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Synthesis and radiosynthesis of N^5-[^{18}F]fluoroethyl-Pirenzepine and its metabolite N^5-[^{18}F]fluoroethyl-LS 75

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The well established M_1 selective muscarinergic antagonist Pirenzepine 11-[2-(4-methyl-piperazin-1-yl)-acetyl]-5,11-dihydro-benzo[e]pyrido[3,2-b][1,4]diazepin-6-one (1) exhibits an unusual behaviour *in vivo*, which cannot be explained with M_1 antagonism exclusively. One of the aspects discussed is a specific interaction with poly ADP-ribose polymerase (PARP-1). 1 undergoes metabolism to form LS 75 5,11-dihydro-benzo[e]pyrido[3,2-b][1,4]diazepin-6-one (2). In order to study deviations in Pirenzepine efficacy from pure M_1 binding *in vivo* using PET, appropriate positron emitter labelled analogues of 1 and 2 were synthesised. Non-radioactive reference compounds 3 and 4 were tested for PARP-1 inhibition. The *n*-octanol–water partition coefficients of compounds 1, 2, 3 and 4 at pH 7.4 ($logD_{7.4}$) were determined. Both, 3 and 4 were labelled with ^{18}F *via* 2-[^{18}F]fluoroalkylation in position 5 of the benzodiazepinone moiety to obtain N^5-[^{18}F]fluoroethyl Pirenzepine [^{18}F]-3 and N^5-[^{18}F]fluoroethyl LS 75 [^{18}F]-4. Radiotracers [^{18}F]-3 and [^{18}F]-4 were obtained in radiochemical yields of 15 ± 4 % and 30 ± 5% after 120 and 110 min, respectively. Metabolism of both compounds was investigated *in vitro* in human and rat plasma, respectively. Compound 3 did not show activity as an inhibitor of PARP-1. Contrary, 4 displays moderate PARP-1 inhibition potency. The new radiotracer [^{18}F]-4 can be applied for molecular imaging using autoradiography and PET.

Keywords: apoptosis; Pirenzepine; N-^{18}F-fluoroalkylation; neuroprotection; PARP-1

Introduction

Pirenzepine **1**, namely 11-[2-(4-methyl-piperazin-1-yl)-acetyl]-5,11-dihydro-benzo[e]pyrido[3,2-b][1,4]diazepin-6-one, has originally been developed as M_1 selective muscarinergic antagonist.[1] After its well accepted efficacy as gastric ulcer therapeuthic has become obsolete due to the development of selective proton-pump inhibitors,[1] **1** has recently been re-introduced as potential neuro-protective *via* PARP-1 modulation. *In vivo*, **1** is metabolised to 5,11-dihydro-benzo[e]pyrido[3,2-b][1,4]diazepin-6-one (**2**), also known as LS 75. The latter compound was found to be a moderate inhibitor of PARP-1, a key enzyme directly related to aging and many age-related human diseases by its central role in neuronal signal transduction, cellular energy homeostasis and in particular to the regulation of key events in apoptotic cascades.[2,3]

With respect to the growing interest in a detailed investigation of this second Pirenzepine-related mode of action on a physiological level,[4] we propose the use of an intermediate to low-affinity radioligand for molecular imaging *in vivo*. Therefore our aim was to obtain ^{18}F-fluorinated analogues of **1** and **2**, to provide basic tools for *in vivo* PET-studies (Chart 1).

Results and discussion

The first approach for incorporation of ^{18}F into **1** was based on a routine methyl to 2-[^{18}F]fluoroethyl substitution at the terminal piperazine nitrogen of **1** to afford 11-[2-(4-(2-[^{18}F]fluoroethyl)-piperazin-1-yl)-acetyl]-5,11-dihydro-benzo[e]pyrido[3,2-b][1,4]diazepin-6-one (**5**).

Nor-Pirenzepine **6** was designed as labelling precursor. However, although an ^{18}F-labelled product was rapidly formed in high yield on the addition of 2-[^{18}F]fluoroethyl tosylate to a stirred solution of potassium carbonate and **6** at 110°C, it was found that this ^{18}F-labelled product did not correspond to **5**. In contrast, the unknown product was assigned 11-[2-(4-methyl-piperazin-1-yl)-acetyl]-5-[^{18}F]fluoroethyl-11-hydro-benzo[e]pyrido[3,2-b][1,4]diazepin-6-one (**7**), after a separately prepared non-radioactive reference sample showed perfect co-elution with the simultaneously injected radioactive product (Figure 1).

As a consequence, an alternative labelling approach was carried out, utilising the good accessibility of the benzamide nitrogen for 2-[^{18}F]fluoroethylation. Reference compounds **3** and **4** were synthesised to facilitate labelling of the benzamide nitrogen with 2-[^{18}F]fluoroethyl tosylate. Compounds **3** and **4** were prepared following a modified procedure, published elsewhere.[5] Compound **2** was synthesised in two steps from ethyl-2-amino-benzoate and 3-amino-2-chloro-pyridine. After forming the benzamide intermediate, which was isolated to verify the structure, ring closure was perfomed in a microwave-supported reaction at 220°C. Following this procedure, **2** was

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obtained in 65 % yield. Compound **1** was obtained by reacting **2** with 2-chloro acetic acid chloride followed by 4-methyl piperazine, again in one pot (Figure 2).

Alkylation of **1** and **2** with 2-fluoroethanol under Mitsunobu conditions afforded reference compounds **3** and **4** in excellent yields of 95 and 90 %, respectively (Figure 3). In comparison, only low to moderate yields (>25 %) of **3** and **4** were found using a variety of aprotic solvent and base systems in combination with 2-fluoroethyl bromide or 2-fluoroethyl tosylate.

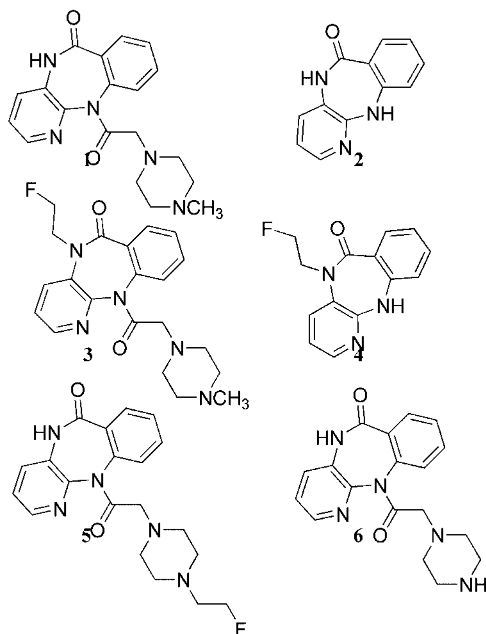

Chart 1. Pirenzepine and analogues.

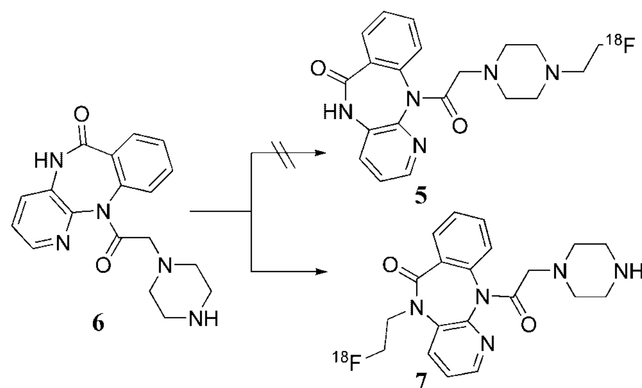

Figure 1. Course of fluoroethylation of **6** with 2-[^{18}F]fluoroethyl tosylate.

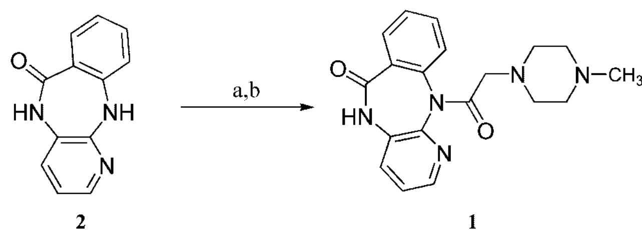

Figure 2. Synthesis route to **1** from **2**, two steps in one pot; (a) 2-chloroacetic acid chloride, CH_2Cl_2, 0°C; (b) 4-methyl piperazine, MeCN, N,N-diisopropyl-N-ethylamine, 45°C, 14 h, 75 % (2 steps).

From enzymatic testing comparatively moderate affinities of **1** and **2** and of respective fluorinated analogues **3** and **4** were expected. Experimentally obtained PARP-1 inhibition potencies K_i, revealed a loss of about one order of magnitude in potency for the fluorinated analogues compared with their non-fluorinated congeners (cf. Table 1). However, the activity of [^{18}F]FE-LS-75 ([^{18}F]-**4**) remains in the same range as the potency of Pirenzepine (**1**) itself.

To assess the lipophilicity of the novel tracers, the $logD_{7.4}$ was determined *via* a modified HPLC-method. The reference curve demonstrated good correlation (R^2 from linear regression = 0.997) between retention (as capacity factor k') and $logD_{7.4}$ (cf. Table 1).[6] Although $logD_{7.4}$ values might indicate a higher brain uptake of piperazines **1** and **3** compared with tricycles **2** and **4**, it has been shown that **3** is rarely present in rat brain. In earlier studies, analogue findings have been ascribed to the P-glycoprotein efflux pump.[7]

[^{18}F]-**3** was synthesised from **1** in DMSO at 120°C, furnishing an average radiochemical yield of 30 ± 5 % after a reaction time of 20 min. In contrast, [^{18}F]-**4** was obtained in 70% yield, after a reaction time of 15 min, although an unusual low precursor amount of less than one milligram was used.

Final tracer formulations were prepared in a semi-automated procedure, yielding 15% of [^{18}F]-**3** in a specific radioactivity of 34–58 GBqμmol^{-1} after 120 min starting from [^{18}F]fluoride. [^{18}F]-**4** was synthesised in a radiochemical yield of 30% and a specific radioactivity of 67–89 GBqμmol^{-1} after 110 min starting from [^{18}F]fluoride. Both compounds were obtained in radiochemical purities >98% after HPLC purification and formulation in PBS solution.

An *in vitro* metabolic study of [^{18}F]-**1** and [^{18}F]-**2** in both, human and rat plasma, was performed. It was found that both radiotracers remain stable in human and rat blood over 120 min. In addition, both radiotracers remained intact in injectable phosphate buffered saline for more than 4 h (Figure 4).

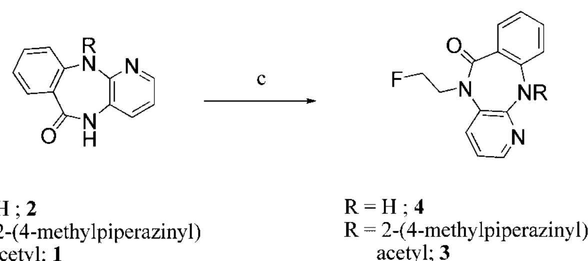

Figure 3. Synthesis of reference compounds *via* Mitsunobu conditions; (c) diethyl azodicarboxylate (DEAD), triphenyl phosphine (TPP), 2-fluoroethanol, THF, 90-95 %.

Table 1. PARP-1 inhibition K_i-values octanol/water partition coefficients at pH 7.4 (log $D_{7.4}$) of **1–4**

Compound	K_i/nmol/ml	$logD_{7.4}$
1	550	2.38
2	18	1.2
3	>3000	2.41
4	200	1.4

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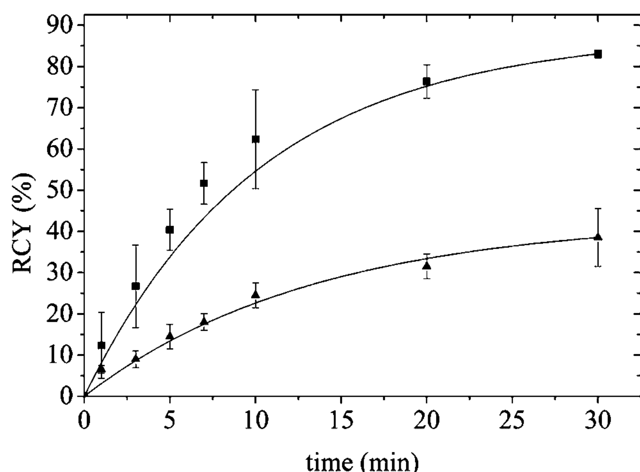

Figure 4. Plot of radiochemical yield as a function of reaction time. (triangles: [^{18}F]-**3**; squares: [^{18}F]-**4**) Conditions: [^{18}F]-**3**: 3 mg **1**, DMSO (1 ml), 120°C, anhydrous K_2CO_3 (3 eq.). [^{18}F]-**4**: 0.75 mg **2**, DMSO (1 ml), 120°C, anhydrous K_2CO_3 (1 eq.).

Conclusion

Pirenzepine (**1**), its metabolite LS 75 (**2**) and their N^5-fluoroethyl analogues were prepared and labelled with ^{18}F *via* 2-[^{18}F]fluoroalkylation in position 5 of the benzodiazepinone moiety to obtain N^5-[^{18}F]fluoroethyl Pirenzepine [^{18}F]-**3** and N^5-[^{18}F]fluoroethyl LS 75 [^{18}F]-**4**. Both compounds were obtained in a radiochemical purity greater than 98% after HPLC purification and solid phase extraction. The slow metabolism of **1** to **2** prohibits the use of [^{18}F]-**3** as a prodrug,[8] despite its advantageous permeative properties may result in an increased brain uptake compared with **4**. Because of its low affinity of $>$ 3 μmol, [^{18}F]-**3** is not useful as a PARP-1 marker. In contrast, [^{18}F]-**4** offers a 0.2 μM affinity and a $\log D_{7.4}$ within the range required for efficient penetration of the blood–brain barrier. No radioactive metabolites were detected *in vitro*, indicating sufficient stability. It thus may become a candidate of molecular imaging of PARP-1-related processes both using autoradiography and PET-imaging *in vivo*. Therefore, we decided to promote [^{18}F]-**4** ([^{18}F]FE-LS-75) as a moderate affinity radiotracer for PARP-imaging in living subjects, thus taking advantage from the relatively high abundance of active PARP-1 binding sites compared with low abundant proteins (e.g. kinases).

Experimental

All reactants and reagents used in this study were obtained from Sigma-Aldrich, Acros Organics b.v., Merck KGaA, TCI or Alfa-Caesar. Solvents were of bulk quality and used without further purification unless necessary. Proton NMR spectra were recorded on a Bruker AC300 300 MHz spectrometer, mass spectra were measured on a Finnigan MAT90 FD spectrometer, HPLC was performed on a Dionex HPLC-system equipped with a Raytest Gina Star radioactivity monitor. Merck Kieselgel 60 F_{256} silica plates were used for TLC, detection was conducted at 256 and 360 nm. Iodine on silica gel and potassium permanganate solution were used for chemical staining. Preparative chromatography was performed on silica gel 0.063–0.2 Å.

5,11-dihydro-benzo[e]pyrido[3,2-b][1,4]diazepin-6-one (2)

Potassium *tert*-butoxide was added in portions to a stirred solution of 2-chloro-3-aminopyridine in 1,2,4-trichlorobenzene. The resultant mixture was heated to 85°C and 1 eq. of 2-aminobenzoeic acid ethyl ester was added. After stirring at 85°C for four additional hours, the alcohol was removed *in vacuo* and the mixture was heated to 215°C for 14 h. The product was precipitated and recrystallised from water/acetone. Yield: 65 %.

^{1}H-NMR (300 MHz, $CDCl_3$): δ(ppm): 9.9 (s, 1H,CON*H*), 8.5 (s, 1H, NH), 7.9 (d, $J = 6$ Hz, 1H), 7.7 (d, $J = 8$ Hz, 1H) 7.35 (t, $J = 6$ Hz, 1H), 7.28 (d, $J = 8$ Hz, 1H) 7.1 (d, $J = 7$ Hz, 1H), 6.95 (d, $J = 7$ Hz, 1H), 6.83 (t, 1H). MS (FD) M(rel. int.): 211.2(100).

11-[2-(4-methyl-piperazin-1-yl)-acetyl]-5,11-dihydro-benzo[e]-pyrido[3,2-b][1,4]diazepin-6-one (1)

2-chloroacetic acid chloride was added to a stirred solution of **4** in methylene chloride at 0°C. After warming to r.t. with stirring, the solvent was removed *in vacuo* and the residue was taken up in acetonitrile (dried). 1-methylpiperazine was added and the mixture was heated to 45°C for 14 h. The solvent was removed and the residue was purified *via* flash chromatography on silica gel. Yield: 58 %. ^{1}H-NMR (300 MHz, $CDCl_3$): δ(ppm): 9.9 (s, 1 H, CON*H*), 8.5 (s, 1 H, N*H*), 7.9 (d, 1H), 7.7 (d, 1H) 7.35 (t, 1H), 7.28 (d, 1H) 7.1 (d, 1H), 6.9 (d, 1H), 6.93 (t, 1H), 3.3 (s, 2H), 3.0 (brs, 4H), 2.8 (brs, 4H) 2.1 (s, 3H). MS (FD) M(rel. int.): 351.4(100).

11-[2-4-BOC-piperazin-1-yl)-acetyl]-5,11-dihydro-benzo[e]-pyrido[3,2-b][1,4]diazepin-6-one (8)

2-chloroacetic acid chloride was added to a stirred solution of **4** in methylene chloride at 0° C. After warming to r.t. with stirring, the solvent was removed *in vacuo* and the residue was taken up in acetonitrile. DiPEA was added (1.1 eq.) before the dropwise addition of 1-BOC-piperazine.[9] The resultant mixture was heated to 45°C for 14 h. The solvent was removed and the residue was purified *via* flash chromatography on silica gel. Yield: 75 %. ^{1}H-NMR (300 MHz, $CDCl_3$): δ(ppm): 7.75 (d, 1H), 7.33 (d, 1H), 7.1 (t, 1H) 6.78 (d, 1H), 6.63–6.75 (m, 3H) 4.01 (brs, 2H), 3.71 (brs, 4H), 3.58 (brs, 4H) 1.38 (s, 9H). MS (FD) M(rel. int.): 375.2(100).

11-[2-piperazin-1-yl)-acetyl]-5,11-dihydro-benzo[e]pyrido-[3,2-b][1,4]diazepin-6-one (7)

Compound **7** was taken up in a small amount of TFA and stirred at r.t. for 24 h. The solvent was removed *in vacuo* and the residue was purified *via* cation exchange chromatography on a XAD 16 resin. Yield: 55 %. ^{1}H-NMR (300 MHz, $CDCl_3$): δ(ppm): 7.75 (d, 1H), 7.33 (d, 1H), 7.1 (t, 1H) 6.78 (d, 1H), 6.61–6.74 (m, 3H) 3.93 (brs, 2H), 3.63 (brs, 4H), 3.54 (brs, 4H). MS (FD) M(rel.int.): 337.2(100).

General procedure for Mitsunobu alkylations (1 and 2)

A THF solution of benzamide precursor was added to a stirred solution of triphenylphosphine, diethyl azodicarboxylate (DEAD) and fluoroethanol in benzene and stirred at r.t. for 24 h. Hexane was added and the mixture was filtered through pad of silica gel. The solvent was removed and the residue was taken up in 0.8 M HCl prior to extraction with diethyl ether. The aqueous phase was basified (5 M NaOH) and extracted with methylene chloride. The methylene chloride was dried above sodium sulphate and concentrated *in vacuo* to afford the product as off-white powder. Yield 90–95 %. ^{1}H-NMR (300 MHz, $CDCl_3$): (**4**): δ(ppm): 8.0 (d, 1H), 7.8 (d, 1H) 7.75 (t, 1H), 7.3 (t, 1H), 7.0–7.1 (m, 2H), 6.85 (d, 1H), 6.5 (brs, 1H, ArN*H*), 4.8 (dt, 2H) 4.0 (dt, 2H). MS (FD) M(rel.int.): 257.1(100). (**3**): δ(ppm): 8.1 (d, 1H), 7.85 (d, 1H) 7.78 (t, 1H), 7.36 (t, 1H), 7.0–7.1 (m, 2H), 6.80 (d, 1H), 4.01 (brs,

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1H), 4.75 (dt, 2H) 4.0 (dt, 2H) 3.5 (brs, 4H), 3.3 (brs, 4H), 2.11 (s, 1H). MS (FD) M(rel.int.): 397.5(100).

Automated synthesis of 2-[^{18}F]fluoroethyltosylate (9)

All operations were performed in a lead-shielded cell, containing a self-build automated synthesis module. Mass flow was driven by He 5.0 gas at a pressure of 1.4×10^5 Pa. [^{18}F]fluoride in $H_2[^{18}O]O$ was trapped on a waters light QMA strong anion exchange cartridge. Elution from the QMA resin was performed using a solution of 15 mg of Kryptofix K 222 and 15 μmol of potassium carbonate in 1 ml of dry MeCN. The eluate was concentrated to dryness in 14 min, using a 5-step drying procedure. Ethylene-1,2-ditosylate, solved in 1 ml of MeCN was added subsequently and the mixture was stirred at 88°C for 3 min. The reaction mixture was diluted with 50% water in acetonitrile (1 ml) and transferred into a 2 ml inject loop of a Knaur preparative HPLC system, equipped with a lichrospher RP18 5 μm column, running with MeCN/water at 5 ml/min. The product fraction was collected, diluted with water (30 ml) and driven above a Merck-lichrospher EN cartridge. After drying of the product *via* passing a stream of helium through the cartridge, the product was eluted in DMSO (1 ml) warmed to 65°C, directly into the pre-heated second reaction vessel, equipped with the appropriate amounts of base and precursor (neat). 2-[^{18}F]fluoroethyl tosylate (**9**) was obtained in a decay corrected yield of 60 ± 8% after 55 min.

Radiosynthesis of [^{18}F]-3 and [^{18}F]-4

After the addition of **9** to the reaction vial the reaction mixture was stirred at 120°C for 15–20 min. The reaction was quenched *via* the addition of water (4 ml) to the reaction vessel. The resultant mixture was taken up into a syringe preloaded with water (5 ml) and passed through a Merck SCX cation exchange cartridge to remove the DMSO prior to HPLC purification. The trapped radioactivity was liberated *via* elution with HPLC eluent and injected into a Dionex HPLC equipped with a semi-preparative Phenomenex Luna 5 μ RP18 column (10×250). After HPLC purification the product fraction was trapped on a cation exchange cartridge, washed with 3 ml of water and eluted in 1 ml of sterile PBS. Average, non-decay corrected yields were 15% for [^{18}F]-**3** and 30% for [^{18}F]-**4**, respectively.

Quality control of [^{18}F]-3 and [^{18}F]-4

Quality control of [^{18}F]-**3** and [^{18}F]-**4** was performed under the HPLC conditions given for lipophilicity determination. The corresponding retention times of [^{18}F]-**3** and [^{18}F]-**4** were 9.8 and 5.7 min, respectively.

Determination of log $D_{7.4}$ values

The 1-octanol/water partition coefficient at pH 7.4 was determined using a modified HPLC method.[6] The mobile phase was prepared from 250 ml deionised water, 62 mg KH_2PO_4 and 450 mg $Na_2HPO_4 \times 2\ H_2O$. The pH-value was set to 7.4 *via* the addition of phosphoric acid 85%, prior to the addition of 750 ml methanol. A Merck LiChroCart 250-4, LiChrosorb RP-18 7 μ HPLC-column was used as stationary phase. A total of 1 mg/ml samples of **1** to **4** were prepared in a 1 mM PBS-solution containing 25% MeOH and injected into the HPLC.

In vitro metabolisation study of [^{18}F]-3 and [^{18}F]-4

A total of 5 ml of human or rat blood were heparinised and centrifuged at 15000 rpm for 15 min. The obtained clear solution of rat/human plasma was partitioned into aliquots of 200 μl in 500 μl screw cap vials. Approximately 1 MBq of [^{18}F]-**3** or [^{18}F]-**4** radioactivity in 10 μl of PBS was added and the samples were slowly shaken at 37°C. Samples of 20 μl were withdrawn at the start, after 5, 10, 20, 30, 45, 60, 90 and 120 min, triturated with 80 μl of MeCN, centrifuged at 15000 rpm for 5 min and spotted onto TLC plates for analysis. (Silica gel 60, MeOH/chloroform 1:6).

PARP-1 inhibition by Pirenzepine (1), LS 75 (2) and fluorinated derivatives (3, 4)

A PARP inhibition assay from R&D Systems (TA 4669; sold in licence from Trevigene) was used.[10] Respective K_i-values for compounds **1**, **2**, **3** and **4** were 550, 18, > 3000 (not active) and 200 μM, respectively.

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