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SPECIAL REPORT

Characterization of two new ET_B selective radioligands, [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 in human heart

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Two new endothelin receptor radioligands, [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1, were characterized in tissue sections of human right atrium and left ventricle. Both radioligands had high affinity ([^{125}I]-BQ3020 right atrium: $K_D = 0.145 \pm 0.037$ nM, left ventricle: $K_D = 0.107 \pm 0.004$ nM; [^{125}I]-[Ala^{1,3,11,15}]ET-1 right atrium: $K_D = 0.239 \pm 0.036$ nM, left ventricle: $K_D = 0.199 \pm 0.027$ nM). Competition binding experiments were performed in the left ventricle. The selective ET_A receptor compound BQ123 competed with low affinity against [^{125}I]-BQ3020 ($K_D = 28.7 \pm 2.7$ μM) and [^{125}I]-[Ala^{1,3,11,15}]ET-1 ($K_D = 28.5 \pm 4.2$ μM). The selective ET_B receptor compound BQ3020 competed with high affinity against [^{125}I]-BQ3020 ($K_D = 40.8 \pm 6.6$ pM) and [^{125}I]-[Ala^{1,3,11,15}]ET-1 ($K_D = 0.276 \pm 0.099$ nM). Another selective ET_B receptor compound, [Ala^{1,3,11,15}]ET-1 also competed with high affinity against [^{125}I]-BQ3020 ($K_D = 0.663 \pm 0.120$ nM) and [^{125}I]-[Ala^{1,3,11,15}]ET-1 ($K_D = 0.643 \pm 0.124$ nM). These results indicate that [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 are selective ET_B receptor radioligands. [Ala^{1,3,11,15}]ET-1 competed with the non-selective radioligand [^{125}I]-ET-1 in left ventricle and revealed the presence of ET_A and ET_B receptors in the proportions of 76:24% respectively in the human left ventricle.

Keywords: Endothelin; BQ3020, [Ala^{1,3,11,15}]ET-1; BQ123; [^{125}I]-BQ3020; [^{125}I]-[Ala^{1,3,11,15}]ET-1; [^{125}I]-ET-1; ET_A receptor; ET_B receptor; human heart

Introduction Endothelin-1 produces a number of important effects in the heart including positive inotropic and chronotropic responses (Davenport *et al.*, 1989; Reid *et al.*, 1989), stimulation of myocyte hypertrophy and renin release (Shubeita *et al.*, 1990). These effects may be produced by one or more endothelin receptors; two subtypes, ET_A and ET_B have been cloned from cDNA libraries (Sakurai *et al.*, 1992). Recently three highly selective ET_A and ET_B receptor compounds have become available, BQ123 cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp-] (ET_A, Ihara *et al.*, 1992; Nakamichi *et al.*, 1992), [Ala^{1,3,11,15}]ET-1 (ET_B, Saeki *et al.*, 1991) and BQ3020 [Ala^{11,15}]Ac-ET-1(6-21) (ET_B). These compounds were used to characterize two new radioligands, [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 in human heart.

Methods Human right atrium and left ventricular free wall were obtained from recipient patients undergoing cardiac transplantation at the Papworth Everard Hospital. Four hearts in total were used, three with ischaemic heart disease and one with the Eisenmengers syndrome. Cardiac tissue was snap frozen in liquid nitrogen and stored at − 70°C until use. Tissues were mounted in O.C.T. compound and sections cut (10 μm) and mounted onto gelatin/chromic potassium sulphate coated microscope slides. Slide-mounted tissue sections (10 μm) were incubated with (3-[^{125}I]iodotyrosyl^{13})-endothelin (ET)-1, (3-[^{125}I]iodotyrosyl^{13})-[Ala^{11,15}]Ac-ET-1(6-21) or (3-[^{125}I]iodotyrosyl^{13})-[Ala^{1,3,11,15}]ET-1 (Amersham International plc, U.K.) in the absence or presence of competing agents for 120 min, except for association experiments where increasing time periods (0–240 min) were used at 22°C as previously described for [^{125}I]-ET-1 (Davenport *et al.*, 1989). Non-specific binding was determined with the corresponding unlabelled ligand (1 μM). Sections were rinsed in Tris-HCl (0.05 M), pH 7.4, 4°C (3 × 5 min) and counted in a gamma counter. Association binding data were analysed with REAP (Gamma Research Systems, Knoxfield, Australia), saturation and competition binding data were analysed with EBDA (McPherson, 1983) and LIGAND (Munson & Rodbard, 1980). Data files were run simultaneously with LIGAND to obtain final parameter estimates. The presence of 1 or 2 sites was tested using the *F*-ratio test in LIGAND. The model adopted was that which provided the significantly best fit ($P < 0.05$).

Drugs BQ3020 and [Ala^{1,3,11,15}]ET-1 were synthesized by solid phase *t*-Boc chemistry, purified by gel filtration and the sequences confirmed by amino acid analysis. Peptide concentration was determined by u.v. spectrophotometry. BQ123 was a gift from Parke-Davis Pharmaceutical Division, Ann Arbor, Michigan, U.S.A.

Results [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 binding to sections of human left ventricle was time-dependent and reached equilibrium after 120 min at 18°C with observed association rate constants of 0.0245 ± 0.0003 min^{-1} and 0.0205 ± 0.0002 min^{-1} respectively.

[^{125}I]-BQ3020 (2 pM–8 nM) and [^{125}I]-[Ala^{1,3,11,15}]ET-1 (6 pM–7 nM) binding to sections of human right atrium and left ventricle was concentration-dependent. Non-specific binding for [^{125}I]-BQ3020 ranged from 24–52% at 2 pM, 19–30% at 0.15 nM and 53–93% at 8 nM and for [^{125}I]-[Ala^{1,3,11,15}]ET-1, 21–62% at 6 pM, 13–22% at 0.3 nM and 70–74% at 7 nM. The Hill coefficients for both radioligands were less than unity (Table 1) suggesting the presence of multiple binding sites. Analysis of binding over the lower concentrations ([^{125}I]-BQ3020 2 pM–0.6 nM; [^{125}I]-[Ala^{1,3,11,15}]-ET-1 6 pM–1.5 nM) resulted in Hill coefficients close to unity. Analysis with LIGAND revealed one binding site for both radioligands (Table 1). A two-binding site model was tested for each radioligand but was not preferred to a one-site model ($P > 0.05$).

[^{125}I]-BQ3020 (0.1–0.2 nM) and [^{125}I]-[Ala^{1,3,11,15}]ET-1 (0.1–0.7 nM) were used in competition binding experiments

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Table 1 Saturation binding analysis for endothelin receptor radioligands in human right atrium and left ventricle

	n	*nH*	K_D	B_{max}
[^{125}I]-BQ3020	RA 3	0.46 ± 0.14	0.145 ± 0.037	25.7 ± 6.8
	LV 3	0.43 ± 0.06	0.107 ± 0.004	18.9 ± 4.8
[^{125}I]-[Ala^{1,3,11,15}]ET-1	RA 3	0.77 ± 0.01	0.239 ± 0.036	22.2 ± 3.4
	LV 3	0.72 ± 0.03	0.199 ± 0.027	12.4 ± 2.2

Hill coefficients (nH), dissociation constants (K_D, nM) and maximal density of receptors (B_{max}, fmol mg^{-1} protein) for [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 in human right atrial (RA) and left ventricular (LV) sections. Values are mean ± s.e.mean from *n* experiments.

against ET_A and ET_B selective compounds in human left ventricle. These concentrations were selected as they provide a clear signal with a high proportion of specific binding to total binding. These concentrations also lie within the range which provided Hill coefficients close to unity in saturation binding experiments. The ET_B-selective compound, BQ3020, competed with [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 with high affinity. Another ET_B-selective compound, [Ala^{1,3,11,15}]ET-1, also competed with [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 with high affinity (Table 2). The ET_A-selective compound, BQ123, competed with low affinity for both radioligands in human left ventricle (Table 2). BQ123 (100 μM) produced 55 ± 2% and 78 ± 2% inhibition of [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 binding.

[Ala^{1,3,11,15}]ET-1 was investigated further in human left ventricle. It competed with the non-selective radioligand, [^{125}I]-ET-1, and revealed the presence of a high affinity binding site corresponding to the ET_B receptor and a lower affinity binding site corresponding to the ET_A receptor in the proportions of 24% (ET_B) and 76% (ET_A, Table 2).

Discussion The new endothelin receptor radioligands, [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1, selectively label ET_B receptors with high affinity. The results of this study suggest that [Ala^{1,3,11,15}]ET-1 is 2500 fold selective for the ET_B receptor. Similar studies (Molenaar *et al.*, unpublished observations) indicate that BQ3020 is approximately 1500 fold selective for the ET_B receptor and BQ123 is 33000 fold selective for the ET_A receptor.

Hill coefficients for [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 in saturation binding experiments were less than unity suggesting the presence of another binding site at higher concentrations. This site is unlikely to be an ET_A receptor in view of the affinities of [Ala^{1,3,11,15}]ET-1 (4.52 μM) and BQ3020 (2.04 μM, Molenaar *et al.*, unpublished observations) for the ET_A receptor. Higher concentrations of [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 than those used in the present study will be needed to characterize fully the low affinity site. The concentrations of [^{125}I]-BQ3020 (0.1–0.2 nM) and [^{125}I]-[Ala^{1,3,11,15}]ET-1 (0.1–0.7 nM) used in competition binding experiments were considered optimal for labelling the ET_B receptor in human cardiac sections. Lower concentrations result in higher levels of non-specific binding and a low 'signal' while higher concentrations also result in an increase in non-specific binding and multiple receptor binding. In other studies using rat cerebellum which has a higher proportion of ET_B receptors (> 85%, Davenport *et al.*, unpublished observations) Hill coefficients for both radioligands are close to unity. The shallow competition binding curves produced by the ET_A-selective antagonist ligand, BQ123, against [^{125}I]-BQ3020 and [^{125}I]-[Ala^{1,3,11,15}]ET-1 remain to be explained but may indicate heterogeneity of binding sites.

In the human left ventricular tissue studied both ET_A and ET_B receptors were detected in the proportions of 76%:24% respectively. Although hearts of two pathological conditions were used in the present study no differences were detected in the radioligand binding assays.

Human heart comprises myocytes, specialized pacemaker and conducting regions, blood vessels, neuronal, connective and adipose tissue; however, the precise anatomical localization of endothelin receptor subtypes is unclear at the present time. The availability of highly selective radioligands and compounds will assist in the determination of the function, cellular localization and quantitation of these receptor subtypes.

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Table 2 Competition binding data analysis between endothelin receptor radioligands and selective competing agents

	n	*nH*	K_D			
[^{125}I]-BQ3020						
BQ3020	3	0.97 ± 0.18	40.8 ± 6.6 pM			
[Ala^{1,3,11,15}]ET-1	3	0.91 ± 0.03	0.66 ± 0.120 nM			
BQ123	3	0.53 ± 0.08	28.7 ± 2.7 μM			
[^{125}I][Ala^{1,3,11,15}]ET-1						
BQ3020	3	0.92 ± 0.15	0.276 ± 0.099 nM			
[Ala^{1,3,11,15}] ET-1	3	0.92 ± 0.06	0.643 ± 0.124 nM			
BQ123	3	0.62 ± 0.01	28.5 ± 4.2 μM			
[^{125}I]-ET-1						
	n	*nH*	$K_D ET_B$	$K_D ET_A$	$\%ET_B$	$\%ET_A$
[Ala^{1,3,11,15}]ET-1	4	0.45 ± 0.04	1.82 ± 1.35 nM	4.52 ± 0.49 μM	24 ± 3	76 ± 3

Pseudo Hill coefficient values (nH) and dissociation constant values (K_D) for BQ3020 and BQ123 and [Ala^{1,3,11,15}]ET-1 at endothelin-1 receptors in human left ventricle. Shown also is the percentage of ET_A and ET_B receptors determined with [Ala^{1,3,11,15}]ET-1. Values are mean ± s.e.mean from *n* experiments.

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