***In vivo* targeting of ERG potassium channels in mice and dogs by a positron-emitting analogue of fluoroclofilium**

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Abbreviations: cERG, canine ether a go-go related gene; hEAG, human ether a go-go; hERG, human ERG; PET, positron emission tomography

Abstract

The antiarrhythmic clofilium is an efficient blocker of hERG1 potassium channels that are strongly expressed in the heart. Therefore, derivatives of clofilium that emit positrons might be useful tools for monitoring hERG1 channels *in vivo*. Fluoroclofilium (F-clofilium) was synthesized and its channel-blocking properties were determined for hERG1 and hEAG1 channels expressed in HEK 293 cells and in *Xenopus* oocytes. When applied

extracellularly in the whole-cell patch-clamp configuration, F-clofilium exhibited a slower onset of block when compared with clofilium, presumably owing to its lower membrane permeability. When applied in the inside-out configuration at the intracellular membrane side, it blocked hEAG1 channels almost as efficiently as clofilium (IC₅₀ 1.37 nM and 0.83 nM, respectively). Similar results were obtained for hERG1, showing F-clofilium is a potent hERG1 and hEAG1 channel blocker once it has reached the intracellularly accessible target site at the channel. Using the ¹⁸F-labeled analog we studied the *in vivo* binding and distribution of F-clofilium in mice and a dog. Greatest activity was found in kidneys and bones. A small but significant enrichment of activity in the dog myocardium known for its expression of cERG1 channels allowed to depict the myocardium of a living dog by PET. Thus, F-clofilium is a useful tool for imaging hERG channels in living organisms.

Keywords: anti-arrhythmia agent; clofilium; dog; ERG1 potassium channel; heart; positron-emission tomography

Introduction

The quaternary ammonium derivative clofilium is well known for its class III antiarrhythmic properties (Borzak *et al.*, 1997). hERG K⁺ channels are blocked by clofilium in a nanomolar range and this binding is believed to be the reason for its antiarrhythmic action (Suessbrich *et al.*, 1997). However, block of hERG1 K⁺ channels in the ventricles can also cause a prolongation of the QT-interval ultimately leading to fatal torsades de pointes (Vandenberg *et al.*, 2001).

Recent studies revealed also that hERG K⁺ channels are abundantly expressed in a variety of tumor cells. hERG conductance markedly promotes H₂O₂-induced apoptosis of various tumor cells, whereas hERG expression facilitates the tumor cell proliferation caused by tumor necrosis factor (TNF) ligand (TNF- α) (Wang *et al.*, 2002). Similarly, hEAG1 channels that are closely related to hERG are also found in tumor cell lines and are believed to play a role in malignant cell growth (Pardo *et al.*, 1999; Gavrilova-Ruch *et al.*, 2002). Thus, specific markers for hERG1 and hEAG1 channels could find appli-

cation in both cardiovascular and oncological research.

^{14}C -labeled clofilium showed high myocardial uptake (Su *et al.*, 1984) and radioactive labeling of its aromatic residue by several iodine isotopes has been carried out (Molly *et al.*, 1983). Recently we synthesized fluoroclofilium (F-clofilium, Figure 1A) (Yu *et al.*, 2003) and its ^{18}F -labelled analog (^{18}F -clofilium) as a potential cardiac imaging agent for studies by PET (Positron Emission Tomography), a powerful method to measure specific binding of ligands and metabolism in living species including humans (Hatazawa *et al.*, 1991; Schweiger *et al.*, 1991).

Often substitution of a hydrogen atom by fluorine does not disturb much (Reivich *et al.*, 1985) the binding properties to a binding site but a disturbance by the fluorine cannot be excluded. Also the metabolic fate can be affected (Degrado *et al.*, 1992). Therefore at first we investigated the binding properties of F-clofilium to hERG1 and hEAG1 channels *in vitro*. Subsequently we studied the biodistribution of radioactive ^{18}F -clofilium in mice and dogs.

Materials and Methods

Synthesis of F-clofilium and ^{18}F -clofilium

Synthesis of F-clofilium and ^{18}F -clofilium was carried out as described earlier (Yu *et al.*, 2003).

Distribution coefficients for clofilium and F-clofilium in the biphasic system 1-octanol/water

Three milligrams of clofilium (or F-clofilium) were dissolved in a mixture of 5 ml of 1-octanol and 5 ml of water. The mixture was vortexed for 1 min and was allowed to separate for 4 h into two phases. The ammonium compound content of the organic and aqueous phase was measured by HPLC (50 μl ; Nova-Pak C-18, 3.9×300 mm; 277 nm). As eluent for measurements of the organic phase MeOH / 0.05 M K_2HPO_4 with 10% MeOH in a ratio of 60:40 was used. The aqueous phase was analyzed by the eluents MeOH / 0.05 M K_2HPO_4 with 10% MeOH in a ratio of 90:10. The content of clofilium (or fluoroclofilium) in each phase was 10 times. Calibration of the HPLC system was carried out for clofilium and fluoroclofilium in each of the two applied eluent systems. The mean distribution coefficient in the system 1-octanol/water was 4.3 ± 0.3 for clofilium and 2.6 ± 0.5 for fluoroclofilium.

Expression of hERG1 in mammalian cell lines

hERG1 channels were stably expressed in HEK 293 cells (Schönherr *et al.*, 1999). Cells were cultured in

Dulbecco's modified Eagle's (Invitrogen) / F12 medium (ratio 1:1) supplemented with 10% FCS. The cell line was maintained at 37°C in a humidified atmosphere with 5% CO_2 and subcultured every 3-4 days. All cells were used within passage numbers 3 to 25. For electrophysiological recordings cells were plated on 35-mm petri-dishes.

Expression of hERG1 and hEAG1 in *Xenopus* oocytes

For heterologous expression in *Xenopus* oocytes capped mRNA of hERG1 (Schönherr *et al.*, 1996) and hEAG1 (Schönherr *et al.*, 2000) subcloned in pGEM-HE was synthesized *in vitro* with T7 RNA polymerase (mMESSAGE mMACHINE kit, Ambion, Austin, TX). Oocyte preparation and injection of RNA were performed as described previously (Schönherr *et al.*, 1999).

Electrophysiological recordings

As patch-clamp amplifier we used an EPC 9 (HEKA Elektronik, Lambrecht, Germany). For recordings from mammalian cells the patch pipettes were fabricated from borosilicate glass (Kimble Glass, Vineland, NJ) with resistances of 1-2 M Ω . Unless stated otherwise, the pipettes were filled with (in mM): 130 KCl, 2.56 MgCl_2 , 10 EGTA, 10 HEPES, pH 7.4 (adjusted with KOH). The standard bath solution contained (in mM): 110 NaCl, 40 KCl, 2 MgCl_2 , 2 CaCl_2 , 10 HEPES, pH 7.4 (NaOH). Currents were measured in the whole-cell configuration with access resistances below 3 M Ω . Series resistance compensation was applied to 80-90%.

For macro-patch recordings from *Xenopus* oocytes in the inside-out configuration the patch pipettes were formed from aluminum silicate glass (Hilgenberg, Malsfeld, Germany) with tip resistances of 12 M Ω . Standard external solution was composed of (in mM): 103.6 NaAsp, 11.4 KCl, 1.8 CaCl_2 , 10 HEPES, pH 7.4 (NaOH). Standard internal solution contained (in mM): 120 KAsp, 15 KCl, 10 EGTA, 10 HEPES, pH 7.4 (KOH). Pipette tips for both types of experiments were fire-polished immediately before use. All experiments were performed at room temperature ($20\text{--}23^\circ\text{C}$).

All chemicals used were of high grade, obtained from Sigma (Taufkirchen, Germany).

F-clofilium was dissolved in the bath solution immediately before the experiments. It was applied to the cells via exchange of the bath solution; this was complete in about 1 min. Blocker effects were monitored by repetitive depolarizations. Current block was analyzed by plotting the remaining current, normalized to the control current before blocker application as a function of the blocker concentration. IC_{50} values

were obtained by fitting a Hill equation to these data:

$I / I_{\text{control}} = 1 / (1 + ([\text{blocker}] / IC_{50})^{n_H})$ (1)
with the half-maximum inhibition concentration IC_{50} and the Hill coefficient n_H . To quantify the time course of onset of block an exponential function:

$I(t) = I(0) - (I(0) - I(\infty)) (1 - \exp(-(t - t_0) / \tau_b))$ (2)
was employed. $I(0)$ and $I(\infty)$ are the current amplitudes before blocker application (at time t_0) and after block equilibration, respectively. τ_b is the time constant of onset of block.

Data acquisition and analysis were carried out with Pulse+PulseFit, PatchMaster, and FitMaster (HEKA) and IgorPro (WaveMetrics, Lake Oswego, OR) software. All data are presented as mean $\pm$ SEM (n) with n being the number of independent experiments. Statistical differences between two groups of data were performed using two-sided Student's t -test indicated as $P < 0.05$ (*) or $P < 0.005$ (**).

Biodistribution of radioactivity in mice after injection of ^{18}F -clofilium

Eight Balb/c female mice (25–35 g) were injected with 1.8 MBq ^{18}F -clofilium in 0.1 ml isotonic NaCl solution in the tail vein. Four mice were sacrificed with diethyl ether after 15 min, the other four after 60 min. The organs were excised from each animal and weighed and the radioactivity was measured. The radioactive content of the blood and other organs was determined with a gamma counter. The data were decay corrected and related to activity per gram of tissue in % of injected dose (% ID/g).

PET scan

The PET scan was carried out on an ECAT EXACT 47 scanner (Siemens Medical System, Inc., Hoffman Estates, IL/ CTI, Knoxville, TN; resolution 5 mm).

A dog (25 kg, male, beagle) was anesthetized using thiopental sodium. Self-respiration was sustained and the dog laterally positioned on its right side. A transmission scan was acquired using triple ^{68}Ge sources to correct data for internal γ -absorption. After intravenous injection of 370 MBq ^{18}F -clofilium the radioactive compound was allowed to distribute in the animal for 30 min. Dynamic myocardial PET data were collected during a period of 5 min. Using the system software, two series of image slices, a coronal (horizontal) and vertical (parallel to the long axis), were acquired.

Results

Block efficiency of F-clofilium

To assay the functional activity of F-clofilium as a

potent blocker of hERG potassium channels, we expressed hERG1 channels in HEK 293 cells and measured K^+ currents employing the whole-cell patch-clamp method. Like previously shown for clofilium (Gessner *et al.*, 2003), the onset of current block upon application of F-clofilium was very slow. When determining complete dose-response relationships from an identical cell one has to compromise precision of measurements as only about 10 min could be waited before application of the next higher concentration of blockers. Using such protocols and a holding voltage of -100 mV, data shown in Figure 1C were compiled. The dose-dependent block of hERG1 current was described with a Hill equation (eq. 1) yielding an apparent IC_{50} for F-clofilium of about 43 nM. In comparison, for clofilium 2.6 nM were measured previously (Gessner *et al.*, 2003).

This result could mean that F-clofilium blocks hERG1 channels less efficiently than clofilium. However, since clofilium has to enter the binding site in the channel cavity from the cytosol, the apparent difference could be due to different membrane permeabilities of F-clofilium and clofilium. This is quite conceivable given the extra polar group added to the alkane chain of clofilium (Figure 1A), yielding an octanol/water distribution coefficient of 2.6 compared with 4.3 for clofilium as described under Materials and Methods. Therefore, we also attempted to measure onset of block by application of either 1 μM F-clofilium or clofilium (Figure 1D). The time course of current reduction was faster for clofilium (109 ± 12 s as compared to 179 ± 31 s for F-clofilium), albeit for the experiments shown no statistical significance was reached. However, the time constant at 100 nM was already about 15 min, making it impossible to obtain faithful steady-state block data in the whole-cell configuration. Certainly, the dose-response measurements obtained for 10 min equilibration periods (Figure 1C) clearly results in an overestimation of the apparent IC_{50} .

In order to obtain a more direct access to the blocking properties of F-clofilium, we applied it to inside-out patches from *Xenopus* oocytes expressing hERG1 channels. Under inside-out conditions, hERG channels undergo a rapid rundown, which compromised the determination of accurate steady-state dose-response curves. Therefore, we applied 10 nM of the substances to inside-out patches and observed the gradual reduction of hERG tail currents. As shown in Figure 1E, the channels are blocked to approximately the same extent by clofilium and F-clofilium. The data shown in Figure 1E are compatible with a steady-state block of 82%, which is equivalent to an apparent IC_{50} value of 2.2 nM.

Clofilium is not only a strong blocker of hERG channels - hEAG1 channels are also inhibited by this

Figure 1. Inhibition of hERG1 currents by F-clofilium. **A**, Chemical structures of clofilium and F-clofilium. **B**, Superposition of whole-cell current recordings from HEK 293 cells, expressing hERG1 channels with the indicated concentrations of F-clofilium. Cells were depolarized for 1 s to +50 mV at an interval of 8 s. Peak inward hERG currents were measured during the deactivation phase at -120 mV 10 min after blocker application. **C**, The current magnitudes, normalized to the control currents, as a function of the blocker concentration. The error bars are SEM values for the indicated number of experiments. The superimposed curve is a fit according to the Hill equation (eq. 1) yielding $IC_{50} = 43.5 \pm 5.3$ nM, and $n_H = 1.07 \pm 0.08$ for F-clofilium. As a control, block by clofilium is also indicated as dashed curve (data from Gessner & Heinemann, 2003): $IC_{50} = 2.6$ nM, and $n_H = 0.62$. **D**, The time course of channel block during repetitive depolarizations was assayed by application of 1 μ M F-clofilium (open symbols) and 1 μ M clofilium (filled symbols), respectively. The onset of block develops approximately monoexponentially with a time constant of 179 ± 31 s ($n = 5$) for F-clofilium. The time constant for clofilium was shorter (109 ± 12 s, $n = 4$), but not significantly different ($P = 0.08$). **E**, Time course of hERG1 channel block in inside-out patches of *Xenopus* oocytes upon application of 10 nM of the indicated substances ($n = 4$). 450 s after application of the drugs the current was reduced to 18% of the control value. Membrane patches were depolarized to +50 mV for 1 s with a repetition interval of 8 s. Currents were measured during the repolarizing phase at -120 mV.

substance (Gessner *et al.*, 2003). As hERG1 channels seem to play an important role in malignant growth, we tested whether F-clofilium is a strong

blocker as well. If so, ^{18}F -clofilium might also be useful for localizing small hERG1-expressing tumors. When applied to inside-out patches of hERG1-expressing

Figure 2. Inhibition of hEAG1 currents in the inside-out patch-clamp configuration by F-clofilium. A, Superposition of current recordings from membrane patches of *Xenopus* oocytes expressing hEAG1 channels with the indicated concentrations of F-clofilium. Cells were depolarized for 1 s to +50 mV at an interval of 7 s. Holding potential was -110 mV and mean outward hEAG current was measured during the second half of the depolarization period. B, The current magnitudes, normalized to the control currents, as a function of the blocker concentration. The error bars are SEM values for the indicated number of experiments. The superimposed curve is a fit according to the Hill equation (eq. 1) yielding $IC_{50} = 1.37 \pm 0.11$ nM, and $n_H = 1.14 \pm 0.07$ for F-clofilium. As a control, block by clofilium is also indicated as dashed curve (data from Gessner & Heinemann, 2003): $IC_{50} = 0.83$ nM, and $n_H = 1.01$.

Xenopus oocytes, F-clofilium blocked almost indistinguishably to clofilium: the apparent IC_{50} values were 1.37 nM for F-clofilium as compared to 0.83 nM for clofilium (Gessner *et al.*, 2003) (Figure 2). Thus, the extra fluoride moiety F-clofilium does not strongly impair the binding of the drug inside the EAG channel pore. Given the strong structural similarity of hEAG1 and hERG1 channels (Gessner *et al.*, 2004), these data corroborate the notion that F-clofilium is also a potent blocker of hERG1 channels once it has passed the plasma membrane.

Biodistribution of ^{18}F -clofilium in mice

The biodistribution of F-clofilium was tested in living mice. For that purpose, ^{18}F -clofilium was injected into the tail vein of Balb/c female mice. The mice were

Table 1. Biodistribution of ^{18}F -clofilium in Balb/c female mice. The data indicate the decay-corrected relative activity (% injected dose/g); the errors indicate SD values from four animals each.

Tissue	Activity at 15 min (% ID/g)	Activity at 60 min (% ID/g)
Blood	0.22 ± 0.06	0.05 ± 0.01
Liver	2.36 ± 0.26	1.09 ± 0.14
Lung	1.09 ± 0.10	0.98 ± 0.02
Spleen	0.97 ± 0.19	0.46 ± 0.07
Kidney	11.23 ± 0.07	5.36 ± 0.45
Stomach	0.45 ± 0.14	0.34 ± 0.03
Femur	0.79 ± 0.15	1.37 ± 0.09
Muscle	0.27 ± 0.05	0.26 ± 0.02
Thyroid	0.21 ± 0.03	0.21 ± 0.02
Intestine	2.87 ± 0.38	4.03 ± 0.10
Brain	0.03 ± 0.00	0.02 ± 0.00
Heart	2.64 ± 0.28	2.38 ± 0.23

sacrificed at two different times after injection (15 min or 60 min). The data are shown in Table 1.

The kidneys showed the highest uptake of radioactivity both at 15 min and 60 min when it had dropped down to half of its former value. Relatively high enrichment was found in the intestines with enhancing tendency over the time. Bones represented by the femur showed a relatively small initial uptake but some enhancement after 60 min, which might be a result of metabolism to free radioactive fluoride. This metabolism might be caused by the liver, also showing decreasing activity over time.

In the blood radioactivity had disappeared nearly completely after 60 min. The ratio of activity in heart to blood was 12 after 15 min and 48 after 60 min. A relatively high radioactivity was found in the myocardium with nearly identical values after 15 and 60 min. Based on these initial data, we assumed that imaging of the dog myocardium by PET might be successful.

Biodistribution of ^{18}F -clofilium in a living dog

Subsequently the biodistribution of ^{18}F -clofilium in a living animal was investigated by PET scan. Given the anatomical and physiological similarities to the human heart we chose the canine model. Abundant expression of cERG messenger ribonucleic acid (mRNA), strongly related to hERG1 (Zehelein *et al.*, 2001), led to the expectation of detecting binding of ^{18}F -clofilium to the dog myocardium using PET. Results of this investigation are shown in Figure 3.

Strong enrichment of radioactivity was seen only in the kidneys, the bladder and the bones (Figure 3A). The radioactivity in bones of the dog, as also found in the femur of mice (s. Table 1), is most likely due to an

Figure 3. (A) ^{18}F -clofilium PET scan of a dog (25 kg, male, beagle). Shown is a series of whole-body images as coronal (horizontal) slices of a dog positioned on its left side. High uptake of radioactivity is found in the kidneys, bladder and bone. The first slices show a kidney, the more central slices a kidney and the bladder, then followed by the second kidney. Slice thickness 10 mm. (B) Series of sagittal PET scan slices (vertical, thickness 5 mm) of the thorax of the dog positioned on his left side. High enrichment of radioactivity is found in the ribs, minor activity is found in the myocardium depicted as red circles cut vertical to the short axis of the heart.

uptake of ^{18}F -fluoride, which is a strong bone-seeking ion (Hoh *et al.*, 1993). Therefore also some elimination of fluoride must have taken place (Degrado *et al.*, 1992).

Compared to the biodistribution in mice, enrichment of radioactivity in the kidneys of the dog seemed to be more dominant.

The strong uptake by the kidneys is certainly not due to free ^{18}F -ions, since enrichment of ^{18}F -ions in this amount in organs such as kidneys is not observed (Hoh *et al.*, 1993). Thus, we can assume a strong binding of the ^{18}F -clofilium cation or perhaps of a radioactive metabolite to the kidneys and subsequently a secretion into urine collected in the bladder. The cause of this very strong binding of activity to the kidneys could be due to a high density of high-affinity binding sites in the kidney tissue. These binding sites should be distinct from cERG1 K^+ channels, since such channels are not strongly expressed in the kidney (Zehelein *et al.*, 2001).

Despite the strong uptake and the fast clearance rates of the radioactivity by the kidneys, metabolization to ^{18}F -ions and their uptake by bones, it was possible to depict the myocardium by PET as shown in Figure 3B. The myocardium is shown in form of circles cut vertical to the short axis of the heart.

Neighboring bones (ribs, breastbone) are disturbing the myocardial imaging by the "partial volume effect" (Hatazawa *et al.*, 1991), caused by the limited resolution (5 mm) of the method. This results in an overestimation of radioactivity in parts of the myocardium in the neighborhood of bones taking up high amounts of ^{18}F -ions.

Thinning of the myocardium in the region of the atria results by partial volume effect in an underestimation of the activity in the atrial walls. With this in mind we

conclude that F-clofilium binding sites are nearly homogeneously distributed throughout the myocardium.

Recently, Angelo *et al.* reported an even distribution of the radioiodinated scorpion toxin [^{125}I]-BeKm-1 in an excised rat heart and concluded a homogeneous expression of hERG channels throughout the heart (Angelo *et al.*, 2003). F-clofilium binds as described above not only to hERG1 channels but also in low nanomolar range to hEAG1 channels. The latter have not been detected in the myocardium but only in the brain and tumor cells. Abundant expression of cERG mRNA in dog's heart was detected (Zehelein *et al.*, 2001). Since cERG and hERG channel proteins are identical in their membrane-spanning segments and the pore forming region (Zehelein *et al.*, 2001), harboring the binding site for clofilium, we conclude that the myocardial uptake of radioactivity after injection of ^{18}F -clofilium primarily reflects a binding to the cERG1 K^+ channels. These binding sites seem to be nearly homogeneously distributed throughout the heart and our results are in support of previous result using a different method (Angelo *et al.*, 2003).

Application of ^{18}F -clofilium by PET is able to depict the dog's myocardium probably by binding to cERG channels. These channels seem to be almost homogeneously distributed throughout the myocardium as far depicted. High renal uptake and fast catabolization of ^{18}F -clofilium to radioactive fluoride might compromise its application in humans for PET-scan investigations.

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