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Development of an efficient and selective radioligand for bradykinin B_1 receptor occupancy studies

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Abstract—We have developed an efficient and selective radioligand, the [^{35}S]-radiolabeled dihydroquinoxalinone derivative, **4**, for an ex vivo receptor occupancy assay in transgenic rats over-expressing the human bradykinin B_1 receptor.
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Bradykinin (BK) B_1 and B_2 receptors are G-protein coupled receptors that mediate the action of kinin peptides which are produced by the catalytic action of kallikrein enzymes on plasma and tissue precursors termed kininogens.[1] BK plays an important role in the pathophysiological processes accompanying acute pain, chronic pain, and inflammation. The BK B_2 receptor is constitutively expressed in most cell types, whereas the BK B_1 receptor is induced during inflammatory insults or painful stimuli. In animal models, late phase hyperalgesia can be attenuated by peptide BK B_1 receptor antagonists, such as des-Arg^9-Leu^8-BK (DALBK) and des-Arg^{10}-Leu^9-Kallidin (DALK).[2] Recent data shows that transgenic BK B_1 receptor knockout mice are hypoalgesic in chronic and acute pain models, implicating a role for the BK B_1 receptor in inflammation and algesia.[3] In addition to the accepted peripheral mode of action of the BK B_1 receptor, the BK B_1 receptor has also been accorded a central role on the basis of recent results which demonstrate that the BK B_1 receptor is constitutively expressed in the central nervous system (CNS) of mice and rats.[4] To support our efforts toward the development of human BK B_1 receptor antagonists as novel therapeutic agents for the treatment of pain and inflammation,[5] an efficient and selective radioligand was needed for animal model studies. The commercially available peptide radioligands, [^{3}H]DAK (des-Arg^{10}-Kallidin) and [^{3}H]DALK, provided unsatisfactory results in our occupancy studies (vide infra). Herein we report the development of a novel [^{35}S]-radioligand for the study of human BK B_1 receptor occupancy in an ex vivo transgenic rat model.

In order to achieve good selectivity and an acceptable signal to noise ratio in the animal model, a radioligand candidate must have excellent binding affinity coupled with well-balanced physicochemical properties (e.g., log *P* and protein binding). The radiolabeled compound should also be readily accessible, preferably with introduction of the radiolabel late in the synthesis.

The current study was initiated with the previously reported, potent and selective BK B_1 receptor antagonist, compound **1** (Table 1).[5a] Although compound **1** possesses excellent binding potency and offers the possibility to introduce [^{35}S] as a radiolabel atom, we judged it to be unsuitable as a radioligand because of its chemical intractability[6] and also, in part, because of its physiochemical properties. Therefore, subsequent work focused on modifying the sulfonamide moiety and properties of compound **1** while maintaining potent binding affinity. The protein binding and log *P* properties of **1**

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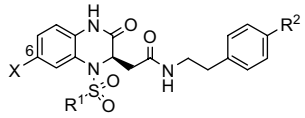
Table 1. Receptor binding affinity and physicochemical properties of dihydroquinoxalinone analogs

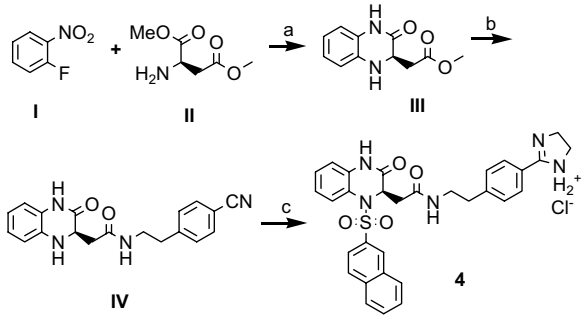


Compound	X	R^1	R^2	hB_1 K_i (nm)[a,b]	Log P[c]	Protein binding[d]
1	H	3,4-Dichlorophenyl		0.034	0.48	36.0
2	H	3,4-Dichlorophenyl		1.09	3.35	ND[e]
3	H	3,4-Dichlorophenyl		3.73	2.35	82.5
4	H	2-Naphthyl		0.02	0.25	79.4
5	H	2-Naphthyl		0.07	3.32	98.5
6	Cl	2-Naphthyl		0.01	1.00	93.6
7	Cl	2-Naphthyl		0.02	0.53	91.7
8	Cl	2-Naphthyl		0.07	3.41	ND[e]

[a] All K_i values are calculated for competition binding assays utilizing cloned human B_1 and B_2 receptors and are the average of at least two experiments.
[b] The hB_2 K_i values for all compounds are greater than 10,000 nM.
[c] Log P values were determined by HPLC analysis in octanol/water.
[d] Human plasma protein.
[e] ND = not done: compound is insoluble in test medium.

were altered with the conversion of the imidazoline to an imidazole (**2**), and triazole (**3**). However, the binding affinities of these analogs were decreased 32- and 110-fold, respectively.[7] Replacement of the 3,4-dichlorophenylsulfonamide moiety in **1** with the more readily accessible [^{35}S]-2-naphthylsulfonamide yielded compound **4**, which had 2-fold increased potency and increased protein binding. However, the log P value for compound **4** is not substantially different from compound **1**. Oxidation of the imidazoline in **4** to imidazole gave **5**, which is highly lipophilic and avidly protein bound. In a related pharmacokinetics study of an analog structurally similar to **4**, we discovered that the 6-position of the dihydroquinoxalinone ring is subject to in vitro oxidation (rat, dog, etc., liver microsomes) to form a phenol derivative. This metabolic pathway can be attenuated with the introduction of a halogen atom at the corresponding metabolic site (data not shown). We speculated that the addition of halogen might also affect the physicochemical properties of analogs. Therefore, a chlorine atom was introduced in **4** to give the equipotent derivative **6**. Although compound **6** exhibits an acceptable log P value, it is highly protein bound. Further modification of the imidazoline ring in **6** (e.g., methylation to give **7** or oxidation to yield **8**) did not result in significant improvement in the properties. Taken together, these data led us to re-assess compound **4**. Based on its overall profile (human B_1 receptor binding potency, chemical accessibility, as well as physicochemical properties) compound **4** was evaluated as a potential radioligand.

Compound **4** was prepared according to the route depicted in Scheme 1. The preparative route is an adaptation of our original synthesis to accommodate the preparation of [^{35}S]-**4**.[5a] Condensation of 2-fluoro nitrobenzene (I) with commercially available methyl aspartate ester (II) afforded the nitrobenzene adduct (Scheme 1). Reduction of the nitro group resulted in intramolecular cyclization to afford the key dihydroquinoxalinone methyl ester III. Saponification of ester III followed by coupling of the resulting acid with 4-(2-



Scheme 1. Reagents and conditions: (a) 1. $NaHCO_3$, MeOH, Reflux. 2. HCl, MeOH, 81% for two steps. 3. H_2, Pd/C, EtOH, 98%. (b) 1. 1 N NaOH, MeOH; 2. 4-(2-aminoethyl)benzonitrile, EDCI, HOAt, Et_3N, CH_2Cl_2, 68% for two steps. (c) 1. 2-naphthyl-SO_2Cl, pyridine, 96%; 2. HCl, EtOH; 3. ethylene diamine, EtOH, 4. HCl, EtOAc, 0 °C, 77% for three steps.

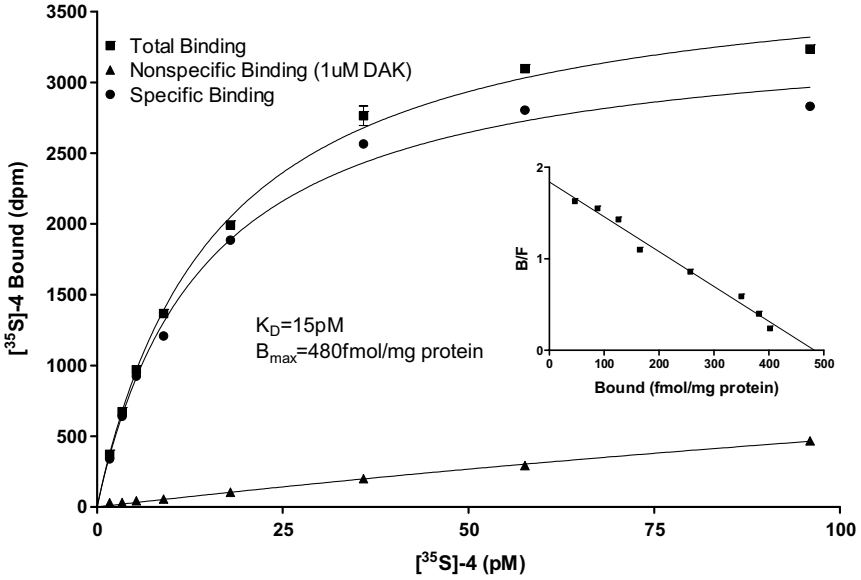


Figure 1. [^{35}S]-**4** Saturation binding to human BK B_1 receptor CHO membranes.

aminoethyl)benzonitrile delivered the desired amide IV. Sulfonylation with 2-naphthylsulfonyl chloride was followed by a three-step sequence which converted the nitrile to the imidazoline ring leading to compound **4** as its HCl salt. The radiolabeled [^{35}S]-**4** was obtained by this route using [^{35}S]-2-naphthylsulfonyl chloride on a 350 mCi scale. The [^{35}S]-2-naphthylsulfonyl chloride was obtained from commercially available [^{35}S]-2-naphthylsulfonic acid by the action of $NaHCO_3$ and oxalyl chloride.[8]

In a panel of assays[9] representing 170 enzymes, receptors, and transporters, compound **4** exhibited over 2700-fold selectivity for the human BK B_1 receptor. Analog **4** demonstrated excellent functional antagonist activity in a fluorescence imaging plate reader (FLIPR) assay (human BK B_1, $IC_{50} = 0.04$ nM) that is in concert with the receptor binding affinity. The binding of [^{35}S]-**4** to the human BK B_1 receptor expressed in CHO membranes was characterized.[10] At 22 °C, [^{35}S]-**4** reaches equilibrium within 2 h and dissociates slowly with a $t_{1/2}$ value of $\sim$ 200 min. In saturation binding studies, [^{35}S]-**4** labels a single site with a K_d value of 15 pM and B_{max} value of 480 fmol/mg protein, a density equivalent to that labeled by [^{3}H]DALK (Fig. 1). There is no specific binding of [^{35}S]-**4** to CHO cell membranes expressing the human B_2 receptor. Data from competition binding studies indicates that the pharmacologies of [^{35}S]-**4** and [^{3}H]DALK binding to the human BK B_1 receptor are similar (data not shown).

Due to significant species differences observed for the binding of BK B_1 peptide and nonpeptide antagonists to BK B_1 receptors,[5a,11,12] we generated and characterized transgenic rats in which the human BK B_1 receptor is constitutively over-expressed.[13] Administration of [^{35}S]-**4** by either intravenous or intra-cerebroventricular injection did not result in the labeling of BK B_1 receptors in the CNS (brain and spinal cord tissue), presumably due to its inability to effectively cross the blood brain barrier from the circulation or to diffuse through the brain tissue from the lateral ventricles. Therefore, this compound was not suitable for the development of an in vivo receptor occupancy assay. Nonetheless, the excellent in vitro binding properties of [^{35}S]-**4** led us to develop an ex vivo method for the determination of human B_1 central receptor occupancy of BK B_1 antagonists.[13] The ex vivo assay involves analysis of the rate of [^{35}S]-**4** association in membranes prepared from tissues harvested from vehicle and BK B_1 antagonist treated animals. In the assay, the association of [^{35}S]-**4** is linear over 10 min and receptor occupancy is directly proportional to the decrease in the slope of the association line compared to the control. The assay works largely because of the high specific activity and low nonspecific binding of [^{35}S]-**4** which allows the assay to be run with minimal tissue over a short time frame. Thus, the association and dissociation are kept to a minimum.[14] This assay provides a rapid and reliable method for determining the extent of a systemically-dosed test compound to occupy the human BK B_1 receptor over-expressed in the CNS of transgenic rats.

In summary, we have developed an efficient and selective radioligand, [^{35}S]-**4**, for the ex vivo human BK B_1 receptor occupancy study in transgenic rats. This assay provides important information in the selection of nonpeptide BK B_1 receptor antagonists for further development.

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Supplementary data

Experimental details for [^{35}S]-**4** and characterization of **4** can be found, in the online version, at doi:10.1016/j.bmcl.2004.09.074.

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6. During the time of this study, vialble methods to incorporate [^{35}S] into the requisite starting material were unavailable.
7. For the assay protocol, see Ref. 5a.
8. See Supplementary data for the preparation of [^{35}S]-**4**. The shelf life of radioligand **4** is limited by the $t_{1/2}$ of ^{35}S (~87 days).
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14. The commercially available [^{3}H]DAK and [^{3}H]DALK, two peptide radioligands with high affinity (0.1–0.2 nM) for the human BK B_1 receptor but relatively low specific activity (75 Ci/μmol), were also tested in the same assay format. Both required long incubation times (leading to dissociation artifacts) and high tissue concentrations (association artifacts) and could not be used for the development of a reliable assay.