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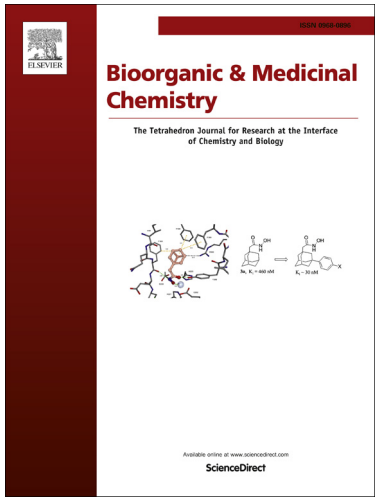

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AFQ056/mavoglurant, a novel clinically effective mGluR5 antagonist: Identification, SAR and pharmacological characterization.

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ABSTRACT

Here we describe the identification, structure-activity relationship and the initial pharmacological characterization of AFQ056/mavoglurant, a structurally novel, non-competitive mGlu5 receptor antagonist. AFQ056/mavoglurant was identified by chemical derivatization of a lead compound discovered in a HTS campaign. In vitro, AFQ056/mavoglurant had an IC_{50} of 30 nM in a functional assay with human mGluR5 and was selective over the other mGluR subtypes, iGluRs and a panel of 238 CNS relevant receptors, transporter or enzymes. In vivo, AFQ056/mavoglurant showed an improved pharmacokinetic profile in rat and efficacy in the stress-induced hyperthermia test in mice as compared to the prototypic mGluR5 antagonist MPEP. The efficacy of AFQ056/mavoglurant in humans has been assessed in L-dopa induced dyskinesia in Parkinson's disease and Fragile X syndrome in proof of principle clinical studies.

Abbreviations

$[Ca^{2+}]i$	Intracellular Ca^{2+} concentration
CDZ	chlordiazepoxide
95% CI	95-percent confidence interval (as obtained with best-curve fitting)
FLIPR	Fluorescence Imaging Plate Reader ™ (assay)
GABAB	Gamma-amino butyric acid receptor, subtype B
L-glu	L-glutamate
HTS	high-throughput screening
IC_{50} (EC_{50})	Concentration at which half-maximal inhibition (stimulation) is observed
hmGluR5a	human metabotropic glutamate receptor, subtype 5a
hmGluR1b	human metabotropic glutamate receptor, subtype 1b
mGluR	metabotropic glutamate receptor
P2Y2	purinoreceptor of the P2Y2 type
PI	phosphoinositol turnover (assay)
SIH	stress induced hyperthermia
s.d.	standard deviation

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1. Introduction

In the last two decades, pharmacological interventions to modulate the glutamate neurotransmitter system have been the focus of intense drug discovery and development efforts aimed at the identification of novel therapeutic solutions to a number of brain disorders. The first attempts were aimed to inhibit the action of the ionotropic glutamate receptors such as the N-Methyl-D-Aspartate receptor (NMDAR) with competitive antagonists for the therapeutic indications stroke[1] and epilepsy.[2] The clinical development of antagonists acting at this ionotropic glutamate receptor, however proved to be challenging due to either a limited efficacy or the emergence of adverse effects that prevented their use as therapeutic agents.[3] The identification of the metabotropic glutamate receptor (mGluR) family and agents capable of selectively modulating a sub-group or even individual subtypes gave rise to new possibilities for pharmacological interventions aimed at treating dysfunctions of the glutamatergic neurotransmission.[4]

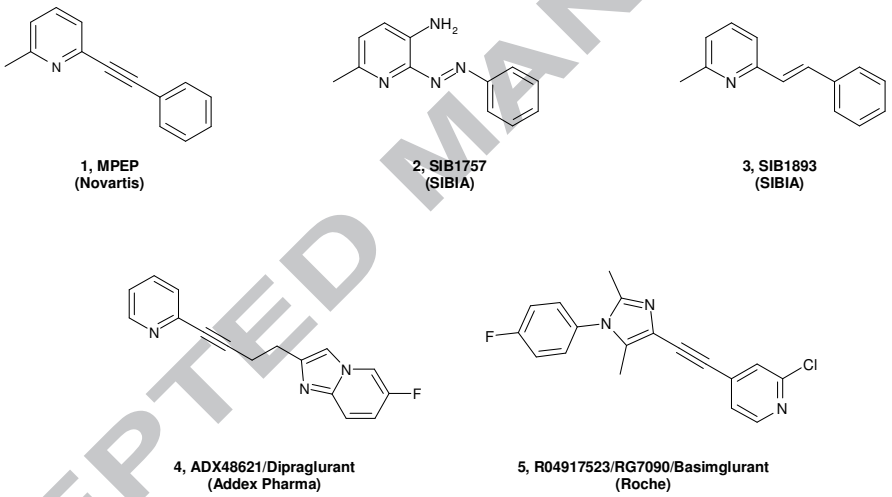


Figure 1. Selected mGluR5 antagonists

For the mGlu5 receptor subtype (mGluR5), significant progress in the understanding of its role and potential therapeutic applications of ligands was made following the disclosure of the first potent and selective non-competitive antagonist MPEP (**1**) and its precursor molecules SIB1757 (**2**) and SIB1893 (**3**).[5,6] Subsequent investigations involving the use of mutated receptor constructs revealed that MPEP and its derivatives interact with a novel binding pocket distal from the glutamate binding site located in the transmembrane domain.[7] More detailed investigations, using site-directed mutagenesis revealed three amino acids, non-conserved between mGluR5 and its sibling mGluR1, that are responsible for the binding and the selectivity of MPEP and its derivatives at the mGluR5 subtype.[7]

Following the reporting of MPEP and its non-competitive mode of action, intense preclinical discovery efforts involving industrial and academic research laboratories led to the identification of a number of preclinical and clinical development candidates.[8,9,10,11,12]

Thanks to its overall favorable properties, MPEP proved to be a very useful tool to explore and reveal the role of antagonizing the mGlu5 subtype in various pathologies such as inflammatory pain, anxiety, Parkinson's disease or Fragile X mental retardation syndrome.[13]

Here we describe the process which led to the identification, the initial preclinical characterization and the selection of AFQ056/mavoglurant, a selective non-competitive antagonist which showed efficacy in the treatment of L-dopa induced dyskinesias in Parkinson's disease and Fragile X mental retardation in proof of principle studies.[14,15] Ongoing clinical programs involving mGluR5 antagonists include ADX48621/Dipraglurant (**4**) (Addex Pharma) (Fig. 1) for Parkinson's disease[16] and RG7090/Basimglurant (**5**) (Roche) for Fragile X and major depressive disorders.[17]

2. Results and discussion

2.1.High throughput screening

With the aim to identify chemical scaffolds differing from the original MPEP structure, we performed a high throughput functional screening using the human mGluR5 recombinantly expressed in L(tk-) cells.[18,19] Glutamate-evoked changes in intracellular calcium concentration $[Ca^{2+}]_i$ were measured using the FLIPR/Fluo-3 method and a total of 214,544 compounds were tested in primary screening as mixtures of 10. 23 validated hits were identified to have good selectivity (>10 fold) over the mGluR1 subtype and an endogenously expressed P2Y receptor. Further filtering of the validated hits allowed the identification of (6) as lead compound (Figure 2).

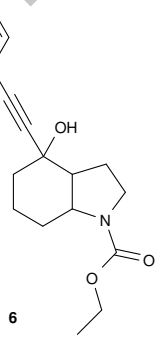


Figure 2. Chemical structure of (±)-(3aRS,4SR,7aRS)-4-Hydroxy-4-phenylethynyl-octahydro-indole-1-carboxylic acid ethyl ester (**5**).

Although sharing part of the structural elements of the original lead MPEP, the newly identified lead (**6**) was considered structurally sufficiently diverse owing to the presence of a tertiary alcohol and a saturated octahydro-indole moiety, which was to be retained for a lead optimization process.

2.2. Lead optimization/structure activity relationships

The previous work in the MPEP series did not allow us to identify a development candidate with an appropriate combination of favorable drug-like properties. Particularly limiting were the poor pharmacokinetic properties observed in rodents such as limited oral bioavailability and high clearance. Therefore, the goal of the lead optimization process described here was to identify a suitable preclinical development candidate with appropriate potency, selectivity, and physico-chemical and drug-like properties. Three main directions for modification of the lead molecule

were defined 1) Explore the nature and the position of substituents on the aromatic ring 2) Investigate the spacer between the aromatic ring and the octahydroindole moiety and 3) Investigate the role of the nitrogen substituent as illustrated in Figure 3:

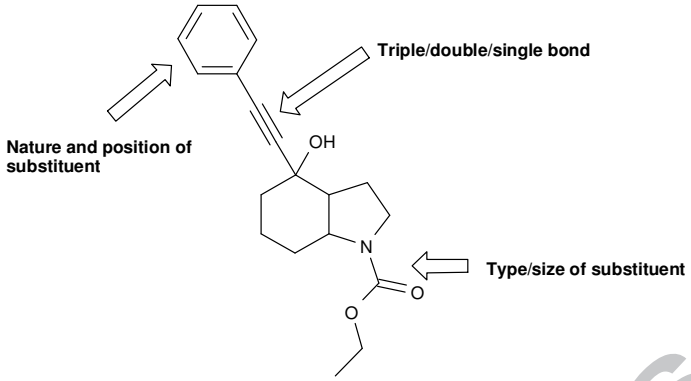


Figure 3. Main modifications directions for exploration of SAR of compound **6**

For the lead optimization phase, the *in vitro* pharmacological characterization was performed with two functional assays (biochemical determination of PI levels[20] and microfluorimetric assessment of $[Ca^{2+}]_i$ levels using a FLIPR™ device) with cells expressing recombinant human mGluR5.[18] Representatives of the new lead series displaced [^{3}H]-M-MPEP binding to preparations of rat brain,[21] with binding affinities roughly in line with functional inhibition, suggesting an allosteric interaction similar to MPEP. The results are reported in the Table 1.

The effect of the position of the substituent on the aromatic ring was investigated by introducing a chlorine atom in ortho (**7**), meta (**8**) and para (**9**) positions. Clearly the meta position resulted in the most active derivative with an IC_{50} of 0.11 µM for (**8**). Variation of the nature of the substituent in the meta position showed that a methyl group (**10**) was more potent than the fluorine (**11**) or methoxy (**12**) substituted derivatives. Double substitution in positions C-3 and C-5 with fluorine (**13**) or methyl (**14**) significantly reduced the activity compared to the corresponding monosubstituted derivatives.

To assess the role of the triple bond as spacer between the aromatic group and the octahydro-indole moiety it was replaced with either a *cis* (**15**) or *trans* (**16**) double bond or a single bond (**17**). This replacement resulted in a significant loss of potency. The exchange of the tertiary alcohol by a methoxy group (**18**) led to a complete loss of activity.

The substituent R2 on the nitrogen was investigated by maintaining the 3-methyl substituent (R1) on the aromatic ring and variation of the length and the nature of the substituent either as a carbamate or an amide function. In the case of the carbamate derivatives introduction of a methyl (**10**) instead of an ethyl substituent (**19**) had only a marginal effect on the potency: IC_{50} of 0.18 µM for (**10**) vs 0.25 µM for (**19**). The change of the carbamate function for an amide gave a contrasting result with no activity for a methoxy-methyl (**20**), weak activity, 43% of inhibition at 1 µM, for an acetamide (**21**) and a higher activity, IC_{50} = 0.22 µM, with a 2-furanyl amide substituent (**22**).

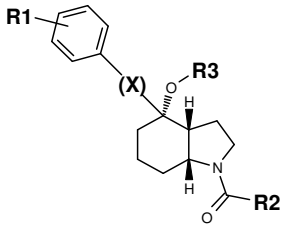


Table 1. Antagonistic activity of derivatives produced via Scheme 1

Cpd	R1	R2	R3	X	IC_{50}[μM] (95% CI) or % inhibition at conc [μM]		
					Ca^{2+}-FLIPR	IP_3 turnover	M-MPEP binding displacement[a]
6	H	OEt	H	C≡C	0.17 (0.14, 0.20)	0.13 (0.04-0.4)	0.37 (0.27, 0.50)
7	2-Cl	OEt	H	C≡C	68% at 10 μM -3% at 1 μM		2 % at 10 μM
8	3-Cl	OEt	H	C≡C	0.11 (0.07-0.17)	0.023	
9	4-Cl	OEt	H	C≡C	62% at 10 μM 10% at 1 μM		16 % at 10 μM
10	3-Me	OMe	H	C≡C	0.18 (0.13-0.25)	0.052 (0.02-0.13)	95% at 1 μM 35 % at 0.11 μM
11	3-F	OEt	H	C≡C	0.28 (0.27-0.29)	0.31(0.16 – 0.64)	0.21 (0.14 – 0.31)
12	3-OMe	OEt	H	C≡C	0.26 (0.21-0.32)	0.13 (0.05 – 0.27)	0.26 (0.18 – 0.38)
13	3,5-F_2	OEt	H	C≡C	1.4 (0.92 - 2.0)	1.52 (1.1 – 2.2)	4 (1.0 - 16)
14	3,5-Me_2	OEt	H	C≡C	48% at 10 μM -15% at 1 μM	54 % at 10 μM	
15	H	OEt	H	(Z)-C=C			53% at 10 μM
16	H	OEt	H	(E)-C=C	98% at 10 μM 54% at 1 μM		81% at 10 μM
17	H	OMe	H	C-C	36% at 10 μM		24% at 10 μM
18	H	OEt	Me	C≡C	-6% at 10 μM		-6% at 10 μM
19	3-Me	OEt	H	C≡C	0.25 (0.13 - 0.35)	0.073 (0.04 – 0.12)	0.072 (0.044 - 0.12)
20	3-Me	CH_2OMe	H	C≡C	-4% at 1 μM		20 % at 1 μM
21	3-Me	Me	H	C≡C	43% at 1 μM		
22	3-Me	2-Furanyl	H	C≡C	0.22 (0.16 - 0.31)	0.036 (0.026 - 0.05)	

[a][^{3}H]$_3$-M-MPEP binding displacement in rat brain membranes[21]

While initially the optimization was performed using the racemic mixtures, the identification of **(±)-10** as the most promising candidate prompted us to investigate whether the chirality played a role for inhibitory activity. The choice of the 3-methyl substituted **(±)-10** over the closely related

and more potent 3-chloro substituted **(±)-8** was made based on the lower lipophilicity and higher solubility of **(±)-10**, which showed a logP of 3.7 compared to 4.8 for **(±)-8**. For this purpose, the two enantiomers of the derivative **10** were synthesized as described on scheme 1.

(-)-10/AFQ056/mavoglurant, the (-)-enantiomer showed IC_{50} of 0.11 µM and 0.03 µM (Ca^{2+} and PI-turnover) whereas the (+)-enantiomer ((+)-**10**) showed only 37% and 18 % inhibition at 10 µM (Table 2 and supplementary data). The preference for the (-)-enantiomeric configuration was confirmed with the two derivatives **(-)-19** and **(-)-22** which showed IC_{50}s of 0.032 and 0.035 µM, respectively (PI-turnover assay; Table 2).

Table 2. Antagonistic activity of AFQ056, its enantiomer and two close analogues

Cpd	R1	R2	R3	X	IC_{50}[µM] (95% CI) or % inhibition at conc [µM]		
					Ca^{2+}-FLIPR	IP_3 turnover	M-MPEP binding displacement
(-)-10 AFQ056/mavoglurant	3-Me	OMe	H	C≡C	0.11 (0.9 – 0.15)	0.03 (0.02 – 0.05)	0.047 (0.03 – 0.07)
(+)-10	3-Me	OMe	H	C≡C	37% at 10 µM	18% at 10 µM	n.d.
(-)-19	3-Me	OEt	H	C≡C	0.25 (0.13 – 0.5)	0.032 (0.015 – 0.07)	0.13 (0.13 – 0.2)
(-)-22	3-Me	2-Furanyl	H	C≡C	0.18 (0.15 – 0.27)	0.035 (0.016 – 0.08)	0.073 (0.06 – 0.09)

The absolute configuration was determined by resolving the crystal structure of a carbamate derivative on the 4-hydroxy of the (+)-rotating enantiomer and was shown to be (3aS,4R,7aS) for ((+)-**10**) and (3aR,4S,7aR) for (**(-)-10**) (Supplementary data, Fig S1).

2.3. In vitro pharmacological characterization

The lead optimization process delivered the compound **(-)-10** as the most promising candidate based on the activity at the mGlu5 receptor, its reduced lipophilicity and increased solubility. We therefore determined its pharmacological profile at the mGluR subtypes, the $GABA_B$ receptor (which is structurally somewhat related to mGluRs) and its selectivity toward a large panel of receptors, enzymes and transporters.

2.3.1. Activity at group I mGluRs

Figure 4 illustrates the in vitro functional activity of AFQ056/mavoglurant. AFQ056/mavoglurant fully antagonized hmGluR5-mediated responses with IC_{50} values of 110 and 30 nM in Ca^{2+}-and PI-turnover assays but did neither inhibit mGluR1-mediated responses nor showed intrinsic agonist-like activity up to 10 µM. Additionally, AFQ056/mavoglurant was assessed for inhibition of ATP-stimulated PI formation mediated through endogenously expressed purinoreceptors (P_2Y_2 type) in the hmGluR5a-expressing cells; no effect was observed in the range between 0.1 to 30 µM.

A	B

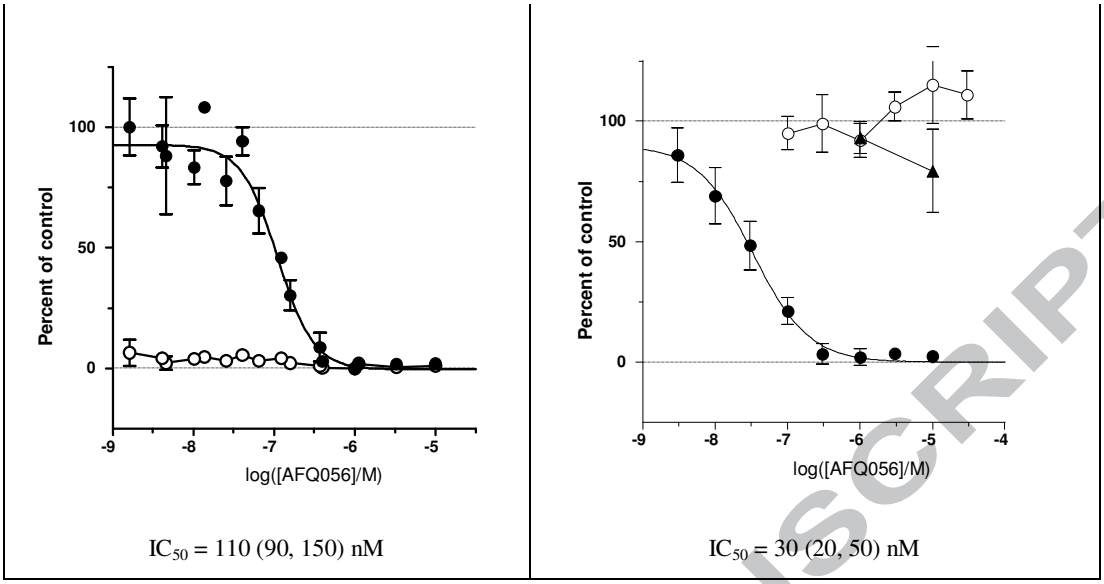
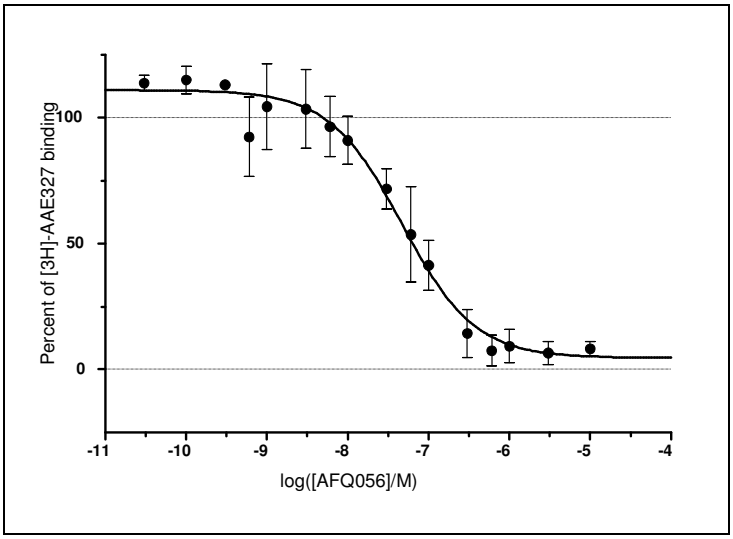


Figure 4. A. Concentration-dependence of effects of AFQ056 on calcium mobilization in L(tk-) cells stably expressing mGluR5a as determined with the FLIPR system. Data represent percentage of amplitude of maximal L-glu-(10 µM)-induced control response (means ± s.d.) of pooled independent determinations (N = 2), one performed in duplicates, one in quadruplicates. Empty symbols (○): effect of test compounds (as indicated in top row) applied alone; filled symbols (●): effect on L-glu-induced Ca2+-mobilization; solid curves: concentration-response curves obtained by fitting the four-parametric logistic equation to the pooled data sets. The respective IC_{50} values (95% CI in brackets) are given below each panel. B. Concentration-dependence of effects of AFQ056 on PI turnover in cells expressing hmGluR5a or hmGluR1b. Data (means ± s.d.) represent percentage of amplitude with respect to maximal agonist-induced control response of N = 3 pooled independent determinations, each performed in triplicates. Filled symbols (●): effect of test compound (as indicated in top row) on quisqualate (0.3 µM)-induced PI formation in hmGluR5a-expressing cells; empty symbols (○): effect on quisqualate (30 µM)-induced PI formation in hmGluR1b-expressing cells, data of N = 2 independent determinations performed in triplicates. Triangles (▲): Effect of AFQ056 on ATP (10 µM)-induced PI formation of N = 2 pooled determinations performed in triplicates. IC_{50} values (95% CI in brackets) are given below each panel.

2.3.2. Site of action

To investigate whether the new series interacts with the previously described allosteric ligand recognition site mediating the inhibition of MPEP and its structural derivatives,[7] we measured binding displacement of [^{3}H]-AAE327/M-MPEP, a ligand interacting at the allosteric binding site.[21] AFQ056/mavoglurant fully displaced the radioligand in a concentration-dependent manner with an IC_{50} of 47 nM, showing that AFQ056 indeed interacts with the same allosteric binding site as MPEP (Figure 5).



IC_{50} = 47 (30,72) nM

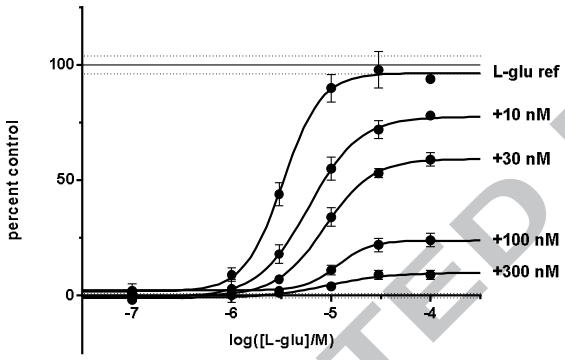
k_i = 26 (17, 40) nM

Figure 5. Concentration-dependence of radioligand displacement by AFQ056 determined in rat brain membranes (mixed hippocampus and cortex). Data (means ± s.d.) represent percentage of specific binding of [^{3}H]-AAE327 (2 nM) of N = 3 pooled independent determinations, each performed in triplicates. IC_{50} and k_i values (95% CI in brackets) are given below.

2.3.3. Mode of action

To assess the inhibitory mode of action of AFQ056/mavoglurant, we performed a Schild-like analysis using the functional PI turnover assay, whereby the effects of the compound at different fixed concentrations on the concentration-response relation of L-glu were determined. In two independent experiments, AFQ056/mavoglurant clearly reduced the response amplitude to L-glu in a concentration-dependent manner (Figure 6A). The EC_{50} of L-glu slightly increased in the presence of increasing concentrations of AFQ056/mavoglurant (from approx. 3 µM to 12 µM), while the Hill coefficient did not vary appreciably (Figure 6B). This result indicated that AFQ056/mavoglurant mediates inhibition at hmGluR5 through a non-competitive mode of action.

A



B

+AFQ056 (nM)	fit parameters, 95% C.I. top (% ctr)	bottom (% ctr)	L-glu EC50 (uM)	nHill
none	97 ± 8	2 ± 10	3.3 (2.6, 4.2)	2.3 ± 1.4
10	78 ± 5	-1 ± 5	6.0 (4.9, 7.4)	1.7 ± 0.5
30	59 ± 3	0 ± 2	8.6 (7.5, 9.9)	1.8 ± 0.4
100	24 ± 4	2 ± 2	12.0 (8.3, 16)	2.7 ± 2.8
300	10 ± 9	-2 ± 4	8.6 (1.2, 63)	1.2 ± 2.4

Figure 6. A. Effects of AFQ056 on L-glu-stimulated PI turnover in L(tk-) cells expressing hmGluR5a. The graph illustrates one of two independent experiments yielding equivalent results. B. Data (means ± s.d., normalized to control) and concentration-response curves obtained by fitting the four-parametric logistic equation (determining parameters: top, bottom, logEC_{50} and Hill coefficient) to the data sets obtained in the absence (L-glu ref) and presence of AFQ056 added at 10, 30, 100 and 300 nM.

2.3.4. Selectivity

AFQ056/mavoglurant was tested for agonistic/positive modulatory and antagonistic activity at representative subtypes of group II/III mGlu and $GABA_B$ receptors, using a GTPγ^{35}S based assays and did not show agonist/positive modulator activity or antagonist action at 1-10 µM at the hmGluR2, 3, 4, 6, 7, 8 or h$GABA_B$R1/2 (Table 3 and supplementary data).

Table 3. Selectivity profile of AFQ056/mavoglurant

Targets	Activity	Selectivity vs IC_{50} at hmGluR5 (IP_3)
mGluR1,2,4,7	NE at 10 µM	> 300

hmGluR3,6,8 (Tested at Euroscreen)	NE at 10 µM	> 300
hGABA$_B$R1/2	NE at 10 µM	> 300
MDS Pharma screen (238 targets)	NE at 10 µM for all targets except for the 5HT$_{1A}$ receptor 75 % Inh at 10 µM (IC$_{50}$ = 13.2 µM)	> 300

NE: No effect

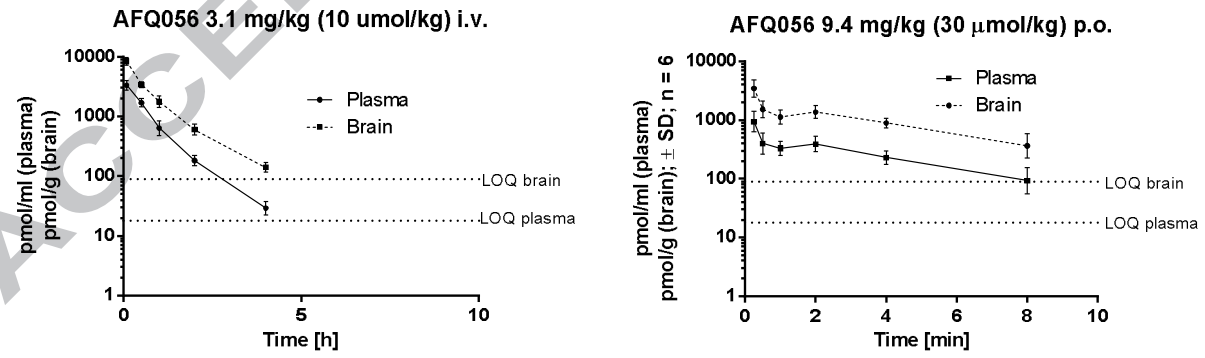
The selectivity profile of AFQ056/mavoglurant was further assessed in a panel of 238 targets including receptors, enzymes, ion channels and transporters. AFQ056 was tested at a single concentration of 10 µM in duplicates. Of the 238 targets tested, AFQ056 showed a weak affinity only to the 5HT$_{1A}$ receptor (75% inhibition at 10 µM). Further investigation in a concentration response mode revealed an IC$_{50}$ of 13.2 µM. To the other tested targets, AFQ056 did not exhibit any significant binding to any of the receptor sites tested up to a concentration of 10 µM. Taken together, AFQ056 showed a > 300 fold selectivity for the mGluR5 over all targets tested (Table 3 and supplementary data for list of targets).

2.4. In vivo characterization

2.4.1. Pharmacokinetics

The pharmacokinetic properties of AFQ056/mavoglurant were determined in rats after a single intravenous (3.1 mg/kg; 10 µmol/kg) or oral (9.4 mg/kg; 30 µmol/kg) administration (Figure 7A). For comparison, the prototypic mGluR5 antagonist MPEP was tested under similar conditions (2.3 mg/kg; 10 µmol/kg, i.v. and 6.9 mg/kg; 30 µmol/kg, p.o.) (Figure 7B).

A



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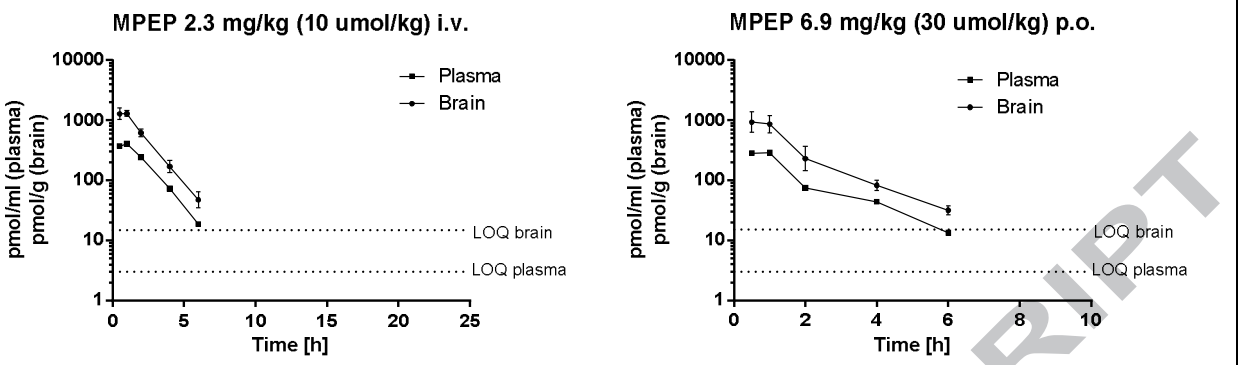


Figure 7: A) Concentration of AFQ056 in rat plasma and brain following i.v. drug administration (3.1 mg/kg; 10 µmol/kg) (left) or p.o. (9.4 mg/kg; 30 µmol/kg) (right) Limit of quantification (LOQ): < 18 pmoles/ml plasma or < 90 pmoles/g brain. B) Concentration of MPEP•HCl in rat plasma and brain following i.v. drug administration (2.3 mg/kg; 10 µmol/kg) (left) or p.o. (6.9 mg/kg; 30 µmol/kg) (right). LOQ: 3 pmol/ml plasma and 15 pmol/g brain. Each point represents the mean ± SD of n=6 rats.

The maximum concentration of AFQ056/mavoglurant after oral administration in plasma and brain was reached at/or before 0.25 h, indicating that AFQ056/mavoglurant readily and significantly penetrated into the brain with a brain/plasma concentration ratio between ~ 3 and 4. An apparent terminal half-life of 2.9 hours was estimated for the removal of orally administered AFQ056/mavoglurant from the plasma compartment. Following intravenous administration, AFQ056/mavoglurant was quickly taken up in the brain, as it reached Tmax at/or before 0.08 h (Figure 7; Table 4). The bioavailability of 32 % was clearly superior to that of MPEP, which showed only 17 %. In a liver microsomal stability assay, both molecules demonstrated a fast clearance in the rat tissue whereas AFQ056/mavoglurant showed a significantly lower clearance in the human microsomes compared to MPEP (see Table 3). Overall, in rats AFQ056/mavoglurant demonstrated a superior pharmacokinetic profile after oral administration compared to MPEP.

	Dose	**Cmax (plasma/brain)**	**Tmax**	**$T_{1/2}$**	**Clearance**	**Oral BAV**	**B/P Ratio**	**Clearance in microsomes**	
								Human	**Rat**
	µmol/kg; mg/kg	pmol/ml (p) pmol/g (b)	h	h	ml•min^{-1}•kg^{-1}	%		µl•min^{-1}•mg^{-1}	
AFQ056								113	277
Oral	30 / 9.4	950/3500	≤ 0.25	2.9		32	3.4-3.9		
Intravenous	10 / 3.1	3330/8400	≤ 0.08	0.69	65		2.0-4.8		
MPEP								578	254
Oral	30 / 6.8	290/930	1	1.6		17	2.4-3.3		
Intravenous	10 / 2.3	410/1300	1	1.1	149		2.3-3.4		

Table 4: Summary of PK parameters for AFQ056/mavoglurant and MPEP after i.v. and p.o. administration to rats. Results were determined using nonlinear curve fitting procedures (software TOPFIT;[22] Non-compartmental model).

Based on the *in vivo* properties in rat and the in vitro metabolic stability in human microsomes for AFQ056 we extrapolated a potential favorable PK profile in human, these predicted favorable properties were confirmed in human studies published recently.[23]

2.4.2. Activity in Stress induced hyperthermia in mice

The prototypic mGluR5 antagonist MPEP showed robust anxiolytic-like effects in rodents, and anxiety was one of the earliest therapeutic uses proposed for mGlu5 antagonists.[24,25] To assess anxiolytic-like properties of AFQ056/mavoglurant we have chosen the stress induced hyperthermia in rodents, a test which was shown to be sensitive to the effect of mGluR5 antagonists[25] and to the genetic ablation of the mGlu5 receptor.[26] The study was performed in mice with oral administration of 10/1/0.1 mg/kg as described by Spooren et al.[27] and using the benzodiazepine chlordiazepoxide (CDZ) at a dose of 10 mg/kg p.o. as a positive control.

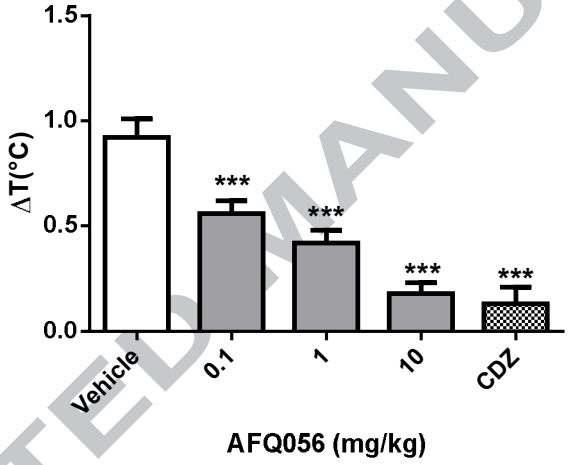


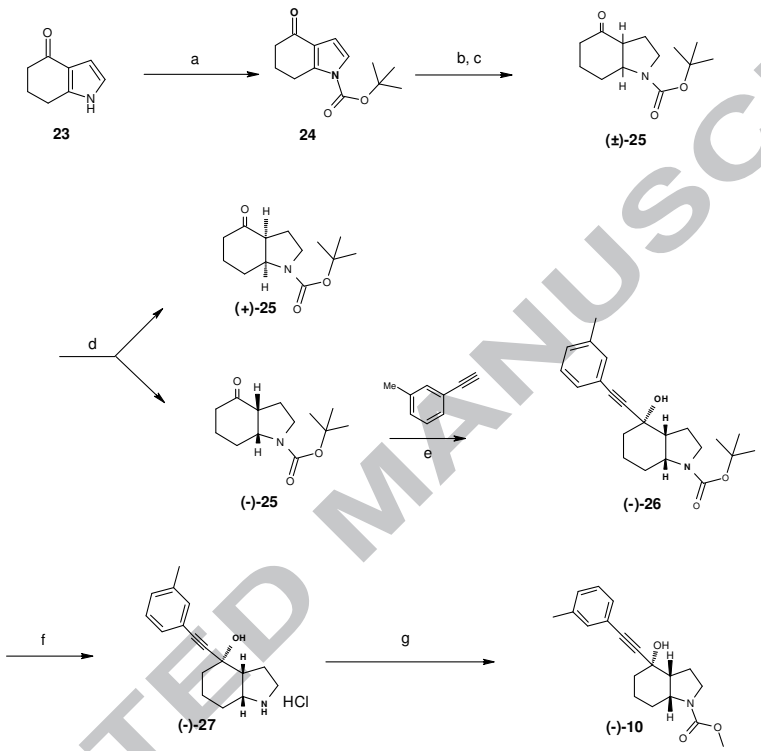
Figure 8: Stress induced hyperthermia in mice. Individually housed mice were treated with AFQ056 at the indicated doses, CDZ (10 mg/kg p.o.) or with vehicle (n=24 per group) and one hour later rectal temperature was measured (T1). In control subjects, stress caused by this procedure resulted in a significant hyperthermia as measured 15 minutes later (T2). Stress-induced hyperthermia (SIH) is defined as T2-T1. Bars represent mean (± s.e.m.). Levels of statistical significance versus the vehicle group are shown with *** $p< 0.001$ (Dunnett's multiple comparison of means).

The results, shown in Figure 8, demonstrate a dose-dependent effect of AFQ056/mavoglurant on SIH at 0.1, 1 and 10 mg/kg p.o. The efficacy of AFQ056/mavoglurant in attenuating the SIH was comparable to the positive control CDZ and was achieved without affecting motor performance as measured in the rotarod assay (data not shown). We attempted to determine the PK/PD relationships by measuring the brain levels of AFQ056 following the SIH assessment (90 minutes post dose). We could only measure brain concentrations of 780 pmol/mg and 104 pmoles/mg in mice dosed with 10 and 1 mg/kg respectively, whereas no drug could be detected in animals dosed at 0.1 mg/kg. Only after improvement of the sensitivity of the detection, we were able to determine that the total brain concentration achieved after administration of the 0.1 mg/kg dose was 8 ± 3 pmol/mg. The observed significant inhibition of the SIH achieved with such a low brain level can be explained with the very short half-life of AFQ056 in mice ($T_{1/2}$ = 0.2 h; data not shown) and the effect on SIH results from an action of the compound in the brain between 60

and 75' post dose whereas the PK was measured 90' post dose. Additionally, as demonstrated by other mGluR5 antagonists such as CTEP, the effect on SIH can be achieved at very low brain concentrations far below the levels needed to achieve full receptor occupancy.[28]

2.5. Chemistry

The examples reported have been synthesized using a linear synthesis starting with the commercially available 1,5,6,7-Tetrahydro-indol-4-one (**23**) as illustrated on scheme 1.



Scheme 1. Reagents and conditions: (a) BOC2, tert-BuOK, THF, reflux, 2 h, 92 %; (b) H2, Pt/C, MeOH, 1 bar, 92 h, 67 %; (c) (COCl)2, DMSO, TEA, THF, 65 %; (d) Chromatographical separation of enantiomers on Chiralpak AD; (e) 1) BuLi, THF, - 20°C; 2) **(-)-25**, 0°C, 2 h, 85 %; (f) HCl, AcOEt, RT, 6 h, 93%; (g) MeOCOCl, TEA, THF, 2 h, 90 %.

1,5,6,7-Tetrahydro-indol-4-one (**23**) is treated with di-tert–butyldicarbonate in the presence of KOtBu in THF to obtain the protected tetrahydro-indolone **24**. **24** is exhaustively hydrogenated with Pt (5%) on charcoal and the resulting alcohol is oxidized with oxalyl chloride and DMSO in THF to afford racemic 4-oxo-octahydro-indole-1-carboxylic acid tert-butyl ester (±)-**25**. The racemate (±)-**25** is separated into its enantiomers by chromatography on a Chiralpak AD column. The (+) enantiomer (+)-**25** and the (-) enantiomer (-)-**25** are obtained with an enantiomeric purity of 99% and 99.9% respectively.

Lithiated 1-ethynyl-3-methyl-benzene is added to (-)-**25** stereoselectively to give the tertiary alcohol (-)-**26** which is deprotected with dry hydrochloride in ethyl acetate. The resulting hydrochloride salt of octahydro-indole (-)-**27** is treated with methylchloroformate in the presence of triethylamine to afford after crystallization pure (-)-(3aR,4S,7aR)-4-hydroxy-4-m-tolylethynyl-octahydro-indole-1-carboxylic acid methyl ester ((-)-**10**, AFQ056/mavoglurant).

3. Summary/Conclusions

At the time this work was initiated, MPEP was the only selective mGluR5 antagonist described.[5] Using MPEP as a scaffold, we conducted extensive derivatization efforts with the aim to identify and develop suitable candidates for various therapeutic indications.[29] Unfortunately, these efforts did not succeed due to various reasons, such as preclinical safety issues or poor pharmacokinetic properties.

In order to identify new chemical matter differing from MPEP, we conducted a HTS campaign yielding 23 validated hits. Further filtering using molecular weight limits, functional potency and selectivity over the sibling mGlu1 receptor subtype and the P2Y receptor allowed the identification of the novel lead compound **6**. Despite sharing some of the structural elements with MPEP, the presence of a saturated carbocyclic element, a tertiary alcohol group and the overall tri-dimensional structure were considered significantly different and the hit molecule **6** was retained for a hit-to-lead program. The derivatization of **6** was conducted along three main directions and allowed the identification of the meta substitution, a triple bond and a methylcarbamate as the optimal combination as illustrated with the derivative (±)-**10** as the best derivative of the series considering its activity at the receptor and lower lipophilicity compared to the 3-chloro derivative **8**. Separation of the enantiomers of (±)-**10** and their characterization revealed the (-)-(3aR,4S,7aR) stereochemical conformation of the octahydro-indole as the most active conformation as illustrated with (-)-**10**/AFQ056/mavoglurant.

Further *in vitro* characterization demonstrated that AFQ056/mavoglurant is a functional antagonist with a potency of 30 nM (IC_{50} in the PI_3-turnover assay) with a non-competitive mode of action. AFQ056/mavoglurant completely displaced the binding of the allosteric binding ligand [^{3}H]-AAE327 demonstrating that it exerts its antagonistic action by interacting with the site located in the transmembrane domain characterized by Pagano et al.[7] This finding was very recently confirmed with the publication of a co-crystal structure of the TM domain of mGluR5 with AFQ056 by Doré et al.[30] AFQ056/mavoglurant is selective for the mGlu5 subtype without significant functional activity at any of the other mGluR subtypes or the $GABA_B$ receptor up to a concentration of 10 μM. Additionally, the activity of AFQ056/mavoglurant was assessed at a large panel of receptors, transporters and enzymes and demonstrated a highly selective profile. With the exception of weak antagonistic activity at the $5HT_{1A}$ receptor (IC_{50} = 13.2 μM), no activity was detected up to 10 μM at any of the other targets.

In vivo, AFQ056/mavoglurant demonstrated superior pharmacokinetic elements, such improved bioavailability as compared to MPEP in rats, and the significantly reduced in vitro clearance in human liver microsomes. In the stress induced hyperthermia model in mice, AFQ056 demonstrated significant reduction of the SIH after single oral administrations of 0.1 and 1 mg/kg with an almost complete inhibition at 10 mg/kg, which was comparable to the effects of the prototypical anxiolytic chlordiazepoxide (CDZ).

Based on the in vitro and in vivo profiles described here, AFQ056/mavoglurant was selected as preclinical candidate, further pre-clinically developed and successfully assessed clinically in proof-of-principle studies.[14,15]

4. Experimental part

4.4. General methods

4.5. General procedure for the synthesis of AFQ056/mavoglurant derivatives

4.5.1. 4-Oxo-4,5,6,7-tetrahydro-indole-1-carboxylic acid tert-butyl ester (24)

1,5,6,7-Tetrahydro-indol-4-one **23** (Aldrich # 35,783-9; 38.4 g, 28.1 mmol), di-tert-butyldicarbonate (66 g; 302 mmol) and potassium tert-butylate (6 g; 62.5 mmol) in 1 l tetrahydrofuran are heated under reflux for 2 h. After cooling at room temperature the reaction mixture is poured on brine (1 l) and extracted with tert.-butylmethylether (4 X 500 ml). The combined organic phases are dried over Na_2SO_4, filtered and evaporated in vacuo. 51 g of a yellowish oil are isolated and purified by column chromatography on silica gel (600 g; eluent hexane/ethyl acetate 8:2 v/v). 30.5 g (92 %) of **24** as white crystals are isolated (mp 84-86°C).

4.5.2. (±)-(3aRS,7aRS)-4-Oxo-octahydro-indole-1-carboxylic acid tert-butyl ester ((±)-25)

Hydrogenation:

A mixture of **24** (60 g; 255 mmol) and 15 g of 5% Pt on charcoal (given in three portions of 5 g each; 24h, 48, 72h) in 1 l of methanol are hydrogenated (1 bar) at room temperature under stirring for 92 h. The mixture is filtered and the solvent evaporated in vacuo. The residual brownish oil is purified by chromatography on silica gel to yield (±)-(3aRS,4SR,7aRS)-4-hydroxy-octahydro-indole-1-carboxylic acid tert-butyl ester as a yellowish oil (41.3 g; yield = 67 %). This mixture was used without purification for the next step.

Oxidation of alcohols:

To a solution of oxalylchloride (1.54 ml; 17.6 mmol) in THF (320 ml) cooled to –60°C a solution of DMSO (2.28 ml; 32 mmol) in THF (32 ml) is added dropwise under stirring. After 5 min a solution of (±)-(3aRS,4SR,7aRS)-4-hydroxy-octahydro-indole-1-carboxylic acid tert-butyl ester (3.96 g; 16.4 mmol) in THF (64 ml) is added and the reaction mixture is stirred for 100 min at –60°C. Triethylamine (11.2 ml; 80 mmol) is added and the cooling bath removed and the reaction mixture stirred for further 60 min. The reaction mixture is diluted with ethyl acetate (1 l) and washed with sat. $NaHCO_3$(150 ml). The water phase is extracted with ethyl acetate (300 ml). The combined organic phases are dried over Na_2SO_4, filtered and evaporated in vacuo. The residue is purified by column chromatography on silica gel (150 g) and the fractions containing the desired compound are collected and evaporated in vacuo to yield **(±)-25/**(±)-(3aRS,7aRS)-4-Oxo-octahydro-indole-1-carboxylic acid tert-butyl ester (2.51 g; yield = 65 %).

4.5.3. (-)-(3aR,7aR)-4-Oxo-octahydro-indole-1-carboxylic acid tert-butyl ester ((-)-25) (Separation on chiral column)

4 g of **(±)-25/**(±)-(3aRS,7aRS)-4-oxo-octahydro-indole-1-carboxylic acid tert-butyl ester are dissolved in 200 ml of hexane-ethanol 80:20 (v/v). This solution is injected via the pump on a 5 cm by 50 cm Chiralpak AD column (Daicel Chemical Industries). The chromatography is achieved at room temperature at a flow-rate of 100 ml/min and UV detection is performed at 210 nm. The mobile phase consists of a mixture of hexane-ethanol 80:20 (v/v). Under the applied

chromatographic conditions, the (+)-enantiomer is isolated from a first fraction collected between 11 and 18 min, and the (-)-enantiomer from a second fraction collected between 20 and 40 min. After 6 injections of a total of 27 g of the racemate, the fractions containing the corresponding enantiomers are combined to yield 12.6 g of the (+)-enantiomer and 12.2 g of the (-)-enantiomer, with an enantiomeric purity of 99% and 99.9%, respectively. The enantiomeric purity is determined on an analytical Chiralpak AD column (0.4 x 25 cm); mobile phase, hexane-ethanol 90:10 (v/v). **(-)-25/**(-)-(3aR,7aR)-4-oxo-octahydro-indole-1-carboxylic acid tert-butyl ester ($[\alpha]_D$= -111.6, ethanol, c = 0.96 %); **(+)-25/**(+)-(3aS,7aS)-4-oxo-octahydro-indole-1-carboxylic acid tert-butyl ester ($[\alpha]_D$= +105, ethanol, c = 1.0 %).

4.5.4. (-)-(3aR,4S,7aR)-4-Hydroxy-4-m-tolylethynyl-octahydro-indole-1-carboxylic acid tert-butyl ester ((-)-26)

To a solution of 1-ethynyl-3-methyl-benzene (3.248 g; 28 mmol) in THF (168 ml) cooled to –20°C, a solution of butyllithium (17.5 ml; 28 mmol; 1.6M in hexane) is added. The reaction mixture is further stirred at –20°C for 2 h then a solution of **(-)-25** (-)-4-oxo-octahydro-indole-1-carboxylic acid tert-butyl ester (3.346 g; 14 mmol) in THF (70 ml) is added and the reaction mixture further stirred at 0-5°C. After 2 h the reaction mixture is diluted with ethyl acetate (900 ml) and washed with sat. $NaHCO_3$ (2 x 90 ml). The aqueous phase is extracted with ethyl acetate (400 ml). The combined organic phases are dried over Na_2SO_4, filtered and evaporated in vacuo. The residue is purified by column chromatography on silica gel (300 g) and the fractions containing the desired compound are collected and evaporated in vacuo to yield **(-)-26/**(-)-(3aR,4S,7aR)-4-Hydroxy-4-[2-(3-methylphenyl)ethynyl]-octahydro-1*H*-indole-1-carboxylic acid tert-butyl ester (4.27 g; yield = 85 %). ^{1}H-NMR (400 MHz; DMSO-d_6): δ 7.3-7.1 (m, 4H), 5.5 (d, J = 5 Hz, 1H), 3.85-3.65 (m, 1H), 3.35-3.25 (m, 1H), 3.25-3.1 (m, 1H), 2.6-2-45 (m, 1H), 2.28 (s, 3H), 1.9-1.4 (m, 7H), 1.36 (s, 9H), 1.13-0.98 (m, 1H). $[\alpha]_D$ = - 21 (ethanol c = 1.15 %).

4.5.5. (-)-(3aR,4S,7aR)-4-m-Tolylethynyl-octahydro-indol-4-ol hydrochloride salt ((-)-27)

In a 1M solution of HCl in ethyl acetate (249 ml) **(-)-26** (-)-(3aR,4S,7aR)-4-Hydroxy-4-m-tolylethynyl-octahydro-indole-1-carboxylic acid tert-butyl ester (4.27 g; 12 mmol) is dissolved and stirred at room temperature for 6 h. After completion of the hydrolysis (TLC) the solvent is evaporated in vacuo to yield (-)-**27/**(-)-(3aR,4S,7aR)-4-hydroxy-4-m-tolylethynyl-octahydro-indole hydrochloride (3.39 g; yield = 93 %). m.p. = 181-183°C. ^{1}H-NMR (400 MHz; DMSO-d_6): δ 7.28-7.14 (m, 4H), 5.94 (broad s), 3.6-3.53 (m, 1H), 3.37-3.25 (m, 1H), 3.21-3.11 (m, 1H), 2.56-2.45 (m, xH), 2.28 (s, 3H), 2.16-2.04 (m, 1H), 2.02-1.92 (m, 1H), 1.91-1.41 (m, 6H). ES-MS (+): 256.2 $[M-H_2O+1]^+$. $[\alpha]_D$ = -72.7 (ethanol c = 1 %).

4.5.6. (-)-(3aR,4S,7aR)-4-Hydroxy-4-m-tolylethynyl-octahydro-indole-1-carboxylic acid methyl ester ((-)-10/AFQ056/mavoglurant)

Triethylamine (3.6 ml; 25.52 mmol) is added to a suspension of **(-)-27/**(-)-(3aR,4S,7aR)-4-hydroxy-4-m-tolylethynyl-octahydro-indole hydrochloride (3.38 g; 11.6 mmol) in CH_2Cl_2 (174 ml) and the mixture is cooled to 5°C. Methylchloroformate (1.2 ml; 15.1 mmol) is added dropwise. After completion of the addition, the cooling bath is removed and the solution stirred for 2 h. The reaction mixture is diluted with CH_2Cl_2 (250 ml) and washed with brine (1 x 50 ml). The aqueous phase is extracted with CH_2Cl_2 (50 ml), the combined organic phases are dried over Na_2SO_4, filtered and the solvent evaporated in vacuo. The residue is column chromatographed on silica gel (240 g), eluent toluene/acetone 9:1 v/v. The fractions containing the desired compound are collected and evaporated in vacuo to yield 3.39 g of **(-)-10/**(-)-(3aR,4S,7aR)-4-hydroxy-4-m-tolylethynyl-octahydro-indole-1-carboxylic acid methyl ester (yield = 90 %). m.p. = 110-112 °C.

^{1}H-NMR (600 MHz; DMSO-D6): δ 7.29-7.23 (m, 2H), 7.23-7.15 (m, 2H), 5.76 (br. s., 1H), 3.82 (dddd, *J* = 23.7, 11.4, 6.1, 5.8 Hz, 1H), 3.45-3.37 (m, 1H), 3.55 (s, 3H), 3.31-3.2 (m, 1H), 2.63-2.52 (m, 1H), 2.29 (s, 3H), 1.96-1.77 (m, 3H), 1.72 (d, *J* = 11.3 Hz, 1H), 1.66 – 1.45 (m, 3H), 1.18-1.03 (m, 1H). ES-MS (+): 314.1 $[M+H]^+$. $[\alpha]_D$= -21.2 (methanol, c = 1%).

4.5.7. (±)-(3aRS,4SR,7aRS)-4-Hydroxy-4-phenylethynyl-octahydro-indole-1-carboxylic acid ethyl ester ((±)-6)

To a solution of phenyl acetylene (6.2 ml, 57 mmol) in THF (100 ml) is added a solution of n-butyllithium in hexane (35 ml, 57 mmol) at 0 °C dropwise via a syringe. After stirring for 75 min at 0°C a solution of 10 g of (±)-(3aRS,7aRS)-4-oxo-octahydro-indole-1-carboxylic acid ethyl ester[31] (10 g, 47 mmol) in THF (50 ml) is added dropwise at 0 °C and the reaction mixture is allowed to warm to room temperature. Stirring is continued for 90 minutes. The solvent is removed in vacuo, the residue is dissolved in CH_2Cl_2 and washed two times with aqueous ammonium chloride solution and with brine. The combined organic phases are dried over Na_2SO_4, filtered and the solvent evaporated in vacuo. The residue is flash chromatographed on silica gel, eluent hexane / CH_2Cl_2 / acetone 5:4:1 v/v. The fractions containing the desired compound are collected and the product is crystallized from ether / hexane to afford (±)-**6** (±)-(3aRS,4SR,7aRS)-4-hydroxy-4-phenylethynyl-octahydro-indole-1-carboxylic acid ethyl ester (yield = 56 %), m.p. = 109 – 110 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.42 (b, 2H), 7.33 (m, 3H), 4.15 (q, 2H), 4.0 (m, 1H), 3.55 (b, 1H), 3.40 (b, 1H), 2.63 (m, 1H), 2.05-1.1 (m, 9H), 1.25 (t, 3H).

4.5.8. (±)-(3aRS,4SR,7aRS)-4-(2-Chloro-phenylethynyl)-4-hydroxy-octahydro-indole-1-carboxylic acid ethyl ester ((±)-7)

To a solution of 1-chloro-2-ethynyl benzene (0.388 g, 2.84 mmol) in THF (5 ml) is added a solution of n-butyllithium in hexane (1.78 ml, 2.84 mmol) at 0 °C dropwise via a syringe. After stirring for 60 min at 0°C a solution of (±)-(3aRS,7aRS)-4-oxo-octahydro-indole-1-carboxylic acid ethyl ester[31] (0.5 g, 2.37 mmol) in THF (1 ml) is added dropwise at 0 °C and the reaction mixture is allowed to warm to room temperature. Stirring is continued for 30 minutes. The solvent is removed in vacuo, the residue is dissolved in CH_2Cl_2 and washed three times with aqueous ammonium chloride solution and with brine. The combined organic phases are dried over $MgSO_4$, filtered and the solvent evaporated in vacuo. The residue is flash chromatographed on silica gel, eluent hexane / ethyl acetate 2:1 v/v. The fractions containing the desired compound are collected and the product is crystallized from hexane / ethyl acetate to afford (±)-**7** (±)-(3aRS,4SR,7aRS)-4-(2-Chloro-phenylethynyl)-4-hydroxy-octahydro-indole-1-carboxylic acid ethyl ester (yield = 47 %), m.p. = 134 – 135 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.46 (d, 1H), 7.41 (d, 1H), 7.30 – 7.18 (m, 2H), 4.12 (q, 2H), 4.10 (m, 1H), 3.55 (b, 1H), 3.40 (b, 1H), 2.65 (m, 1H), 2.1 - 1.1 (m, 9H), 1.25 (t, 3H). ES-MS (+): 348.2 $[M+H]^+$.

4.5.9. (±)-(3aRS,4SR,7aRS)-4-(3-Chloro-phenylethynyl)-4-hydroxy-octahydro-indole-1-carboxylic acid ethyl ester ((±)-8)

To a solution of 1-chloro-3-ethynyl-benzene (0.5 g, 3.66 mmol) in THF (10 ml) is added a solution of n-butyllithium in hexane (2.9 ml, 3.6 mmol) at -5 °C dropwise via a syringe. After stirring for 90 min at -5°C a solution of (±)-(3aRS,7aRS)-4-oxo-octahydro-indole-1-carboxylic acid ethyl ester[31] (0.386 g, 1.83 mmol) in THF (1 ml) is added dropwise at -5 °C and the reaction mixture is allowed to warm to room temperature and diluted with diethylether (150 ml). The resulting solution is washed two times with aqueous sat $NaHCO_3$, H_2O and dried over $MgSO_4$, filtered and the solvent evaporated in vacuo. The residue is flash chromatographed on silica gel,

eluent hexane / Ethyl acetate 3:1 v/v. The fractions containing the desired compound are collected and **8** is obtained as yellowish oil (Yield = 27%). The product is obtained in 27 % yield. ^{1}H NMR (400MHz; $CDCl_3$): δ 7.40 (m, 1H), 7.22-7.31 (m, 3H), 4.10 (m, 2H), 3.54 (m, 1H), 3.38 (m, 1H), 2.27 (m, 1H), 1.88-2.11 (m, 5H), 1.60-1.80 (m, 4H), 1.27 (t, 3H).

4.5.10. (±)-(3aRS,4SR,7aRS)-4-(4-Chloro-phenylethynyl)-4-hydroxy-octahydro-indole-1-carboxylic acid ethyl ester ((±)-9)

To a solution of 4-chloro-phenyl acetylene (0.388 g, 2.84 mmol) in THF (5 ml) is added a solution of n-butyllithium in hexane (1.78 ml, 2.84 mmol) at 0 °C dropwise via a syringe. After stirring for 30 min at 0°C this solution is added dropwise to a solution of (±)-(3aRS,7aRS)-4-oxo-octahydro-indole-1-carboxylic acid ethyl ester[31] (0.5 g, 2.37 mmol) in THF (2 ml) cooled at -78°C. After 15' at -78°C, the reaction mixture is allowed to warm to room temperature. The solvent is removed in vacuo, the residue is dissolved in CH_2Cl_2 and washed three times with aqueous ammonium chloride solution and with brine. The combined organic phases are dried over $MgSO_4$, filtered and the solvent evaporated in vacuo. The residue is flash chromatographed on silica gel, eluent hexane / ethyl acetate 1:1 v/v. The fractions containing the desired compound are collected and the product is crystallized from hexane / ethyl acetate to afford (±)-**9**, mp. 108-109 °C, yield 40 %. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.35 (d, 2H), 7.30 (d, 2H), 4.12 (q, 2H), 3.97 (m, 1H), 3.55 (b, 1H), 3.40 (b, 1H), 2.62 (m, 1H), 2.1 - 1.1 (m, 9H), 1.25 (t, 3H). ES-MS (+): 348.2 $[M+H]^+$.

4.5.11. (±)-(3aRS,4SR,7aRS)-4-Hydroxy-4-m-tolylethynyl-octahydro-indole-1-carboxylic acid methyl ester ((±)-10)

Triethylamine (0.44 ml; 3.18 mmol) is added to a suspension of (±)-(3aR,4S,7aR)-4-hydroxy-4-m-tolylethynyl-octahydro-indole hydrochloride (0.54 g; 2.12 mmol) in CH_2Cl_2 (5 ml) and the mixture is cooled at 0°C. Methylchloroformate (0.4 ml; 5.1 mmol) is added dropwise. After completion of the addition, the cooling bath is removed and the reaction mixture allowed to warm at room temperature. The reaction mixture was washed with a 10% solution of citric acid, brine and dried over K_2CO_3. After filtration the solvent evaporated in vacuo. The residue is column chromatographed on silica gel (240 g), eluent toluene/acetone 9:1 v/v. The fractions containing the desired compound are collected, evaporated in vacuo and the product is crystallized from ethyl ether / hexane to afford (±)-**10**, mp. 107.5- 108 °C, yield 63 %. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.26-7.07 (m, 4H), 5.26 (s, 1H), 4.09 (m, 1H), 3.94 (m, 1H), 3.65 (s, 3H), 3.52 (m, 1H), 3.36 (m, 1H), 2.68 (m, 1H), 2.3 (s, 3H), 2.14-1.61 (m, 6H), 1.07 (m, 1H). ES-MS (+): 336.2 $[M+Na]^+$.

4.5.12. (±)-(3aRS,4SR,7aRS)-4-(3-Fluoro-phenylethynyl)-4-hydroxy-octahydro-indole-1-carboxylic acid ethyl ester ((±)-11)

To a solution of 1-ethynyl-3-fluoro-benzene (0.18 g, 1.5 mmol) in THF (2.5 ml) is added a solution of n-butyllithium in hexane (1.05 ml, 1.5 mmol) at -20 °C dropwise via a syringe. After stirring for 60 min at -20°C a solution of (±)-(3aRS,7aRS)-4-oxo-octahydro-indole-1-carboxylic acid ethyl ester[31] (0.22 g, 1 mmol) in THF (0.5 ml) is added dropwise at -20 °C. The reaction mixture is allowed to warm to room temperature and after 2 h diluted with diethylether. The resulting solution is washed with aqueous sat NH_4Cl, H_2O and dried over $MgSO_4$, filtered and the solvent evaporated in vacuo. The residue is flash chromatographed on silica gel, eluent hexane / ethyl acetate 7:1 v/v. The fractions containing the desired compound are collected and (±)-**11** is

obtained as colorless foam (Yield = 46 %). ^{1}H-NMR (400 MHz; DMSO-D6): δ 7.44-7.31 (m, 1H), 7.29-7.18 (m, 3H), 5.79 (d, J = 5 Hz, 1H), 4.05-3.88 (m, 2H), 3.85-3.74 (m, 1H), 3.43-3.17 (m, 2H), 2.62-2.47 (m, 1H), 1.92-1.76 (m, 3H), 1.73-1.65 (m, 1H), 1.64-1.44 (m, 3H), 1.17-1.01 (m, 4H). HPLC-MS: 354 $(M+Na)^+$.

4.5.13. (±)-(3aRS,4SR,7aRS)-4-Hydroxy-4-(3-methoxy-phenylethynyl)-octahydro-indole-1-carboxylic acid ethyl ester ((±)-12)

To a solution of 1-ethynyl-3-methoxy-benzene (0.2 g, 2 mmol) in THF (3.5 ml) is added a solution of n-butyllithium in hexane (1.3 ml, 2.1 mmol) at -20 °C dropwise via a syringe. After stirring for 60 min at -20°C a solution of (±)-(3aRS,7aRS)-4-oxo-octahydro-indole-1-carboxylic acid ethyl ester[31] (0.22 g, 1 mmol) in THF (0.5 ml) is added dropwise at -20 °C. The reaction mixture is allowed to warm to room temperature and after 2 h diluted with diethylether. The resulting solution is washed with aqueous sat NH_4Cl, H_2O and dried over $MgSO_4$, filtered and the solvent evaporated in vacuo. The residue is flash chromatographed on silica gel, eluent hexane / ethyl acetate 7:1 v/v. The fractions containing the desired compound are collected and (±)-**12** is obtained as colorless gum (Yield = 64 %). ^{1}H-NMR (400 MHz; DMSO-d_6): δ 7.33-7.24 (m, 1H), 7.03-6.89 (m, 3H), 5.75 (d, J = 5 Hz, 1H), 4.08-3.92 (m, 2H), 3.87-3.72 (m, 1H), 3.75 (s, 3H), 3.44-3.19 (m, 2H), 2.61-2.48 (m, 1H), 1.9-1.79 (m, 3H), 1.76-1.67 (m, 1H), 1.64-1.46 (m, 3H), 1.19-1.03 (m, 4H). HPLC-MS: 366 $(M+Na)^+$.

4.5.14. (±)-(3aRS,4SR,7aRS)-4-(3,5-Difluoro-phenylethynyl)-4-hydroxy-octahydro-indole-1-carboxylic acid ethyl ester ((±)-13)

To a solution of 1-ethynyl-3,5-difluoro-benzene (0.26 g, 1.9 mmol) in THF (2 ml) is added a solution of n-butyllithium in hexane (1.3 ml, 2.1 mmol) at -20 °C dropwise via a syringe. After stirring for 60 min at -20°C a solution of (±)-(3aRS,7aRS)-4-oxo-octahydro-indole-1-carboxylic acid ethyl ester[31] (0.22 g, 1 mmol) in THF (0.5 ml) is added dropwise at -20 °C. The reaction mixture is allowed to warm to room temperature and after 2 h diluted with diethylether. The resulting solution is washed with aqueous sat NH_4Cl, H_2O and dried over $MgSO_4$, filtered and the solvent evaporated in vacuo. The residue is flash chromatographed on silica gel, eluent hexane / ethyl acetate 7:1 v/v. The fractions containing the desired compound are collected and (±)-**13** is obtained as colorless oil (Yield = 86 %). ^{1}H-NMR (400 MHz; DMSO-D6): δ 7.33-7.26 (m, 1H), 7.21-7.14 (m, 2H), 5.82 (d, *J* = 5 Hz, 1H), 4.08-3.88 (m, 2H), 3.86-3.74 (m, 1H), 3.43-3.18 (m, 2H), 2.6-2.49 (m, 1H), 1.89-1.77 (m, 3H), 1.76-1.41 (m, 1H), 1.63-1.43 (m, 3H), 1.21-1.0 (m, 4H). HPLC-MS: 350 $(M+H)^+$.

4.5.15. (±)-(3aRS,4SR,7aRS)-4-(3,5-Dimethyl-phenylethynyl)-4-hydroxy-octahydro-indole-1-carboxylic acid ethyl ester ((±)-14)

4.5.15.1. (±)-(3aRS,4SR,7aRS)-4-Hydroxy-4-trimethylsilyl-ethynyl-octahydro-indole-1-carboxylic acid ethyl ester

Prepared from (±)-(3aRS,7aRS)-4-oxo-octahydro-indole-1-carboxylic acid ethyl ester and trimethylsilyl acetylene according to the same procedure as in 4.5.7. The product is crystallized from CH_2Cl_2 / hexane. ES-MS (+): 332.2 $[M+Na]^+$.

4.5.15.2.(±)-(3aRS,4SR,7aRS)-4-Ethynyl-4-hydroxy-octahydro-indole-1-carboxylic acid ethyl ester

A mixture of 3.3 g (10.7 mmol) of (±)-(3aRS,4SR,7aRS)-4-hydroxy-4-trimethylsilyl-ethynyl-octahydro-indole-1-carboxylic acid ethyl ester and 1.6 g (11.7 mmol) K_2CO_3 in 15 ml methanol is stirred for 2 h at r.t. The solvent is removed in vacuo and the residue is partitioned between CH_2Cl_2 and water. The organic phases are washed with brine, dried over Na_2SO_4, filtered and evaporated in vacuo. The product is obtained as a white solid (2.5 g, 98 %), ^{1}H-NMR (300 MHz, $CDCl_3$): δ 4.12 (q, 2H), 4.0 (b, 1H), 3.52 (bt, 1H), 3.38 (bq, 1H), 2.63 (m, 1H), 2.53 (s, 1H), 2.1 - 1.0 (m, 9H), 1.25 (t, 3H).

4.5.15.3.(±)-(3aRS,4SR,7aRS)-4-(3,5-Dimethyl-phenylethynyl)-4-hydroxy-octahydro-indole-1-carboxylic acid ethyl ester

A mixture of 100 mg (0.42 mmol) (±)-(3aRS,4SR,7aRS)-4-ethynyl-4-hydroxy-octahydro-indole-1-carboxylic acid ethyl ester, 117 mg (0.63 mmol) 1-bromo-3,5-dimethyl-benzene, 15 mg (0.021 mmol) bis(triphenylphosphine)palladium(II) dichloride, 8 mg (0.042 mmol) copper(I) iodide and of 64 mg (0.46 mmol) K_2CO_3 in 0.5 ml DME and 0.5 ml water are heated at 90 °C under argon for 18 h. The reaction mixture is partitioned between diethyl ether and water. The organic phases are washed with brine, dried over Na_2SO_4, filtered and evaporated in vacuo. The residue is flash chromatographed on silica gel, eluent hexane / ethyl acetate 85:15 v/v. The fractions containing the desired compound are collected to afford (±)-**14** (±)-(3aRS,4SR,7aRS)-4-(3,5-dimethyl-phenylethynyl)-4-hydroxy-octahydro-indole-1-carboxylic acid ethyl ester as a white solid (yield = 21 %). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.04 (s, 2H), 6.93 (s, 1H), 4.15 (q, 2H), 3.97 (b, 1H), 3.52 (b, 1H), 3.40 (b, 1H), 2.88 (b, 1H), 2.70 (m, 1H), 2.36 (t, 1H), 2.27 (s, 6H), 2.07-1.1 (m, 7H), 1.25 (t, 3H). ES-MS (+): 364.2 $[M+Na]^+$.

4.5.16. (±)-(3aR,4R,7aR)-4-Hydroxy-4-((Z)-styryl)-octahydro-indole-1-carboxylic acid ethyl ester ((±)-15)

A solution of 150 mg **(±)-6/**(±)-(3aRS,4SR,7aRS)-4-hydroxy-4-phenylethynyl-octahydro-indole-1-carboxylic acid ethyl ester (4.5.7) in 6 ml of a mixture of THF and ethanol (3:1 v/v) is hydrogenated in the presence of 30 mg of a Lindlar catalyst (1 bar/ r.t.) for 30 min. The mixture of starting material and product is purified by flash chromatography with hexane / CH_2Cl_2 / acetone (6:3:1) as the eluent. Pure fractions are collected to yield the product (±)-**15/**(±)-(3aR,4R,7aR)-4-Hydroxy-4-((Z)-styryl)-octahydro-indole-1-carboxylic acid ethyl ester (38 mg) as an oil. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.48 (d, 2H), 7.32 (t, 2H), 7.24 (t, 1H), 6.53 (d, 1H), 5.93 (d, 1H), 4.10 (q, 2H), 3.92 (m, 1H), 3.47 (m, 1H), 3.31 (m, 1H), 2.1 – 1.1 (m, 9H), 1.24 (t, 3H). ES-MS (+): 316.3 $[M+H]^+$.

4.5.17. (±)-(3aR,4R,7aR)-4-Hydroxy-4-((E)-styryl)-octahydro-indole-1-carboxylic acid ethyl ester ((±)-16)

To a mixture of 0.145 ml (1.14 mmol) (E)- and (Z)-(2-bromovinyl)benzene (85:15) in 3 ml THF is added a solution of 0.715 ml (1.14 mmol) butyllithium in hexane at -78 °C. To the red solution is added dropwise a solution of 200 mg (0.95 mmol) of (±)-(3aRS,7aRS)-4-oxo-octahydro-indole-1-carboxylic acid ethyl ester in 1 ml THF at -78 °C. The cooling is removed and stirring is

continued for 1 h. The solvent is removed in vacuo and the mixture is quenched with aqueous ammonium chloride solution and extracted with CH_2Cl_2. The organic phases are washed with brine, dried over Na_2SO_4, filtered and evaporated in vacuo. The residue is flash chromatographed on silica gel, eluent hexane / ethyl acetate 2:1 v/v to afford the product (±)-**16** as an oil (21 mg), ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.40 (d, 2H), 7.32 (t, 2H), 7.26 (t, 1H), 6.73 (d, 1H), 6.47 8d, 1H), 4.12 (q, 2H), 3.96 (m, 1H), 3.53 (m, 1H), 3.38 (q, 1H), 2.5-1.1 (m, 9H), 1.26 (t, 3H). ES-MS (+): 338.2 $[M+Na]^+$.

4.5.18. (±)-(3aR,4R,7aR)-4-Hydroxy-4-phenethyl-octahydro-indole-1-carboxylic acid methyl ester ((±)-17)

Prepared by hydrogenation of **(±)-6**/(±)-(3aRS,4SR,7aRS)-4-hydroxy-4-phenylethynyl-octahydro-indole-1-carboxylic acid ethyl ester (4.5.7.) using Pd/C 5% as the catalyst to give (±)-(3aR,4R,7aR)-4-hydroxy-4-phenethyl-octahydro-indole-1-carboxylic acid ethyl ester, m.p. 91-92 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.29 (t, 2H), 7.26 (d, 1H), 7.20 (d, 2H), 4.10 (q, 2H), 3.85 (b, 1H), 3.52 (m, 1H), 3.35 (q, 1H), 2.70 (m, 2H), 2.33 - 1.1 (m, 11H), 1.15 (t, 3H). ES-MS (+): 340.2 $[M+Na]^+$. The ethyl carbamate (500 mg, 1.58 mmol) is treated with 5 ml of a solution of NaOMe in methanol (5.4 M, 27 mmol) at 100 °C in an autoclave for 4 h. The solvent is removed in vacuo. The residue is partitioned between CH_2Cl_2 and 1 M aqueous citric acid solution. The organic phases are washed with brine, dried over Na_2SO_4, filtered and evaporated in vacuo. The residue is flash chromatographed on silica gel, eluent hexane / ethyl acetate 1:1 v/v to afford the (±)-17 as a white foam after drying (345 mg, 72 %), ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.27 (t, 2H), 7.24 (d, 1H, 7.19 (d, 2H), 3.97 (b, 1H), 3.68 (s, 3H), 3.52 (m, 1H), 3.35 (m, 1H), 2.70 (m, 2H), 2.25 (q, 1H), 2.15 - 1.05 (m, 11H). ES-MS (+): 326.2 $[M+Na]^+$.

4.5.19. (±)-(3aRS,4SR,7aRS)-4-Methoxy-4-phenylethynyl-octahydro-indole-1-carboxylic acid ethyl ester ((±)-18)

To a solution of 100 mg (0.32 mmol) (±)-(3aRS,4SR,7aRS)-4-hydroxy-4-phenylethynyl-octahydro-indole-1-carboxylic acid ethyl ester in 3 ml DMF is added a suspension of 15 mg (0.38 mmol) NaH in 1.5 ml DMF under argon at 0 °C. After stirring for 30 minutes at 0°C 40 µl methyl iodide (0.64 mmol) is added dropwise via a syringe. After 20 minutes stirring is continued at room temperature for 30 min. The reaction mixture is quenched with water and extracted with CH_2Cl_2. The organic phases are washed with brine, dried over Na_2SO_4, filtered and evaporated in vacuo to afford (±)-18 as an oil (yield = 96 %), ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.46 (m, 2H), 7.33 (m, 3H), 4.12 (q, 2H), 3.98 (m, 1H), 3.57-3.33 (m, 3H), 3.46 (s, 3H), 2.77 (m, 1H), 2.15-1.1 (m, 7H), 1.25 (t, 3H). ES-MS (+): 328.3 $[M+H]^+$.

4.5.20. (±)-(3aRS,4SR,7aRS)-4-Hydroxy-4-m-tolylethynyl-octahydro-indole-1-carboxylic acid ethyl ester ((±)-19)

To a solution of 1-ethynyl-3-methyl benzene (0.33 g, 2.84 mmol) in THF (5 ml) is added a solution of n-butyllithium in hexane (1.78 ml, 2.84 mmol) at 0 °C dropwise via a syringe. After stirring for 60 min at 0°C a solution of (±)-(3aRS,7aRS)-4-oxo-octahydro-indole-1-carboxylic acid ethyl ester[31] (0.5 g, 2.37 mmol) in THF (1 ml) is added dropwise at 0 °C and the reaction mixture is allowed to warm to room temperature. Stirring is continued for 60 minutes. The

solvent is removed in vacuo, the residue is dissolved in CH_2Cl_2 and washed three times with aqueous ammonium chloride solution and with brine. The combined organic phases are dried over $MgSO_4$, filtered and the solvent evaporated in vacuo. The residue is flash chromatographed on silica gel, eluent hexane / ethyl acetate 2:1 v/v. The fractions containing the desired compound are collected and the product is crystallized from hexane / ethyl acetate to afford (±)-**19,** 123-124°C. ^{1}H-NMR (400 MHz; $CDCl_3$): δ 7.29-7.11 (m, 4H), 4.17-4.04 (m, 3H), 3.97 (m, 1H), 3.55 (m, 1H), 3.39 (m, 1H), 2.71 (m, 1H), 2.33 (s, 3H), 2.08-1.84 (m, 4H), 1.76-1.65 (m, 3H), 1.25 (m, 3H), 1.1 (m, 1H). ES-MS (+): 350.2 $[M+Na]^+$.

4.5.21. (±)-((3aRS,4SR,7aRS)-4-Hydroxy-4-m-tolylethynyl-octahydro-indol-1-yl)-2-methoxy-ethanone ((±)-20)

Triethylamine (0.053 ml; 0.376 mmol) is added to a suspension of (±)-(3aR,4S,7aR)-4-hydroxy-4-m-tolylethynyl-octahydro-indole hydrochloride (0.08 g; 0.31 mmol) in CH_2Cl_2 (1.5 ml) and the mixture is cooled at 0°C. 2-methoxy-acetyl chloride (0.03 ml; 0.33 mmol) is added dropwise. After completion of the addition, the cooling bath is removed and the reaction mixture allowed to warm at room temperature. The reaction mixture was washed with a 10% solution of citric acid, brine and dried over $MgSO_4$. After filtration the solvent evaporated in vacuo and (±)-**20** is obtained as an oil in 91 % yield. ^{1}H-NMR (400 MHz, $CDCl_3$, rotamers 1:1): δ 7. 25 – 7.10 (m, 4H), 4.45 (m, 0.5H), 4.01, 4.0 (2s, 2H), 4.0 (m, 0.5H), 3.62 (m, 1H), 3.43 (m, 1H), 3.45 (s, 3H), 2.75, 2.67 (2m, 1H), 2.35, 2.32 (2s, 3H), 2.3 – 1.25 (m, 9H). ES-MS (+): 328.3 $[M+H]^+$.

4.5.22. (±)-1-((3aRS,4SR,7aRS)-4-Hydroxy-4-m-tolylethynyl-octahydro-indol-1-yl)-ethanone ((±)-21)

Triethylamine (0.053 ml; 0.376 mmol) is added to a suspension of (±)-(3aR,4S,7aR)-4-hydroxy-4-m-tolylethynyl-octahydro-indole hydrochloride (0.08 g; 0.31 mmol) in CH_2Cl_2 (1.5 ml) and the mixture is cooled at 0°C. Methylchloroformate (0.03 ml; 0.33 mmol) is added dropwise. After completion of the addition, the cooling bath is removed and the reaction mixture allowed to warm at room temperature. The reaction mixture was washed with a 10% solution of citric acid, brine and dried over $MgSO_4$. After filtration the solvent evaporated in vacuo. The residue is column chromatographed on silica gel eluent hexane / ethyl acetate 1:3 v/v. The fractions containing the desired compound are collected, evaporated in vacuo to afford (±)-**21** as an oil in 86 % yield.^{1}H-NMR (400 MHz, $CDCl_3$, rotamers): δ 7.25 – 7.10 (m, 4H), 4.40 (m, 0.5H), 3.92 (m, 0.5H), 3.65 – 3.40 (2m, 2H), 2.78, 2.70 (2m, 1H), 2.35, 2.33 (2s, 3H), 2.08, 2.01 (2s, 3H), 2.5 – 1.0 (m, 9H). ES-MS (+): 298.1 $[M+H]^+$.

4.5.23. (±)-Furan-2-yl-((3aRS,4SR,7aRS)-4-hydroxy-4-m-tolylethynyl-octahydro-indol-1-yl)-methanone ((±)-22)

Triethylamine (0.053 ml; 0.376 mmol) is added to a suspension of (±)-(3aR,4S,7aR)-4-hydroxy-4-m-tolylethynyl-octahydro-indole hydrochloride (0.08 g; 0.31 mmol) in CH_2Cl_2 (1.5 ml) and the mixture is cooled at 0°C. Furan-2 carbonyl chloride (0.07 ml; 0.07 mmol) is added dropwise. After completion of the addition, the cooling bath is removed and the reaction mixture allowed to warm at room temperature. The reaction mixture was washed with a 10% solution of citric acid, brine and dried over $MgSO_4$. After filtration the solvent evaporated in vacuo, the residue is

crystallized from CH_2Cl_2 / ethyl ether to afford (±)-**22**, mp 195-196 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.5 (s, 1H), 7.31 – 7.04 (m, 5H), 4.40 (m, 0.54H), 3.92 (m, 0.46H), 3.65 – 3.40 (2m, 2H), 2.78, 2.70 (2m, 1H), 2.35, 2.33 (2s, 3H), 2.08, 2.01 (2s, 3H), 2.5 – 1.0 (m, 9H). ES-MS (+): 298.1 $[M+H]^+$.

4.5.24. (+)-(3aS,4R,7aS)-4-Hydroxy-4-m-tolylethynyl-octahydro-indole-1-carboxylic acid methyl ester ((+)-10)

(±)-10/(±)-(3aRS,4RS,7aRS)-4-hydroxy-4-m-tolylethynyl-octahydro-indole-1-carboxylic acid methyl ester (1.12 g) was separated into its enantiomers by chromatography on a Chiralpak AD column (Daicel Chemical Industries, 5 x 50 cm). The chromatography is achieved at room temperature at a flow-rate of 150 ml/min and UV detection is performed at 210 nm. The mobile phase consists of a mixture of hexane-ethanol 95:5 (v/v) + 0.1 % TFA. After iterative recycling of fractions, the (+)-enantiomer is isolated first and the (-)-enantiomer second.

Crystallization of the (+)-enantiomer from diethyl ether / n-hexane gave 365 mg of (+)-(3aS,4R,7aS)-4-hydroxy-4-m-tolylethynyl-octahydro-indole-1-carboxylic acid methyl ester (**(+)-10**), m.p. 114.9-115.2 °C, $[\alpha]_D$= +21.3 (methanol 1%, T=21 °C, 589 nm). Optical purity was determined on an analytical Chiralpak AD column, 250x 4.6 mm with n-hexane / EtOH (95:5 + 0.1 % TFA, flow 1 ml/min) as the mobile phase: 100 % ee (RT = 13.4 min, UV detection 210 nm). 1H-NMR (400 MHz, $CDCl_3$): δ 7.25-7.10 (m, 4H), 4.17-3.90 (b, 1H), 3.68 (s, 3H), 3.53 (b, 1H), 3.40 (b, 1H), 2.80 (m, 1H), 2.33 (s, 3H), 2.20-1.50 (m, 9H). ES-MS (+): 336.2 $[M+Na]^+$.

4.5.25. (-)-(3aR,4S,7aR)-4-Hydroxy-4-m-tolylethynyl-octahydro-indole-1-carboxylic acid ethyl ester ((-)-19)

Triethylamine (0.9 ml; 0.6.49 mmol) is added to a suspension of (-)-(3aR,4S,7aR)-4-hydroxy-4-m-tolylethynyl-octahydro-indole hydrochloride (0.86 g; 2.95 mmol) in CH_2Cl_2 (44 ml) and the mixture is cooled at 5°C. Ethylchloroformate (0.366 ml; 3.835 mmol) is added dropwise. After completion of the addition, the cooling bath is removed and the reaction mixture stirred for 2h30' at RT. The reaction mixture is diluted with CH_2Cl_2, washed brine and dried over Na_2SO_4. After filtration the solvent evaporated in vacuo, the residue is column chromatographed on silica gel eluent toluene / acetone 9:1 v/v. The fractions containing the desired compound are collected, evaporated in vacuo to afford **(-)-19**, white crystals: mp 118-121°C. $[\alpha]_D$ = -21.6 (Ethanol 1.09 %). ^{1}H-NMR (400 MHz; DMSO-d_6): δ 7.67-7.14 (m, 4H), 5.73(d, J = 5 Hz, 1H), 4.05-3.93 (m, 2H), 3.85-3.73 (m, 1H), 3.41-3.32 (m, 1H), 3.28-3.17 (m, 1H), 2.6-2-48 (m, 1H), 2.27 (s, 3H), 1.9-1.77 (m, 3H), 1.74-1.66 (m, 1H), 1.64-1.45 (m, 3H), 1.17-1.03 (m, 4H). ES-MS (+): 328.2 $[M+H]^+$

4.5.26. (-)-Furan-2-yl-((3aR,4S,7aR)-4-hydroxy-4-m-tolylethynyl-octahydro-indol-1-yl)-methanone ((-)-22)

Triethylamine (0.53 ml; 3.8 mmol) is added to a suspension of (-)-(3aR,4S,7aR)-4-hydroxy-4-m-tolylethynyl-octahydro-indole hydrochloride (0.5 g; 1.7 mmol) in CH_2Cl_2 (5 ml) and the mixture is cooled at 0°C. Furan-2 carbonyl chloride (0.19 ml; 1.9 mmol) is added dropwise. After completion of the addition, the cooling bath is removed and the reaction mixture allowed to warm

at room temperature. The reaction mixture was washed with a 10% solution of citric acid, brine and dried over $MgSO_4$. After filtration the solvent evaporated in vacuo, the residue is crystallized from CH_2Cl_2 / hexane to afford (-)-**22**, mp 195-196 °C. $[\alpha]_D$= -9.6 (methanol 0.5%, T=20 °C, 589 nm). Optical purity was determined on an analytical Chiralpak AD column, 250x 4.6 mm with n-hexane / EtOH (9:1 + 0.1 % TFA, flow 1 ml/min) as the mobile phase: 100 % ee (R_T = 11.1 min, UV detection 210 nm). ^{1}H-NMR (500 MHz, DMSO-d_6, rotamers 2:1): δ 7.85 (d, 1H), 7.30 – 7.15 (m, 4H), 7.06 (d, 1H), 6.62 (m, 1H), 5.8 (b, 1H), 4.49 (m, 0.33 H), 4.30 (m, 0.67 H), 3.95 (t, 0.67 H), 3.25 (q, 0.67 H), 3.58 (m, 0.66 H), 2.70, 2.58 (2m, 1H), 2.30, 2.29 (2s, 3H), 2.10 – 1.10 (m, 8H).

4.6. Assessment of inhibitory activity at the human mGluR5

Generation of stable cell line and cell culture:

The generation and the culturing of the cell lines stably expressing hmGluR1b, hmGluR2, hmGluR4, hmGluR5a and hmGluR7 were described in detail previously.[18,32,33,34]

Cell culture was performed essentially as described by Flor et al.[32] Briefly, cells were cultured in glutamate free medium composed of DMEM lacking L-glutamate (without phenol red, Gibco, #11880-028) containing a reduced concentration of 2 mM L-glutamine (Gibco, #25030-024), supplemented with 0.046 mg/ml proline (Sigma, #P0380), 10% dialyzed fetal calf serum (Gibco, #26300-061) and 50 mg/ml geneticin (G-418 sulphate, Gibco, #11811-031).

High throughput screening:

Functional antagonists of human mGluR5 were identified by their ability to block glutamate-induced intracellular calcium transients in L(tk-)/hmGluR5 cells utilizing a 96-well FLIPR assay platform in combination with the fluorescent calcium indicator Fluo-3. For a description of the methodology see e.g. Widler et al.[19] In brief, L(tk-) cells were seeded at 60,000 cells/well in clear bottom black plates for 24 h in the presence of 2 μM dexamethasone to up-regulate mGluR5. Cells were loaded with Fluo-3 and washed twice to remove excess of the dye. Cells were then kept at room temperature for 2 hours until prior to testing in the FLIPR device.

Primary assay:

Assessment of hmGluR5 antagonism during the lead optimization phase was also assessed with a FLIPR-based assay using a slightly modified protocol and the fluorescent calcium indicator Fluo-4. Prior to testing, L(tk-)/hmGluR5 cells were cultured and treated essentially as described above, then seeded at 40'000 cells/well, left to incubate with fluo-4 for 75 minutes, washed twice, left to recover for 60 minutes at room temperature, washed once more and left to recover for another 30 minutes. In the FLIPR device, test compounds were dispensed to the cells and left to incubate for 10 minutes, following which cells were challenged by injecting L-glutamate (final assay concentration 10 μM). Concentration-response relations of test compounds were typically derived from 8-point serial dilutions utilizing 2-4 replicates per concentration. IC_{50} values were obtained by fitting the four-parametric logistic equation to the data sets utilizing GraphPad Prism software.

Secondary assay:

The functional phosphoinositol (PI) turnover assays using hmGluR5a- and hmGluR1b-expressing cell lines were performed as described previously by Litschig et al.[20]

Radioligand displacement:

The radioligand displacement assay using a membrane preparation of rat brain (mixture of hippocampus and cortex) was performed as described previously Gasparini et al.[21]

Data analysis and curve fitting

If not stated otherwise, results are given as percentages (mean ± s.d.) normalized to positive and negative controls (i.e. maximal agonist-stimulated effect = 100% and unstimulated [basal] effect = 0%); the percentages represent data pooled replicatewise from at least two independent determinations. To quantify concentration-response relations (regardless of assay), the four-parametric logistic equation was fitted to the pooled means. Where appropriate, the best-fit parameters are quoted with their corresponding 95% confidence intervals (95% CI) as obtained with the fitting procedure (GraphPad Prism 2.0).

4.7. Assessment of in vivo properties

Pharmacokinetic

Animals: Male Sprague-Dawley rats (175-250g) were obtained from Iffa Credo, France. They were housed in groups of three. The animals had access to water and food (Ecosan, Eberle Nafag AG, Gossau, Switzerland), ad libitum.

Drug treatment:

AFQ056/mavoglurant was administered as a microemulsion (made from a mixture of corn oil-mono-di-triglycerides, polyoxyl 40 hydrogenated castor oil NF, DL-α-tocopherol USP, propylene glycol USP and 12 % ethyl alcohol) at a dose of 3.1 mg/kg (10 µmol/kg; application volume 1 ml/kg) intravenously and 9.4 mg/kg (30 µmol/kg; application volume 2 ml/kg) orally. After 0.08, 0.5, 1, 2, 4, 8 or 24 hours (i.v.) and 0.25, 0.5, 1, 2, 4, 8 or 24 (p.o.) (N=6 rats per time-point per injection procedure) the animals were decapitated and trunk-blood was collected in EDTA-containing tubes and the brain was removed and immediately frozen on dry ice. Thereafter, plasma samples and brains were stored at -80°C until analysis.

MPEP was administered to rats intravenously (suspension in 50 % PEG200/50 % H_2O; volume 1 ml/kg) at the dose of 10 µmol/kg (~2.3 mg/kg salt) or per os (suspension in 0.5% methylcellulose; volume 2 ml/kg) at the dose of 30 µmol/kg (~6.9 mg/kg salt). After 0.5, 1, 2, 4, 6 or 24 hours (N=4 rats per time-point per injection procedure) the animals were decapitated and trunk-blood was collected in EDTA-containing tubes and the brain was removed and immediately frozen on dry ice. Thereafter, blood samples and brains were stored at -80°C until analysis.

Determination of brain/blood levels of AFQ056 in mice following SIH

Ninety minutes post dose (10/1/0.1 mg/kg) (N=6 mice) the animals were decapitated and trunk-blood was collected in EDTA-containing tubes and the brain was removed and immediately frozen on dry ice. Thereafter, blood samples and brains were stored at -80°C until analysis.

Analytical methodology, preparation of samples:

Frozen plasma and brain samples were thawed out. To 100 µL plasma 10 µL internal standard (AAG561)[35] (0.1 pmol) was added and extracted three times with 500 µL ethylacetate. The combined extracts were then dried under a stream of nitrogen and redissolved in 100 µL acetonitrile. Brains were weighed and then homogenized in water (1:5 w/v) using an Ultra-Turax

Mod T8 for 30 sec. Two 100 µL aliquots of each homogenate + 10 µL internal standard (0.1 pmol) were extracted three times with 500 µL ethylacetate and further processed as the plasma samples.

Data processing:

The disposition and absorption parameters of AFQ056/mavoglurant and MPEP in rats were analyzed by non-compartmental approach using the software TOPFIT (Tanswell et al 1993)[22] and using the plasma concentration versus time profiles after intravenous or oral administration. The area under the curve (AUC) was obtained from the measured plasma concentrations versus time using the linear trapezoidal rule and extrapolated to infinite time. The terminal half-life and total clearance were calculated by the program from the plasma concentrations after i.v. or p.o. administration versus time.

Stress induced hyperthermia

Animals:

Male OF1/IC mice (Iffa Credo, France) were housed in groups of twelve, in macrolon cages (Type IV, 33 x 55 x 19 cm) in the experimental room of the laboratory, which was temperature controlled and equipped with artificial illumination (6:00 hr - 18:00 hr, lights on). Animals had always free access to food (Ecosan, Eberle Nafag AG, Gossau, Switzerland) and water.

Experimental protocol:

The test procedure for the modified stress-induced hyperthermia was adapted from Van der Heyden et al. (1997)[36] as described by Spooren et al (2002).[27] Approximately 18 hours before testing (i.e. on the afternoon before the test day) animals were individually housed in smaller macrolon cages (26 x 21 x 14 cm) and transferred to the experimental room. The next morning, test-compounds (or vehicle) were administered orally and one hour later rectal temperature was measured (T1). Core temperatures were measured to the nearest 0.1 °C by an ELLAB instruments thermometer (Copenhagen, Denmark) via a lubricated thermistor probe (2 mm diameter) inserted 20 mm into the rectum while the mouse was hand held near the base of the tail. The probe was left in place until steady readings were obtained (± 15 seconds). Immediately after the measurement was completed the probe was removed and the mouse released in his cage. Hyperthermia induced by this procedure was assessed 15 minutes later by a second temperature measurement (T2). The dependent variable, i.e. stress-induced hyperthermia (SIH), was defined as the difference T2 - T1. In addition, T1 was used to evaluate whether the test-compound by itself would have a potential effect on basal body temperature.

Drug treatment:

AFQ056 was administered to mice orally as a suspension in 0.5% methylcellulose (volume 2 ml/kg) at the doses of 0.1/1/10 mg/kg.

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Supplementary Material

Crystal structure determination of a derivative of **(+)-10**, selectivity profile at the mGluR subtypes, binding battery, ^{1}H-NMR spectra.

5. References and notes

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AFQ056/Mavoglurant a novel clinically effective mGluR5 antagonist: Identification, SAR and pharmacological characterization.

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