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Synthesis and Use of FSCPX, an Irreversible Adenosine A_1 Antagonist, as a 'Receptor Knock-Down' Tool

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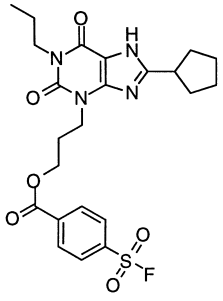
Abstract—A new preparative synthetic route for the irreversible adenosine A_1 antagonist 8-cyclopentyl-3-*N*-[3-((3-(4-fluorosulphonyl)benzoyl)-oxy)-propyl]-1-*N*-propyl-xanthine (FSCPX, **1**) is described. The availability of ample amounts of the irreversible antagonist FSCPX allowed us to use FSCPX as a research tool for adenosine A_1 receptors in in vivo experiments. After verification of the irreversible antagonistic function of FSCPX in in vitro experiments, FSCPX was used successfully as a 'receptor knock-down' tool in in vivo experiments on conscious rats.

Introduction

Adenosine is a purine nucleoside that is widely distributed throughout the mammalian body, modulating a variety of physiological functions via extracellular adenosine receptors. The currently known adenosine receptors are classified into four subtypes, called A_1, A_{2A}, A_{2B} and A_3 receptors.[1,2] Activation of the adenosine A_1 receptor has been shown to slow heart rate,[3] to reduce blood pressure,[4] to inhibit lipolysis,[5,6] and to inhibit neurotransmitter release.[7] Although the actions of adenosine mediated by the adenosine A_1 receptors are well characterized, no selective ligands for this receptor subtype are in clinical use yet. Due to the wide distribution of adenosine A_1 receptors in the body, many agonists show undesired effects.

Irreversible antagonists (photoaffinity as well as chemoreactive ligands) have been used to measure receptor reserve,[8–10] and to identify ligand binding sites.[11,12] Chemoreactive compounds were made through attachment of reactive electrophilic groups to xanthine derivatives, for example with an isothiocyanate moiety in *meta*-1,3-phenylenediisothiocyanate xanthine amine congener(*m*-DITC-XAC)[13] and 8-(3-isothiocyanatostyryl) caffeine (ISC),[14] or via a *p*-(fluorosulfonyl)benzoyl group in 8-cyclopentyl-3-*N*-[3-((3-(4-fluorosulphonyl)-benzoyl)oxy)-propyl]-1-*N*-propylxanthine (FSCPX, **1**) (Fig. 1).[15,16]

The present study demonstrates that pretreatment of animals with the irreversible antagonist FSCPX (**1**) is a useful alternative or complement for knock-out animals in in vivo experiments. When FSCPX is used as a 'receptor knock-down' tool, relatively large amounts of FSCPX are needed. However, the available synthetic



FSCPX (**1**)

Figure 1.

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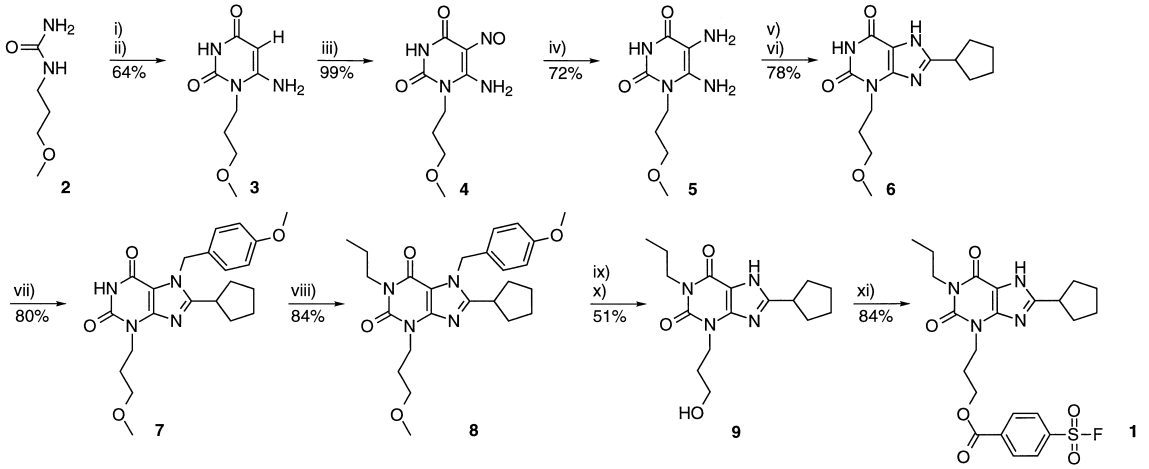

Scheme 1. Reagents: (i) NaOEt; (ii) cyanoacetic ethyl ester, △; (iii) $NaNO_2$, acetic acid , 6 M HCl; (iv) $Na_2S_2O_4$, 50% NH_4OH; (v) cyclopentanecarboxylic acid, EDCI, DMAP, DMF; (vi) 2 N NaOH, △; (vii) *p*-methoxybenzylchloride, K_2CO_3, DMF, △; (viii) K_2CO_3, *n*-propyl iodide, DMF, △; (ix) BBr_3, −78 °C, CH_2Cl_2; (x) TFA, anisole, H_2SO_4 (conc); (xi) 4-(fluorosulfonyl)benzyl chloride, dioxane, △.

method[15] for FSCPX has a very low overall yield and comprises two preparative HPLC separations in the last steps preventing the scaling up of this synthetic method. To obtain FSCPX on a preparative scale we developed a new, eight-step, synthesis route for FSCPX (Scheme 1) with an overall yield of 10%. In this paper, we describe the synthesis of FSCPX and the measurement of its irreversible binding to adenosine A_1 receptors in binding studies. Subsequently, we describe the 'knock-down' approach which was verified in in vitro (cAMP production in time), and in vivo (heart rate in conscious rats) studies.

Chemistry

FSCPX (**1**) was synthesized according to Scheme 1. *N*-3-Methoxypropylureum (**2**)[17] was condensed with cyanoacetic ethylester to give uracil **3** (64%).[18] At this stage, we attempted a selective alkylation of the N3 of the uracil **5** by silylation with HMDS followed by reaction with propylbromide according to the procedure of Müller et al.[19] However, starting material remained present after the silylation step even after prolonged heating. We tried therefore a direct alkylation of the uracil with propyl iodide using several solvents, bases and reaction times, but the expected product, besides di- and trialkylated products, could only be detected (^{1}H NMR, N3-CH_2 δ = 3.7 ppm, 25% in a mixture) after reaction with sodium ethoxide. Likewise, in EtOH with KOH as base[20] low amounts of the product could be measured. Isolation of the pure product was tedious and gave only low yields of product. We therefore decided to alkylate this nitrogen atom in the xanthine stage after selective protection of the N7 position. Standard nitrosation with sodium nitrite and reduction with sodium dithionite, followed by condensation of the resulting diaminouracil **5** with cyclopentanecarboxylic acid using EDCI and DMAP afforded 3-(3-methoxypropyl)-8-cyclopentylxanthine **6** in a good yield (78%). After protection of N7 with 4-methoxybenzyl chloride, the N1 position was alkylated with propylbromide.[21] Deprotection of the methoxy groups with BBr_3 at −78 °C[22] gave a mixture of the expected N3-3-hydroxypropyl derivative and the N3-3-bromopropyl derivative. This mixture was deprotected at the N7 position to give pure xanthine **9** in 51% yield. Esterification of the N3-3-hydroxypropyl group gave FSCPX (**1**) in 10% overall yield.[23]

Biology, Results and Discussion

FSCPX (**1**) was tested for its affinity at, and irreversible binding to rat brain adenosine A_1 receptors. Cortical membranes were preincubated with or without (control) 10 nM FSCPX for 1 h at 37 °C. Membranes were washed 6 times by centrifugation for 10 min at 12,000 g and were resuspended in 50 mM Tris/HCl buffer (pH = 7.4). In subsequent radioligand binding studies the percentage binding to the membranes of the radiolabelled antagonist [^{3}H]-DPCPX was measured at different concentrations of the reference A_1 receptor agonist N^6-cyclopentyladenosine (CPA).

Figure 2 shows that FSCPX binds irreversibly to the rat adenosine A_1 receptors. The maximum level of [^{3}H]-DPCPX binding was decreased, indicating that even after the extensive washing procedure receptors were still occupied by FSCPX. In Table 1, the affinity of CPA for the adenosine A_1 receptor is presented. CPA recognized a high and a low affinity site with affinities of 9.2 and 322 nM, respectively. After pretreatment with 10 nM FSCPX, CPA still recognized these sites (K_i = 11.5 nM and K_i = 230 nM, respectively). Moreover, the percentage of high affinity sites had not been affected by the pretreatment with FSCPX. These data indicate that the remaining receptors had unchanged binding characteristics.

The 'knock-down' approach was tested in vitro in a study on cAMP production in time. CHO cells, stably expressing the human adenosine A_1 receptor, were treated with rolipram (50 μM), cilostamide (50 μM) and adenosine deaminase (10 IU/mL) during 40 min, followed by 15 min incubation with 10 μM forskolin and

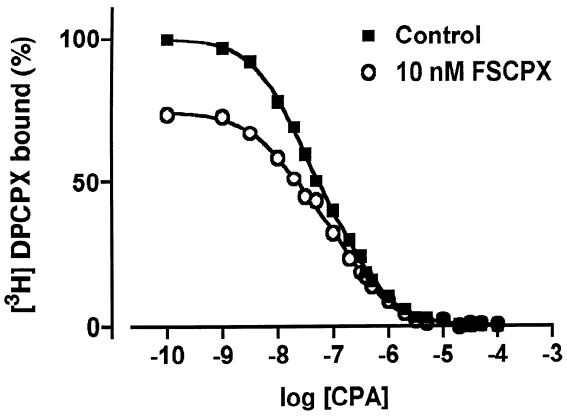

Figure 2. Displacement by CPA of [^{3}H]-DPCPX binding to FSCPX pretreated and non-treated rat cortical membranes. The data in Table 1 were calculated using a curve fitting procedure according to a two-state receptor model (H = high affinity state, L = low affinity state).

Table 1.

	Control	+ 10 nM FSCPX
$K_{i,H}$	9.2 ± 0.4 nM	11.5 ± 7.3 nM
$K_{i,L}$	322 ± 227 nM	230 ± 91 nM
f_{high}	72 ± 10%	61 ± 14%

10 μM CPA. The antagonist FSCPX (5 μM) or 8-cyclopentyltheophylline (CPT, 50 μM) was added and at different time points cAMP formation was stopped by aspiration of the medium and addition of 200 μL 0.1 N HCl. The amount of cAMP was determined in a competition binding assay with [^{3}H]cAMP and protein kinase A (PKA). The results are given in Figure 3 (FSCPX (□), reversible antagonist CPT (○)).

The adenosine A_1 receptor agonist CPA almost fully inhibited the forskolin-stimulated cAMP production (data not shown). Treatment with the reversible antagonist CPT reduced this decrease slightly, and after 10 min the cAMP production was stabilized because of the settled equilibrium between the agonist- and antagonist-bound receptors. However, treatment of the cells with FSCPX counteracted the induced decrease in cAMP production. A decreased inhibition of cAMP production was perceived, most probably due to FSCPX binding irreversibly overtime to the adenosine A_1 receptors. In other words, FSCPX 'knocked-down' the adenosine A_1 receptors.

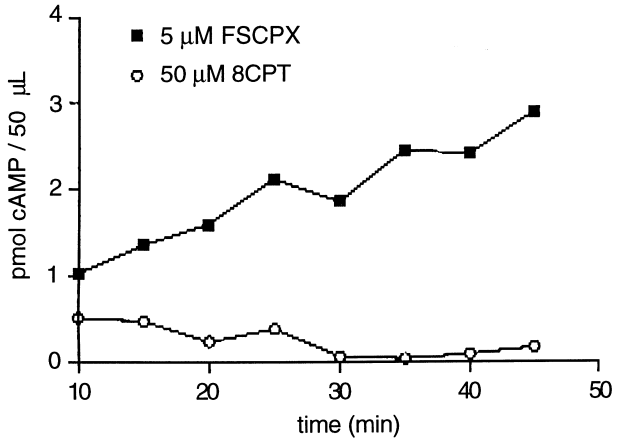

Figure 3. Time course for inhibition of the CPA-induced decrease of cAMP production in CHO cells expressing the human adenosine A_1 receptor.

A series of experiments were performed to demonstrate that FSCPX also acts as an irreversible adenosine A_1 receptor antagonist in vivo. First, even at the lowest (0.3 mg/kg iv) dose tested, FSCPX directly and completely antagonised the decrease in heart rate induced by a 15 min iv infusion of 0.2 mg/kg of the selective adenosine A_1 receptor agonist, CPA (data not shown). Subsequently, the irreversible nature of the antagonism by FSCPX was demonstrated by pretreating rats with different doses of FSCPX (0, 1, 3 and 5 mg/kg given as an iv infusion over 5 min) 20 h prior to administration of CPA. FSCPX had no effect on basal heart rate but the CPA-mediated bradycardia was markedly attenuated 20 h after the treatment in a dose-dependent manner, as determined by the area under the time–effect relationship (Fig. 4). Remarkably, in some rats of the 5 mg/kg treatment group, CPA did not display any bradycardiac effect at all, while basal heart rate was unchanged. The antagonism by FSCPX was not due to a nonspecific effect, because the decrease in heart rate produced by the cholinergic agonist, carbachol, was not affected by FSCPX pretreatment (data not shown).

FSCPX was tested for stability in rat whole blood by a method described by Pavan and IJzerman[24] and analyzed using TLC. We observed a rapid degradation at 37 °C, that is after 2.5 min only the hydrolysed product was detected. Therefore, it is very unlikely that the loss of effect of CPA was due to the presence of significant concentrations of FSCPX in the circulation during the time of the experiments. The short half-life makes this compound useful as 'knock-down' tool in in vivo studies by iv infusion.[25] Overall, therefore, these experiments show that specific and gradual 'knock-down' of adenosine A_1 receptor-mediated responses can also be achieved in vivo with FSCPX.

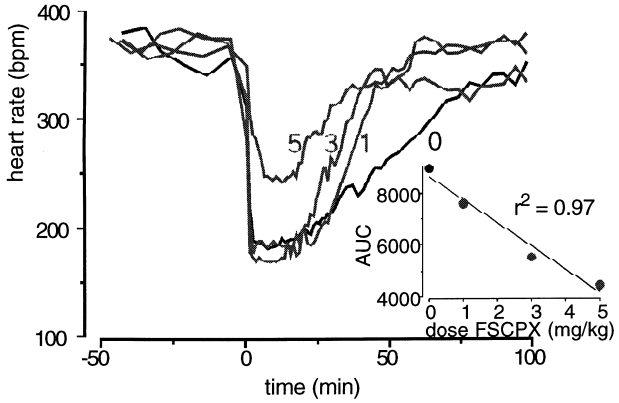

Figure 4. Dose-dependent in vivo pharmacological knockdown of cardiac adenosine A_1 receptors. In vivo effect on heart rate in conscious rats ($n = 6$–9) of a 15 min infusion of the selective adenosine A_1 receptor agonist, CPA (0.2 mg/kg iv) administered 20 h after pretreatment with 0, 1, 3 and 5 mg/kg FSCPX (5 min iv infusion). The inserted graph shows the corresponding relationship between dose of FSCPX and area under the CPA time–effect curve (AUC).

Conclusions

The in vivo and in vitro studies described in this paper reveal that the irreversible adenosine A_1 receptor antagonist FSCPX is effective as a 'knock-down' tool. For in vivo studies, FSCPX is needed in relatively large amounts. Therefore, we developed a new route for the preparative synthesis of this irreversible adenosine A_1 receptor antagonist.

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