2.91 (d, J = 11.6 Hz, 2H), 2.49−2.42 (m 1H), 2.20 (t, J = 11.2 Hz, 2H), 1.68−1.63 (m, 2H), 1.53 (d, J = 6.4 Hz, 6H); mass (m/z): 285.4 $(M + H)^+$.

*1-Isopropyl-1H-indazole-3-carboxylic Acid (3-Aza-bicyclo[3.1.0]-hex-6-ylmethyl)-amide (**6g**).* Following the procedure of Boc deprotection as mentioned in general procedure 2, compound 6-{[(1-isopropyl-1*H*-indazole-3-carbonyl)-amino]-methyl}-3-aza-bicyclo[3.1.0]hexane-3-carboxylic acid *tert*-butyl ester **5g** (806.7 mg, 3.90 mmol) was converted to title compound **6g** (555.0 mg) in 92.0% yield with HPLC purity 96.8%. ^{1}H NMR (DMSO-d_6): δ 8.15 (d, J = 8.0 Hz, 1H), 7.95 (d, J = 8.4 Hz, 1H), 7.77 (d, J = 8.8 Hz, 1H), 7.43 (t, J = 7.6 Hz, 1H), 7.25 (t, J = 7.6 Hz, 1H), 5.07−5.04 (m, 1H), 2.91 (d, J = 11.6 Hz, 2H), 2.71 (d, J = 12.0 Hz, 2H), 2.49−2.42 (m, 1H), 2.20 (t, J = 11.2 Hz, 2H), 1.68−1.63 (m, 2H), 1.53 (d, J = 6.4 Hz, 6H); mass (m/z): 299.1 $(M + H)^+$.

*1-Isopropyl-1H-indazole-3-carboxylic Acid (8-Aza-bicyclo[3.2.1]-oct-3-yl)-amide (**6h**).* Following the procedure of Boc deprotection as mentioned in general procedure 2, compound 3-[(1-isopropyl-1*H*-indazole-3-carbonyl)-amino]-8-aza-bicyclo[3.2.1]octane-8-carboxylic acid *tert*-butyl ester **5h** (340.7 mg, 1.82 mmol) was converted to title compound **6h** (340.7 mg) in 90.0% yield with HPLC purity 97.1%. ^{1}H NMR ($CDCl_3$): δ 8.37 (d, J = 8.4 Hz, 1H), 7.52 (d, J = 6.0 Hz, 1H), 7.43 (d, J = 8.4 Hz, 1H), 7.41 (t, J = 6.8 Hz, 1H), 4.91−4.86 (m, 1H), 4.35−4.34 (m, 1H), 3.65 (bs, 1H), 2.85−2.75 (m, 1H), 2.72−2.65 (m, 1H), 2.41−2.25 (m, 2H), 2.21−2.08 (m, 2H), 2.05−1.9 (m, 2H), 1.83−1.80 (m, 2H), 1.62 (d, J = 6.4 Hz, 6H); mass (m/z): 313.4 $(M + H)^+$.

*1-Isopropyl-1H-indazole-3-carboxylic Acid [1-(3-Hydroxy-propyl)-piperidin-4-yl]-amide Tartarate (**7a**).* 1-Isopropyl-1*H*-indazole-3-carboxylic acid piperidin-4-ylamide **6a** (580.0 mg, 2.02 mmol) was N-alkylated with 3-bromo-propan-1-ol by following general procedure 3 to obtain title compound **7a** free base which was converted to its tartarate salt by using the general procedure 8 to obtain title compound (685.6 mg) as cream color solid in 88.2% yield with HPLC purity 95.7%. ^{1}H NMR (DMSO-d_6): δ 8.15 (t, J = 8.0 Hz, 2H), 7.78 (d, J = 8.8 Hz, 1H), 7.44 (t, J = 7.6 Hz, 1H), 7.26 (t, J = 7.6 Hz, 1H), 5.10−5.03 (m,1H), 4.05 (s, 2H), 3.98−3.96 (m, 1H), 3.90 (bs, 1H), 3.49 (t, J = 7.2 Hz, 2H), 3.20 (d, J = 11.2 Hz, 2H), 2.72 (d, J = 6.4 Hz, 2H), 2.52 (bs, 2H), 1.91 (d, J = 10.8 Hz, 2H), 1.81 (d, J = 11.2 Hz, 2H), 1.67 (t, J = 6.0 Hz, 2H), 1.54 (d, J = 6.8 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 174.4, 167.3, 160.6, 139.6, 129.1, 127.0, 122.8, 122.5, 121.9, 109.6, 81.0, 62.7, 57.9, 52.1, 51.6, 31.9, 28.2, 27.0, 22.0; DSC: (RAMP 5 °C/min): 143.2 °C; mass (m/z): 345.1 $(M + H)^+$; $C_{23}H_{34}N_4O_8$; % C, H, N: calculated C; 55.86, H; 6.93, N; 11.33; found C; 55.62, H; 6.93, N; 11.52.

*1-Isopropyl-1H-indazole-3-carboxylic Acid [1-(3-Methoxy-propyl)-piperidin-4-ylmethyl]-amide Oxalate (**7l**).* By following general procedure 3, 1-isopropyl-1*H*-indazole-3-carboxylic acid (piperidin-4-ylmethyl)-amide **6b** (190.0 mg, 0.63 mmol) was N-alkylated with 1-bromo-3-methoxypropane to obtain **7l** free base as a gummy mass which was converted to its oxalate salt by using the general procedure 8 to obtain title compound **7l** (188.6 mg) as off-white solid in 80.0% yield with HPLC purity 98.7%. ^{1}H NMR (DMSO-d_6): δ 8.16 (d, J = 7.6 Hz, 1H), 7.79 (d, J = 7.6 Hz, 1H), 7.44 (t, J = 7.6 Hz, 1H), 7.26 (t, J = 7.6 Hz, 1H), 5.10−5.06 (m, 1H), 4.11−3.99 (m, 1H), 3.80 (d, J = 10.8 Hz, 1H), 3.62−3.50 (m, 1H), 3.34 (t, J = 9.6 Hz, 2H), 3.18 (s, 3H), 2.79 (d, J = 10.4 Hz, 2H), 2.69 (d, J = 10.4 Hz, 4H), 2.29 (bs, 2H), 1.97−1.76 (m, 2H), 1.64−1.61 (m, 1H), 1.53 (d, J = 6.8 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.9, 160.6, 139.6, 129.1, 127.0, 122.8, 122.5, 121.9, 109.6, 69.9, 57.9, 52.1, 51.6, 31.9, 28.2, 27.0, 22.0; DSC: (RAMP 5 °C/min): 157.1 °C; mass (m/z): 373.0 $(M + H)^+$; $C_{23}H_{34}N_4O_6$; % C, H, N: calculated C; 59.72, H; 7.41, N; 12.11; found C; 59.81, H; 7.61, N; 12.23.

*1-Isopropyl-1H-indazole-3-carboxylic Acid [4-Hydroxy-1-(3-methoxy-propyl)-piperidin-4-ylmethyl]-amide Oxalate (**7o**).* 1-Isopropyl-1*H*-indazole-3-carboxylic acid (4-hydroxy-piperidin-4-ylmethyl)-amide **6c** (1.1 g, 3.47 mmol) was N-alkylated with 1-bromo-3-methoxy-propane by following general procedure 3 to obtain **7o** free base as a gummy mass which was converted to its oxalate salt by using the general procedure 8 to obtain title compound in 92.1% yield (1.32 g) with HPLC purity 97.5%. ^{1}H NMR (CD_3OD): δ 8.31 (bs, 1H), 8.22 (d, J = 8.0 Hz, 1H), 7.68 (d, J = 8.4 Hz, 1H), 7.47 (t, J = 7.6 Hz, 1H), 7.28 (t, J = 7.6 Hz, 1H), 5.08−5.02 (m, 1H), 3.65 (bs, 2H), 3.54 (s, 3H), 3.51- 3.46 (m, 6H), 3.23−2.219 (m, 2H), 2.02−1.95 (m, 6H), 1.62 (d, J = 6.4 Hz, 6H); mass (m/z): 389.2 $(M + H)^+$; $C_{23}H_{34}N_4O_6$; % C, H, N: calculated C; 59.72, H; 7.41, N; 12.11; found C; 59.39, H; 7.61, N; 12.23.

*1-Isopropyl-1H-indazole-3-carboxylic Acid [4-Fluoro-1-(3-methoxy-propyl)-piperidin-4-ylmethyl]-amide (**7q**).* 1-Isopropyl-1*H*-indazole-3-carboxylic acid (4-hydroxy-piperidin-4-ylmethyl)-amide **6d** (0.5 g, 1.67 mmol) was N-alkylated with 1-bromo-3-methoxy-propane by following general procedure 3 to obtain **7q** as off-white powder in 88.2% yield (0.49 g) with HPLC purity 95.8%. ^{1}H NMR ($CDCl_3$): δ 8.36 (d, J = 8.2 Hz, 1H), 7.45 (d, J = 8.1 Hz, 2H), 7.38 (t, J = 8.4 Hz, 1H), 7.21 (t, J = 7.6 Hz, 1H), 4.26 (bs, 3H), 3.55−3.43 (m, 4H), 3.54 (s, 3H), 2.73 (m, 2H), 2.04−1.88 (m, 6H), 1.64 (d, J = 6.8 Hz, 6H); mass (m/z): 391.1 $(M + H)^+$; $C_{21}H_{31}FN_4O_2$; % C, H, N: calculated C; 64.59, H; 8.0, N; 14.35; found C; 64.02, H; 7.69, N; 14.26.

*1-Isopropyl-1H-indazole-3-carboxylic Acid [4-(3-Methoxypropyl)-morpholin-2-ylmethyl]-amide Tartarate (**7r**).* 1-Isopropyl-1*H*-indazole-3-carboxylic acid (4-fluoro-piperidin-4-ylmethyl)-amide was N-alkylated with 1-bromo-3-methoxy-propane by following general

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procedure 3 to obtain compound **7r** free base as a gummy mass which was converted to its tartarate salt using general procedure 8 to obtain title compound **7r** (851.8 mg) in 83% yield with HPLC purity 95.5%. ^{1}H NMR (DMSO-d_6): δ 8.16 (d, J = 7.6 Hz, 2H), 7.79 (d, J = 8.4 Hz, 1H), 7.44 (t, J = 7.6 Hz, 1H), 7.26 (t, J = 7.6 Hz, 1H), 5.08−5.05 (m, 1H), 3.80−3.44 (m, 3H), 3.35−3.29 (m, 2H), 3.18 (s, 3H), 2.79−2.76 (m, 2H), 2.65−2.62 (m, 4H), 2.37−2.29 (m, 2H), 1.64−1.61 (m, 2H), 1.53 (d, J = 6.8 Hz, 6H); mass (m/z): 375.1 (M + H)$^+$; $C_{24}H_{36}N_4O_9$; % C, H, N: calculated C; 54.95, H; 6.92, N; 10.68; found C; 55.01, H; 6.73, N; 10.43.

*1-Isopropyl-1H-indazole-3-carboxylic Acid [3-(3-Methoxy-propyl)-3-aza-bicyclo[3.1.0]hex-6-yl]-amide Oxalate (**7s**).* 1-Isopropyl-1*H*-indazole-3-carboxylic acid (3-aza-bicyclo[3.1.0]hex-6-yl)-amide **6f** (998.4 mg, 3.51 mmol) was N-alkylated with 1-bromo-3-methoxy-propane by following general procedure 3 to obtain compound **7s** free base as a gummy mass which was converted to its oxalate salt using general procedure 8 to obtain title compound **7s** (1.1 g) as off-white powder in 93% yield with HPLC purity 95.5%. ^{1}H NMR (DMSO-d_6): δ 8.37 (d, J = 3.2 Hz, 1H), 8.15 (d, J = 8.4 Hz, 1H), 7.78 (d, J = 8.4 Hz, 1H), 7.44 (t, J = 7.2 Hz, 1H), 7.27 (t, J = 7.6 Hz, 1H), 5.07−5.04 (m, 1H), 3.75 (s, 3H), 3.68 (t, J = 9.2 Hz, 2H), 3.52−3.33 (m, 4H), 3.22- 3.14 (m, 6H), 3.14−2.99 (m, 2H), 2.06 (s, 2H), 1.81−1.78 (m, 2H), 1.52 (d, J = 6.8 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.2, 163.0, 139.9, 136.4, 126.3, 122.9, 122.6, 122.5, 109.3, 69.0, 58.7, 55.9, 54.3, 51.0, 39.8, 26.1, 22.1, 21.1; DSC: (RAMP 5 °C/min): 172.6 °C; mass (m/z): 357.2 (M + H)$^+$; $C_{22}H_{30}N_4O_6$; % C, H, N: calculated C; 59.18, H; 6.77, N; 12.55; found C; 59.01, H; 6.71, N; 12.83.

*1-Isopropyl-1H-indazole-3-carboxylic Acid [3-(3-Methoxy-propyl)-3-aza-bicyclo[3.1.0]hex-6-ylmethyl]-amide Oxalate (**7v**).* 1-Isopropyl-1*H*-indazole-3-carboxylic acid (3-aza-bicyclo[3.1.0]hex-6-yl-methyl)-amide **6g** (555.0 mg, 1.86 mmol) was N-alkylated with 1-bromo-3-methoxy-propane by following general procedure 3 to obtain compound **7v** free base as a gummy mass which was converted to its oxalate salt using general procedure 8 to obtain title compound **7v** (640.0 mg) as a gummy mass in 91.7% yield with HPLC purity 97.2%. ^{1}H NMR (DMSO-d_6): δ 8.41 (bs, 1H), 8.17 (d, J = 8.0 Hz, 1H), 7.80 (d, J = 8.3 Hz, 1H), 7.45 (t, J = 7.21 Hz, 1H), 7.27 (t, J = 7.5 Hz, 1H), 5.09−5.06 (m, 1H), 3.32 (t, J = 11.0 Hz, 4H), 3.19 (s, 6H), 3.05 (bs, 4H), 1.75 (bs, 4H), 1.54 (d, J = 6.4 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 163.9, 163.1, 139.9, 136.4, 126.3, 122.9, 122.6, 122.5, 109.3, 69.0, 58.7, 55.9, 54.3, 51.0, 47.1, 39.8, 26.1, 22.1, 21.0; DSC: (RAMP 5 °C/min): 163.8 °C; mass (m/z): 371.2 (M + H)$^+$; $C_{22}H_{33}N_4O_6$; % C, H, N: calculated C; 59.99, H; 7.00, N; 12.17; found C; 60.20, H; 6.98, N; 12.23.

*1-Isopropyl-1H-indazole-3-carboxylic Acid [8-(2-Fluoro-ethyl)-8-aza-bicyclo[3.2.1]oct-3-yl]-amide (**7aa**).* 1-Isopropyl-1*H*-indazole-3-carboxylic acid (8-aza-bicyclo[3.2.1]oct-3-yl)-amide **6h** (340.0 mg, 1.08 mmol) was N-alkylated with 1-bromo-3-methoxy-propane by following general procedure 3 to obtain title compound **7aa** (351.0 mg) as off-white color powder in 90% yield with HPLC purity 96.7%. ^{1}H NMR ($CDCl_3$): δ 8.38 (d, J = 8.4 Hz, 1H), 7.58 (d, J = 6.0 Hz, 1H), 7.46 (d, J = 8.4 Hz, 1H), 7.41 (t, J = 6.8 Hz, 1H), 4.91−4.86 (m, 1H), 4.70−4.60 (m, 1H), 4.55−4.48 (m, 1H), 4.35−4.34 (m, 1H), 3.65 (bs, 1H), 3.34 (bs, 2H), 2.85−2.75 (m, 1H), 2.72−2.65 (m, 1H), 2.41−2.25 (m, 2H), 2.21−2.08 (m, 2H), 2.05−1.9 (m, 2H), 1.83−1.80 (m, 2H), 1.62 (d, J = 6.4 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.9, 139.8, 136.4, 126.3, 122.9, 122.6, 122.5, 109.3, 84.1, 58.7, 55.9, 54.3, 51.0, 39.8, 26.1, 22.1, 21.8; DSC: (RAMP 5 °C/min): 159.2 °C; mass (m/z): 359.2 (M + H)$^+$; $C_{20}H_{27}FN_4O$; % C, H, N: calculated C; 67.01, H; 7.59, N; 15.63; found C; 67.18, H; 7.23, N; 15.61.

*Piperidine-1,4-dicarboxylic Acid 1-tert-Butyl Ester 4-Ethyl Ester (**8a**).* By following general procedure 1, ethyl isonipecotate (6.5 g, 41.35 mmol) was converted to the title compound **8** (9.54 g) in 90% yield with GC purity 98.8%. ^{1}H NMR ($CDCl_3$): δ 4.16−4.11 (m, 2H), 2.86 (t, J = 12.0 Hz, 2H), 2.43−2.43 (m, 1H), 1.88−1.85 (bs, 2H), 1.67−1.57 (m, 4H), 1.47 (s, 9H), 1.27 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.0, 159.3, 71.1, 51.2, 30.9, 28.4, 26.5; mass (m/z): 258.2 (M + H)$^+$.

*4-Hydrazinocarbonyl-piperidine-1-carboxylic Acid tert-Butyl Ester (**9a**).* By following general procedure 5, compound **8a** (5.0 g, 19.45 mmol) was converted to the titled compound **9a** (4.1 g) as an off-white crystalline powder in 87.2% yield with GC purity 99.79%. ^{1}H NMR ($CDCl_3$): δ 8.12 (bs, 1H), 2.86 (t, J = 12.0 Hz, 2H), 2.43−2.43 (m, 1H), 2.05 (bs, 2H), 1.88−1.85 (bs, 2H), 1.67−1.57 (m, 4H), 1.47 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.9, 159.3, 69.2, 51.0, 30.5, 28.4, 26.5; DSC: (RAMP 5 °C/min): 102.1 °C; mass (m/z): 244.4 (M + H)$^+$.

*4-[N′-(1-Isopropyl-1H-indazole-3-carbonyl)-hydrazinocarbonyl]-piperidine-1-carboxylic Acid tert-Butyl Ester (**10a**).* By following general procedure 6, 4-hydrazinocarbonyl-piperidine-1-carboxylic acid *tert*-butyl ester **9a** (3.2 g, 13.48 mmol) was reacted with 1-isopropyl-1*H*-indazole-3-carbonyl chloride **3** (3.0 g, 13.48 mmol) to obtain the above titled compound **10a** (5.3 g) as an off-white crystalline powder in 92% yield with HPLC purity 98.75%. ^{1}H NMR (DMSO-d_6): δ 10.02 (bs, 1H), 9.86 (bs, 1H), 8.12 (d, J = 8.4 Hz, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.45 (t, J = 7.6 Hz, 1H), 7.30 (t, J = 7.6 Hz, 1H), 5.13−5.06 (m, 1H), 2.80 (bs, 2H), 2.71 (bs, 3H), 1.76 (d, J = 6.8 Hz, 2H), 1.55 (d, J = 6.4 Hz, 6H), 1.40 (s, 9H), 1.28 (t, J = 10.8 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 177.9, 167.3, 159.3, 139.9, 128.9, 127.5, 123.5, 121.9, 121.3, 111.1, 69.2, 53.7, 51.0, 30.5, 28.4, 26.5, 22.2; DSC: (RAMP 5 °C/min): 123.2 °C; mass (m/z): 430.5 (M + H)$^+$.

*4-Hydroxy-4-[N′-(1-isopropyl-1H-indazole-3-carbonyl)-hydrazinocarbonyl]-piperidine-1-carboxylic Acid tert-Butyl Ester (**10b**).* By following general procedure 6, 4-hydrazinocarbonyl-4-hydroxy-piperidine-1-carboxylic acid *tert*-butyl ester **9b** (2.0 g, 8.98 mmol) was reacted with 1-isopropyl-1*H*-indazole-3-carbonyl chloride **3** (2.3 g, 8.98 mmol) to obtain the above titled compound **10b** (1.2 g) as an off-white crystalline powder in 63.1% yield with HPLC purity 98.7%. ^{1}H NMR (DMSO-d_6): δ 10.02 (bs, 1H), 9.86 (bs, 1H), 8.12 (d, J = 8.4 Hz, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.45 (t, J = 7.6 Hz, 1H), 7.30 (t, J = 7.6 Hz, 1H), 5.13−5.06 (m, 1H), 2.80 (bs, 2H), 2.71 (bs, 4H), 1.76 (d, J = 6.8 Hz, 2H), 1.55 (d, J = 6.4 Hz, 6H), 1.40 (s, 9H), 1.28 (t, J = 10.8 Hz, 2H); mp: 106.1−107.2 °C; mass (m/z): 446.3 (M + H)$^+$.

*4-{2-[N′-(1-Isopropyl-1H-indazole-3-carbonyl)-hydrazino]-2-oxo-ethyl}-piperidine-1-carboxylic Acid tert-Butyl Ester (**10c**).* By following general procedure 6, 4-hydrazinocarbonylmethylpiperidine-1-carboxylic acid *tert*-butyl ester **9c** (3.4 g, 13.47 mmol) was reacted with 1-isopropyl-1*H*-indazole-3-carbonyl chloride **3** (3.0 g, 13.47 mmol) to obtain the above titled compound **10c** (4.7 g) as brown color gummy mass in 80.0% yield with HPLC purity 95.6%. ^{1}H NMR (DMSO-d_6): δ 10.02 (bs, 1H), 9.86 (bs, 1H), 8.12 (d, J = 8.4 Hz, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.45 (t, J = 7.6 Hz, 1H), 7.30 (t, J = 7.6 Hz, 1H), 5.13−5.06 (m, 1H), 3.98 (d, J = 12.0 Hz, 2H), 2.80 (bs, 2H), 2.71 (bs, 4H), 2.45 (bs, 2H),1.76 (d, J = 6.8 Hz, 2H), 1.55 (d, J = 6.4 Hz, 6H), 1.40 (s, 9H), 1.28 (t, J = 10.8 Hz, 2H); mass (m/z): 444.2 (M + H)$^+$.

*6-[N′-(1-Isopropyl-1H-indazole-3-carbonyl)-hydrazinocarbonyl]-3-aza-bicyclo[3.1.0]hexane-3-carboxylic Acid tert-Butyl Ester (**10d**).* By following general procedure 6, 6-hydrazinocarbonyl-3-aza-bicyclo[3.1.0]hexane-3-carboxylic acid *tert*-butyl ester **9d** (1.01 g, 4.50 mmol) was reacted with 1-isopropyl-1*H*-indazole-3-carbonyl chloride **3** (1.0 g, 4.50 mmol) to obtain the above titled compound **10d** (1.5 g) as crystalline solid in 78% yield with HPLC purity 95.2%. ^{1}H NMR (DMSO-d_6): δ 10.02 (bs, 1H), 9.86 (bs, 1H), 8.12 (d, J = 8.4 Hz, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.45 (t, J = 7.6 Hz, 1H), 7.30 (t, J = 7.6 Hz, 1H), 5.13−5.06 (m, 1H), 3.98 (d, J = 12.0 Hz, 2H), 2.80 (bs, 2H), 2.71 (bs, 2H), 1.55 (d, J = 6.4 Hz, 6H), 1.40 (s, 9H), 1.28 (t, J = 10.8 Hz, 2H); mp: 152.3−154.1 °C; mass (m/z): 428.4 (M + H)$^+$.

*3-[N′-(1-Isopropyl-1H-indazole-3-carbonyl)-hydrazinocarbonyl]-8-aza-bicyclo[3.2.1]octane-8-carboxylic Acid tert-Butyl Ester (**10e**).* By following general procedure 6, 3-hydrazinocarbonyl-8-aza-bicyclo[3.2.1]octane-8-carboxylic acid *tert*-butyl ester **9d** (2.4 g, 8.98 mmol) was reacted with 1-isopropyl-1*H*-indazole-3-carbonyl chloride **3** (2.4 g, 8.98 mmol) to obtain the above titled compound **10e** (3.89 g) as an off-white crystalline powder in 95.1% yield with HPLC purity

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98.7%. ^{1}H NMR (DMSO-d_6): δ 10.02 (bs, 1H), 9.86 (bs, 1H), 8.12 (d, J = 8.4 Hz, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.45 (t, J = 7.6 Hz, 1H), 7.30 (t, J = 7.6 Hz, 1H), 5.13−5.06 (m, 1H), 3.98 (d, J = 12.0 Hz, 2H), 2.71 (bs, 4H), 1.76 (d, J = 6.8 Hz, 2H), 1.55 (d, J = 6.4 Hz, 6H), 1.40 (s, 9H), 1.35−1.28 (m, 3H); mp: 117.9−118.8 °C; mass (m/z): 456.2 (M + H)$^+$.

*1-Aza-bicyclo[2.2.2]octane-3-carboxylic Acid N′-(1-Isopropyl-1H-indazole-3-carbonyl)-hydrazide (**10f**).* By following general procedure 6, 1-aza-bicyclo[2.2.2]octane-3-carboxylic acid hydrazide **9f** (3.0 g, 12.34 mmol) reacts with 1-isopropyl-1*H*-indazole-3-carbonyl chloride **3** (2.7 g, 12.34 mmol) to obtain the above titled compound **10f** (4.24 g) as an off-white crystalline powder in 74.5% yield with HPLC purity 96.2%. ^{1}H NMR (DMSO-d_6): δ 10.02 (bs, 1H), 9.86 (bs, 1H), 8.12 (d, J = 8.4 Hz, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.45 (t, J = 7.6 Hz, 1H), 7.30 (t, J = 7.6 Hz, 1H), 5.13−5.06 (m, 1H), 2.80 (m, 4H), 2.71 (m, 3H), 1.76 (d, J = 6.8 Hz, 2H), 1.55 (d, J = 6.4 Hz, 6H), 1.28 (m, 3H); mp: 137.3−138.8 °C; mass (m/z): 356.4 (M + H)$^+$.

*1-Isopropyl-3-(5-piperidin-4-yl-[1,3,4]oxadiazol-2-yl)-1H-indazole (**11a**).* By following general procedure 7, 4-[*N*′-(1-isopropyl-1*H*-indazole-3-carbonyl)-hydrazinocarbonyl]-piperidine-1-carboxylic acid *tert*-butyl ester **10a** (5.3 g, 12.33 mmol) was converted to above titled compound **11a** (3.6 g) as brown color solid in 95% yield with HPLC purity 99.3%. ^{1}H NMR (DMSO-d_6): δ 8.19 (d, J = 8.0 Hz, 1H), 7.91 (d, J = 8.8 Hz, 1H), 7.56 (t, J = 7.6 Hz, 1H), 7.40 (t, J = 7.6 Hz, 1H), 5.20−5.13 (m,1H), 3.22−3.16 (m, 1H), 3.02 (d, J = 12.4 Hz, 2H), 2.67 (d, J = 8.8 Hz, 2H), 2.01(d, J = 10.8 Hz, 2H), 1.73−1.65 (m, 2H), 1.56 (d, J = 6.4 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 160.1, 139.8, 128.6, 127.5, 123.4, 121.9, 121.3, 111.0, 51.0, 45.4, 33.4, 30.3, 22.2; DSC: (RAMP 5 °C/min): 109.6 °C; mass (m/z): 312.1(M + H)$^+$.

*4-[5-(1-Isopropyl-1H-indazol-3-yl)-[1,3,4]oxadiazol-2-yl]-piperidin-4-ol (**11b**).* By following general procedure 7, 4-hydroxy-4-[*N*′-(1-isopropyl-1*H*-indazole-3-carbonyl)-hydrazinocarbonyl]-piperidine-1-carboxylic acid *tert*-butyl ester **10b** (1.2 g, 2.69 mmol) was converted to above titled compound **11b** (705.4 mg) which was isolated as a gummy mass in 90.0% yield with HPLC purity 92.3%. ^{1}H NMR (DMSO-d_6): δ 10.12 (bs, 1H), 9.84 (bs, 1H), 8.10 (d, J = 8.4 Hz, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.45 (t, J = 7.6 Hz, 1H), 7.30 (t, J = 7.6 Hz, 1H), 5.13−5.06 (m, 1H), 2.80 (bs, 2H), 2.71 (bs, 2H), 1.76 (d, J = 6.8 Hz, 2H), 1.55 (d, J = 6.4 Hz, 6H), 1.28 (t, J = 10.8 Hz, 2H); mass (m/z): 328.1 (M + H)$^+$.

*1-Isopropyl-3-(5-piperidin-4-ylmethyl-[1,3,4]oxadiazol-2-yl)-1H-indazole (**11c**).* By following general procedure 7, 4-{2-[*N*′-(1-isopropyl-1H-indazole-3-carbonyl)-hydrazino]-2-oxo-ethyl}-piperidine-1-carboxylic acid *tert*-butyl ester **10c** (4.7 g, 10.6 mmol) was converted to above titled compound **11c** (3.1 g) as brown color solid compound in 89% yield with HPLC purity 99.3%. ^{1}H NMR (DMSO-d_6): δ 10.02 (bs, 1H), 9.86 (bs, 1H), 8.12 (d, J = 8.4 Hz, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.45 (t, J = 7.6 Hz, 1H), 7.30 (t, J = 7.6 Hz, 1H), 5.13−5.06 (m, 1H), 2.80 (bs, 2H), 2.71 (bs, 2H), 2.45 (bs, 2H),1.76 (d, J = 6.8 Hz, 2H), 1.55 (d, J = 6.4 Hz, 6H), 1.28 (t, J = 10.8 Hz, 2H); mp: 134.6−134.9 °C; mass (m/z): 325.1 (M + H)$^+$.

*3-[5-(3-Aza-bicyclo[3.1.0]hex-6-ylmethyl)-[1,3,4]oxadiazol-2-yl]-1-isopropyl-1H-indazole (**11d**).* By following general procedure 7, 6-[*N*′-(1-isopropyl-1H-indazole-3-carbonyl)-hydrazinocarbonyl]-3-aza-bicyclo[3.1.0]hexane-3-carboxylic acid *tert*-butyl ester **10d** (1.5 g, 3.51 mmol) was converted to above titled compound **11d** (1.0 g) as a gummy mass in 93.2% yield with HPLC purity 95.2%. ^{1}H NMR (DMSO-d_6): δ 10.02 (bs, 1H), 9.86 (bs, 1H), 8.12 (d, J = 8.4 Hz, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.45 (t, J = 7.6 Hz, 1H), 7.30 (t, J = 7.6 Hz, 1H), 5.13−5.06 (m, 1H), 2.80 (bs, 2H), 2.71 (bs, 4H), 1.55 (d, J = 6.4 Hz, 6H), 1.28 (t, J = 10.8 Hz, 2H); mp: 127.3−128.9 °C; mass (m/z): 324.4 (M + H)$^+$.

*3-[5-(8-Aza-bicyclo[3.2.1]oct-3-yl)-[1,3,4]oxadiazol-2-yl]-1-isopropyl-1H-indazole (**11e**).* By following general procedure 7, 3-[*N*′-(1-isopropyl-1*H*-indazole-3-carbonyl)-hydrazinocarbonyl]-8-aza-bicyclo[3.2.1]octane-8-carboxylic acid *tert*-butyl ester **10e** (3.8 g, 8.342 mmol) was converted to above titled compound **11e** (2.71 g) as white color solid in 98% yield with HPLC purity 95.2%. ^{1}H NMR (DMSO-d_6): δ 10.02 (bs, 1H), 9.86 (bs, 1H), 8.12 (d, J = 8.4 Hz, 1H), 7.82 (d, J = 8.4 Hz, 1H), 7.45 (t, J = 7.6 Hz, 1H), 7.30 (t, J = 7.6 Hz, 1H), 5.13−5.06 (m, 1H), 2.71 (bs, 4H), 1.76 (d, J = 6.8 Hz, 2H), 1.55 (d, J = 6.4 Hz, 6H), 1.35−1.28 (m, 3H); mp: 123.1−124.1 °C; mass (m/z): 339.5 (M + H)$^+$.

*1-Isopropyl-3-{5-[1-(3-methoxypropyl)-piperidin-4-yl]-[1,3,4]-oxadiazol-2-yl}-1H-indazole oxalate (**12l**).* By following general procedure 3, 1-isopropyl-3-(5-piperidin-4-yl-[1,3,4]oxadiazol-2-yl)-1*H*-indazole **11a** (1.8 g, 6.07 mmol) was N-alkylated with 1-bromo-3-methoxypropane to obtain 1-isopropyl-3-{5-[1-(3-methoxypropyl)-piperidin-4-yl]-[1,3,4]oxadiazol-2-yl}-1*H*-indazole (1.84 g) as light yellow gummy mass in 80.0% yield with HPLC purity 99.0%. The obtained free base was converted to oxalate salt by following general procedure 8 to obtain the titled compound **12l** as off-white crystalline powder with HPLC purity 99.83%. IR (cm^{-1}): 3435, 2974, 2932, 2890, 2697, 2537, 1710, 1604, 1564, 1465, 1193, 1113, 992, 750; ^{1}H NMR (DMSO-d_6): δ 8.20 (d, J = 8.4 Hz, 1H), 7.92 (d, J = 8.4 Hz, 1H), 7.55 (t, J = 7.2 Hz, 1H), 7.39 (t, J = 7.6 Hz, 1H), 5.25−5.12 (m, 1H), 3.50−3.37 (m, 5H), 3.24 (s, 3H), 3.08−2.97 (m, 4H), 2.37−2.27 (m, 2H), 2.18−2.05 (m, 2H), 1.95−1.85 (m, 2H), 1.56 (d, J = 6.8 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 167.2, 164.9 (2C), 160.3, 139.9, 128.8, 127.6, 123.5, 121.9, 121.3, 111.2, 69.5, 58.3, 54.1 (2C), 51.0, 50.7, 30.5, 26.6 24.3 (2C), 22.4 (2C); DSC: (RAMP 5 °C/min): 208.2 °C; mass (m/z): 384.2 (M + H)$^+$; oxalic acid content 18.34% (average of two titration experiments; corresponds to mono oxalate); $C_{23}H_{31}N_5O_6$; % C, H, N: calculated C; 58.34, H; 6.60, N; 14.79; found C; 58.49, H; 6.49, N; 14.54.

*4-[5-(1-Isopropyl-1H-indazol-3-yl)-[1,3,4]oxadiazol-2-yl]-1-(3-methoxypropyl)-piperidin-4-ol (**12v**).* By following general procedure 3, 4-[5-(1-isopropyl-1*H*-indazol-3-yl)-[1,3,4]oxadiazol-2-yl]-piperidin-4-ol **11b** (705.0 mg, 2.15 mmol) was N-alkylated with 1-bromo-3-methoxypropane to obtain above titled compound **12v** (585.6 mg) as cream color powder in 68% yield with HPLC purity 98.2%. ^{1}H NMR ($CDCl_3$): δ 8.37 (d, J = 8.1 Hz, 1H), 7.53 (d, J = 8.4 Hz, 1H), 7.47 (t, J = 6.9 Hz, 1H), 7.33 (t, J = 7.6 Hz, 1H), 5.0−4.93 (m, 1H), 3.10−2.98 (m, 2H), 1.67 (d, J = 6.6 Hz, 6H), 1.45−1.40 (m, 2H), 0.92 (d, J = 6.5 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 167.1, 164.9, 160.4, 139.8, 128.8, 127.6, 123.5, 121.9, 121.3, 111.0, 74.1, 69.5, 58.3, 54.1, 51.0, 50.7, 26.6 24.3, 22.4; DSC: (RAMP 5 °C/min): 197.3 °C; mass (m/z): 400.1 (M + H)$^+$; $C_{21}H_{29}N_5O_3$; % C, H, N: calculated C; 63.14, H; 7.32, N; 17.53; found C; 62.91, H; 7.51, N; 17.64.

*3-[5-(1-Cyclobutyl-piperidin-4-ylmethyl)-[1,3,4]oxadiazol-2-yl]-1-isopropyl-1H-indazole Oxalate (**12x**).* By following general procedure 3, 1-isopropyl-3-(5-piperidin-4-ylmethyl-[1,3,4]oxadiazol-2-yl)-1*H*-indazole **11c** (3.1 g, 9.53 mmol) was N-alkylated using general reductive amination procedure 9 with cyclobutane to obtain compound **12x** free base which was converted to its oxalate salt by following the general procedure 8 to obtain above titled compound **12x** (2.5 g) as light yellow color powder in 72.0% yield with HPLC purity 99.1%. ^{1}H NMR (DMSO-d_6): δ 8.19 (d, J = 8.0 Hz, 1H), 7.91 (d, J = 8.4 Hz, 1H), 7.56 (t, J = 7.6 Hz, 1H), 7.40 (t, J = 7.6 Hz, 1H), 5.19−5.15 (m, 1H), 3.15−3.09 (m, 5H), 3.01 (d, J = 6.8 Hz, 2H), 2.06−1.99 (m, 5H), 1.88−1.85 (m, 2H), 1.69−1.62 (m, 2H), 1.55 (d, J = 6.8 Hz, 6H), 1.48−1.42 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.4, 170.6, 165.0, 161.5, 160.3, 139.9, 139.8, 135.5, 128.8, 127.6, 126.9, 123.5, 123.0, 122.9, 121.8, 121.8, 121.3, 111.2, 110.9, 72.5, 58.5, 51.0, 50.7, 48.4, 48.2, 32.0, 31.2, 30.7, 28.4, 25.4, 25.3, 22.4, 22.4, 13.7; DSC: (RAMP 5 °C/min): 179.1 °C; mass (m/z): 380.2 (M + H)$^+$; $C_{24}H_{31}N_5O_5$; % C, H, N: calculated C; 61.39, H; 6.65, N; 14.92; found C; 61.74, H; 6.49, N; 14.78.

*1-Isopropyl-3-[5-(3-isopropyl-3-aza-bicyclo[3.1.0]hex-6-yl)-[1,3,4]oxadiazol-2-yl]-1H-indole Oxalate (**12z**).* By following general procedure 3, 3-[5-(3-aza-bicyclo[3.1.0]hex-6-yl)-[1,3,4]oxadiazol-2-yl]-1-isopropyl-1*H*-indole **11d** (1.0 g, 3.26 mmol) was N-alkylated with 2-iodo propane compound **12z** free base which was converted to its oxalate salt by following the general procedure 8 to obtain above titled compound **12z** (758.6 mg) as pale yellow color powder in 70.0 yield with HPLC purity 96.8%. ^{1}H NMR (DMSO-d_6): δ 8.16 (d, J = 8.0 Hz, 1H), 7.91 (d, J = 8.4 Hz, 1H), 7.55 (t, J = 7.6 Hz, 1H), 7.39 (t, J =

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7.6 Hz, 1H), 5.18−5.15 (m, 1H), 3.79−3.73 (m, 1H), 3.28−3.23 (m, 4H), 2.68 (bs, 1H), 1.54 (d, J = 6.4 Hz, 6H), 1.22 (d, J = 6.0 Hz, 6H), 1.03 (d, J = 6.0 Hz, 2H); DSC: (RAMP 5 °C/min): 184.2 °C; mass (m/z): 351.0 $(M + H)^+$; $C_{23}H_{28}N_4O_5$; % C, H, N: calculated C; 62.71, H; 6.41, N; 12.72; found C; 63.04, H; 6.23, N; 12.44.

*1-Isopropyl-3-[5-(8-methyl-8-aza-bicyclo[3.2.1]oct-3-yl)-[1,3,4]-oxadiazol-2-yl]-1H-indazole Oxalate (**12ac**).* By following general procedure 3, 3-[5-(8-aza-bicyclo[3.2.1]oct-3-yl)-[1,3,4]oxadiazol-2-yl]-1-isopropyl-1*H*-indazole **11e** (2.7 g, 8.00 mmol) was N-alkylated with iodomethane to obtain **12ac** free base which was converted to its oxalate salt by following the general procedure 8 to obtain above titled compound **12ac** (2.5 g) as off-white color powder in 90.0% yield with HPLC purity 97.9%. ^{1}H NMR (DMSO-d_6): δ 8.20 (d, J = 8.0 Hz, 1H), δ 7.92 (d, J = 8.8 Hz, 1H), 7.56 (t, J = 7.2 Hz, 1H), 7.40 (t, J = 7.2 Hz, 1H), 5.19−5.16 (m, 1H), 2.67 (s, 3H), 2.53−2.49 (m, 2H), 2.34−2.27 (m, 4H), 2.13−2.06 (m, 4H), 1.99−1.93 (m, 1H), 1.55 (d, J = 6.8 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 175.4, 174.1, 172.7, 171.2, 161.4, 161.2, 139.8, 135.6, 126.8, 124.4, 123.1, 123.0, 122.9, 121.8, 10.8, 73.3, 72.3, 72.2, 62.5, 61.4, 61.2, 50.6, 32.3, 32.0, 31.5, 30.7, 24.4, 24.2, 24.0, 22.4, 17.1; DSC: (RAMP 5 °C/min): 178.1 °C; mass (m/z): 352.0 $(M + H)^+$; $C_{22}H_{27}N_5O_5$; % C, H, N: calculated C; 59.85, H; 6.16, N; 15.86; found C; 60.01, H; 6.02, N; 15.43.

*1-(3-Methoxypropyl)-piperidine-4-carbonitrile (**14a**).* By following general procedure 3, piperidine-4-carbonitrile **13** (2.0 g, 18.18 mmol) was N-alkylated using 1-bromo-3-methoxy propane (3.0 g, 20.01 mmol) to obtain titled compound **14a** in 63.6% yield. ^{1}H NMR ($CDCl_3$): δ 3.42 (t, J = 6.3 Hz, 2 H), 3.32 (s, 3H), 2.64 (bs, 3H), 2.43 (t, J = 7.3 Hz, 2 H), 1.96−1.83 (m, 4 H), 1.77−1.70 (m, 2 H); mass (m/z): 183.1 $(M + H)^+$.

*1-(Tetrahydropyran-4-ylmethyl)-piperidine-4-carbonitrile (**14b**).* By following general procedure 3, piperidine-4-carbonitrile **13** (1.0 g, 9.09 mmol) was N-alkylated using 4-bromomethyltetrahydropyran (1.7 g, 9.54 mmol) to obtain titled compound **14b** in 59.2% yield. ^{1}H NMR ($CDCl_3$): δ 3.92−3.86 (m, 2H), 3.86−3.82 (m, 2H), 2.69−2.63 (m, 3H), 2.46−2.42 (m, 4H), 2.42−2.39 (m, 1H), 1.98−1.81 (m, 4H) 1.77−1.70 (m, 4H); mass (m/z): 209.2 $(M + H)^+$.

*1-Isopropyl-piperidine-4-carbonitrile (**14c**).* By following general procedure 3, piperidine-4-carbonitrile **13** (1.0 g, 9.09 mmol) was N-alkylated using 2-iodopropane (1.62 g, 9.54 mmol) to obtain titled compound **14c** in 77.8% yield. ^{1}H NMR ($CDCl_3$): δ 4.32−4.22 (m, 1H), 2.71−2.65 (m, 3H), 2.49−2.43 (m, 1H), 1.96−1.83 (m, 3H), 1.76−1.71 (m, 2H), 1.23 (d, J = 6.2 Hz, 6H); mass (m/z): 153.1 $(M + H)^+$.

*N-Hydroxy-1-(3-methoxypropyl)-piperidine-4-carboxamidine (**15a**).* To the stirred solution of compound **14a** (800.0 mg, 4.39 mmol) in ethanol (20.0 mL) at r.t., hydroxylamine hydrochloride (636.0 mg, 9.21 mmol) and K_2CO_3 (1.27 g, 9.21 mmol) were added. The reaction was gradually heated to reflux for 16 h. After completion of the reaction, it was cooled to r.t. and filtered through a Celite bed. The filtrate was concentrated under vacuum to afford title compound **15a** in quantitative yield. ^{1}H NMR ($CDCl_3$): δ 8.77 (bs, 1H), 5.32 (s, 2H), 4.02−3.99 (m, 1H), 3.29 (bs, 2 H), 3.20 (s, 3 H), 2.85 (d, J = 11.2 Hz, 3H), 2.70 (t, J = 10.4 Hz, 3 H), 1.92 (d, J = 3.6 Hz, 2H), 1.83 (t, J = 1.8 Hz, 4H); mass (m/z): 216.4 $(M + H)^+$.

*N-Hydroxy-1-(tetrahydropyran-4-ylmethyl)-piperidine-4-carboxamidine (**15b**).* By following the procedure as mentioned for compound **15a**, compound **15b** was prepared. ^{1}H NMR ($CDCl_3$): δ 8.72 (bs, 1H), 5.34 (bs, 2H), 3.92−3.86 (m, 2H), 3.86−3.82 (m, 2H), 2.69−2.63 (m, 3H), 2.46−2.42 (m, 4H), 2.42−2.39 (m, 1H), 1.98−1.81 (m, 4H) 1.77−1.70 (m, 4H); mass (m/z): 242.2 $(M + H)^+$.

*N-Hydroxy-1-(isopropyl)-piperidine-4-carboxamidine (**15c**).* By following the procedure as mentioned for compound **15a**, compound **15c** was prepared. ^{1}H NMR ($CDCl_3$): δ 8.92 (bs, 1H), 5.27 (bs, 2H), 4.32−4.22 (m, 1H), 2.71−2.65 (m, 3H), 2.49−2.43 (m, 1H), 1.96−1.83 (m, 3H), 1.76−1.71 (m, 2H), 1.23 (d, J = 6.2 Hz, 6H); mass (m/z): 186.3 $(M + H)$.

*1-Isopropyl-1H-indazole-3-carboxylic acid methyl ester (**16**).* To the stirred solution of compound **2** (1.0 g, 4.91 mmol) in methanol (20.0 mL) cooled at 0 °C, thionyl chloride (1.2 mL, 17.22 mmol) was added. The reaction temperature was gradually warmed to r.t., and the reaction was stirred for 6 h. After completion of the reaction, the volatiles were evaporated. The crude mass thus obtained was quenched with aqueous $NaHCO_3$ and diluted with ethyl acetate, and the two layers were separated. The organic layer was washed with brine solution and dried over Na_2SO_4, and the solvent was evaporated under vacuum to afford the title compound **16** in quantitative yield. ^{1}H NMR ($CDCl_3$): δ 8.25 (d, J = 8.1 Hz, l H), 7.53 (d, J = 8.4 Hz, l H), 7.45 (t, J = 7.1 Hz, 1H), 7.33 (t, J = 7.6 Hz, 1H), 5.02−4.93 (m, l H), 4.04 (s, 3H) 1.67 (d, J = 6.7 Hz, 6H); mass (m/z): 219 $(M + H)^+$.

*1-Isopropyl-3-{3-[1-(3-methoxy-propyl)-piperidin-4-yl]-[1,2,4]-oxadiazol-5-yl}-1H-indazole (**17a**).* To a stirred solution of compound **15a** (500.0 mg, 2.32 mmol) in DMF (10.0 mL) at r.t., a DMF (5.0 mL) solution of sodium hydride (116.0 mg, 3.0 mmol) followed by compound **16** (659.0 mg, 3.02 mmol) was added, and the reaction mass was stirred at 120 °C for 16 h. After completion of the reaction, the reaction mass was quenched with ice cold water and extracted with ethyl acetate, and two layers were separated. The organic layer was washed with brine solution and dried over Na_2SO_4, and the solvent was evaporated under vacuum to afford above titled compound **17a** in quantitative yield. ^{1}H NMR ($CDCl_3$): δ 8.33 (d, J = 8.1 Hz, 1H), 7.57 (d, J = 8.5 Hz, 1H), 7.50 (t, J = 7.2 Hz, 1H), 7.38 (t, J = 7.6 Hz, 1H), 5.03−4.97 (m, 1H), 3.47 (t, J = 6.4 Hz, 2H), 3.35 (s, 3H), 3.06−3.03 (m, 2H), 2.88 (s, 1H), 2.43−2.40 (m, 2H), 2.17 (bs, 1H), 2.05 (bs, 2H), 1.85−1.84 (m, 2H), 1.76 (d, J = 6.4 Hz, 6H), 1.68−1.60 (m, 4H) in 17.9% yield with HPLC purity 98.1%. DSC: (RAMP 5 °C/min): 154.7 °C; mass (m/z): 384.1 $(M + H)^+$; $C_{21}H_{29}N_5O_2$; % C, H, N: calculated C; 65.77, H; 7.62, N; 18.26; found C; 65.39, H; 7.33, N; 18.18.

*1-Isopropyl-1H-indazole-3-carboxylic Acid Amide (**18**).* To a stirred solution of compound **2** (1.0 g, 4.91 mmol) in acetonitrile (20.0 mL) at r.t., pyridine (425.9 mg, 5.39 mmol) and Boc anhydride (1.28 g, 5.88 mmol) were added. After stirring for 1 h at r.t., NH_4HCO_3 (627.0 mg, 7.93 mmol) was added to the reaction mixture, and the reaction was stirred for 16 h. The reaction mass was diluted with ethylaceate and water, and the two layers were separated. The organic layer was washed with 1 N HCl followed by 1 N NaOH solution and dried over anhydrous Na_2SO_4, and the solvent was evaporated under vacuum to obtain title compound **18** (830.0 mg) in 83.4% yield. ^{1}H NMR (DMSO-d_6): δ 8.17 (d, J = 8.2 Hz, l H), 7.89 (d, J = 8.6 Hz, 1H), 7.57 (bs, 1H), 7.44 (t, J = 7.4 Hz, 1H), 7.34 (bs, 1 H), 7.24 (t, J = 7.3 Hz, 1H), 5.09−5.03 (m, 1H), 1.53 (d, J = 6.5 Hz, 6H); mass (m/z): 204.0 $(M + H)^+$.

*1-Isopropyl-1H-indazole-3-carbonitrile (**19**).* To a stirred solution of compound **18** (1.0 g, 5.71 mmol) in THF (22.0 mL) at 0 °C, trimethylamine (1.73 g, 17.1 mmol) followed by trifluoroacetic anhydride (1.79 g, 8.55 mmol) were added. The reaction temperature was gradually warmed to r.t., and the reaction was stirred for 2 h. The reaction was quenched by adding ice cold water and ethyl acetate. The two layers were separated and the organic layer was washed with sat. $NaHCO_3$ solution and dried over Na_2SO_4. The solvent was evaporated under vacuum to obtain title compound **19** (933.0 mg) in 86% yield. ^{1}H NMR ($CDCl_3$): δ 7.85 (d, J = 8.1 Hz, l H), 7.57 (d, J = 8.5 Hz, l H), 7.50 (t, J = 7.0 Hz, l H), 7.36 (t, J = 7.5 Hz, l H), 5.09−5.03 (m, l H), 1.64 (d, J = 6.6 Hz, 6H); mass (m/z): 186.1 $(M + H)^+$.

*N-Hydroxy-1-isopropyl-1H-indazole-3-carboxamidine (**20**).* To a stirred solution of compound **19** (770.0 mg, 4.16 mmol) in ethanol (16.0 mL) at r.t., hydroxylamine hydrochloride (603.0 mg, 8.74 mmol) and K_2CO_3 (1.2 g, 8.74 mmol) were added. The reaction mixture was gradually heated to reflux for 16 h. The reaction mass was cooled to r.t. and filtered through Celite, and the filtrate was dried over Na_2SO_4 and evaporated under vacuum to afford title compound **20** (923.6 mg) in quantitative yield. ^{1}H NMR (DMSO-d_6): δ 9.76 (bs, 1H), 8.09 (d, J = 8.1 Hz, 1H), 7.70 (d, J = 8.4 Hz, 1H), 7.40 (t, J = 7.3 Hz, 1H), 7.18 (t, J = 7.5 Hz, 1H), 5.60 (bs, 2H), 5.03- 4.96 (m, 1H), 1.51 (d, J = 6.5 Hz, 6H); mass (m/z): 219.0 $(M + H)^+$.

*1-(3-Methoxypropyl)-piperidine-4-carboxylic Acid Ethyl Ester (**22a**).* By following general procedure 3, ethylisonipecotate **21** was

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alkylated using 1-bromo-3-methoxy propane to obtain compound **22a** as a gummy liquid. ^{1}H NMR ($CDCl_3$): δ 4.12 (q, 2H), 3.41 (t, J = 6.4 Hz, 2H), 2.90−2.85 (m, 2H), 2.38 (t, J = 7.4 Hz, 2H), 2.34−2.20 (m, 1H), 2.05−1.93 (m, 2H), 1.92−1.85 (m, 2H), 1.80−1.70 (m, 2H), 1.23 (t, J = 7.1 Hz, 3H); mass (m/z): 230.2 $(M + H)^+$.

*1-(Tetrahydropyran-4-ylmethyl)-piperidine-4-carboxylic Acid Ethyl Ester (**22b**).* By following general procedure 3, ethyl-isonipecotate **21** was alkylated using 4-bromomethyltetrahydropyran to obtain compound **22b** as a gummy liquid. ^{1}H NMR ($CDCl_3$): δ 4.12 (q, 2H), 3.89 (t, J = 6.1 Hz, 2H), 3.81 (t, J = 6.1 Hz, 2H), 3.20−3.12 (m, 3H), 2.90−2.85 (m, 3H), 2.36−2.26 (m, 1H), 2.11−2.07, (m, 1H), 2.05−1.93 (m, 4H), 1.92−1.85 (m, 2H), 1.80−1.70 (m, 2H), 1.25 (t, J = 6.5 Hz, 3H); mass (m/z): 256.3 $(M + H)^+$.

*1-(Isopropyl)-piperidine-4-carboxylic Acid Ethyl Ester (**22c**).* By following general procedure 3, ethylisonipecotate **21** was alkylated using 2-iodopropane to obtain compound **22c** as a gummy liquid. ^{1}H NMR ($CDCl_3$): δ 4.12 (q, 2H), 4.02−3.98 (m, 1H), 3.20−3.12 (m, 3H), 2.90−2.85 (m, 1H), 2.36−2.26 (m, 1H), 1.92−1.85 (m, 2H), 1.80−1.70 (m, 2H), 1.62 (d, J = 6.7 Hz, 6H), 1.21 (t, J = 6.5 Hz, 3H); mass (m/z): 200.2 $(M + H)^+$.

*1-Isopropyl-3-{5-[1-(3-methoxypropyl)-piperidin-4-yl]-[1,2,4]-oxadiazol-3-yl}-1H-indazole (**23a**).* To a stirred solution of compound **22a** (685.0 mg, 2.9 mmol) in DMF (6.0 mL) at 0 °C, a DMF solution of sodium hydride (119.0 mg, 2.9 mmol) followed by compound **20** (500.0 mg, 2.29 mmol) was added. The reaction temperature was gradually raised to 120 °C for 16 h. The reaction mass was cooled to r.t. and filtered through Celite, the filtrate was dried over Na_2SO_4, and the solvent was evaporated under vacuum to afford title compound **23a** (105.6 mg) in 95% yield. ^{1}H NMR ($CDCl_3$): δ 8.31 (d, J = 8.1 Hz, 1H), 7.55 (d, J = 8.4 Hz, 1H), 7.46 (t, J = 7.1 Hz, 1H), 7.32 (t, J = 7.5 Hz, 1H), 5.01−4.96 (m, 1H), 3.46 (t, J = 6.3 Hz, 2H), 3.35 (s, 3H), 3.01 (bs, 2H), 2.48 (t, J = 7.2 Hz, 2H), 2.20−2.10 (m, 2H), 2.05 (bs, 1H), 1.83−1.76 (m, 2H), 1.69 (d, J = 6.7 Hz, 6H), 1.25−1.23 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 172.4, 170.7, 164.9, 139.9, 128.8, 127.7, 124.1, 122.4, 121.1, 111.5, 69.5, 58.3, 54.2, 51.3, 51.1, 31.3, 27.1, 24.4, 22.4; DSC: (RAMP 5 °C/min): 167.3 °C; mass (m/z): 384.4 $(M + H)^+$; $C_{21}H_{29}N_5O_2$; % C, H, N: calculated C; 65.77, H; 7.62, N; 18.26; found C; 65.93, H; 7.84, N; 18.01.

Animals and Ethics. Adult male Wistar rats (8−12 weeks; 200−300 g; in-house bred) were used in all the studies. Naive animals were used for experimentation. Rats were housed in groups of 3−4 in hanging polycarbonate cages (dimension: length, 43.2 cm × width, 25.4 in. × height, 21.2 cm) with a bedding of clean corn cob. For all the experiments, animals were introduced to the animal house at least 7 days prior to the commencement of the study. The animal house was maintained at 21 ± 3 °C, 30−70% relative humidity and 12 h light/dark cycle (lights on at 7:00 AM and off at 7:00 PM). Food (pelleted rodent feed, Rayan's Biotechnologies Pvt. Ltd. Hyderabad) and water were made available *ad libitum* unless specified otherwise for the experiment. The test compound was administered orally, and scopolamine was administered through the intraperitoneal route. For the object recognition task, each group had 12 rats, and in the radial arm maze, each group had 6 rats. All experimental procedures were performed in accordance with the Institutional Animal Ethics Committee of Suven Life Sciences Ltd., Hyderabad, constituted as per the directions of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Determination of EC_{50} Values for 5-HT_4R. The compounds were screened in the cell-based luciferase reporter gene luminescence assay which measures the levels of cAMP inside cells upon activation or inhibition of the receptor. Serotonin (5-HT) and luciferin was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). T4 DNA ligase and high fidelity Taq polymerase were procured from Roche (Basel, Switzerland). Superscript reverse transcriptase and mammalian expression vector pcDNA3.1 were purchased from Invitrogen (Carlsbad, California, USA). CRE-Luc reporter gene construct was supplied by Strata gene (La Jolla, California, USA). Cell culture media and sera were procured from Invitrogen (Carlsbad, California, USA). All other DNA restriction and modification enzymes were from New England Biolabs (Ipswich, Massachusetts, USA). All other reagents and common chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Human 5-HT_4R cDNA clone was purchased from Origene (Rockville, Maryland, USA). The coding sequence was amplified by PCR using gene specific primers. Amplified DNA was cloned in to mammalian expression vector pcDNA 3.1. The authenticity of the cloned genes was determined by restriction analysis and nucleotide sequencing. CHO cell line was purchased from American Type Culture Collection, Manassas, Virginia, USA. The cells were routinely cultured in Hams F12 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. The cells were transfected with 5-HT_4R isoforms along with 3-fold excess of CRE-Luc reporter gene using lipofectamine as recommended by the supplier. Cells were further cultured in the same medium supplemented with 800 μg/mL G418. Individual colonies were picked up after 2 weeks of selection and screened for serotonin-induced luciferase activity. Colonies exhibiting maximum serotonin-induced luciferase activity were selected and amplified for further investigations. The recombinant CHO cells were plated in 96-well clear bottom white plates (Corning) and cultured overnight as described above. Cells were grown overnight in serum-free medium before incubation with compounds to prevent receptor desensitization due to presence of endogenous ligands in the serum.

The reference endogenous agonist serotonin and test compounds in Opti-MEM medium at 11 different test concentrations, starting from 10 μM until 0.1 nM in 3-fold serial dilutions, were incubated with the cells separately in individual wells for 4 h at 37 °C in 5% CO_2. Compounds whose potency was <1 nM were tested in a dose−response study with concentrations starting from 1 μM to 0.01 nM. After incubation, the medium was removed, and the cells were washed with phosphate buffered saline and lysed in lysis buffer (Tris, 10 mM (pH-8.0); NaCl, 50 mM; DTT, 1 mM; protease inhibitor cocktail 1×, NP 40−0.1%). The luciferase activity was measured in individual wells using luciferin substrate in a Victor Light Luminometer, PerkinElmer.

The maximum response produced by each drug was normalized to the 5-HT-induced maximum response. Data were analyzed using Graphpad prism software to derive EC_{50} values that corresponded to the concentration of agonists required to obtain half-maximal stimulation of adenylyl cyclase. Values were expressed as the mean of two independent experiments performed in duplicates. Reference compound PF-04995274, a reported partial agonist[9], showed partial agonist activity in our 5-HT_{4E} cell-based reporter gene assay with an EC_{50} value of 1.3 nM and E_{max} 18%, which was very well in agreement with published data of EC_{50}/E_{max}: 0.26 nM/7%. Some of other reported compounds EC_{50} values were also determined using our assay and compared with the published data. The EC_{50} values obtained versus reported values are tabulated in the Supporting Information of this manuscript.

Microsomal Metabolic Stability. Compounds were incubated with microsomes at 0.5 mg protein/mL in 100 mM phosphate buffer pH 7.4 at 37 °C. Reactions were initiated by addition of NADPH. All incubations were carried out for 30 min. At 30 min, an aliquot of the sample was removed and transferred into 300 μL of acetonitrile. The samples were stored at −20 °C until analysis. Samples were analyzed using LC-MS/MS. Results were expressed as % metabolized at 30 min postincubation.

Protein Binding. Unbound fractions of test compound in plasma, brain homogenate, and liver microsomes were determined using high-throughput dialysis (HT dialysis). Briefly, dialysis membranes were soaked in deionized water for 20 min, then in deionized water with 30% ethanol for 15 min, and finally in phosphate buffer until use. Membranes were rinsed in phosphate buffer before assembling. The membranes were layered between Teflon bars of dialysis assembly. Stock solution of test compound was prepared at 10 mM in DMSO, diluted to 1 mM in acetonitrile, and further diluted to 100 μM in mixture of water and acetonitrile (1:1 v/v). Rat blood and brains were isolated on the day of the study, and brains were homogenized with two volumes of phosphate buffer. The blood was centrifuged at 4000 rpm for 10 min to obtain plasma. Human plasma (pool of 3) was

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prepared from human blood (3 donors) by centrifuging at 4000 rpm for 10 min at 4 °C. Suspension of liver microsomes was prepared at 0.5 in phosphate buffer (100 mM, pH 7.4). The dialysate chambers were loaded with 150 μL of 100 mM phosphate buffer (pH 7.4) in triplicates. The matrix chambers were loaded with 150 μL of the plasma or brain homogenate or microsomal suspension spiked with test compound at a final concentration of 1 μM. 50 μL of the sample was removed from both of the chambers at 0 h. The plate was sealed and incubated at 37 °C for 6 h at 100 rpm. After 6 h, 50 μL of the sample was removed from both the chambers. Equal volumes of buffer or matrix were added to the matrix and buffer samples, respectively, to create identical sample matrices for analysis. The supernatants were transferred to vials and were analyzed by LC-MS/MS.

Pharmacokinetic Study in Rats. Male Wistar rats (225 ± 25 g) were used for evaluation of pharmacokinetics. Three animals were housed in each cage. Two days prior to dosing day, male Wistar rats were anesthetized with isoflurane for surgical placement of jugular vein catheter. Animals were fasted overnight before oral dosing (p.o.), and food pellets were allowed 2 h postdosing, whereas during intravenous dosing food and water were provided as *ad libitum*. Three rats were dosed with test compound orally (3 mg/kg) or intravenously (i.v., 1 mg/kg). Dose formulation was prepared by using water as a vehicle (10 mL/kg for oral and 2 mL/kg for intravenous dosing). At each time point, 200 μL of blood was collected through jugular vein cannula and immediately replenished with an equivalent volume of normal saline in freely moving rats. Collected blood was transferred into a labeled eppendorf tube containing 10 μL of heparin as anticoagulant. The time points for blood collection were predose and 0.08 (only i.v.), 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h postdose (n = 3). The collected blood was centrifuged at 4000 rpm for 10 min at 4 °C. Plasma was separated in prelabeled tubes and stored below −70 °C until analysis. The test compound concentrations were extracted from plasma samples by protein precipitation using four volumes of acetonitrile (v/v) containing predetermined internal standard. The precipitants were centrifuged (at 11292×g and 10 °C for 10 min), and supernatant was transferred and injected in LC-MS/MS. The analytes of interest were quantified using a suitable multiple reaction monitoring mode in the range of 2−2000 ng/mL or ng/g against a set of calibration standards and quality control samples. Study samples were analyzed using calibration samples in the batch and quality control samples spread across the batch. Pharmacokinetic parameters were determined by using standard noncompartmental analysis (Phoenix WinNonLin 6.2 version or higher software) using linear trapezoidal with linear interpolation method.

Rodent Brain Penetration Study. Male Wistar rats (225 ± 25 g) were used as experimental animals. Three animals were housed in each cage. Animals were given water and food *ad libitum* throughout the experiment and maintained on a 12 h light/dark cycle. Brain penetration was determined in a discrete manner in the rats. The compounds were formulated in water and administered orally at 10 mg/kg (free base equivalent). Blood samples were removed via cardiac puncture under isoflurane anesthesia at 1 h postdose. The animals were sacrificed to collect the brain tissue. Plasma was separated, and brain samples were homogenized and stored at −70 °C until analysis. The concentrations of the compound in plasma and brain were determined using a LC-MS/MS method. The test compound was extracted from brain homogenate by protein precipitation using four volumes of acetonitrile (v/v) containing predetermined internal standard. The precipitants were centrifuged (at 11292g at 10 °C for 10 min), and the supernatant was transferred and injected in LC-MS/MS. The compounds were quantified in the calibration range of 1−2000 ng/mL in plasma and brain homogenate. Study samples were analyzed using calibration samples in the batch and quality control samples spread across the batch. The extent of brain to plasma ratio was calculated (C_{brain}/C_{plasma}).

***In Vivo* PK Profile in Male Beagle Dogs.** A single dose, crossover study design with 7 days washout period between the treatments was used to administer compound **12l** to three male beagle dogs. All dogs were fasted overnight, food was offered at regular time intervals for an intravenous route of administration, and food was offered after 4 h of postoral dose. On the day of dosing (day 0), each dog was administered intravenously as 5 min infusion at 1 mg/kg and oral administration as gavage at 3 mg/kg on day 7. Dose formulations were prepared on the day of treatment. At each time point, 0.5 mL of blood sample was collected from saphenous/cephalic vein at designated time points, and collected blood samples were transferred to prelabeled sodium heparin-coated sampling tubes. The time points for blood collection from each animal were predose and 0.08 (only i.v.), 0.16 (only i.v.), 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h post dose (n = 3). The collected blood samples were centrifuged at 4000 rpm for 10 min at 4 °C within 30 min of collection, and plasma was separated in prelabeled tubes and stored at −70 °C until analysis. Compound **12l** concentrations in plasma samples were determined using a qualified LC-MS/MS method with ACN protein precipitation technique and quantified in the calibration range of 1.013−1013 ng/mL. The PK evaluation was performed using noncompartmental analysis (WinNonLin version 5.0.3 Pharsight Corporation, Mountain View, California 94040/USA) and using the linear trapezoidal with linear interpolation calculation method.

CYP 3A4 and 2D6 Inhibition.[50] CYP 3A4 and 2D6 inhibitory potential of the test compounds was studied using human liver microsomes. Inhibitory activity was evaluated by incubating the test compound (0.1−45 μM) in duplicates for 2 (3A4) and 12 min (2D6) with human liver microsomes in the presence of isoform-specific substrate (midazolam-3A4 or dextromethorphan-2D6) and NADPH regeneration system. Incubations were terminated by adding ice-cold acetonitrile containing an internal standard, and metabolites were quantified using LC-MS/MS. Peak area ratios of analyte versus internal standards were used for calculating IC_{50}.

hERG Patch Clamp Assay IC_{50} Determination.[52] The HEK293-hERG cells for patch clamp assay were cultured in Dulbecco's modified Eagle's medium (DMEM):Ham's F12 (1:1 ratio) with 10% FBS containing 0.5 mg/mL of selective antibiotic, G-418 sulfate (Calbiochem), and 0.1% penicillin and streptomycin (Thermofisher, MA, USA) in a T-75 flask. Cells were harvested at 70−80% confluency by washing with HBSS lacking Mg^{2+} and Ca^{2+} ions and subsequent addition of accumax (2.5 mL) (Sigma-Aldrich, MO, USA) and incubated in the flask at 5% CO_2 and 37 °C until cells start sliding on the surface. After cells have completely detached from the surface, about 10 mL of complete media without selective antibiotic was added and pipetted to detach the cell clumps. Additional 7.5 mL of complete media was added to collect the residual cells in the flask, and cell suspension was incubated at 5% CO_2 and 37 °C for 30 min. Centrifuged at 200g for 2 min, the supernatant was discarded, and the cell pellet was resuspended in external solution which was used for the patch clamp assay. hERG patch clamp assay was performed using the external solution of 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM D-glucose monohydrate, 10 mM HEPES/NaOH, pH 7.4, osmolarity: 298 mOsmol and internal solution of 50 mM KCl, 10 mM NaCl, 60 mM KF, 20 mM EGTA, 10 mM HEPES/KOH, pH 7.2, osmolarity 285 mOsmol. Whole cell patch clamp recordings were performed by using the $I-V$ protocol of holding HEK293-hERG cells at −80 mV and stepping to +40 mV for 500 ms and followed by 500 ms step to −40 mV before stepping back to the holding potential. The peak current is measured at −40 mV tail currents using the Patchliner instrument. The $I-V$ pulse protocol was pulsed every 10 s, online analyses were recorded by substituting the leak current from the peak current, and IC_{50} values were calculated from IGOR PRO software (WaveMetrics, Inc. Portland, OR, USA).

Determination of *In Vivo* Receptor Occupancy. Male Wistar rats (n = 4−6/group) were administered with vehicle or test compounds (compound **12l** (0.1, 0.3, 1, 3, 10, and 30 mg/kg, p.o.), PF-04995274 (0.003, 0.01, 0.03, 0.1, 0.3, 1, and 3 mg/kg, s.c.) or RS-67333 (1, 3, 10, 20, and 30 mg/kg, p.o.). After 1 h of treatment with test compounds or vehicle, rats were restrained and administered with tracer (SB207145, 1 μg/kg) intravenously. After 30 min of tracer administration, rats were sacrificed, and the brain was separated for isolation of cerebellum (nonspecific region) and striatum (specific

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region). Isolated brain tissues were transferred to prelabeled tubes and stored on dry ice until quantification of tracer concentrations using LC-MS/MS. Blood and remaining brain were also collected from the rats treated with compound **12l** for measurement of test compound concentrations. Percentage R.O. of test compound was calculated using the concentrations of tracer in the striatum and cerebellum.

Object Recognition Task. Male Wistar rats 10−12 weeks old were used. The arena was an open field made up of acrylic (50 × 50 × 50 cm). Twenty-4 h prior to testing, the rats were habituated to individual test arenas for 20 min in the absence of any objects. Twenty-4 h after the habituation, rats (12 animals per group) were administered respective treatments. After the post-treatment interval of 30 min, rats were subjected to familiarization phase (T1). Rats were placed individually in the open field for 3 min, containing two identical objects (a1 and a2). Choice trial (T2) was carried out after 24 h after the T1 trial. Rats were administered respective treatments. After the post treatment interval of 30 min rats were subjected to choice trial (T2). Rats were allowed to explore the open field for 3 min in the presence of one familiar object (a3) and one novel object (b). Exploration time was noted. Recognition index was calculated as time spent with the novel object and time spent with the familiar object in the choice trial. Rats which did not explore the familiar object for ≥15 s in the familiarization trial and ≥10 s in the recognition trial were not considered for statistical evaluation. The object was such that it could not be moved from its place. The familiar object was a bright yellow colored bottle of dimension 12 × 4 cm, while the novel object was an ambered colored bottle of dimension 11 × 3.5 cm. After each of the familiarization or recognition trials, the bottles were cleaned with 20% alcohol to mask olfactory cues.

Radial Arm Maze. Rats were fasted until they reached 85% of their body weight and then trained to eat pelleted food in the home cage. On days 1 and 2, the rats were habituated in the radial arm maze to find and eat the pellets. On the subsequent 4 days, the rats had to find and eat the pellets which were exclusively placed in the hopper of the arm. Each hopper was baited once. Re-entry into an arm from which the food was retrieved or exited from an arm without eating the pellet was considered as an error. % Choice accuracy was calculated as

$$\%\text{choice accuracy} = 100 - \{(\text{TE}/\text{TA}) \times 100\}$$

where TE is the total error and TA is the total number of choices made. A rat could make a total of 16 arm visits. The trial was ended when all the pellets were retrieved or 16 arms were visited or a time of 10 min had elapsed. Total error was calculated as sum of number of repeat entries into an arm after food retrieval + number of arms omitted + exit from an arm without food retrieval. Following each trial, the radial maze was cleaned with soap solution to mask olfactory cues.

Estimation of sAPPα Levels Secreted by Cell Line. The measurement of sAPPα by Western blot was performed according to Fei Shen[48] with slight modifications. 5-HT$_4$R agonist-evoked stimulation of sAPPα secretion *in vitro* was monitored using tsa201 cells transiently transfected with h5-HT$_{4E}$ receptor and human APP695. The amount of sAPPα released into the culture medium was determined using a semiquantitative Western blot assay with an antibody purified anti-β-amyloid, 1−16 monoclonal antibody (clone 6E10) to amino acid residues 1−17 of amyloid beta (Aβ) in the C terminus of sAPPα.

Cell culture medium, serum, and transfection reagent were purchased from Invitrogen (Carlsbad, California, USA). The human kidney cell line tsa201 was purchased from ECACC Sigma-Aldrich (St. Louis, Missouri, USA). Primary antibody clone 6E10 was purchased from Covance (Princeton, New Jersey, USA). Secondary antibody antimouse IgG-alkaline phosphatase, detection reagent, protease inhibitor, and chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Cell Culture and Transient Transfection. The human kidney cell line tsa201 was grown at 37 °C and 5% CO_2 in DMEM supplemented with 10% FBS, 2 mM glutamine, and antibiotics. For transient transfection experiments, tsa201 cells were transfected with expression constructs encoding the human APP695 gene and 5-HT$_{4E}$ gene using lipofectamine 2000 according to the manufacturer's instructions. Briefly, 50−60% confluent cell cultures were incubated for 4 h in a mixture of a cationic transfection reagent and expression constructs. At the end of the incubation period, media were changed, and cells were cultured for an additional period of 40 h before the determination of sAPPα.

Measurement of sAPPα by Western Blot. Cells were cultured as described above and 40 h prior to assay were seeded into 6-well poly-D-lysine-coated multiwell dishes and incubated in a humidified 5% CO_2 incubator at 37 °C. Cells were grown to approximately 90% confluency and, following aspiration of the growth medium, incubated in serum-free DMEM for 4 h. Cells then were washed with phosphate-buffered saline prior to incubation with test compound (0.001−10 μM in DMEM) for 30 min at 37 °C. For antagonist study, the cells were preincubated for 15 min and then treated with 5-HT$_4$R agonist for 30 min. A buffer control and 5-HT (1 μM) were included as controls on every plate. For each condition, determinations were made in duplicate. At the end of the assay incubation period, an aliquot of extracellular medium was transferred from the respective wells into a tube containing protease inhibitor cocktail. The sample was centrifuged, and the supernatant collected into a new tube and stored at −80 °C prior to assay by Western blot. Samples were separated on SDS-PAGE 10% and transferred to PVDF membrane from Millipore (Burlington, Massachusetts, USA) in a mini trans blot cell apparatus from Bio-Rad (Hercules, California, USA) with transfer conditions of 10−15 V for overnight at 2−8 °C using transfer buffer (25 mM Tris, 192 mM glycine and 10% methanol). Membrane was preactivated in 100% methanol for 20 s and equilibrated in transfer buffer for 5 min before subjecting for transfer. After transfer, membrane was washed for 5 min in Tris buffered saline (TBS). The membrane was blocked in blocking buffer (TBS containing 0.1% Tween 20 and 5% nonfat dry milk) for 1 h at r.t. After blocking, the membrane was incubated with primary antibody purified anti-β-amyloid, 1−16 monoclonal antibody (clone 6E10) raised in a mouse for overnight at 2−8 °C. Membrane was washed three times each for 5 min in wash buffer (TBS containing 0.1% Tween 20). Membrane was incubated with secondary antibody antimouse IgG-alkaline phosphatase raised in a goat for 1 h at r.t. Membrane was washed three times each for 5 min in wash buffer (TBS containing 0.1% Tween 20). Membrane was developed with BCIP/NBT detection reagent, and images were captured in GelDoc imaging System (Bio-Rad). Electrophoresis molecular weight markers were used to verify the size of the protein, that is, sAPPα 98 kDa. Images were analyzed using ImageJ software (NIH).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00703.

Characterization data of final compounds, comparative *in vitro* affinities of 5-HT$_4$R reference standards, data from functional assays, selectivity profile and elemental analysis (PDF)

Additional data (PDF)

Molecular formula strings (CSV)

Additional data (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AD, Alzheimer's disease; SAR, structure−activity relationship; CNS, central nervous system; 5-HT_4R, 5-hydroxytryptamine 4 receptor; p.o., per oral; s.c., subcutaneous; i.v., intravenous; min, minute; mm, millimeter; rpm, rotations per minute; cps, counts per second; mp, melting point; DSC, differential scanning calorimetry; hERG, human ether-à-go-go-related gene; sAPPα, soluble amyloid precursor protein α; R.O., receptor occupancy; LC-MS/MS, liquid chromatography−mass spectrometry; HPLC, high-performance liquid chromatography; Ach, acetylcholine; EC_{50}, concentration giving a 50% response; ED_{50}, dose giving a 50% response; E_{max}, maximal effect; GPCR, G-protein-coupled receptor; HEK, human embryonic kidney; HLM, human liver microsome; RLM, rat liver microsome; SEM, standard error of the mean; cAMP, cyclic adenosine monophosphate; CHO, Chinese hamster ovary; THP, tetrahydropyran; DCM, dichloromethane; TLC, thin-layer chromatography; IPA, isopropanol; EtOAc, ethyl acetate; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate; HATU, 1-[bis(dimethylamino)-methylene]-1H-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; TFA, trifluoroacetic acid; TFAA, trifluoroacetic anhydride; DMAP, *N*,*N*-dimethylaminopyridine; ICH, International Council for Harmonization; OECD, Organization for Economic Cooperation and Development

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