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Original scientific paper

μ-Opioid/D_2 dopamine receptor pharmacophore containing ligands: Synthesis and pharmacological evaluation

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Abstract: Herein, the synthesis and pharmacological evaluation of 13 novel compounds, designed as potential heterobivalent ligands for μ-opioid receptor (MOR) and dopamine D_2 receptors (D_2DAR), are reported. The compounds consisted of anilido piperidine and *N*-aryl piperazine moieties, joined by a variable-length methylene linker. The two moieties represent MOR and D_2DAR pharmacophores, respectively. The synthesis encompassed four steps, securing the final products in 28–42 % overall yields. The approach has a considerable synthetic potential, providing access to various related structures. Pharmacological tests involved *in vitro* competitive assay for D_2DAR using [^{3}H] spiperon, as a standard radioligand, and *in vivo* antinociceptive tests for MOR. The measured dopamine affinities were modest to low, while antinociceptive activity was completely absent. Therefore, the compounds of the general structure prepared in this research are unlikely to be useful as opioid–dopamine receptor heterobivalent ligands.

Keywords: piperidine; piperazine; heterobivalent; opioids; analgesics; dopaminergic.

INTRODUCTION

Pain relief is one of the major goals of modern drug development. Currently, there are several pharmacologically distinct groups of drugs, acting on various types and intensities of pain. Two groups of drugs are particularly prominent, *i.e.* NSAIDs (non-steroidal anti-inflammatory drugs) and opioids. While the former are highly useful in the treatment of many milder to moderate painful conditions,

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opioids are indispensable for alleviating severe acute and chronic pain, especially in clinical settings.[1]

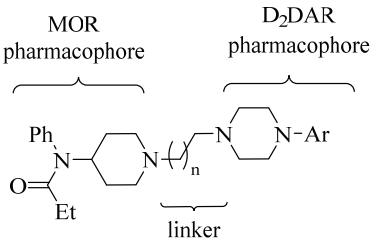
Almost all approved opioids are MOR agonists, including morphine and its numerous semi-synthetic derivatives, as well as many structurally diverse synthetic compounds. Among the synthetic MOR opioids, especially potent are anilido piperidines, including the clinically significant drugs fentanyl (Actiq®, Abstral®), sufentanil (Sufenta®), alfentanil (Alfenta®) and remifentanil (Ultiva®).

Agonists of κ- and δ-opioid receptors (KOR and DOR, respectively) have minimal medical use, due to the low potency and/or side effects. However, the side effects of the MOR agonists are also quite severe, often causing acute life-threatening respiratory depression as well as tolerance and addiction.

Extensive experimental results have indicated that opioid receptors in the central nervous system may form homodimers.[2,3] In addition, heterodimerization was inferred, primarily involving MOR and other opioid or non-opioid receptors. The main tools in these have been numerous homo and heterobivalent ligands, designed as specific pharmacological probes. Some of the representative heterodimers include MOR/DOR,[2] MOR/KOR,[4] MOR/cannabinoid receptor type 1,[5] MOR/metabotropic glutamate receptor type 5,[6] MOR/chemokine-receptor type 5,[7] MOR/NSAIDs[8] and MOR/*N*-type Ca^{2+} channels.[9]

It is known that opioid and dopamine receptors are co-distributed in many brain tissues, indicating possible functional interaction.[10,11] Thus, there is significant evidence of opioid-dopaminergic cross regulation, especially in reward processes associated with opioid addiction. Five dopamine receptor subtypes are known to exist in the CNS, divided into two groups: D_1likeDAR (D_1DAR and D_5DAR) and D_2likeDAR (D_2DAR, D_3DAR and D_4DAR). Evidence of direct interactions between D_2likeDAR and MOR was shown in several *in vivo* and *in vitro* tests.[12] Recently, reported series of compounds were designed to act as bivalent ligands for MOR/D_2likeDAR heterodimers, especially for D_2DAR/MOR and D_4DAR/MOR heterodimers the existence of which was observed experimentally.[13]

The D_2DAR and MOR system, whether it exists in the form of heterodimers or monomers, represents a potential target in the treatment of opioid addiction. Therefore, a series of potential heterobivalent opioid–dopamine receptor ligands were designed and synthesized as a part of ongoing synthetic and pharmacological research in functionalized heterocycles.[14] The compounds included the both pharmacophores, joined by methylene linkers of various lengths (general structure **1**, Fig. 1). The fentanyl scaffold (*i.e.*, anilido piperidine moiety) was selected as MOR pharmacophore because of the known high affinity for this receptor type. Likewise, the *N*-aryl piperazine moiety represents a well known structural motif with high affinity for D_2DAR receptors.[15,16]



1

n= 2-6; Ar= Ph, methoxy or halogen subsituted phenyl groups

Fig. 1. Bivalent ligand of the general structure **1**, possessing 4-anilido piperidine and *N*-aryl piperazine moieties.

EXPERIMENTAL

General information

Unless otherwise stated, all solvents were freshly distilled under Ar prior to use. All reagents were purchased from a commercial vendor and used as supplied.

^{1}H- and ^{13}C-NMR spectra were recorded on Bruker Avance III spectrometer, at 500 MHz for the proton (^{1}H) and at 126 MHz for the carbon (^{13}C). Chemical shifts are given in parts per million from tetramethylsilane (TMS) as the internal standard in $CDCl_3$. 2D-NMR spectra (HSQC) were recorded at 500 MHz. The coupling constants (*J*) are reported in Hz. Unless otherwise stated, all spectra were recorded at 25 °C. High resolution mass spectra (HRMS) were obtained with a heated ESI (HESI)-Orbitrap spectrometer.

All reactions were monitored by thin layer chromatography (TLC). Flash and dry--column flash chromatography were realized using silica gel (10–18 or 18–32 μm, ICN--Woelm). Melting points were obtained at a heating rate of 4 °C min^{-1} and are uncorrected.

IR spectra were recorded by using a Thermo Scientific Nicolet 6700 Fourier-transform spectrometer operated in the ATR mode.

The structures of all new compounds were determined by 1D-, 2D-NMR and IR spectroscopy. The structures of the three final compounds were additionally confirmed by high-resolution mass spectrometry (HRMS).

Analytical and spectral data are given in Supplementary material to this paper.

Syntheses

*General procedure for the synthesis of aryl piperazino carboxamides **3a**–**m**.* To a magnetically stirred solution of aryl piperazine hydrochloride **2a**–**c** (3.5 mmol) in CH_2Cl_2 (10 mL), Et_3N (4.2 mmol) and *ω*-bromo-acylchloride (5.25 mmol) were added at 0 °C. The mixture was then allowed to stir. Reaction was monitored by TLC, with mixture of CH_2Cl_2–––MeOH (95:5) as the eluent. After 40 min of stirring at 0 °C, the mixture was stirred for an additional l20 min at 25 °C to complete the reaction. MeOH (10 mL) was then added, and the mixture was concentrated on a rotary evaporator. A solution of K_2CO_3 (1.5 M) was added (pH ≈11) and the mixture was extracted with 2×25 mL of CH_2Cl_2. Organic layers were collected and concentrated on a rotary evaporator. The crude product was used in the next step without further purification.

*General procedure for the synthesis of aryl piperazino carboxamide-anilino piperidine **5a**–**m**.* To a magnetically stirred solution of anilino piperidine **4** (1.6 mmol) and K_2CO_3 (4.08

mmol) in MeCN (12 mL), aryl piperazino carboxamide **3a**–**m** (2.04 mmol) was added. The mixture was allowed to stir at 70 °C. Reaction was monitored by TLC, with mixture of CH_2Cl_2–MeOH (95:5) as an eluent. The reaction was completed after 8 h of reflux and additional stirring for 10 h at 25 °C. The mixture was then concentrated on a rotary evaporator. CH_2Cl_2 (50 mL) was added and the mixture was extracted with 2×25 mL of brine. The organic phase was concentrated on a rotary evaporator. The crude product was purified by dry-column flash chromatography (SiO_2; CH_2Cl_2/MeOH 10:0:95:5).

*General procedure for the synthesis of aryl piperazino-anilino piperidine adducts **6a**–**m**.* To a magnetically stirred suspension of aryl piperazino-anilino piperidine adduct **5a**–**m** (1.0 mmol) in dry THF (12 mL), 1M solution of BH_3 in dry THF (2.5 mmol) was added at 0 °C. The mixture was then allowed to stir at 25 °C. After the spontaneous boiling had stopped, the mixture was heated at 70 °C. Reaction was monitored by TLC, with mixture of CH_2Cl_2––MeOH (95:5) as the eluent. After 4 h of reflux, water (1 mL) was added dropwise, followed with addition of 5.5 M HCl (2 mL) at 25 °C. The reflux was continued for an additional 4 h. The mixture was then concentrated on a rotary evaporator. CH_2Cl_2 (50 mL) was added, the layers were separated, and the organic phase was washed with 2×25 mL of brine. The organic phase was concentrated on a rotary evaporator. The crude product was used in the next step without further purification.

*General procedure for the synthesis of aryl piperazino-anilido piperidine adducts **1a**–**m**.* To a magnetically stirred solution of aryl piperazino-anilino piperidine adducts **6a**–**m** (0.6 mmol) in CH_2Cl_2 (10 mL), Et_3N (0.9 mmol) followed by addition of propionyl chloride (EtCOCl, 1.8 mmol) were added. Mixture was then allowed to stir at 25 °C. Reaction was monitored by TLC, with mixture of CH_2Cl_2–MeOH (95:5) as the eluent. After 3.5 h, MeOH (10 mL) was added, and the mixture was concentrated on a rotary evaporator. A solution of K_2CO_3 (1.5 M) was added (pH ≈11) and the mixture was extracted with 2×25 mL of CH_2Cl_2. The organic layers were collected, washed with 2×25 mL of brine, and concentrated on a rotary evaporator. The crude product was purified by dry-column flash chromatography (SiO_2; CH_2Cl_2–MeOH, 10:0 to 95:5).

Biological assays

Membrane preparation. The preparation of rat caudate nuclei synaptosomal membranes for D_2DAR binding experiments was described in detail in previous publications.[17,18]

Radioligand binding assay. The [^{3}H] spiperone binding assay was performed in 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM KCl, 120 mM NaCl, 25 mM Tris–HCl solution, pH 7.4, at a membrane protein concentration of 0.7 mg mL^{-1} at 37 °C for 10 min in a total volume of 0.4 mL of the incubation mixture. Binding of the radioligand to the 5-HT2a receptors was prevented by 50 μM ketanserin. The K_i values of the tested compounds were determined by competition binding at 0.2 nM of the radioligand and eight to ten different concentrations of each compound (10^{-4} to 10^{-10} M). Nonspecific binding was measured in the presence of 10 μM spiperone. The reaction was terminated by rapid filtration through Whatman GF/C filters, which were further washed three times with 3.0 mL of ice-cold incubation buffer. Each point was determined in triplicate. The retained radioactivity was measured by introducing the dry filters into 3 mL of toluene-based scintillation liquid and counting in a 1219 Rackbeta Wallac scintillation counter (EG & G Wallac, Turku, Finland) at an efficiency of 51–55 % for tritium. The results were analyzed by nonlinear curve fitting of the inhibition curves of the compounds utilizing the Graph-Pad Prism program.[4,6] Hill slope coefficients were fixed to unity during the calculation.

Animal preparation. The study was carried out on 75 adult male Wistar rats (200–250 g) obtained from the Military Medical Academy (Belgrade, Serbia). The animals were housed in groups of three per cage (42.5 cm×27 cm×19 cm) under standard conditions of temperature (22±1 °C), relative humidity (60 %) and a 12 h light/dark cycle, with lights on at 8:00 a.m. Food and water were freely available, except during the experimental procedures. The animals were fed standard rat pellets obtained from the Veterinary Institute Subotica, Serbia. The experiments were conducted by the same experimenter on consecutive days, always at the same time of the day, between 8:00 a.m. and 2:00 p.m., to avoid diurnal variation in the behavioral tests. The animals were unrestrained during testing. Each animal was used only once and killed at the end of the experiments by an intraperitoneal (i.p.) injection of sodium thiopental (200 mg/kg). Prior to each experiment, the animals were habituated to the handling and experimental procedure for three consecutive days. The antinociceptive activity was determined by tail-immersion.[19]

All compounds were dissolved in saline and injected i.p. in a final volume of 2 ml kg^{-1}.[19]

Tail-immersion test. The rat was placed in a cylindrical rat holder with its tail hanging freely outside the cage. The distal 5 cm of the tail was immersed in a warm water bath (55±0.5 °C) and the time for tail-withdrawal was measured as response latency. To minimize tissue damage by repeated testing, a 10-s cutoff time was imposed for all animals that failed to respond to the stimulus. This means that the maximal duration of a single exposure of rat tail to hot water was 10 s. Pre-drug response latency was obtained 5 min before i.p. drug administration. Post-drug response latency was measured after i.p. administration of the test compound at 5, 10, 15, 20, *etc.* min. To test whether drug injection in rats has effect on the tail immersion latency, the *t*-test for paired values was used.[20] A P value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

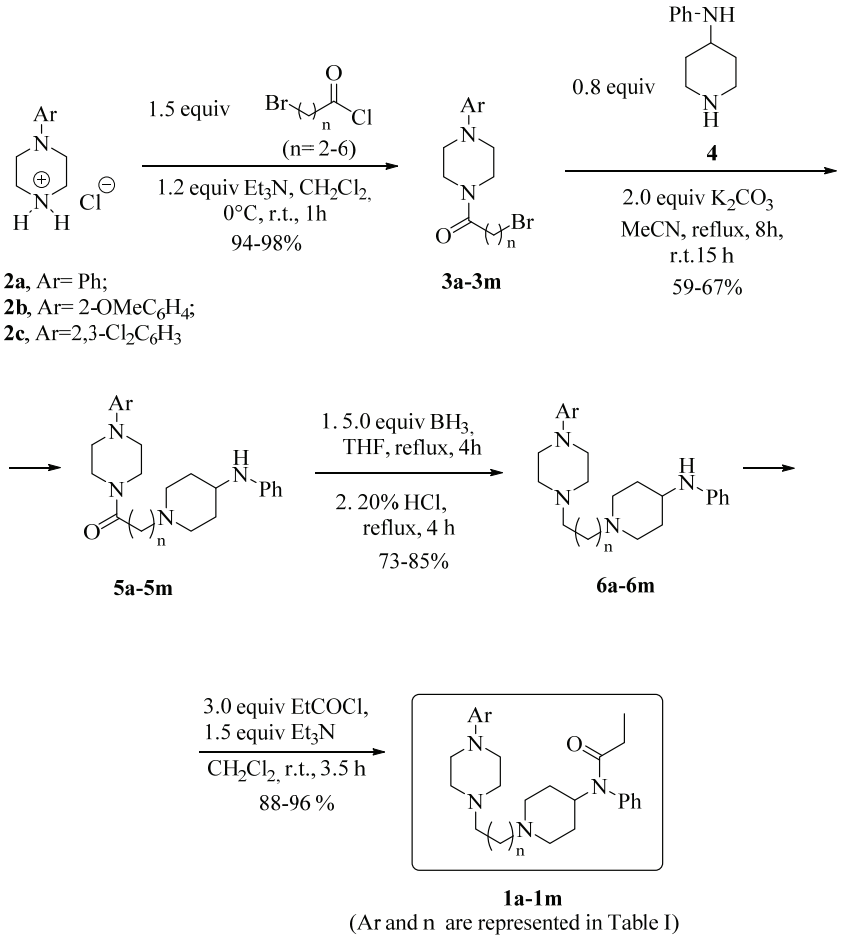
The initial research of the D_2DAR/MOR heterobivalent ligands involved the synthesis of only three compounds of general formula **1**, as proof of the synthetic concept.[14]

Herein, the optimized synthesis of 13 novel compounds of the general formula **1**, having 3 to 7 methylene groups in the linker, were synthesized according to the published procedure,[14] (Scheme 1). 2-Methoxyphenylpiperazine was chosen here rather than 3-methoxyphenylpiperazine[16], since it is a more abundant structural unit in D2-like-DAR pharmacophores.

Additionally, 15 compounds as D_2DAR and/or MOR ligands were assayed including the previously prepared compounds **1n** and **1o**[14] (Table I).

The synthesis encompassed 4 steps (Scheme 1). Commercial *N*-aryl piperazines **2a**–**c** were acylated with 3-bromopropanoyl chloride or higher homologues, furnishing *ω*-bromo amides **3a**–**m**, almost quantitatively. The compounds were then reacted with anilinopiperidine **4** providing *N*-alkylated piperidines **5a**–**m** in moderate to good yields. Although the alkylation of the piperidine nitrogen predominated, small amounts of *N*,*N*-dialkylated products were also formed, and removed chromatographically. It is noteworthy that the dialkylation was unexpected, because of the estimated weak nucleophilicity of the anilino nitrogen. The

subsequent borane reduction of the tertiary carboxamido group proceeded cleanly to amines **6a**–**m**. The final propanamides **1a**–**m** were obtained by direct *N*-propionylation (Table I). The overall synthetic approach is concise and practical, affording structurally diverse final products in 28–42 % overall yields, depending on the reactant structures.



Scheme 1. Synthetic route to the potential MOR/D_2DAR heterobivalent ligands **1a–1m**.

The prepared propanamides, having linkers of various lengths, are potential ligands of D_2DAR, MOR as well as D_2DAR/MOR heterodimers. In this research, the pharmacological testing was limited to *in vitro* assaying of D_2DAR binding as well as estimating the opioid activity using *in vivo* rodent tests.

The affinity of propanamides **1a**–**m** towards D_2DAR was assessed by a competitive displacement assay using [^{3}H] spiperon as the standard radioligand. The

results are summarized in Table I. Compounds **1a**–**c**, possessing three methylene groups in the linker, displayed moderate D_2DAR affinity. Substituents on the piperazine aryl group did not play a significant role (Table I, entries 1–3). However, longer linkers, having 4–7 methylene groups, strongly reduce the affinity (Table I, entries 4–15). Thus, it is evident that most ligands of the general structure **1** cannot form stable complexes with D_2DAR, likely because they cannot fit into the binding site of the receptor. More detailed insight into D_2DAR–ligand interactions could be gained from future docking studies, using the known D_2DAR crystalline structure.[21]

TABLE I. Structure and pharmacological activity of the ligands of the general formula **1**

Entry	Cmpd.	Ar	*n*	Yield[a] %	Binding for D_2DAR receptor K_i D_2DAR, nM[b]	Opioid activity[c]
1	**1a**	Ph	2	32	869	Inactive[e]
2	**1b**	2-OMeC_6H_4	2	31	800	Inactive[e]
3	**1c**	2,3-$Cl_2C_6H_3$	2	28	594	Inactive[e]
4	**1d**	2-OMeC_6H_4	3	37	7992	Inactive[e]
5	**1e**	Ph	4	37	7083	Inactive[e]
6	**1f**	2-OMeC_6H_4	4	32	4436	Inactive[e]
7	**1g**	2,3-$Cl_2C_6H_3$	4	30	2376	Inactive[e]
8	**1h**	Ph	5	40	8105	Inactive[e]
9	**1i**	2-OMeC_6H_4	5	34	3778	Inactive[e]
10	**1j**	2,3-$Cl_2C_6H_3$	5	30	1500	Inactive[e]
11	**1k**	Ph	6	42	1853	Inactive[e]
12	**1l**	2-OMeC_6H_4	6	35	5454	Inactive[e]
13	**1m**	2,3-$Cl_2C_6H_3$	6	32	5326	Inactive[e]
14[d]	**1n**	Ph	3	–	1357	Inactive[e]
15[d]	**1o**	2,3-$Cl_2C_6H_3$	3	–	6956	Inactive[e]
16	Haloperidol	–	–	–	5.3	–

[a]Overall isolated yield for four steps; [b]values are the means of three independent experiments performed in triplicate; [c]examined *in vivo* in rats by the rat-tail immersion test; [d]prepared in previous research;[14] [e]in doses up to 2 mg kg^{-1}

Opioid activity of propanamides **1a**–**m** was estimated using *in vivo* tests, since these tests provide more clinically reliable information about the analgesic activity of ligands in animal models than *in vitro* experiments in the laboratory. The complete absence of opioid activity (Table I), does not rule out affinity to MOR, as the ligands may not be able to reach the MOR due to physicochemical characteristics and/or metabolism. Therefore, their opioid affinity requires *in vitro* reassessment, in order to correlate it to the future molecular docking studies. It should be noted that such theoretical studies could be performed with good accuracy because the crystalline structure of MOR is known.[3,22,23]

CONCLUSION

The general procedure presented in this paper is useful for the synthesis of structurally diverse, heterobifunctional compounds. Thus, any two secondary amino groups can be joined selectively, using various linkers. Besides polymethylene chains, the linkers may include oligo (ethylene glycol) units as well as more complex groups. The obtained heterobifunctional compounds are potential bivalent receptor ligands, and may have numerous applications in bioconjugation chemistry.[24] The synthesized compounds were inactive as bidentate ligands, as no antinociceptive activity was observed. It is practically certain that the structure of the ligands prevented effective binding to the receptors. However, ligand structural requirements for effective binding can only be deduced from the extensive ligand docking, corroborated by experimental results.

SUPPLEMENTARY MATERIAL

Analytical and spectral data are available electronically from http://www.shd.org.rs/ /JSCS/, or from the corresponding author on request.

Conflict of Interest. The Authors declare that there are no conflicts of interest.

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ИЗВОД

ЛИГАНДИ КОЈИ САДРЖЕ ФАРМАКОФОРЕ ЗА μ-ОПИОИДНЕ И D_2 ДОПАМИНСКЕ РЕЦЕПТОРЕ: СИНТЕЗА И ФАРМАКОЛОШКО ИСПИТИВАЊЕ

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У овом раду је приказана синтеза и фармаколошко испитивање 13 нових једињења, дизајнираних са циљем да буду потенцијални бивалентни лиганди за μ -опиоидни рецептор (MOR) и допамински D_2 рецептор (D_2DAR). Једињења се састоје од анилидо-пиперидинских (MOR фармакофора) и *N*-арилпиперазинских остатака (D_2DAR фармакофора), повезаних метиленским ланцем променљиве дужине. Синтеза је обухватала четири корака, обезбеђујући крајње производе у укупним приносима од 28 до 42 %. Афинитет везивања за D_2DAR одређен је *in vitro* тестом компетиције користећи [^{3}H] спиперон као стандардни радиолиганд док је антиноцицептивна (опиоидна) активност испитана *in vivo* антиноцицептивним тестом. Активности новосинтетисаних једињења ка D_2DAR биле су умерене до ниске, док је антиноцицептивна активност у потпуности изостала.

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