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Short communication

Muscarinic interactions of bisindolylmaleimide analogues

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Abstract

We have used radioligand binding studies to determine the affinities of seven bisindolylmaleimide analogues, six of which are selective inhibitors of protein kinase C, at human muscarinic M_1–M_4 receptors. The compounds were most potent at M_1 receptors, and Ro-31-8220 was the most potent analogue, with a K_d of 0.6 μM at M_1 receptors. The weakest compounds, bisindolylmaleimide IV and bisindolylmaleimide V, had K_d values of 100 μM. If it is necessary to use protein kinase C inhibitors at concentrations of 10 μM or more in studies involving muscarinic receptors then bisindolylmaleimide IV may be the most appropriate inhibitor to use.

Keywords: Muscarinic receptor; Protein kinase C inhibitor; Bisindolylmaleimide

1. Introduction

Certain bisindolylmaleimide analogues, such as GF 109203X and Ro-31-8220, are widely used as selective inhibitors of protein kinase C. Recently, however, Willars et al. (1996) have reported that Ro-31-8220 has antimuscarinic properties, inhibiting radioligand binding to the human M_3 receptor with an apparent log affinity of 4.8. Here we report on interactions of seven bisindolylmaleimide analogues with human muscarinic M_1–M_4 receptor subtypes.

2. Materials and methods

2.1. Materials

[^{3}H]*N*-methyl scopolomine (83–86 Ci/mmol) was from Amersham International, UK. The test compounds (Fig. 1A), 3-[1-[-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl)maleimide methane sulfonate (Ro-31-8220), [2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2*a*]indol-3-yl}-3-(1-methylindol-3-yl)maleimide, hydrochloride (Ro-32-0432), 2-[1-(3-dimethylaminopropyl)1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide (GF 109203X/bisindolylmaleimide I), 2-[1-(3-dimethylaminopropyl)1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide, hydrochloride (bisindolylmaleimide I HCl), 2-[1-[2-(1-methylpyrrolidino)ethyl]-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide (bisindolylmaleimide II), 2-[1-[3-aminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)maleimide, HCl (bisindolylmaleimide III), 2,3-bis(1*H*-indol-3-yl)maleimide (bisindolylmaleimide IV) and 2,3-bis(1*H*-indol-3-yl)-*N*-methylmaleimide (bisindolylmaleimide V), were obtained from Calbiochem, Nottingham, UK.

2.2. Practical procedures

Membranes were prepared from Chinese hamster ovary (CHO) cells stably expressing cDNA encoding human muscarinic M_1–M_4 receptors (generously provided by Dr. N.J. Buckley, University College London) as described (Lazareno et al., 1998). Aliquots of frozen membranes were thawed, resuspended in a buffer containing 20 mM HEPES + 100 mM NaCl + 10 mM $MgCl_2$ + 0.2 mM GTP (pH 7.4) and incubated with radioligand and unlabelled drugs for 2 h at 30°C in a volume of 1 ml. Membranes were collected by filtration over glass fibre filters (Whatman GF/B) presoaked in 0.1% polyethylenimine, using a Brandel cell harvester (Semat, Herts, UK), extracted overnight in scintillation fluid (ReadySafe, Beckman) and counted for radioactivity in Beckman LS6000 scintillation

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counters. Membrane protein concentrations (5–50 μg/ml) were adjusted so that not more than about 15% of added radioligand was bound. Nonspecific binding was measured in the presence of 1 μM quinuclidinyl benzilate (an antagonist with picomolar potency) and accounted for 1–5% of total binding. Compounds were dissolved in dimethyl sulfoxide which, at the highest final concentration of 1%, did not affect binding.

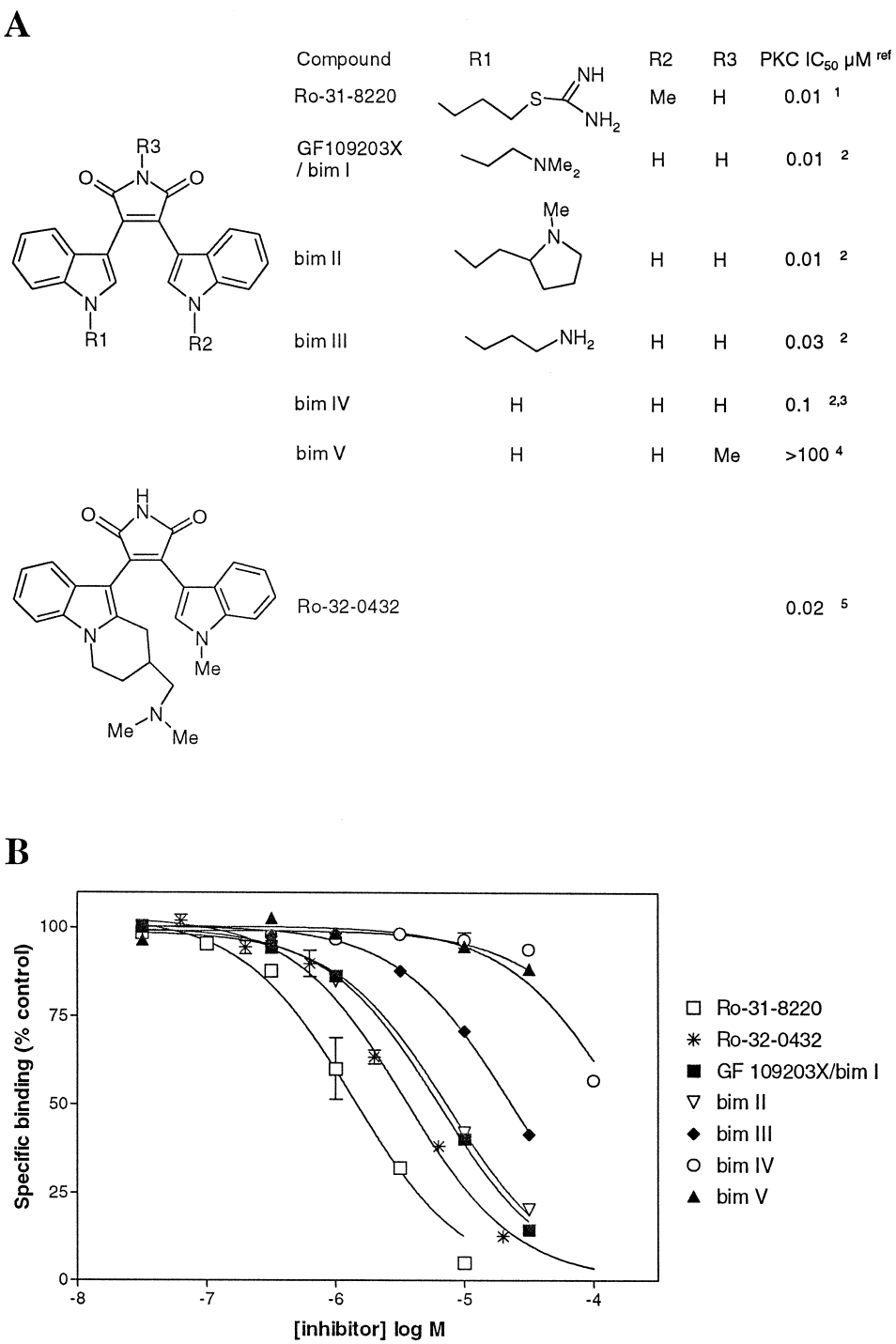

Fig. 1. (A) Structures and reported inhibitory potency at protein kinase C of bisindolylmaleimide (bim) analogues used in this study. References: (1) Davis et al., 1992a; (2) Toullec et al., 1991; (3) Davis et al., 1992b; (4) Sancelme et al., 1994; (5) Wilkinson et al., 1993. (B) Inhibition of specific [^{3}H]*N*-methyl scopolamine (0.25 nM) binding to M_1 receptors by bisindolylmaleimide (bim) analogues. The data are the mean and range of duplicate measures from single assays. The K_d of [^{3}H]*N*-methyl scopolamine at M_1 receptors, measured independently in each assay, was 0.11 ± 0.01 nM ($n = 3$). The lines show the fit to a logistic function with Hill slope fixed to 1. In general, across the receptor subtypes, inhibition curves for most of the compounds had Hill slopes of 1, but steeper slopes were seen with Ro-31-8220 (1.1–1.3) and bim IV (1.5–2.4).

Table 1
Apparent log affinity values of bisindolylmaleimide (bim) analogues at muscarinic receptors (log M^{-1})

Compound	M_1	M_2	M_3	M_4
Ro-31-8220	6.24 ± 0.12 (2)	5.90 ± 0.03 (2)	5.61 ± 0.06 (2)	5.82 ± 0.03 (2)
Ro-32-0432	5.90 ± 0.05 (2)	5.10 ± 0.06 (2)	5.02 ± 0.07 (2)	5.16 ± 0.06 (2)
GF 109203X[a]	5.75 ± 0.05 (3)	5.20 ± 0.03 (3)	5.09 ± 0.03 (3)	5.02 ± 0.04 (3)
bim II	5.73 ± 0.01 (2)	5.26 ± 0.02 (2)	5.16 ± 0.02 (2)	5.10 ± 0.02 (2)
bim III	5.14 ± 0.16 (3)	4.58 ± 0.07 (3)	4.43 ± 0.03 (3)	4.48 ± 0.06 (3)
bim IV	4.31 ± 0.12 (4)	4.00 ± 0.09 (2)	4.31 ± 0.29 (2)	4.36 ± 0.12 (2)
bim V	4.09 ± 0.11 (2)	4.16 ± 0.01 (2)	4.21 ± 0.05 (2)	4.12 ± 0.06 (2)

Affinity estimates were obtained from radioligand binding assays using [^{3}H]*N*-methyl scopolamine and membranes from CHO cells stably expressing human muscarinic receptors. The data are the mean ± S.E. of (n) observations, or mean ± range/2 when $n = 2$.
[a] Pooled data from GF 109203X (bisindolylmaleimide I) and bisindolylmaleimide I HCl, a more soluble form of the compound.

2.3. Experimental design and analysis

Binding of a low concentration of [^{3}H]*N*-methyl scopolamine (0.1–0.3 nM) was measured alone and in the presence of six concentrations of test agent. Binding of a high concentration of [^{3}H]*N*-methyl scopolamine (2–4 nM) was also measured, as well as nonspecific binding with both [^{3}H]*N*-methyl scopolamine concentrations. All points were measured in duplicate. The K_d of [^{3}H]*N*-methyl scopolamine was estimated within each assay by inserting the values of specific binding (B_1 and B_2) obtained with low and high concentrations of [^{3}H]*N*-methyl scopolamine alone ([L_1] and [L_2], respectively) into the equation:

$$K_d = \frac{(B_2 - B_1)[L_1][L_2]}{B_1[L_2] - B_2[L_1]}$$

Concentrations of test agent which inhibited specific [^{3}H]*N*-methyl scopolamine binding by 50% (IC_{50} values) were obtained by fitting specific binding data to a one-site binding model using the nonlinear regression facility of Prism (Graphpad, San Diego, CA, USA). Estimates of the log affinity of the test agent were obtained from the values of K_d, [L_1] and IC_{50} using the equation

$$\log \text{affinity} = -\log\left(\frac{IC_{50}}{1 + [L_1]/K_d}\right)$$

3. Results

Fig. 1A shows the structures and reported inhibitory potency at protein kinase C of the compounds used in this study. All the compounds inhibited [^{3}H]*N*-methyl scopolamine binding. Fig. 1B shows representative data for M_1 receptors and Table 1 shows the log affinity (pK_i) of the compounds at M_1–M_4 receptors. Bisindolylmaleimide IV and bisindolylmaleimide V were very weak and inhibited [^{3}H]*N*-methyl scopolamine binding only at the highest concentration used. The compounds containing a sidechain were more potent and showed the same pattern of affinities across the receptor subtypes, with the highest affinity at M_1 receptors, about 2-fold weaker at M_2 receptors, and a further 2–3-fold weaker at M_3 and/or M_4 receptors. The highest log affinity was seen with Ro-31-8220 at M_1 receptors (6.24, corresponding to a K_d of 575 nM). Ro-32-0432, GF 109203X and bisindolylmaleimide II were 2–4-fold weaker than Ro-31-8220, and bisindolylmaleimide III was about 4-fold weaker still.

4. Discussion

Our results confirm the report of Willars et al. (1996) that Ro-31-8220 has significant affinity for the M_3 receptor, but the compound is about 6-fold more potent in our study. This discrepancy probably reflects the different experimental conditions: the above studies were conducted with membrane preparations while the study of Willars et al. measured binding to intact cells.

Except for bisindolylmaleimide V, which is used as a negative control in studies of protein kinase C, the bisindolylmaleimide analogues studied here have been reported to have potencies of 10–100 nM for inhibiting isoforms of protein kinase C (Toullec et al., 1991; Davis et al., 1992a,b; Wilkinson et al., 1993; Sancelme et al., 1994). In many studies, however, much higher concentrations are needed to overcome the effects of protein kinase C activators and/or high levels of ATP (e.g., McKenna and Hanson, 1993), and the use of 10 μM Ro-31-8220 in studies of muscarinic function is not uncommon (e.g., Chung and Fleming, 1995; Purkiss, 1995; Willars et al., 1996; Shariot-Madar et al., 1997). As Willars et al. (1996) have pointed out, this concentration of Ro-31-8220 may not interact with muscarinic receptors in the presence of a high concentration of agonist, but probably will in the presence of a low, or zero, concentration of agonist. Ro-31-8220 may also block nicotinic receptors (Marley and Thomson, 1996).

Of the bisindolylmaleimide analogues we have studied, those containing a sidechain block muscarinic receptors with apparent K_d values in the μM–10 μM range and with a small selectivity for the M_1 subtype. The most

potent effects were seen with Ro-31-8220 which had a K_d of 0.6 μM at M_1 receptors. Analogues without a sidechain (bisindolylmaleimide IV and bisindolylmaleimide V) were very weak inhibitors of [^{3}H]*N*-methyl scopolamine binding, and would seem to be the most suitable of these analogues for use with studies of muscarinic receptor function.

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