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SCIENCE DIRECT
Bioorganic & Medicinal Chemistry
Bioorganic & Medicinal Chemistry 14 (2006) 1910–1917

Synthesis, in vitro pharmacology and biodistribution studies of new PD 156707-derived ET_A receptor radioligands

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Received 12 January 2005; accepted 16 August 2005
Available online 10 November 2005

Abstract—It is assumed that the regulation of cardiac endothelin (ET) receptor density is abnormal in heart diseases. From that perspective, an ET receptor radioligand is needed to assess ET receptor density in vivo. The nonpeptidyl ET_A receptor antagonist PD 169390 was labelled with radioiodine to give a putative radioligand for SPECT. Labelling with [^{125}I]iodide and [^{123}I]iodide was accomplished with good to excellent radiochemical yields. The affinities of the nonradioactive reference and those of selected precursor compounds for ET_A receptors were determined, using [^{125}I]iodine labelled endothelin-1 with mouse ventricular membranes. All employed substances exhibited potent in vitro pharmacological characteristics with K_i values comparable to that of the lead compound PD 156707. Biodistribution studies and scintigraphic imaging experiments in mice, however, showed no significant uptake of the [^{123}I] derivative in the heart.

1. Introduction

Since endothelin (ET) was first described by Hickey et al.[1] and subsequently isolated by Yanagisawa et al.[2] as a 21-amino acid peptide with vasoactive potential, the role of the endothelins in several diseases, including atherosclerosis, congestive heart failure and pulmonary hypertension, has been increasingly investigated.[3–7] At least three isoforms of endothelin exist (ET-1, ET-2 and ET-3), exerting their effects via two different G-protein coupled receptors (ET_A, ET_B). ET_A receptors are primarily located on vascular smooth muscle cells and are responsible for vasoconstriction and cell proliferation, whereas ET_B receptors are located on smooth muscle cells and vascular endothelial cells, cause vasodilation by the release of nitric oxide and prostacyclin and are responsible for the clearance of ET-1 from plasma.[8–13] The affinity of ET-1 and ET-2 to the ET_A receptor is about a 100-fold higher than the affinity of ET-3. The affinity to the ET_B receptor, however, is equal for all three isoforms.[14–17] All native endothelins, therefore, represent nonselective ET receptor substrates.

Plasma levels of ET-1 are elevated in many cardiovascular diseases. In this connection, it has been shown that ET receptor distribution is abnormally regulated in diseased human tissue.[18a,b] In mouse models of human atherosclerosis (i.e., ApoE$^{-/-}$) ET_A receptor density has been found to be upregulated in atherosclerotic plaques.[19a,b] To overcome the unwanted effects of an abnormally regulated ET system, ET receptor antagonists are used in the treatment of these diseases, and a number of different peptidyl and nonpeptidyl ligands, both selective and nonselective, have been developed in attempts to improve efficacy.[20–27] In addition to its role in cardiovascular diseases, ET-1 is also an important factor in the pathophysiology of certain human cancers, especially prostate cancer,[28] promoting tumour growth by inducing cell proliferation and angiogenesis and inhibiting apoptosis.[29,30]

A noninvasive method for the visualisation of ET receptor density in affected tissue would be invaluable for clinical diagnosis and the evaluation of therapy. The

Keywords: Radioiodination; Endothelin receptor antagonists; SPECT; Biodistribution studies.
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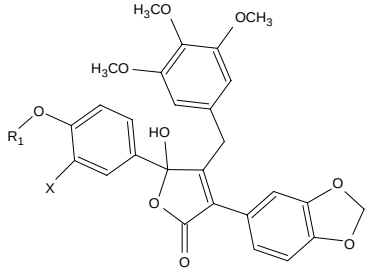
doi:10.1016/j.bmc.2005.10.039

methods of choice for imaging receptor distribution in vivo are positron emission tomography (PET) and single photon emission computed tomography (SPECT).[31–33] Radiolabelled ET receptor antagonists could be used to assess ET receptor density with these techniques. The radiolabelling of ET receptor agonists and antagonists in connection with in vivo studies has been reported.[34–42] Very recently, Johnstroem et al.[40b] have shown the importance of ET_B receptors for the clearance of ET-1 from circulation using [^{18}F]ET-1 for PET imaging in rats. Aleksic et al.[42a] have used [^{11}C]L-753037, a 1,3-bisarylindane-2-carboxylic acid mixed ET_A/ET_B antagonist, for the imaging of ET receptors in a dog heart in vivo. Johnstroem et al.[42b] have [^{18}F]fluorinated another representative of this class of compounds (SB-209670) and imaged ET receptor distribution in rats. Furthermore, the here described lead compound PD 156707 has been synthesised as a [^{11}C]methoxy analogue, but no imaging experiments were performed.[39] Interestingly, none of these approaches have been utilised for the in vivo imaging of ET receptors in humans so far, so there is still a strong motivation for research towards new ET receptor radioligands for use in the human body.

PD 156707 **3** (3-benzo[1,3]dioxol-5-yl-5-hydroxy-5-(4-methoxyphenyl)-4-(3,4,5-trimethoxybenzyl)-5*H*-furan-2-one, Scheme 1) and related butenolide derivatives are potent ET receptor antagonists with high affinity and ET_A selectivity.[43–48] In this study, we describe the radioiodination of PD 169390 **5** (Scheme 1), the 4-hydroxyphenyl-analogue of **3**. The structure of **5** has been described but its synthesis has not been reported.[39] [^{125}I]NaI was used to develop the radiosynthesis, yielding derivative **7**. A related compound, [^{125}I]PD 164333, with an *ortho*-radioiodinated hydroxyphenyl group attached via a small amide-linker to a butenolide based structure, was synthesised by Davenport et al. and used for in vitro determination of ET receptors in human tissue.[35] Subsequently the ^{123}I-derivative **8** was prepared for biodistribution studies and initial scintigraphic imaging experiments using wild type mice. The affinities of the nonradioactive 4-hydroxy-3-iodo-derivative **6**, the benzyloxy-precursor **4** and PD 169390 **5** to ET receptors were evaluated. Compound **6** is the corresponding nonradioactive counterpart of the radioiodinated and structurally identical target radioligands **7** and **8** and is used for the evaluation of its binding potency to ET receptors in vitro. PD 156707 **3** was also synthesised and used for comparison of the affinity values.



R_1= CH_3, X = H	**3** (PD 156707)
R_1 = $CH_2C_6H_5$, X = H	**4**
R_1 = H, X = H	**5** (PD 169390)
R_1 = H, X = I	**6**
R_1 = H, X = ^{125}I	**7**
R_1 = H, X = ^{123}I	**8**

Scheme 1. Lead structure PD 156707 **3** and its derivatives.

2. Results and discussion

2.1. Chemistry

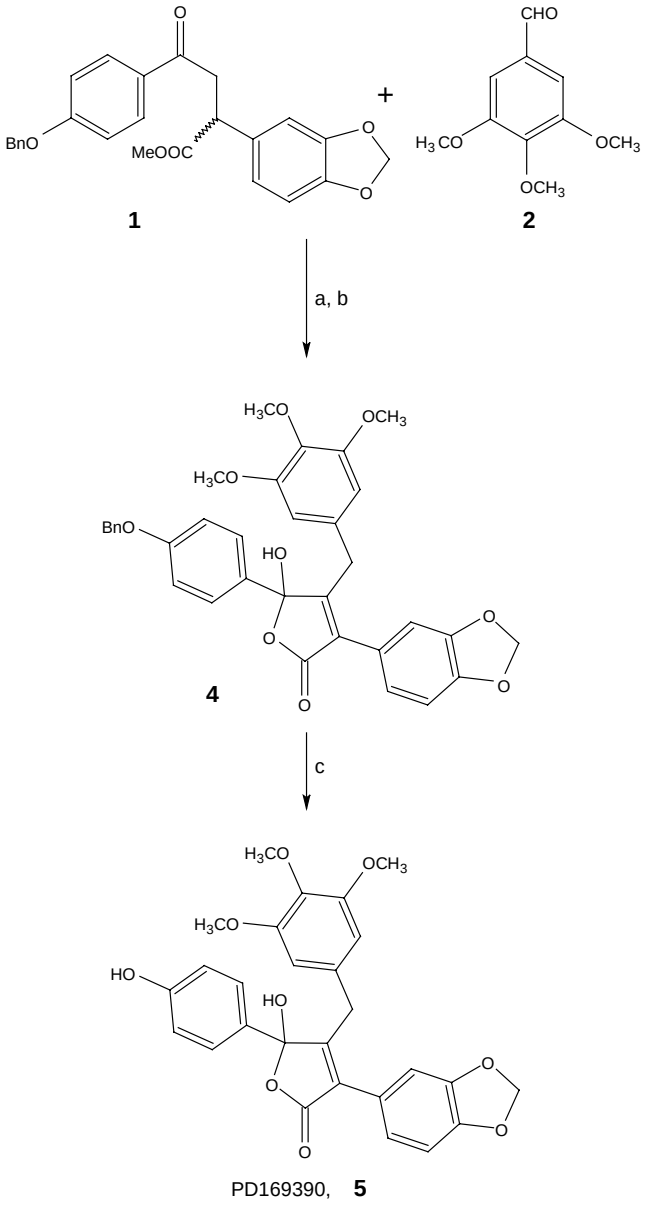
PD 156707 **3** (Scheme 1) and related butenolide derivatives are known to be potent ET_A receptor antagonists.[43–48] To use butenolide-derived structures for the development of a radioiodinated ET_A ligand for SPECT, we used the 4-hydroxy-derivative PD 169390 **5** as a precursor for radioiodination. The synthesis of PD 169390 **5** was accomplished via a route using a benzyloxy-protecting group, starting from keto-ester **1** and 3,4,5-trimethoxybenzaldehyde **2** (Scheme 2). Deprotection of the benzyloxy butenolide **4** was achieved by hydrogenolysis in alcoholic solution, resulting in precursor PD 169390 **5** in moderate yield (39%). Hydrogenation of the double-bond of the butenolide five-membered ring occurred as a side reaction. PD 169390 **5** was iodinated with iodine and ammonia in alcoholic solution, yielding the monoiodinated compound **6** as a nonradioactive reference for the target radioligands **7** and **8** (Scheme 3). The method described below for the *ortho*-radioiodination resulting in compounds **7** and **8** could not be transferred to this reaction. Because of longer reaction times needed for the complete conversion of the reactants to give compound **6**, unwanted byproducts resulted. To be able to compare the receptor binding potencies of the newly developed compounds, PD 156707 **3** was synthesised as a reference, using literature procedures.[45]

2.2. Radiochemistry

The radioiodination of PD 169390 **5** was performed using chloroamine T as oxidant and [^{125}I]NaI or [^{123}I]NaI (Scheme 3). The radiolabelled products were purified by reversed-phase radio-HPLC to yield the compounds **7** or **8** with radiochemical yields between 70% and 90%. The radiochemical purities of the compounds were >99%, with specific radioactivities of 121.7 MBq/μg (75.0 GBq/μmol) in case of the ^{125}I-derivative **7** and 14.04 GBq/μg (8.63 TBq/μmol) for the ^{123}I-derivative **8** at the end of synthesis (EOS). The iodinated nonradioactive analogue **6** was used to verify the chemical identities of the radiolabelled compounds **7** and **8** ($t_{R,(6/7/8)}$ = 21.5 min).

2.3. Biology

2.3.1. In vitro assays. The affinities of the prepared butenolide derivatives **3**–**6** towards endothelin receptors

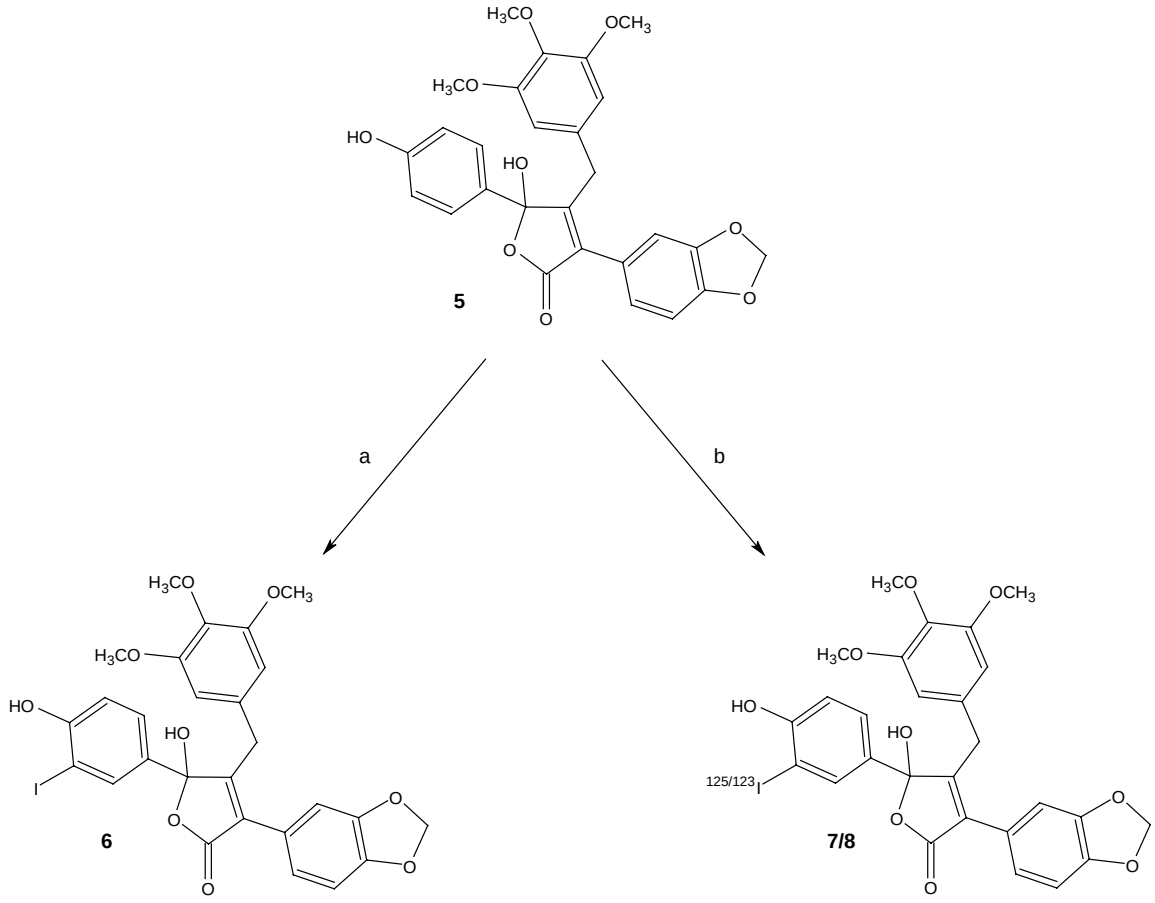


Scheme 2. Synthesis of PD 169390 via the benzyloxy derivative. Reagents: (a) NaOMe, methanol; (b) HOAc, methanol; (c) H_2/Pd/C, ethanol/isopropanol 2:1.

were determined by competition binding studies using [^{125}I]iodine labelled endothelin-1 ([^{125}I]ET-1) (Perkin-Elmer Live Sciences Inc., Billerica, MA, USA) and mouse ventricular membrane preparations (Fig. 1). The binding of the nonselective ET receptor antagonist [^{125}I]ET-1 to ventricular membranes was specific, saturable and of high affinity. Scatchard[46,47] transformation of the saturation data yielded values for the dissociation constant (K_D = 208 ± 2 pM) and the maximum number of binding sites (B_{max} = 300 ± 3 fmol/mg protein). Different concentrations of the prepared compounds (1.0 pM–100 μM) were incubated with 40 pM [^{125}I]ET-1 and a fixed amount of mouse ventricular membrane preparation (10 μg) at 37 °C for 4 h. The membrane bound radioactivity was evaluated using a γ-scintillation counter. Nonlinear regression analysis showed that the data of the compounds fitted a two-site model. All compounds were found to have a significantly higher affinity to ET_A than to ET_B receptors (Table 1). The high- and low-affinity IC_{50} values were converted into the high- and low-affinity inhibition constants ($K_{i/ETA}$ and $K_{i/ETB}$) by the method of Cheng–Prusoff[47,48] using the experimentally determined K_D value of [^{125}I]ET-1 (208 ± 2 pM). The ratios of the low- to high-affinity inhibition constants ($K_{i/ETB}/K_{i/ETA}$) yield the ET_A selectivities of the derivatives **3**–**6** (Table 1). Additionally, the calculated log *P* values (LogD Suite, ACD-software) for compounds **3**–**6** are also listed in Table 1 to indicate the changes in lipophilicities caused by the chemical modifications of the lead compound PD 156707.

Recent structure–activity relationship (SAR)studies[45,49–51] have shown that at least two aromatic moieties surrounding a carboxylic group are necessary for ET receptor binding, although most ligands, like the lead compound **3**, contain a third aromatic group. The investigation of substitution patterns of some butenolide derivatives[45] indicates that additional increase of the steric bulk on these aromatic moieties leads to a decrease in receptor affinity. As predicted, the benzyloxy derivative **4** shows the poorest affinity to the ET_A receptor ($K_{i/ETA}$ = 32.3 ± 22.1 nM) and also the poorest ET_A selectivity ($K_{i/ETA}/K_{i/ETB}$ = 141 ± 32). The affinity values of the compounds **3**, **5** and **6** to the ET_A receptor all lie in the subnanomolar range, whereas the affinity to the ET_B receptors is only micromolar. Compound **5**, as an exception, also shows nanomolar affinity to the ET_B receptor, probably due to the polar phenolic hydroxy group, although selectivity is still >500. The nonradioactive reference compound **6** shows K_i values of ≈1 nM for the ET_A receptor and ≈1.25 μM for the ET_B receptor, respectively. Therefore, with the corresponding highly affine and selective ET_A receptor radioligand **8** in hand, first in vivo evaluation studies were performed.

2.3.2. Biodistribution studies. The ^{123}I-radioiodinated compound **8** was chosen for biodistribution studies and scintigraphic imaging experiments in BL6 mice. With the exception of the liver, uptake indices for the tissues dissected were lower than those for plasma at all time points (5, 10, 20, 30, 40 and 130 min). Values for the liver were comparable to those for plasma. Uptake indices decreased with time after injection, for example indices for myocardium and liver were 0.19–0.46 and 0.9–1.5, respectively, at 20–30 min and fell to 0.07–0.11 and 0.3–0.4 at 130 min. Predosing with PD 156707 had no effect (Fig. 2). Radioactivity accumulated in the urine which showed uptake indices between 5 and 8 at 20–30 min and 12 and 20 at 130 min. Scintigraphic imaging confirmed uptake of radioactivity in the liver and bladder but the heart was not visualised (Fig. 3). Radioactivity in the three regions of interest, chest, liver and bladder, are shown in Figure 3. Predosing with PD 156707 had no effect on tissue radioactivity assessed either ex vivo (Fig. 2) or by scintigraphic imaging (Fig. 3). Radioactivity accumulated in the urine and HPLC analysis of urine taken from one mouse at 5 min after injection of **8** (data not shown) predominantly showed a radioactive polar fraction and, to a lesser extent, the parent radioligand (ratio ~ 9:1).



Scheme 3. Iodination of PD 169390 **5**. Reagents: (a) I_2, NH_3, ethanol; (b) [$^{123/125}$I]NaI n.c.a., chloroamine T, K_2HPO_4-buffer, water, methanol.

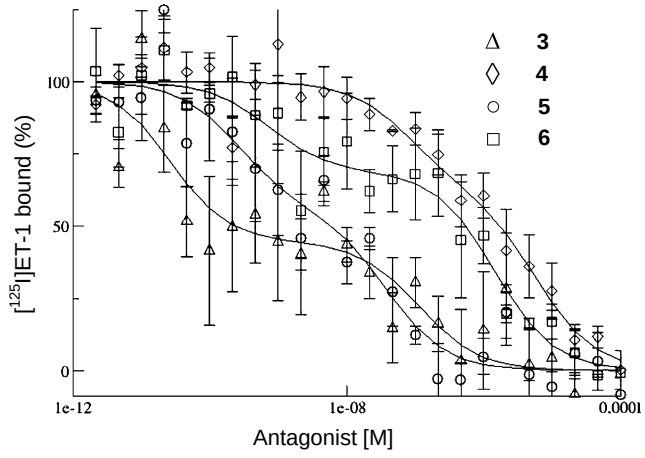


Figure 1. Competition of [^{125}I]ET-1 binding. Mouse ventricular membranes were incubated with 40 pM [^{125}I]ET-1. Membrane bound radioactivity is expressed as percentage of [^{125}I]ET-1 binding. Lines for best fit (nonlinear regression analysis) are shown.

Table 1. Receptor affinities and calculated log *P* values (LogD Suite, ACD-software) of prepared compounds

Compound	$K_{i/ETA}$ (nM)	$K_{i/ETB}$ (μM)	Selectivity	log P^a
3	0.04 ± 0.02	4.5 ± 3.1	>50,000	3.30
4	32.3 ± 22.1	4.6 ± 3.1	140 ± 32	4.95
5	0.11 ± 0.09	0.06 ± 0.05	780 ± 170	2.65
6	0.97 ± 0.13	1.3 ± 0.2	1300 ± 43	3.81

ET receptor affinities were determined by competition binding studies using [^{125}I]ET-1 and mouse ventricular membrane preparations. K_i values were calculated by nonlinear regression analysis using the *XMGRACE* program (Linux software), given as means ± SEM. The ratios of the low- over the high-affinity inhibition constants ($K_{i/ETA}/K_{i/ETB}$) yield the ET_A selectivities of the derivatives, given as means ± SEM. Note the differences in concentration dimensions in $K_{i/ETA}$ and $K_{i/ETB}$.

[a] Calculated by LogD Suite program (ACD-software).

3. Conclusion

The endothelin receptor antagonist 3-benzo[1,3]dioxol-5-yl-5-hydroxy-5-(4-hydroxy-phenyl)-4-(3,4,5-trimethoxybenzyl)-5*H*-furan-2-one (PD 169390) was radiolabelled with [^{125}I]iodide and [^{123}I]iodide, respectively. The radioligands were prepared in good to excellent radiochemical yields. The radiochemical purities were >99% with specific radioactivities of 121.7 MBq/μg (75.0 GBq/μmol) for the ^{125}I-derivative and 14.04 GBq/μg (8.63 TBq/μmol) for the ^{123}I-compound (EOS). The affinities of the nonradioactive reference as well as those of selected precursor compounds towards ET receptors were determined. Inhibition constants were all in the nanomolar range and are comparable to that of the known ET_A receptor antagonist PD 156707. The affinity and selectivity of the nonradioactive ligand **6** to ET_A receptors evidence the potency of this compound. The derived radioligands **7** and **8**, therefore, are promising

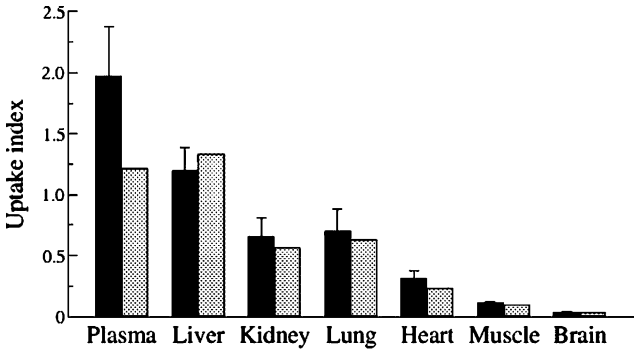


Figure 2. Radioactivity in tissues 20–30 min after iv injection of **8** given alone (■, 4 mice) or 5 min after iv injection of 1 μmol kg^{-1} **3** (▨, 1 mouse). Bars indicate standard errors.

tools for in vitro and in vivo studies. First biodistribution studies with the ^{123}I-derivative **8** in mice, however, showed no significant uptake of the ^{123}I-labelled compound in the heart after different periods of time. With the exception of the liver, uptake indices for the tissues dissected were lower than those for plasma. Altogether, scintigraphic imaging confirmed uptake of radioactivity in the liver and bladder but the heart was not visualised. Predosing with PD 156707 had no significant effect. The radioligand supposably metabolises in vivo very rapidly without approaching the target receptors. The use of the ^{125}I-radioligand **7** as a substitute for [^{3}H]BQ-123 as a ET_A selective radioligand for in vitro studies is currently under investigation. Also, work is in progress to design more stable derivatives of PD 156707 and use these compounds for further in vivo studies with the aim to develop a suitable radioligand for human in vivo imaging.

4. Experimental

4.1. General

All chemicals, reagents and solvents for the synthesis of the compounds were of analytical grade and purchased from commercial sources. PD 156707 **3** and 2-(benzo-[1,3]-dioxol-5-yl)-1-(4-benzyloxyphenyl)-4-oxobutyric acid methyl ester **1** were synthesised as reported.[45] Melting points (uncorrected) were determined with a Stuart Scientific SMP3 capillary melting point apparatus. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker ARX 300, and AMX 400 spectrometer, respectively. Mass spectrometry was performed using a Varian MAT 212 (EI = 70 eV) spectrometer and a Bruker MALDI-TOF-MS Reflex IV (matrix: DHB). Elemental analysis was realised by a Vario EL III analyser. Separation of the radiosynthesised compounds, analyses of radiochemical yields and radiochemical purities were performed by gradient radio-RP-HPLC with a RP-HPLC column Nucleosil 100 C-18 5 μ $250 \times 4.6\ mm^2$, a corresponding $20 \times 4.6\ mm^2$ precolumn, a Knauer K-500 and a Latek P 402 pump, a Knauer K-2000 UV-detector (wavelength 254 nm) and a Crismatec Na(Tl) Scintibloc 51 SP51 γ-detector. Sample injection was carried out using a Rheodyne injector block (type 7125 incl. 200 μl loop). The

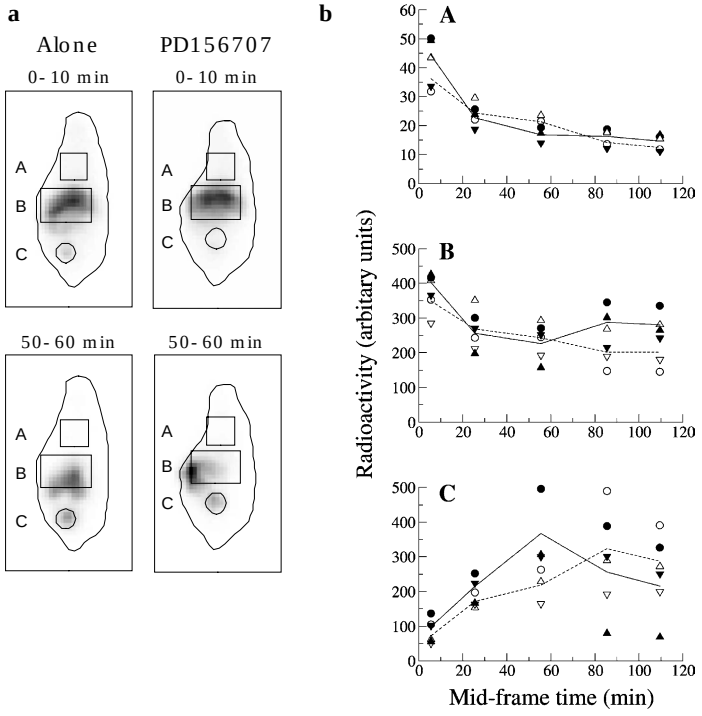


Figure 3. (a) Representative scintigraphic images of two mice after retro-orbital injection of **8** given alone or 30 min after retro-orbital injection of **3** at 1 μmol kg^{-1}. Two time frames (1–10 min and 51–60 min) are shown. Regions of interest were drawn over the chest (A), liver (B) and bladder (C) in the early images for each of 6 mice (3 mice with **8** alone, 3 mice with **8** after administration of unlabelled **3**) and radioactivity in each region for each of 5 time frames was calculated using in-house software. (b) The uptake of radioactivity (arbitrary units) is shown in the right-hand panel; solid symbols, **7** alone (● mouse 1, ▲ mouse 2 and ▼ mouse 3): open symbols, **8** after administration of unlabelled **3** (○ mouse 4, △ mouse 5 and ▽ mouse 6). Lines are drawn between mean values; solid line, **8** alone; dashed line, **8** after administration of unlabelled **3**. Note the differences in the scales for radioactivity.

following conditions were used: eluent A: CH_3CN/H_2O/TFA (950:50:1); eluent B: CH_3CN/H_2O/TFA (50:950:1); isocratic time-programme with 55% eluent B for 40 min; flow rate: 2.0 ml/min; $\lambda = 254$ nm. The recorded data were processed by the NINA radio-HPLC software (GE Functional Imaging GmbH). Radioactivity of tissue was determined using an automated γ-counter (Wallac Wizard 3″, Perkin-Elmer Life Sciences, Boston, USA). All animal experiments were conducted in accordance with Local Institutional Guidelines for the Care and Use of Laboratory Animals.

4.2. Synthesis of butenolide derivatives

4.2.1. 3-Benzo[1,3]dioxol-5-yl-5-hydroxy-5-(4-benzyloxyphenyl)-4-(3,4,5-trimethoxybenzyl)-5*H*-furan-2-one 4. To a solution of 700 mg (13.0 mmol) sodium methoxide in 50 ml methanol, 5.00 g (11.9 mmol) 2-(benzo[1,3]dioxol-5-yl)-1-(4-benzyloxyphenyl)-4-oxobutyric acid methyl ester **1** and 2.45 g (12.5 mmol) 3,4,5-trimethoxybenzaldehyde **2** were added. The solution was refluxed for 12 h and treated with 6.5 ml acetic acid. After another 4 h of reflux, the solvent was removed by evaporation. Water and ethyl acetate were added and the mixture was neutralised by the dropwise addition of 2.5 N hydrochloric acid. The organic phase was separated and the aqueous phase was extracted twice with ethyl acetate. The combined organic extracts were washed with water and brine and dried over sodium sulfate. After filtration and evaporation, an off-white foam remained, which was purified by silica gel chromatography (light petroleum/ethyl acetate 2:1), yielding 5.13 g (8.81 mmol, 74%) of a white crystalline solid, mp 123 °C. ^{1}H NMR (DMSO-d_6) $\delta = 8.18$ (s, br s, 1H), 7.39 (m, 7H), 6,94 (m, 5H), 5.99 (s, 2H), 5.97 (s, 2H), 5.07 (s, 2H), 3.60 (s, br s, 1H), 3.40 (s, 6H), 3.19 (s, 3H), 3.06 (s, br s, 1H) ppm. ^{13}C NMR $\delta = 171.4$, 162.0, 159.5, 152.9, 148.0, 147.7, 137.6, 136.4, 132.4, 130.1, 129.2, 128.6, 128.5, 128.4, 128.3, 127.5, 124.1, 123.8, 115.2, 110.0, 108.8, 107.0, 101.9, 70.1, 60.5, 56.2, 32.2 ppm. MS: $m/z = 582$ $[M^+]$, 91 $[C_7H_7]^+$. Anal. calcd for $C_{34}H_{30}O_9 \cdot \frac{1}{2}$ EtOAc: C, 69.00; H, 5.47. Found: C, 69.07; H, 5.41.

4.2.2. 3-Benzo[1,3]dioxol-5-yl-5-hydroxy-5-(4-hydroxyphenyl)-4-(3,4,5-trimethoxybenzyl)-5*H*-furan-2-one, PD 169390, 5. 6.35 g (10.9 mmol) 3-benzo[1,3]dioxol-5-yl-5-hydroxy-5-(4-benzyloxyphenyl)-4-(3,4,5-trimethoxybenzyl)-5*H*-furan-2-one **4** was suspended in 100 ml absolute ethanol and 50 ml isopropanol. 440 mg of catalyst (Pd/C) was added and the mixture was stirred under hydrogen atmosphere. After completion of the reaction (tlc, 12 h), the catalyst was filtered off and the filtrate was evacuated to remove the solvent. The remaining off-white foam was purified by silica gel chromatography (light petroleum/ethyl acetate, 1:1) to yield 2.09 g (4.2 mmol, 39%) of the desired product as a white powder, mp 172 °C (decomp). ^{1}H NMR (DMSO-d_6) $\delta = 9.80$ (s, br s, 1H), 8.25 (s, br s, 1H), 7.47 (m, 2H), 7.12 (m, 3H), 6,92 (m, 2H), 6.20 (s, 2H), 6.17 (s, 2H), 3.90 (m, 2H), 3.71 (s, 6H), 3.70 (s, 3H) ppm. ^{13}C NMR $\delta = 170.9$, 161.7, 158.1, 152.3, 147.4, 147.0, 135.9, 131.9, 127.7, 127.4, 126.6, 123.6, 123.2, 115.1, 109.4, 108.2, 106.4, 106.1, 101.3, 60.0, 55.7, 31.6 ppm. MS: $m/z = 492$ $[M]^+$, 474 $[M-H_2O]^+$. Anal. calcd for $C_{27}H_{24}O_9$: C, 65.85; H, 4.91. Found: C, 65.70; H, 4.55.

4.2.3. 3-Benzo[1,3]dioxol-5-yl-5-hydroxy-5-(4-hydroxy-3-iodophenyl)-4-(3,4,5-trimethoxybenzyl)-5*H*-furan-2-one 6. Two hundred forty-six milligram (0.5 mmol) 3-benzo[1,3]dioxol-5-yl-5-hydroxy-5-(4-hydroxyphenyl)-4-(3,4,5-trimethoxybenzyl)-5*H*-furan-2-one **5** was dissolved in 15 ml of a 2.0 M solution of ammonia in ethanol. 2.5 millilitres of a 0.4 N solution of iodine in ethanol (1.0 mmol) was added dropwise. After completion of the addition, the mixture was stirred for 60 min at ambient temperature. The solvent was evaporated and the residue was adsorbed on silica gel and purified by silica gel chromatography (light petroleum/ethyl acetate 3/2→1/1) to yield 170 mg (0.275 mmol, 55%) of an off-white powder, mp 216 °C (decomp). ^{1}H NMR (DMSO-d_6) $\delta = 10.43$ (s, br, 1H), 8.13 (s, br, 1H), 7.67 (s, 1H), 7.19 (d, 1H, $J = 8.5$ Hz), 6.94 (m, 3H), 6.76 (d, 1H, $J = 8.5$ Hz), 6.00 (s, 2H), 5.96 (s, 2H), 3.53 (s, 6H), 3.51 (s, 3H) ppm. ^{13}C NMR $\delta = 170.8$, 161.3, 157.4, 152.5, 147.7, 147.4, 136.9, 136.0, 131.8, 129.8, 127.9, 127.2, 123.6, 123.3, 114.7, 109.5, 108.5, 106.3, 105.2, 101.5, 84.2, 60.2, 55.9 ppm. MS: $m/z = 641$ $[M+Na]^+$, 618 $[M^+]$, 601 $[M-H_2O]^+$. Anal. calcd for $C_{27}H_{23}IO_9$: C, 52.44; H, 3.75. Found: C, 52.60; H, 3.90.

4.3. Radiosyntheses

4.3.1. General procedure for the synthesis of [$^{125/123}$I]3-benzo[1,3]dioxol-5-yl-5-hydroxy-5-(4-hydroxy-3-iodophenyl)-4-(3,4,5-trimethoxybenzyl)-5*H*-furan-2-one 7, 8. To a solution of 57 μg (0.115 μmol) **5** in 39 μl MeOH in a conical glass-vial, 39 μl of 0.1 M K_2HPO_4-buffer (pH 7.34), a solution of 32 μg (0.141 μmol) chloroamine-T·H_2O in 39 μl of 0.1 M K_2HPO_4-buffer and 3–4 μl [^{123}I]NaI n.c.a. solution or 6–22 μl [^{125}I]NaI n.c.a. solution (0.05 M NaOH) were added. The reaction mixture was vortexed and allowed to stand for 5 min at RT. Then 50 μl of 10% $Na_2S_2O_5$-solution was added. After 5 min at RT, the mixture was injected into a radio-RP-HPLC-chromatograph to isolate the radiolabelled compounds **7** or **8**, respectively. Yields: [^{125}I] **7**: 84% (average, $n = 3$, decay-corrected, EOS), [^{123}I] **8**: 74% (average, $n = 4$, decay-corrected, EOS). HPLC: $t_R = 21.5$ min.

4.3.2. Radioanalytical data

4.3.2.1. Quality control. Fifty to two hundred microlitres of the product fraction was re-injected into the HPLC column. The quality control did not show any impurities within the γ-range. Only the injection peak was detectable within the UV-range.

4.3.2.2. Reference control. The radioiodinated products **7** and **8** were verified by concentrating 200 μl of the isolated γ-fraction with 50 μg of the nonradioactive reference **6**. The concentrated mixtures were again injected into the HPLC column. The radiolabelled product and the nonradioactive reference co-eluted.

4.4. Biology

4.4.1. Tissue preparation. Microsomes were prepared by homogenizing ventricles from DBA mice at 4 °C for 90 s

in 1 ml buffer A (10 mM EDTA, 10 mM Hepes and 0.1 mM benzamidine, pH 7.4), using a Polytron PT 3000 (Kinematica, Lucerne, Switzerland). Homogenates were centrifuged at 45,000g_{max} for 15 min at 4 °C. The pellets were resuspended in 1 ml buffer B (1 mM EDTA, 10 mM Hepes and 0.1 mM benzamidine, pH 7.4) and recentrifuged at 45,000g_{max} for 15 min at 4 °C. The pellets were resuspended in 1 ml buffer B and centrifuged at 10,000g_{max} for 10 min at 4 °C. The supernatants were recentrifuged at 45,000g_{max} for 15 min at 4 °C. The pellets, partially enriched membranes, were resuspended in buffer C (50 mM Tris · HCl, 5 mM $MgCl_2$, pH 7.4) and stored frozen at −80 °C.

4.4.2. In vitro assays. For competition binding studies, the prepared membranes were resuspended in buffer D (10 mM Tris · HCl, 154 mM NaCl and 0.1 mM ascorbic acid, pH 7.4) at 0 °C. Ten micrograms of membranes was incubated with a constant concentration of [^{125}I]ET-1 (40 pM) and with varying concentrations (10 pM–100 μM) of ET antagonist **3**–**6** at 37 °C for 4 h. Reactions were stopped by filtering onto Whatman GF/B filters and washed with 0.9% NaCl at 4 °C. The membrane bound radioactivity was determined in a γ-scintillation counter. Competition binding curves were analyzed by nonlinear regression analysis using the *XMGRACE* program (Linux software).

4.4.3. Biodistribution studies. Adult male BL6 mice (20–25 g) were anaesthetised by isoflurane/O_2 (2%, flow 1.0 L/min) and one lateral tail was cannulated (27 G VenofixA needle with 20 cm polythene catheter tubing, od 1 mm, id 0.38 mm). Animals were allowed to recover from the anaesthesia for ~1 h and during the studies they were conscious but under light restraint. 0.25 MBq of the radioligand **8** was injected as a bolus (50 μl, 0.9% NaCl) via the tail vein. Aliquots of each injectate were diluted in ethanol:saline and measured to determine the radioactivity injected into each animal. In three animals, PD 156707 **3** (1 μmol/kg) was injected as a bolus (1 μl g^{-1} body weight) via the tail vein 5 min before the injection of **8**. At selected times after injection of the radioligand, animals were killed by intravenous injection of sodium pentobarbitone (Euthatal) at 200 mg · (kg body weight)$^{-1}$, blood was taken by cardiac puncture, tissues were rapidly removed and a sample of urine was taken from the bladder. Tissue samples were blotted and transferred to weighed vials for reweighing and measurement of radioactivity using an automated γ-counter. Radioactivity was expressed as cpm · (g wet tissue)$^{-1}$. Results were expressed as an uptake index, defined as:

$$\text{Uptake index} = \frac{\text{Tissue radioactivity(cpm)/Tissue wet weight(g)}}{\text{Radioactivity injected(cpm)/Body weight(g)}}.$$

4.4.4. Scintigraphic imaging. Six mice (20–25 g) were anaesthetised by isoflurane/O_2 (2%, flow 1.0 L/min) for scintigraphic scanning. Three mice received PD 156707 **3** (100 μl at 200 nmol/ml, 0.9% NaCl) by retro-orbital injection 30 min before scanning. A solution of **8** (8.5–12.9 Mbq in 100 μl of 0.9% NaCl) was injected retro-orbitally and the scan was started within 5 min. Images (115 frames) were added in 10 min time frames, namely 1–10, 21–30, 51–60, 81–90 and 104–115 min. Rectangular regions of interest were drawn to represent the chest, liver and bladder and radioactivity in each region was expressed as arbitrary units using in-house software.

Acknowledgments

Financial support of the Deutsche Forschungsgemeinschaft (SCHA 758/5-1) is gratefully acknowledged. C.H. wishes to thank Sandra Schröer for the in vitro experiments.

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