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Synthesis and Pharmacological Evaluation of [^{3}H]HS665, a Novel, Highly Selective Radioligand for the Kappa Opioid Receptor

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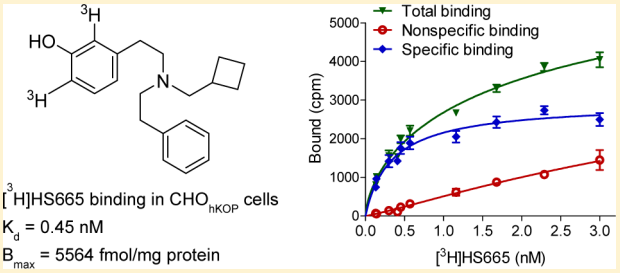
ABSTRACT: Herein we report the radiolabeling and pharmacological investigation of a novel radioligand, the *N*-cyclobutylmethyl substituted diphenethylamine [^{3}H]HS665, designed to bind selectively to the kappa opioid peptide (KOP) receptor, a target of therapeutic interest for the treatment of a variety of human disorders (i.e., pain, affective disorders, drug addiction, and psychotic disorders). HS665 was prepared in tritium-labeled form by a dehalotritiated method resulting in a specific activity of 30.65 Ci/mmol. Radioligand binding studies were performed to establish binding properties of [^{3}H]HS665 to the recombinant human KOP receptor in membranes from Chinese hamster ovary cells stably expressing human KOP receptors (CHO_{hKOP}) and to the native neuronal KOP receptor in guinea pig brain membranes. Binding of [^{3}H]HS665 was specific and saturable in both tissue preparations. A single population of high affinity binding sites was labeled by [^{3}H]HS665 in membranes from CHO_{hKOP} cells and guinea pig brain with similar equilibrium dissociation constants, K_d, 0.45 and 0.64 nM, respectively. Average receptor density of [^{3}H]HS665 recognition sites were 5564 and 154 fmol/mg protein in CHO_{hKOP} cells and guinea pig brain, respectively. This study shows that the new radioligand distinguishes and labels KOP receptors specifically in neuronal and cellular systems expressing KOP receptors, making this molecule a valuable tool in probing structural and functional mechanisms governing ligand−KOP receptor interactions in both a recombinant and native in vitro setting.



KEYWORDS: *κ opioid receptor, κ opioid ligand, radiolabeling, receptor binding assay, binding affinity, selectivity*

The opioid receptor family consists of three structurally homologous but functionally distinct receptors, the mu opioid peptide (MOP), delta opioid peptide (DOP), and kappa opioid peptide (KOP).[1] They are all seven-transmembrane G protein-coupled receptors (GPCRs), and are widely distributed throughout the central nervous system and the periphery. Intense interest in the pharmacology of the KOP receptor over the past years has revealed its important role in pathways related to pain, affective disorders, drug addiction, and psychotic disorders.[2−10] The KOP receptor is well-known for contribution in mediation of pain, and pain remains a likely indication for KOP agonists, as stimulation of this receptor produces analgesia, while it causes neither physical dependence nor respiratory failure.[4] However, KOP receptor activation has also been implicated in inducing neuropsychiatric effects including sedation and dysphoria.[4,5] Nowadays, the KOP receptor is emerging as an important target for the treatment of a variety of other human disorders. Drugs directed at the KOP receptor as antagonists or partial agonists have potential utility as antidepressants and anxiolytics.[2,6,7,10] Additionally, KOP agonists are gaining attention as potential antiaddiction medications,[2,7,9] and for the treatment of inflammatory[11] and itching skin diseases.[12]

In addition to the evolving pharmacological studies on the KOP receptor, structure elucidation of the KOP receptor at high-resolution by X-ray crystallography has been revealed,[13] along with crystal structures of the other members in the opioid receptor family.[14,15] The present understanding of the KOP receptor function is persistently increasing with the crystal structure now available, which enables to investigate activation mechanisms and ligand−receptor interactions, to develop structure−affinity and structure−function relationships, and ultimately to give significant insights for the design of ligands with improved pharmacological properties targeting the KOP receptor.[16−21]

Several ligands, both small molecules and peptides, interacting with the KOP receptor have been developed over the years.[2,3,6,7,22,23] The major classes of KOP agonists comprise benzomorphans (e.g., bremazocine, pentazocine), morphinans (e.g., nalfurafine), arylacetamides (e.g., U50,488, U69,593), neoclerodane diterpenes (e.g., salvinorin A and analogues), and peptides (e.g., dynorphin analogues). Some notable examples of selective KOP receptor antagonists include

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the morphinans nor-binaltorphimine (nor-BNI)[24] and 5′-guanidinonaltrindole (GNTI),[25] and the structurally distinct molecule JDTic, a *trans*-3,4-dimethyl-4-(3-hydroxyphenyl)-piperidine derivative,[26] the latter was used to elucidate the crystal structure of the human KOP receptor.[13] In contrast to the aforementioned KOP antagonists (nor-BNI, GNTI and JDTic) known to exhibit long-lasting pharmacokinetic properties,[6,7,22] shorter-acting and selective KOP antagonists were developed, such as the new JDTic analogue BU09059[27] and the pyrrolidine derivative LY-2456302,[28] the latter one is currently under development as an augmentation to antidepressant therapy for treatment-resistant depression.[28,29] In addition to dynorphin A analogues, arodyn (Ac[Phe^{1,2,3},Arg^4,D-Ala^8]-dynorphin A-(1-11) amide),[30] zyklophin ([*N*-benzyl-tyr^1,*cyclo*(D-Asp^5,Dap^8)]dynorphin A(1-11)-NH$_2$),[31] and other peptides unrelated to the endogenous opioid peptide were recognized as selective KOP receptor antagonists, for example, the macrocyclic peptide *cyclo*[Phe-D-Pro-Phe-Trp] (CJ-15,208) and derivatives.[32,33] Additionally, KOP receptors located in the periphery were targeted in order to avoid the centrally mediated side effects, and the evolving peripherally selective KOP agonists were reported to have therapeutic potential for the treatment of pain and inflammatory diseases.[2,3,34−38] The KOP peripherally acting tetrapeptide FE200665 (CR665; H-D-Phe-D-Phe-D-Nle-D-Arg-NH-4-Picolyl) has shown benefit in patients with visceral pain.[37] Asimadoline, an arylacetamide derivative agonist with preference for the KOP receptor in the periphery, was efficacious in patients with irritable bowel syndrome.[35] 5-Pyrrolidinyl substituted perhydroquinoxalines were designed as conformationally restricted KOP receptor agonists with limited blood-brain barrier penetration demonstrating analgesic effects in experimental models of visceral pain.[38]

The identification of novel scaffolds which selectively target the KOP receptor could have a major impact on the treatment of several neurological disorders, as well as the use as essential pharmacological tools for the study of this receptor system. We have recently described the design, synthesis, and biological characterization of ligands interacting with the KOP receptor from the class of diphenethylamines.[39] Within the reported series, the *N*-cyclobutylmethyl substituted derivative HS665 (Figure 1) represents a new molecule with a structurally

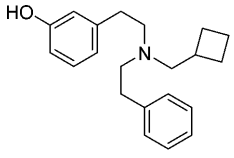


Figure 1. Structure of HS665.

distinct scaffold compared to the so far known KOP ligands, and a highly selective KOP receptor agonist with potent antinociceptive activity.[39] In our previous study,[39] we have presented different procedures for the preparation of HS665 that are efficient, simple, and cost-effective, while the synthesis of the commonly used KOP agonists U50,488 and particularly U69,593 are rather complicated and laborious.[40,41] This fact, accompanied by the structural simplicity, is a notable advantage of HS665 over U50,488 and U69,593. We have shown that HS665 binds to the human KOP receptor with high affinity (K_i value of 0.49 nM), paralleled by high KOP selectivity versus MOP and DOP receptors (>1000 and >20 000, respectively).[39] Although HS665 displays a comparable binding profile to the prototypical KOP agonists U50,488 and U69,593, in vitro functional studies demonstrated an increase in KOP agonist potency by about 3- and 7-fold versus U50,488 and U69,593, respectively, complemented by full agonism at the human KOP receptor of HS665.[39] In vivo, subcutaneous administration of HS665 induces significant analgesic effects in mice in the abdominal stretching model with a potency equivalent to that U50,488, proven to be mediated through selective activation of KOP receptors.[39]

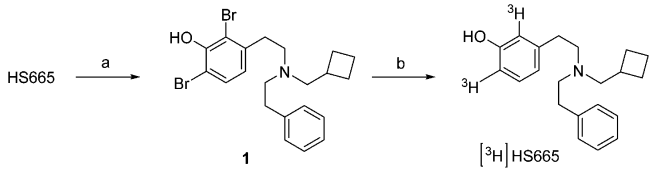
The favorable properties of the new selective KOP agonist HS665 warrant its application in further pharmacological investigations, and this can be facilitated by the availability of the ligand in a radioactive form. The present study describes the radiolabeling and pharmacological investigation of [^{3}H]HS665 to the recombinant human KOP receptor in membranes from Chinese hamster ovary cells stably expressing hKOP receptors (CHO_{hKOP}) and to the native neuronal KOP receptor in guinea pig brain membrane preparations using in vitro binding assays. We show that the new radioligand is binding selectively to the human and rodent KOP receptor, and thereby it may be of significant value in probing functional mechanisms governing ligand-KOP receptor interactions.

RESULTS AND DISCUSSION

The previously reported structure−activity relationship studies performed on differently substituted diphenethylamines[39] guided our selection of the *N*-cyclobutylmethyl substituted analogue HS665 for labeling with tritium and pharmacological evaluation as a potential new radioligand for the KOP receptor, based on its high affinity and excellent selectivity toward the KOP receptor. Through combination of radiochemical and biological approaches, our present work provides a new valuable molecular tool to investigate the neurobiology of KOP receptors that may have important implications for the future development of novel therapeutics targeting KOP receptors.

Chemistry. For preparation of the tritium labeled form of HS665, the compound was first brominated using *N*-bromosuccinimide in dichloromethane to afford the dibrominated analogue **1**. Dehalotritiation of **1** was performed essentially as described earlier,[42,43] and yielded [^{3}H]HS665 (Scheme 1) with a specific activity of 30.65 Ci/mmol.

Scheme 1. Synthesis of [^{3}H]HS665[a]



[a]Reagents and conditions: (a) DIPA, NBS, CH_2Cl_2, RT; (b) TEA, PdO/$BaSO_4$ catalyst, tritium gas, DMF, RT.

Pharmacology. Specific binding properties of the new radioligand [^{3}H]HS665 to the human KOP receptor were first determined in various binding assays using membrane preparations from CHO cells expressing human KOP receptors (CHO_{hKOP}). Receptor binding assays were performed according to the previously described procedures.[39,44] Association binding experiments revealed specific binding of [^{3}H]HS665 to CHO_{hKOP} cell membranes upon incubation at 0 °C, with a

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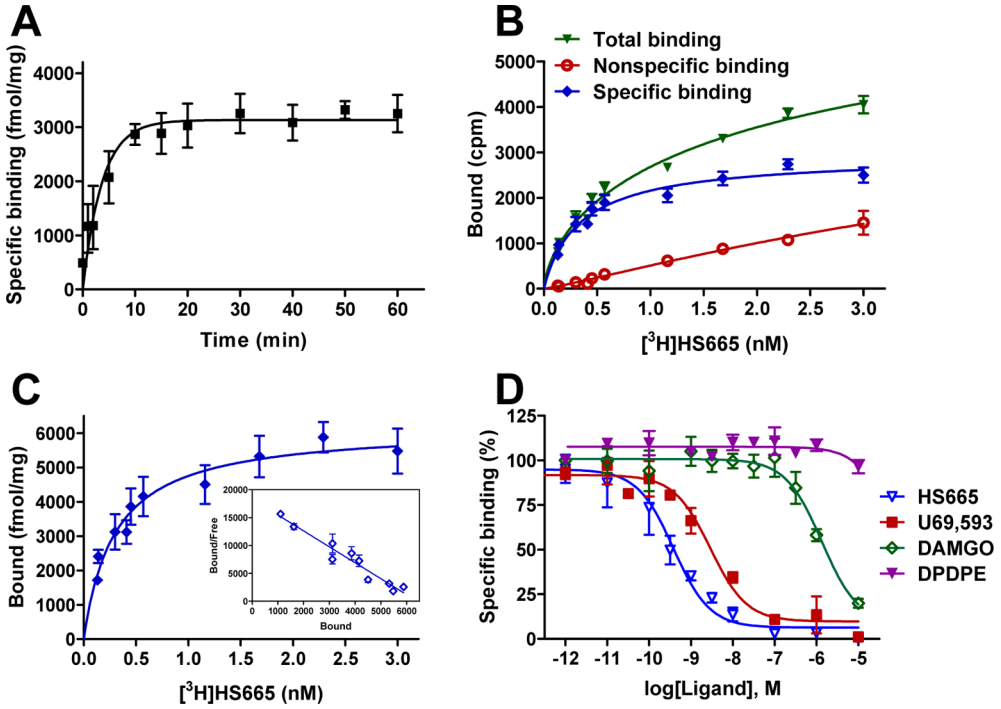

Figure 2. Binding properties of [^{3}H]HS665 to membranes from CHO_{hKOP} cell membranes. (A) Association time course of [^{3}H]HS665 (0.4 nM) at 0 °C for the indicated times. (B) Saturation binding of [^{3}H]HS665 at 0 °C and 30 min incubation; isotherms for total, nonspecific, and specific binding of increasing concentrations of [^{3}H]HS665. (C) Concentration-dependence of bound [^{3}H]HS665; inset: Scatchard plot, where "Bound" is specific binding in fmol/mg protein and "Free" is the free radioligand concentration in nanomoles. (D) Competitive displacement of [^{3}H]HS665 (0.4 nM) binding by opioid ligands at 0 °C and 30 min incubation. In all binding assays, 15 μg of CHO_{hKOP} cell membranes was incubated with [^{3}H]HS665. Nonspecific binding was determined in the presence of 10 μM unlabeled HS665. Data are means ± SEM (n = 3). Nonvisible SEM is within the symbol. Binding data were analyzed with the GraphPad Prism software.

steady-state reached after 15 min (Figure 2A), that remained stable up to 90 min, the longest time examined (not shown). Incubation of [^{3}H]HS665 with cell membranes at room temperature or higher temperatures resulted in an extremely rapid association within less than 5 min. Therefore, all subsequent assays were carried out at 0 °C and 30 min incubation time.

Saturation binding studies were performed with increasing radioligand concentrations for 30 min. As shown in Figure 2B, the specific binding of [^{3}H]HS665 to CHO_{hKOP} cell membranes defined as the difference of the total and nonspecific binding was saturable and of high affinity. Scatchard transformation of the equilibrium binding data is illustrated in Figure 2C. The presence of a homogeneous set of specific binding sites was suggested by the linear Scatchard plot and the Hill coefficient value close to the unity (n_H = 0.94 ± 0.04). The equilibrium binding parameters were calculated (Table 1), where the equilibrium dissociation constant, K_d, was 0.45 nM, and the maximal number of binding sites, B_{max}, labeled by [^{3}H]HS665 in CHO_{hKOP} cells was 5564 fmol/mg protein. Binding affinity and capacity values were also assessed by equilibrium homologous competition experiments using [^{3}H]HS665 at constant concentration and increasing concentrations of unlabeled HS665 as competing ligand (Figure 2D). Nonlinear regression analysis of the sigmoid competition curves indicated the presence of one binding site in CHO_{hKOP} cell membrane preparations. Accordingly, the average Hill slope of the curves was not far from the unity (n_H = 0.94). In homologues displacement studies an affinity (K_d = K_i) of 0.40 ± 0.05 nM and receptor density (B_{max}) of 4839 ± 752 fmol/mg protein were calculated. Notably, the nonspecific binding of

Table 1. Equilibrium Binding Parameters of [^{3}H]HS665[a]

	CHO_{hKOP} cells	guinea pig brain
[^{3}H]HS665		
K_d (nM)	0.45 ± 0.04	0.64 ± 0.16
B_{max} (fmol/mg protein)	5564 ± 473	145 ± 3
n_H	0.94 ± 0.04	1.06 ± 0.03
[^{3}H]U69,593		
K_d (nM)	1.47 ± 0.23	0.90 ± 0.16
B_{max} (fmol/mg protein)	5674 ± 473	123 ± 12
n_H	1.04 ± 0.06	0.94 ± 0.02

[a]Affinity (K_d), receptor density (B_{max}), and Hill coefficient (n_H) values were calculated from saturation binding experiments with [^{3}H]HS665 in membranes from CHO_{hKOP} cells expressing human KOP receptors or guinea pig brain. Binding parameters of the well-known KOP radioligand [^{3}H]U69,593 are shown for comparison. Data are means ± SEM.

[^{3}H]HS665 to membranes from CHO_{hKOP} cells represented less than 10% of total binding at a concentration equal to the K_d value (Figure 2B).

For comparison to the new radioligand [^{3}H]HS665, we also present binding parameters of the well-known KOP radioligand [^{3}H]U69,593 to CHO_{hKOP} cells, and data are summarized in Table 1. Specific binding and a single population of high affinity binding sites was labeled by [^{3}H]U69,593 in CHO_{hKOP} cell membranes, with a K_d value of 1.47 nM, and receptor density (B_{max}) of 5674 fmol/mg protein. When compared to the generally used KOP radioligand [^{3}H]U69,593, [^{3}H]HS665 exhibits about 3-fold increase in binding affinity (K_d = 0.45 nM) to the human KOP receptor, while recognizing a similar

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number of KOP binding sites (B_{max} = 5564 fmol/mg protein) in CHO_{hKOP} cells (Table 1).

Competition binding assays were carried out to evaluate the abilities of several opioid ligands to inhibit binding of [^{3}H]HS665 to membranes from CHO_{hKOP} cells. The specificity of [^{3}H]HS665 binding was measured with increasing concentrations of various unlabeled site-specific opioid ligands, including the general opioid receptor antagonist naloxone, the selective KOP agonists U50,488 and U69,593, the selective KOP antagonist nor-BNI, the MOP receptor selective peptide agonist [D-Ala^2,NMePhe^4,Gly^5-ol]enkephalin (DAMGO) and the selective DOP peptide agonist [D-Pen^2,D-Pen^5]enkephalin (DPDPE) (Table 2). It was observed that the investigated ligands displaced [^{3}H]HS665 binding from CHO_{hKOP} cell preparations in a concentration-dependent manner and with varying affinities. Competition binding curves of U69,593 (KOP), DAMGO (MOP) and DPDPE (DOP) are depicted in Figure 2D. The inhibitory constants (K_i) calculated for each tested opioid derived from the competition curves are summarized in Table 2. Only ligands selective for the KOP receptor, like U50,488, U69,593, and nor-BNI, were found to be very potent inhibitors of [^{3}H]HS665 binding. Also, the nonselective opioid antagonist naloxone efficiently displaced [^{3}H]HS665 with a K_i value of 4.31 nM. The MOP selective agonist DAMGO exhibited very low potency in competing reversibly for the [^{3}H]HS665 binding sites in CHO_{hKOP} cell membranes. Moreover, the specific DOP peptide DPDPE was unable to produce any inhibition of the specific [^{3}H]HS665 binding. In our earlier study,[39] we also demonstrated the very weak ability of the two MOP and DOP specific radioligands, [^{3}H]DAMGO and [^{3}H]DPDPE, respectively, to compete with HS665. Consequently, the KOP receptor specificity of the new radioligand [^{3}H]HS665 to membrane preparations from CHO cells expressing the human KOP receptor is clearly proven by the present competition binding experiments.

Table 2. Inhibition Constants (K_i) of Opioid Ligands against [^{3}H]HS665[a]

ligand	type	K_i (nM)	
		CHO_{hKOP} cells	guinea pig brain
naloxone	nonselective	4.31 ± 0.73	n.t.
HS665	KOP	0.40 ± 0.05	0.72 ± 0.10
U50,488	KOP	0.97 ± 0.12	n.t.
U69,593	KOP	0.61 ± 0.15	0.94 ± 0.12
nor-BNI	KOP	1.26 ± 0.58	1.83 ± 0.86
DAMGO	MOP	542 ± 120	>10 000
DPDPE	DOP	>10 000	>10 000

[a]Membranes from CHO_{hKOP} cells or guinea pig brain were incubated with [^{3}H]HS665 in the presence of increasing concentrations of test opioid ligands. K_i values were calculated by nonlinear curve fitting analysis using the GraphPad Prism software. n.t.: not tested. Values are means ± SEM (n = 3).

Direct binding studies have also established the high affinity and specificity of [^{3}H]HS665 binding to the KOP receptor in membranes from the guinea pig brain, a tissue well-known to express higher density of KOP receptors in comparison to the brain of other rodents i.e. rat and mouse.[45] As initial studies showed that specific binding of [^{3}H]HS665 was much less at 0 °C than at 25 °C, all subsequent experiments were carried out at 25 °C. Kinetic assays revealed that association of [^{3}H]HS665 occurred rapidly, and the steady-state level of specific binding is achieved in about 15 min of incubation at 25 °C, which remained stable up to 60 min (Figure 3A).

Additionally, equilibrium saturation assays confirmed the presence of a single binding site labeled by [^{3}H]HS665 in guinea pig brain membranes after incubation for 30 min at 25 °C. As shown in Figure 3B, the specific binding of the radioligand was saturable and of high affinity, and a linear Scatchard plot was obtained (Figure 3B, inset). The Hill slopes for [^{3}H]HS665 binding was close to the unity (n_H = 1.06), also indicative for ligand interaction with a single population of receptors in guinea pig brain preparations. The calculated K_d value of [^{3}H]HS665 was 0.64 nM, and the B_{max} value was found to be 145 fmol/mg protein in the brain tissue, with a specific binding of about 80% of the total binding.

Binding properties of the prototypical KOP radioligand [^{3}H]U69,593 were also evaluated in guinea pig brain membranes for correlation to the binding of [^{3}H]HS665 in CHO_{hKOP} cells. Similar to human KOP receptors expressed in CHO cells, [^{3}H]U69,593 labels a single class of neuronal KOP receptors with high affinity based on the K_d value of 0.90 nM. As shown in Table 1, [^{3}H]HS665 exhibits overall KOP receptor binding affinity and capacity values comparable to [^{3}H]-U69,593.

Competition studies also demonstrated the KOP receptor selectivity of the radioligand in the guinea pig brain, based on the high potencies of KOP ligands, U69,593 and nor-BNI to displace the bound [^{3}H]HS665, while DAMGO and DPDPE,

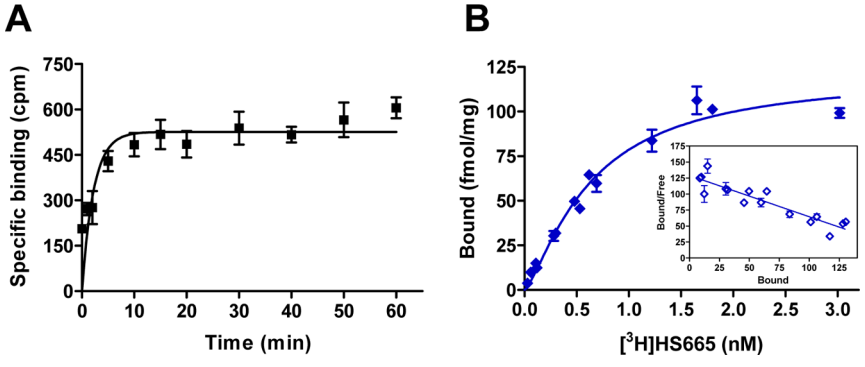

Figure 3. Specific binding of [^{3}H]HS665 to guinea pig brain membranes. (A) Association time course of [^{3}H]HS665 (0.6 nM) at 25 °C for the indicated times. (B) Saturation binding of [^{3}H]HS665 at 25 °C and 30 min incubation. Inset: Scatchard plot. Guinea pig brain membranes (300–500 μg) were incubated with [^{3}H]HS665. Nonspecific binding was determined in the presence of 10 μM unlabeled HS665. Data are means ± SEM (n = 3). Nonvisible SEM is within the symbol. Binding data were analyzed with the GraphPad Prism software.

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MOP, and DOP ligands, respectively, were much weaker inhibitors (Table 2). High binding affinity was also depicted by the unlabeled HS655 in homologues competition studies, with a K_i value of 0.72 nM.

On the basis of our present findings, it is evident that [^{3}H]HS665 displays similar binding properties at the recombinant human KOP receptor in membranes from CHO cells stably expressing hKOP receptors, and to the native neuronal KOP receptor in the guinea pig brain. In both membrane preparations, association of ligand−receptor complexes occurred rapidly, in a monophasic manner, with an equilibrium reached within 15 min. Furthermore, equilibrium binding experiments revealed that a single population of high affinity binding sites is labeled by [^{3}H]HS665 in CHO_{hKOP} cells and guinea pig brain with comparable K_d values, 0.45 nM and 0.64, respectively. Affinities of native and recombinant opioid receptors are reportedly close to one another.[46−48] On the other hand, the binding capacities of [^{3}H]HS665, B_{max}, were significantly higher in CHO_{hKOP} cells than in guinea pig brain preparations, reflecting simply the overexpression of hKOP receptors in transfected epithelial cells. In the present study, it is also shown that in brain membranes, [^{3}H]HS665 specifically labels an opioid receptor site with pharmacological properties analogous to those exhibited by the recombinant hKOP receptor expressed in CHO cells.

Radioligands are essential tools in GPCR research, and the field of opioid drug discovery has benefited greatly from the structural and functional insights afforded by radiolabeled ligands. While in the early studies radioligands were critical in the initial demonstration of opioid receptors, with tritiated ligands labeling all opioid binding sites, later the main purpose was the development of selective ligands to identify different receptor types (KOP, MOP, and DOP) in brain tissue and cell lines. Typically, tritium labeling of small molecules and opioid peptides has a long tradition, and still is a sound method to obtain labeled compounds.[43,49,50] Tritium has become an important tool for several reasons, including the relatively long half-life of tritium (12.37 years), and practical utility of the tritiated compounds with high specific radioactivity for many biological investigations. Tritium labeling chemistry is often simple and the labeling can usually be accomplished very late in the overall synthesis, thus reducing the radiochemistry handling required. As a β-emitter, tritium has low energy and low toxicity. An advantageous feature of tritiated compounds as tracers is the high stability of the tritium label under experimental conditions, especially with tritium bound to a carbon atom being the most stable label.[43] Therefore, on the basis of the mentioned aspects, labeling of HS665 with tritium was selected and effectively accomplished in the current study.

In primary pharmacological studies, the KOP receptor was defined using nonselective opioid radioligands, including [^{3}H]ethylketocyclazocine, [^{3}H]bremazocine, [^{3}H]-diprenorphine, [^{3}H]etorphine, [^{3}H]buprenorphine and [^{3}H]-dynorphins, used in the presence of cold ligands DAMGO, D-Ala-D-Leu-enkephalin, to saturate the MOP and DOP receptors, respectively.[43] The endogenous KOP peptides dynorphins,[51] available in tritium-labeled form, [^{3}H]dynorphins ([^{3}H]dynorphin (1−8) and [^{3}H]dynorphin (1−9)) show also high affinity to MOP and DOP receptors in the guinea pig brain.[43,52−55] Moreover, the dynorphins are rapidly metabolized enzymatically, thereby rendering them of little value as pharmacological agents. Nor-BNI, as a KOP opioid antagonist designed with a bivalent ligand approach,[24] was radiolabeled with tritium,[56] and [^{3}H]nor-BNI proved to have high potency and selectivity to preparations from guinea pig, frog, and rat brain.[56] Its main drawback is the unusual long duration of KOP antagonism that is still not well understood. Of the various existing tritium labeled agonists, the arylacetamide KOP agonist [^{3}H]U69,593[57] is nowadays one of the most commonly used KOP selective radioligands in practice for the characterization of new opioid ligands by means of radioligand binding assays. We were successful in generating a new selective KOP radioligand [^{3}H]HS665, that is a structurally distinct small molecule to the long-time known [^{3}H]U69,593.[57] Furthermore, the structural simplicity, the readily accessible and cost-effective synthesis in comparison to [^{3}H]U69,593, and improved binding affinity to the human KOP receptor as the ultimate target of opioid drugs makes this new radioligand an attractive pharmacological tool for investigating the KOP receptor system, nowadays appraised as an innovative approach for the treatment of several neurological disorders. Thus, the use of [^{3}H]HS665 as a novel selective KOP receptor radioligand combines scientific and economic incentives.

CONCLUSIONS

In summary, the present study describes radiolabeling and pharmacological evaluation of a new, highly potent, and selective KOP receptor radioligand, the *N*-cyclobutylmethyl substituted diphenethylamine [^{3}H]HS665, for the recombinant human KOP receptor in CHO cells stably expressing hKOP receptors, and the native neuronal KOP receptor in the guinea pig brain. This class of opioid receptors is a target of therapeutic interest for the treatment of a variety of human disorders (i.e., pain, affective disorders, drug addiction, and psychotic disorders). The current outcomes from direct binding studies with the tritium-labeled HS665 ([^{3}H]HS665) confirm and extend our observations obtained with the unlabeled ligand in terms of its high affinity and selectivity for the KOP receptor. Importantly, this radioligand satisfies the criteria of saturability, selectivity, and relatively low nonspecific binding necessary for useful radioprobes. As such, tritiated HS665 should prove utility as pharmacological probe for various applications, including studies on binding to opioid receptors, intracellular distribution, and tissue distribution. The present results revealed that the new radioligand recognizes and labels KOP receptors specifically in neuronal and cellular systems expressing KOP receptors, making this molecule a valuable tool in investigating KOP receptor pharmacology in recombinant or native in vitro systems.

METHODS

Materials. Cell culture media and supplements were from Sigma-Aldrich Chemicals (St. Louis, MO) or Life Technologies (Carlsbad, CA). All other chemicals were of analytical grade and obtained from standard commercial sources. Guinea pig brains were obtained frozen from the Institut für Labortierkunde and Laborgenetik, Medizinische Universität Wien (Himberg, Austria).

Chemistry. *General Methods.* Melting points were determined on a Kofler melting point microscope and are uncorrected. ^{1}H NMR (200 MHz) and ^{13}C NMR (50 MHz) spectra were obtained on a Varian Gemini 200 spectrometer. IR spectra were taken on a Bruker Alpha FT-IR spectrometer. For detection, an ATR sensor (room temperature deuterated L-alanine triglycine sulfate, RT DLATGS detector) was used. Mass spectra were recorded on a Bruker-Daltronics Esquire 3000 plus ion trap for electron spray ionization (ESI). For column chromatography (MPLC), silica gel 60 (0.040−0.063 mm, 230−400 mesh ASTM) Fluka, Switzerland was used. TLC was performed on

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silica gel plates Polygram SIL G/UV_{254} (Macherey-Nagel, Germany) with CH_2Cl_2/MeOH/NH_4OH 98.5:0.5:1 as an eluent. All other chemicals were of analytical grade and obtained from standard commercial sources. HPLC: Analytical RP-HPLC column, Atlantis (C_{18}, 3.9 × 150 mm, 5 μm), flow rate of 1 mL/min was used. The mobile phase consisted of 0.1% (v/v) TFA in water and 0.08% (v/v) TFA in acetonitrile with gradient from 20% up to 60% during 20 min. Purities of tested compounds were determined and were ≥95%.

*2,6-Dibromo-3-[2-[(cyclobutylmethyl)(phenethyl)amino]ethyl]-phenol (**1**).* To a stirred solution of HS665 (50 mg, 0.162 mmol) in CH_2Cl_2 (1 mL) and diisopropylamine (0.007 mL, 0.049 mmol), a solution of *N*-bromosuccinimide (57.67 mg, 0.324 mmol) in 2 mL of CH_2Cl_2 was added dropwise over a period of 30 min at room temperature. Stirring was continued for 2 h, then the mixture was diluted to 25 mL with CH_2Cl_2, washed with brine (2 × 15 mL), dried over Na_2SO_4, and evaporated. After column chromatography (silica gel, CH_2Cl_2/MeOH/NH_4OH, 98.5:0.5:1), 28 mg (37%) of **1** was isolated. Mp: 86−88 °C; IR (ATR) 2958 cm^{-1} (OH). ^1H NMR ($CDCl_3$) δ 7.36−7.16 (m, 6 arom. H), 6.67 (d, *J* = 8.4 Hz, 1 arom. H), 2.87−2.60 (m, 10 H), 2.09−1.63 (m, 7 H). ^{13}C NMR ($CDCl_3$) δ 156.92, 141.27, 139.68, 129.93, 128.92, 128.73, 126.47, 120.51, 116.04, 114.07, 59.74, 55.67, 55.59, 33.24, 32.56, 32.20, 28.24, 19.01; MS (ESI) *m*/*z* 468.24 $[M + 1]^+$.

Preparation of [^{3}H]HS665. A mixture of **1** (2.5 mg), *N*,*N*-dimethylformamide, triethylamine (2.0 μL), and PdO/$BaSO_4$ catalyst (10 mg) was reacted with tritium gas at room temperature for 1 h while stirring. The catalyst was filtered off using a Whatmann GF/C glass-fiber filter, and the crude product was obtained with 216 mCi in 20 mL of ethanol. This product was purified by RP-HPLC (Atlantis C_{18}, 3.9 × 150 mm, 5 μm) using a mobile phase of 0.1% (v/v) TFA in water and 0.08% (v/v) TFA in acetonitrile with a gradient from 20% up to 60% to afford [^{3}H]HS665 with a radioactive purity of ≥95% and with a specific activity of 30.65 Ci/mmol. [^{3}H]HS665 was dissolved in spetroscopic ethanol and was stored at a concentration of 1 mCi/mL in liquid nitrogen.

Pharmacology. *Cell Culturing.* CHO cells expressing recombinant human KOP receptors (CHO_{hKOP} cell line) was cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with fetal bovine serum (FBS, 10%), penicillin/streptomycin (0.1%), L-glutamine (2 mM), and Geneticin (400 μg/mL). Cell cultures were maintained at 37 °C in 5% CO_2 humidified air.

Membrane Preparation. Membranes from CHO_{hKOP} cells were prepared in 50 mM Tris-HCl buffer (pH 7.4) according to the previously described procedure.[39] Cells grown to confluency were harvested by scraping tissue culture plates with a rubber policeman and then centrifuged at 500*g* for 10 min. The cell pellet was resuspended in Tris buffer, homogenized using a Dounce homogenizer and centrifuged at 20 000*g* for 20 min at 4 °C. The centrifugation step was repeated, the final pellets were resuspended in the same buffer and stored at −80 °C until use.

Membranes from guinea pig brains were prepared as previously described.[58] Brains (minus cerebella) were homogenized in 50 mM Tris-HCl buffer (pH 7.4) using a Teflon glass homogenizer. The homogenate was centrifuged at 40 000*g* for 20 min at 4 °C. The resulting pellets were resuspended in fresh Tris-HCl buffer, incubated at 37 °C for 30 min, and recentrifuged. The final pellets were suspended in 50 mM Tris-HCl (pH 7.4) containing 0.32 M sucrose and stored at −80 °C until use in binding assays. Protein content of cell and brain preparations was determined by the Bradford method using bovine serum albumin as the standard.[59]

Receptor Binding Assays. Assays were performed in 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 1 mL. Time course of association was determined by incubating of [^{3}H]HS665 with membrane preparations from CHO_{hKOP} cells (15 μg) or guinea pig brain (300−500 μg) at 0 or 25 °C, respectively, for the indicated times. Saturation binding experiments were performed by measuring specific binding over increasing concentrations of [^{3}H]HS665 (0.01−3.0 nM) to membrane preparations from CHO_{hKOP} cells or guinea pig brain. Competition binding experiments were carried out by incubating [^{3}H]HS665 in the presence of increasing concentrations of unlabeled opioid ligands. Nonspecific binding was measured in the presence of 10 μM HS665. Reactions were terminated by rapid filtration through Whatman glass GF/C fiber filters. Filters were washed three times with 5 mL of ice-cold 50 mM Tris-HCl buffer (pH 7.4) with a Brandel M24R cell harvester (Gaithersburg, MD). Radioactivity retained on the filters was counted by liquid scintillation counting using a Beckman Coulter LS6500 (Beckman Coulter Inc., Fullerton, CA). All binding experiments were performed in duplicate and repeated at least three times.

Data Analysis. Experimental data were analyzed and graphically processed using the GraphPad Prism 5.0 Software (GraphPad Prism Software Inc., San Diego, CA). Nonlinear regression analysis of the direct saturation isotherms was performed to obtain the equilibrium K_d and B_{max} (receptor densities) values. The inhibitory constant K_i values were calculated from the displacement curves using nonlinear least-squares curve fitting and the Cheng−Prusoff equation.[60] Data are expressed as means ± SEM.

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Author Contributions

M.S., H.S., and G.T. conceived and designed the experiments. E.G. and J.R.M. synthesized and characterized the compounds. M.S. and E.G. performed the biological assays and analyzed the data. M.S., H.S., and G.T. contributed reagents/materials/analysis tools. M.S., H.S., and G.T. wrote the manuscript. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

B_{max}, maximum number of binding sites, receptor densities or binding capacities; CHO, Chinese hamster ovary; DIPA, diisopropylamine; DOP receptor, delta opioid peptide receptor; DMF, *N*,*N*-dimethylformamide; hKOP receptor, human kappa opioid peptide receptor; K_d, equilibrium dissociation constant; K_i, inhibition constant; n_H, Hill coefficient; MOP receptor, mu opioid peptide receptor; NBS, *N*-bromosuccinimide; RT, room temperature; TEA, triethylamine

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