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Probes for Imidazoline Binding Sites: Synthesis and Evaluation of a Selective, Irreversible I_2 Ligand

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Abstract—An irreversible ligand (**7**) has been prepared based on the selective I_2 ligand 2-BFI. Compound **7** displayed high affinity and selectivity for I_2-sites and has been shown to irreversibly bind to these sites in rat brain. Compound **7** should, therefore, prove an invaluable tool for the further elucidation of I_2-site function.

Introduction

It is now widely recognised that mammalian imidazoline binding sites can be divided into I_1 and I_2 subtypes and recently a third class, I_3 sites, have also been proposed.[1] In order to better determine the role and nature of these sites there is a need for ligands of high affinity and selectivity. To this end we have identified a number of ligands (e.g. **1**–**3**) with exceptional selectivity for I_2 sites over both I_1 sites and α_2-adrenoceptors.[2,3] These included 2-BFI (2-benzofuranyl-2-imidazoline) (**1**) which has been studied in several animal models of brain function and tritiated in order to map the distribution of I_2 sites in a number of species.[4–6] Even with the availability of these highly selective ligands it is still unclear what the imidazoline binding sites represent and what their pharmacological role is. While Parini's group have demonstrated that a significant population of these sites are associated with monoamine oxidase-B (MAO_B),[7] Garcia-Sevilla's group have defined distinct binding proteins corresponding to subgroups of I_2 sites.[8] This raises the possibility that I_2 sites may represent more that one entity and may help to explain the array of effects attributed to I_2 ligands. For example, I_2 effects attributed to the α_2-adrenoceptor antagonist/imidazoline ligand idazoxan include antidepressive effects in man, elevation of monoamines in rat brain, increase of food intake in rats and also neuroprotection in models of stroke.[9,10]

In order to better define the role of I_2-sites we have prepared an irreversible ligand, based on the selective ligand, 2-BFI. Irreversible ligands allow complete knockout of one particular site enabling the role of individual sites to be more easily determined. Additionally an irreversible ligand should function as an antagonist at its site and allow the assessment of efficacy of other ligands at that site i.e. the apparent efficacy of a ligand is reduced as the receptor reserve in a preparation is immobilised. Until now it has not been possible to label, with any certainty, the known I_2 ligands as agonist, partial agonist or antagonist in character.

Previously a radio-iodinated photoaffinity ligand ([^{125}I]AZIPI) has been prepared and shown to label at least three subtypes of I_2 sites that differ in their ligand recognition properties, apparent molecular weight and tissue distribution.[15] However, since a photo-affinity label requires photoactivation it is not possible to use such a ligand in vivo. Furthermore the very high reactivity of the intermediate generated on photo-activation can reduce the selectivity of the labelling. An alternative approach is to use an isothiocyanate moiety as the alkylating function. This group is fairly small and usually has minimum impact on the binding affinity and selectivity of the parent ligand, though examples are known where a change in selectivity has occurred.[11] The group is highly reactive towards amino and sulhydryl groups, while displaying low reactivity to hydroxyl functions, including water. Thus, there is less non-specific

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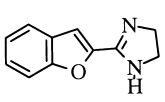
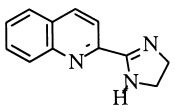
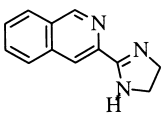


1: 2-BFI



2: BU224



3: BU236

binding compared to more reactive electrophiles. Previously an isothiocyanate containing irreversible ligand, IBI, has been prepared based on the I_2/α_2 ligand tolazoline.[12,14] Although it could be shown to dose-dependently reduce I_2-binding site density, this was only by 41% after a 30 mg/kg ip dose.[16] 2-BFI has higher affinity and selectivity than tolazoline for I_2 sites and, therefore, was anticipated to be a superior framework for the development of a selective, high affinity non-competitive ligand.

It is known that 2-BFI can tolerate the presence of electron withdrawing substituents on the benzo ring,[3] therefore it was decided to target an aryl substituted derivative, and in particular 5-isothiocyanato-2-BFI, as the potential irreversible ligand.

Chemistry

5-Nitrosalicylaldehyde was treated with diethyl bromomalonate in a modification of the procedure for the formation of ethyl 7-nitrobenzofuran-2-carboxylate[13] to give ethyl 5-nitrobenzofuran-2-carboxylate that was converted into the corresponding primary amide **5** by treatment with NH_3. Dehydration to the nitrile with $POCl_3$ was followed by formation of the imidazoline ring using ethylenediamine under acidic conditions. Reduction of the nitro group of **6** was achieved utilising Fe/HCl. By keeping the imidazoline group as its HCl salt,[14] thiophosgene could be reacted selectively with the aryl-NH_2 to yield **7** in 14% yield over 6 steps (Scheme 1).

Results and Discussion

The binding affinities of **7** were determined in rat whole brain membranes (I_2 and α_2) and kidney membranes (I_1) by displacement of [^{3}H]2-BFI (I_2), [^{3}H]clonidine (I_1) and [^{3}H]RX821002 (α_2).[4,5] Compound **7** displayed high (nM) affinity for I_2 sites, but only low (μM) affinity for both I_1 and α_2, resulting in selectivity of greater than three orders of magnitude (Table 1). In comparison with 2-BFI, **7** displayed equivalent affinity for I_2 and I_1 sites but increased selectivity over α_2 adrenoceptors.

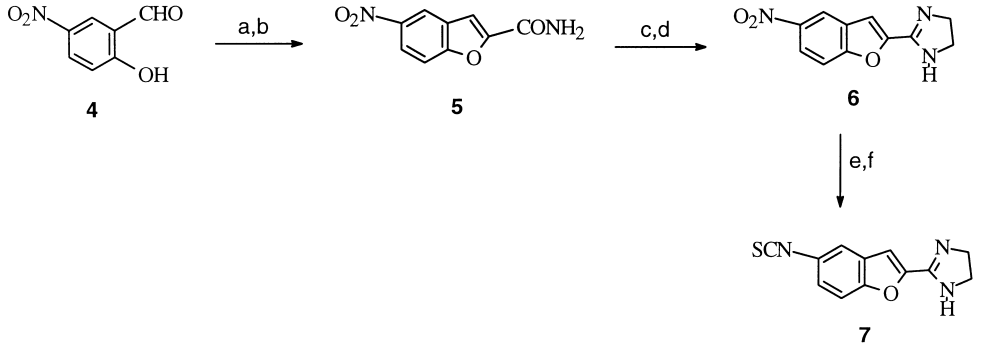
In order to determine if **7** was binding in an irreversible manner, tissue was incubated with BU99006 (**7**) (10 μM), water (vehicle) and BU224 (10 μM) at 37 °C for 20 min. Incubation was terminated by addition of 20 mL ice cold buffer and then centrifugation. The tissue was then washed by repeated centrifugation (twice) to remove any non-irreversibly bound compound. While the vehicle treated tissue displayed specific binding of 65 fmol/mg protein, the tissue treated with **7** showed only 4 fmol/mg (Fig. 1). In contrast pre-treatment with BU224 (**2**) showed no significant change from control (data not shown).

Thus an irreversible ligand (**7**) has been prepared with high affinity and selectivity for I_2-sites. It has been shown to immobilise I_2-sites and should, therefore, prove an invaluable tool for the further elucidation of I_2-site function. Work is in progress to further characterise this ligand and also to prepare a tritiated version

Table 1. Affinities for I_2 sites, I_1 sites and α_2-adrenoceptors

Ligand	K_i (nM) I_2[a]	IC_{50} (nM) I_1[b]	K_i (nM) α_2[c]
1	1.7 ± 0.4	6400 ± 1300	1900 ± 500
7	2.3 ± 0.3	8100 ± 1700	16300 ± 900

[a]I_2 site affinity was determined by competition assays against 1 nM [^{3}H]2-BFI bound to rat whole brain membranes.
[b]I_1 site affinity by competition with 3 nM [^{3}H]clonidine (in the presence of rauwolscine) bound to rat kidney membranes.
[c]α_2-Adrenoceptor affinity by competition with 1 nM [^{3}H]RX821002 bound to rat whole brain membranes.



4 **5** **6** **7**

Scheme 1. (a) $BrCH(COOEt)_2$, K_2CO_3, Bu_4NBr, toluene, reflux, 24 h, 84%. (b) NH_3, EtOH, room temperature, 24 h, 99%. (c) $POCl_3$, reflux, 0.5 h, 74%. (d) 1. HCl, MeOH, room temperature, 1 h; 2. ethylenediamine, reflux, 18 h, 52%. (e) Fe, HCl, EtOH, reflux, 3 h, 59%. (f) $CSCl_2$, acetone–water, room temperature, 1.5 h, 75%.

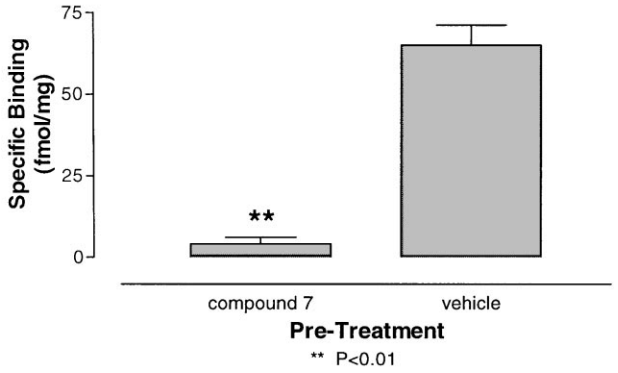


Figure 1. Effect of pretreatment with **7** on the specific binding of [^{3}H]2-BFI (5 nM).

to allow the isolation and identification of the binding proteins recognised by **7**.

Acknowledgements

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