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Supplemental material and data

Article title:

Potentiation of muscarinic M_3 receptor activation through a new allosteric site with a novel positive allosteric modulator ASP8302

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Supplemental material

Primers for constructions of cells expressing of human muscarinic M_1, M_2, M_3, M_4 and M_5 receptors are as follows:

Human muscarinic M_1 receptors

Forward primer: GTGGGATCCATGAACACTTCAGCCCCACCTGC

Reverse primer: CACGCGGCCGCCTATCAGCATTGGCGGGAGG

Human muscarinic M_2 receptors

Forward primer: CCACCATGAATAACTCAACAAACTCCTCTAAC

Reverse primer: GATATTTTACCTTGTAGCGCCTATG

Human muscarinic M_3 receptors

Forward primer: TCACAATGACCTTGCACAATAACAG

Reverse primer: GATACAACCTCATTCTACAAGGCC

Human muscarinic M_4 receptors

Forward primer: CTCGCGGCCGCATGGCCAACTTCACACCTGTCAA

Reverse primer: CTCGGATCCCCTACCTGGCAGTGCCGATGTTCCG

Human muscarinic M_5 receptors

Forward primer: CTCGCGGCCGCATGGAAGGGGATTCTTACC

Reverse primer: CTCGGATCCTCAGGGTAGCTTGCTGTTCC

Supplemental Table 1. Methods, PCR templates and primers used for the construction of plasmids expressing receptor mutants

Chimeric receptor	Methods	1st PCR						2nd PCR			
		Fragment 1				Fragment 2					
		Templates	Primers			Templates	Primers			Templates	Primers		
M3_TM2_M1 M3_TM4_M1	Inverse PCR-based site-directed mutagenesis, Mega primer polymerase chain reaction	pcDNA hM3	P1	P2 P3		NA	NA			pcDNA hM1	Fragment 1	P4	
M3_TM5_M1	PCR-based single-site mutagensis	pcDNA hM3	P5	P6		pcDNA hM1	P7	P8		Fragment 1	Fragment 2	P5	P8
M1_TM5_M3		pcDNA hM1	P9	P10		pcDNA hM3	P11	P12		Fragment 1	Fragment 2	P9	P12
M3_TM4_M1_TM5_M3 M1_TM4_M3_TM5_M1	Inverse PCR-based site-directed mutagenesis	pcDNA hM3 pcDNA hM1	P13 P15	P14 P16		NA	NA			NA	NA	NA	NA

M1: muscarinic M_1 receptor; M3: muscarinic M_3 receptor; hM1: human muscarinic M_1 receptor, hM3: human muscarinic M_3 receptor, TM: transmembrane, NA: not applicable.

The primers used were as follows:

P1 TTTCACAATGACCTTGCACAATAAC

P2 GATGAGGTCAGCACAGGCCAGGCTTAAGAGGAAGTAGTTGTTG

P3 GCCAGAAGAGGATGGCTGGAGCCCAAAGGACAAAGGAGATG

P4 CTTAAGTTTAAACGCTAGCCAGC

P5 ATACAGAATTCGTCACCATGACCTTGCACAATAACAGTAC

P6 CGTGCACATAATGGTGACAGGCATATAAAAAGC

P7 GCCTGTCACCATTATGTGCACGCTCTACTGG

P8 TCCTCGGATCCCTATCAGCATTGGCGGGAGGG

P9 TCGGGGAATTCGCCACCATGAACACTTCAGCCC

P10 GTCATGACTGTGACAGGGAGGTAGAAG

P11 CCTCCCTGTCACAGTCATGACTATTTTATACTGGAGGATC

P12 CTATGGGATCCATTCTACAAGGCCTGCTCGGGTG

P13 CAGTGCTACATTCAGTTCCTCAGTGAGCCC

P14 CCCAGCTAGCACAGTTCTCTTTCCAACAAAG

P15 GAGTGCTTCATCCAGTTCCTCTCCCAGCC

P16 TCCCGGAGGCACTGTCCGCTCCCCTACC

M3_TM2_M1 and M3_TM4_M1 were constructed by inverse PCR-based site-directed mutagenesis and mega primer polymerase chain reaction (Rajiv et al., 2004). N-terminal M_3 receptor sequences were amplified by PCR and purified by agarose gel electrophoresis (shown as 1st PCR in Table 1). Using this fragment as a mega primer, inverse PCR was conducted (shown as 2nd PCR in Table 1). Inverse PCR, digestion of the template plasmid by DpnI, and self-ligation of PCR products (Kinase/Ligase) were conducted using a KOD -Plus- Mutagenesis Kit (Toyobo) according to the manufacturer's instructions.

M3_TM5_M1 and M1_TM5_M3 were constructed by PCR-based single-site mutagenesis (Fanli et al., 2017). M_1 receptor and M_3 receptor sequences were amplified by PCR and purified by agarose gel electrophoresis (shown as 1st PCR in Table 1). Using this fragment as template, 2nd PCR was conducted. The PCR products were digested by EcoRI and BamHI, purified by agarose gel electrophoresis, and inserted into pcDNA 3.1 (-) EcoRI – BamHI sites.

M3_ECL2_M1 and M1_ECL2_M3 were constructed by inverse PCR-based site-directed mutagenesis (BioTechniques, 1997). Inverse PCR (shown as 1st PCR in Table 1), digestion of the template plasmid by DpnI, and self-ligation of PCR products (Kinase/Ligase) were conducted using a KOD -Plus- Mutagenesis Kit (Toyobo) according to the manufacturer's instructions.

Sequences of the mutant constructs were confirmed by DNA sequencing.

Reference for

Rajiv Tyagi, Richard Lai, Ronald G Duggleby (2004) A new approach to 'megaprimer' polymerase chain reaction mutagenesis without an intermediate gel purification step. *BMC Biotechnol*. 26;4:2.

Fanli Zeng, Yujie Zhang, Ze Zhang, Asrar Ahmad Malik, Yibin Lin (2017) Multiple-site fragment deletion, insertion and substitution mutagenesis by modified overlap extension PCR. *Biotechnology & Biotechnological Equipment* 31: 339-348

Modification of a PCRBased Site-Directed Mutagenesis Method (1997) Modification of a PCRBased Site-Directed Mutagenesis Method. *BioTechniques* 23:570-574

Supplemental data

Supplemental Table 2. Inhibitory effect of ASP8302 on radioligand binding to various receptors, ion channels and transporters

Tested molecule	Inhibition (%)	
	ASP8302	Positive substance
Adenosine A1 (Rat)	13.40	99.87 (DPCPX)
α1-Adrenergic (Non-selective) (Rat)	13.93	99.41 (Prazosin)
α2-Adrenergic (Non-selective) (Rat)	26.90	99.92 (Yohimbine)
β-Adrenergic (Non-selective) (Rat)	3.92	100.00 ((±)-Propranolol)
Angiotensin AT1 (Human)	10.30	96.12 (Angiotensin II)
Angiotensin AT2 (Mouse)	0.00	100.00 (Angiotensin II)
Bradykinin B2 (Human)	6.11	98.98 (HOE140)
Ca Channel (Type L, Dihydropyridine) (Rat)	0.08	100.00 (Nitrendipine)
Ca Channel (Type N) (Rat)	0.00	99.81 (ω-Conotoxin GVIA)
CCK A (Human)	0.00	95.13 (CCK-8)
CCK B (Human)	0.00	99.58 (CCK-8)
CRF1 (Human)	5.88	99.90 (Urocortin human)
Dopamine D1 (Rat)	1.67	99.46 (R(+)-SCH-23390)
Dopamine D2 Short (Human)	0.00	100.00 ((+)-Butaclamol)
Dopamine Transporter (Human)	0.00	99.74 (GBR12909)
Estrogen (Rat)	1.29	100.00 (β-Estradiol)
Endothelin ETA (Human)	0.00	97.21 (Endothelin-1)
Endothelin ETB (Human)	9.01	97.89 (Endothelin-1)
GABA A (Agonist Site) (Rat)	2.71	100.00 (Muscimol)
GABA A (BZ Central) (Rat)	25.41	100.00 (Diazepam)
GABA B (Rat)	1.16	100.00 (GABA)
Glutamate (AMPA) (Rat)	4.73	100.00 ((*S*)-AMPA)
Glutamate (Kainate) (Rat)	3.18	97.60 (Kainic acid)
Glutamate (NMDA Agonist Site) (Rat)	6.01	100.00 (L-Glutamic acid)
Glutamate (NMDA Glycine Site) (Rat)	0.00	98.72 (MDL105,519)
Glycine (Strychnine Sensitive) (Rat)	2.48	100.00 (Strychnine)
Histamine H1 (Central) (Guinea pig)	9.28	100.00 (Pyrilamine)
Histamine H2 (Rat)	8.00	100.00 (Cimetidine)
Histamine H3 (Rat)	13.26	99.81 ((R)(−)-α-Methylhistamine)

ASP8302 concentration: 10 μmol/L, Positive substance concentration: 1 μmol/L for HOE140, urocortin human and endothelin-1, or 10 μmol/L for the others in supplemental table 2

Data are expressed as the mean values of duplicate samples.

The inhibition rate was calculated from “100 – binding ratio”.

Binding ratio: $[(B - N)/(B_0 - N)] \times 100$ (%)

B: Bound radioactivity in the presence of test substance (individual value)

B_0: Total bound radioactivity in the absence of test substance (mean value)

N: Non-specific bound radioactivity (mean value)

DPCPX: dipropylcyclopentylxanthine; CCK: cholecystokinin; CRF: corticotropin-releasing factor; GABA: gamma-amino butyric acid; AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA: N-methyl-D-aspartic acid

Supplemental Table 3. Inhibitory effect of ASP8302 on radioligand binding to various receptors, ion channels and transporters

Tested molecule	Inhibition (%)	
	ASP8302	Positive substance
K Channel KATP (Rat)	1.84	100.00 (Glybenclamide)
K Channel SkCa (Rat)	6.46	100.00 (Apamin)
Leukotriene B4 (Guinea pig)	0.00	100.00 (Leukotriene B_4)
Leukotriene D4 (Guinea pig)	0.00	100.00 (Leukotriene D_4)
Melatonin MT1 (Human)	1.50	100.00 (Melatonin)
Muscarinic (Non-selective) (Rat)	8.96	99.94 (Atropine)
Muscarinic M1 (Human)	19.65	100.00 (Atropine)
Muscarinic M2 (Human)	22.86	100.00 (Atropine)
Na Channel Site 2 (Rat)	23.41	99.56 (Dibucaine)
Neurokinin NK1 (Human)	19.89	100.00 (L-703,606)
Neurokinin NK2 (Human)	9.79	100.00 (Neurokinin A)
Neurokinin NK3 (Human)	0.00	100.00 (Senktide)
Norepinephrine Transporter (Human)	7.46	99.11 (Desipramine)
Nicotinic (Neuronal) (Rat)	0.00	99.73 ((±)-Nicotine)
Opiate (Non-selective) (Rat)	8.65	100.00 (Naloxone)
Opiate μ (Human)	0.00	100.00 (DAMGO)
Oxytocin (Rat)	0.00	99.71 (Oxytocin)
PAF (Rabbit)	0.08	100.00 (PAF)
Serotonin 5HT1 (Non-selective) (Rat)	7.80	95.94 (Serotonin)
Serotonin 5HT2B (Human)	10.05	97.07 (Serotonin)
Serotonin Transporter (Human)	7.88	100.00 (Imipramine)
Sigma (Non-selective) (Guinea pig)	27.42	100.00 (Haloperidol)
Testosterone (Human)	14.15	100.00 (Testosterone)
Vasopressin V1 (Rat)	5.54	100.00 ([Arg^8]-Vasopressin)
VIP 1 (Human)	6.76	100.00 (VIP)
Muscarinic M3 (Human)	15.90	100.00 (Atropine)
Muscarinic M4 (Human)	9.27	99.95 (Atropine)
Muscarinic M5 (Human)	8.89	99.82 (Atropine)

ASP8302 concentration: 10 μmol/L, Positive substance concentration: 1 μmol/L for leukotriene B_4, leukotriene D_4 and VIP, or 10 μmol/L for the others in supplemental table 3

Data are expressed as the mean values of duplicate samples.

The inhibition rate was calculated from "100 – binding ratio".

Binding ratio: $[(B - N)/(B_0 - N)] \times 100$ (%)

B: Bound radioactivity in the presence of test substance (individual value)

B_0: Total bound radioactivity in the absence of test substance (mean value)

N: Non-specific bound radioactivity (mean value)

DAMGO: [D-Ala2, N-MePhe4, Gly-ol5]enkephalin; KATP: ATP-sensitive potassium; SkCa: small conductance calcium-activated potassium; PAF: platelet activating factor; VIP: vasoactive intestinal peptide

Supplemental Table 4. Inhibitory effect of ASP8302 on various enzymes

Tested enzyme	Inhibition (%)	
	ASP8302	**Positive substance**
Acetylcholinesterase (Human)	0.00	99.40 (Eserine)
MAO-A (Rat)	11.28	98.25 (Clorgyline)
MAO-B (Rat)	8.09	93.41 (Ro 16-6491)

ASP8302 concentration: 10 μmol/L, Positive substance concentration: 100 μmol/L for Ro 16-6491, or 10 μmol/L for the others in supplemental table 4

Data are expressed as the mean values of duplicate samples.

The inhibition rate was calculated from "100 – reaction ratio".

Reaction ratio: $[(B - N)/(B_0 - N)] \times 100$ (%)

B: Radioactivity or fluorescence intensity in the tube or well for calculation of inhibition ratio (individual value)

B_0: Radioactivity or fluorescence intensity of the tube or well for calculation of total reaction (mean value)

N: Radioactivity or fluorescence intensity of the tube or well for calculation of non-specific reaction (mean value)

MAO: monoamine oxidase