Design, synthesis and biological evaluation of bivalent ligands against A₁–D₁ receptor heteromers

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Aim: To design and synthesize bivalent ligands for adenosine A₁–dopamine D₁ receptor heteromers (A₁–D₁R), and evaluate their pharmacological activities.

Methods: Bivalent ligands and their corresponding A₁R monovalent ligands were designed and synthesized. The affinities of the bivalent ligands for A₁R and D₁R in rat brain membrane preparation were examined using radiolabeled binding assays. To demonstrate the formation of A₁–D₁R, fluorescence resonance energy transfer (FRET) was conducted in HEK293 cells transfected with D₁-CFP and A₁-YFP. Molecular modeling was used to analyze the possible mode of protein-protein and protein-ligand interactions.

Results: Two bivalent ligands for A₁R and D₁R (20a, 20b), as well as the corresponding A₁R monovalent ligands (21a, 21b) were synthesized. In radiolabeled binding assays, the bivalent ligands showed affinities for A₁R 10–100 times higher than those of the corresponding monovalent ligands. In FRET experiments, the bivalent ligands significantly increased the heterodimerization of A₁R and D₁R compared with the corresponding monovalent ligands. A heterodimer model with the interface of helixes 3, 4, 5 of A₁R and helixes 1, 6, 7 from D₁R was established with molecular modeling. The distance between the two ligand binding sites in the heterodimer model was approximately 48.4 Å, which was shorter than the length of the bivalent ligands.

Conclusion: This study demonstrates the existence of A₁–D₁R in situ and a simultaneous interaction of bivalent ligands with both the receptors.

Keywords: G protein-coupled receptors; adenosine; dopamine; A₁ receptor; D₁ receptor; heterodimers; bivalent ligands; radiolabeled binding assay; FRET; molecular modeling

Introduction

G protein-coupled receptors (GPCRs) constitute the largest protein family of cell surface receptors, and they mediate a variety of signaling processes. Over the last 30 years, many researchers have identified that GPCRs can form dimeric and higher-order oligomers in natural tissues[1, 2]. In comparison with the constituting receptors, GPCR heteromers, which have different characteristics, are important for the understanding of receptor function and pharmacology. As a result, bivalent ligands are currently being developed as a promising strategy for drug discovery[3, 4].

The adenosine A₁ and dopamine D₁ receptors belong to the superfamily of GPCRs. Evidence for the existence of A₁R and D₁R heteromers has been presented and is based on immunoprecipitation and double immunolabeling experiments[5]. There are two subtypes of striatal GABAergic efferent neurons, projecting to the thalamus across two distinct pathways: the striato pallidal neurons (indirect pathway) and the striatonigrostriatoentopeduncular neurons (direct pathway). Evidence indicates that A₁R and D₁R are coexpressed in the basal ganglia and prefrontal cortex, which are particularly involved in the direct pathway[6]. In rat models of Parkinson’s disease, the motor activating effects of the D₁ agonist, SKF38393, were enhanced by the A₁ receptor antagonist[7]. This synergistic mechanism may provide some insight into the design of novel agents for the treatment of Parkinson’s disease and neuropsychiatric disorders based on the pharmacological properties of the A₁–D₁ heteromeric complex[5].

Although heteromerization was reported in native tissues, there is no direct evidence to prove the existence of A₁–D₁ receptor heterodimerization. A₁–D₁ bivalent ligands could be important research tools to detect heterodimerization and further explore the biological functions of A₁–D₁ receptor het-
Bivalent ligands are molecules that consist of two pharmacophores linked by a spacer. Recently, synthesis and biological investigations of bivalent ligands were described to target serotonin, muscarinic, opioid and other receptors. Rafael Franco and Miriam Royo designed bivalent ligands targeting A2aR-D2R heterodimerization, which contained a D2R agonist, a A2aR antagonist and their corresponding monovalent control compounds. Their results indicated that the existence of A2aR–D2R heterodimerization and a simultaneous interaction of bivalent ligands with both receptors.

We designed and synthesized a group of bivalent ligands containing a D1R agonist and a A1R antagonist linked by different lengths of PEG linkers. The binding properties of these compounds were determined by radioligand binding studies in membrane preparations from brain striatum. FRET experiments were performed to test if these bivalent ligands can promote the formation of A1R and D1R heterodimers (Figure 1).

**Materials and methods**

**Chemistry**

**Compound design**

A1–D1 bivalent ligands were designed by combining two pharmacophoric entities with different lengths of linkers. First, compound 1, a naphthylxanthine derivative, was chosen as the A1 antagonist pharmacophore. Then, SKF38393, a 1-aryl-3-benzazepine analog that displays high binding affinity at the D1 receptor, was selected as the D1 agonist pharmacophore. Structure-activity relationship indicated that the N-3 site of compound 1 and 4’ site of SKF38393 are not active sites. Thus, linkers were introduced at these sites to combine the A1 antagonist and D1 agonist pharmacophores.

Polyethylene glycol (PEG) is widely used due to its low toxicity, excellent aqueous solubility and low antigenicity. Recently, Kiessling et al found that an unexpected enhancement in the biological activity of a GPCR ligand was induced by a polyethylene glycol substitute. Considering the fact that PEG has a moderate &Delta;Solv (the entropy associated with torsional motions about a single bond), PEG was chosen as the linker for the selected pharmacophores. Recently, Gmeiner et al reported the synthesis and biological investigation of bivalent ligands for D2-like receptors. Bivalent ligands, linked by 5–8 oligoethylene glycol units, showed up to a 70-fold increase of D2 binding affinity compared to monovalent ligand compounds. As a result, we designed bivalent ligands linked by 4 and 6 oligoethylene glycol units.

We also synthesized the corresponding monovalent ligand compounds for the A1 antagonist as a control to determine whether the bivalent ligands showed improved affinity for the A1 receptor compared to the monovalent ligand controls. In 1991, Neumeyer et al synthesized the derivative of SKF38393 to identify effective fluorescent probes. However, coupling of the large group to the 4′ site of SKF38393 resulted in a considerable loss of affinity. As a result, we did not synthesize the corresponding monovalent ligand compounds for the D1 antagonist as a control.

**Preparation of the bivalent ligands and their monovalent ligands**

Bivalent ligands linked by PEG chains were synthesized by the route demonstrated in Scheme 1. Commercially available polyethylene glycol was easily converted to compound 2a–2b using acrylonitrile via the Michael addition reaction. Reduction of compound 2a–2b with borane produced the compound 3a–3b.

The synthesis of the common intermediate, compound 4, has previously been described. Treatment of compound 4 with ethyl 4-bromobutanoate produced compound 5. Then, compound 5 was heated in 20% NaOH (aq), followed by ring closure, to generate compound 6. Compound 6 was coupled with 3a–3b in the presence of PyBop/DIPEA in DMF to yield 7a–7b.

Commercially available nitroacetophenone was transformed into compound 8 using a catalytic amount of Lewis acid. The reaction of 8 with NaBH4 produced compound 9. Compound 9 and commercially available 3,4-dimethoxy phenylethylamine were heated at reflux in THF to yield compound 10. Compound 10 was heated in PPA, followed by ring closure, to produce the cyclization compound 11. The reaction of 11 with iron powder generated compound 12. The addition of a Nosyl group to 12 in the presence of basic conditions yielded compound 13. Compound 13 was converted to 14 via a Mitsunobu reaction. Deprotection of compound 14 with BBr3 at -78 °C produced compound 15 without future purification. The addition of a MOM group to 15 under basic conditions produced compound 16. Compound 17 was easily obtained from 16 via an ester hydrolysis reaction.

Compounds 18a–18b were obtained from 17 and 7a–7b using standard peptide synthesis procedures, with EDCI/HOBt as the catalytic coupling agents. Removal of the Nosyl group in 18a–18b, in the presence of K2CO3 and PhSH, yielded compound 19a–19b. Deprotection of compound 19a–19b with BBr3 at -78°C gave the target compound 20a–20b (Scheme 1).

Monovalent ligands were synthesized, and the route is demonstrated in Scheme 2. Compounds 21a–21b were obtained using hexanoic acid, and 7a–7b were obtained using standard peptide synthesis procedures, with EDCI/HOBt as the catalytic coupling agents.

**Reagents and conditions**

- acrylonitrile, THF, 60 °C, 1.5 h (99%);
- BH3, THF, 60 °C, 3.5 h (97%);
- ethyl 4-bromobutanoate, DBU, DMF, 60 °C, 48 h
Scheme 1. Synthesis of the bivalent ligands linked by PEG.
(44%); d) 20% NaOH (aq), 60℃, 3 h (61%); e) PyBOP, DIPEA, DMF, rt, overnight (60%); f) Br₂, AlCl₃ (Cat.), Et₂O, 0℃, rt, 3 h (95%); g) NaBH₄, 2 mol/L NaOH (aq), THF-MeOH, 0℃, rt, 35 min (69%); h) 3,4-dimethoxyphenylethylamine, THF, reflux overnight (56%); i) PPA, 100°C, 1 h (75%); j) Fe powder, HCl (aq), EtOH-H₂O, reflux for 1 h (40%); k) Nosyl-Cl, Et₃N, DCM, 0℃, rt, overnight (80%); l) Ph₃P, DIAD, hex-4-yn-1-ol, rt, overnight (80%); m) Bromomethyl methyl ether, DIPEA, rt, overnight; n) 2 mol/L NaOH(aq), THF, CH₃OH, rt, 1 h (70%); o) EDCI, HOBt, DIPEA, DMF, rt, overnight (40%); p) PhSH, K₂CO₃, 80℃, overnight (60%); q) TFA, DCM, rt, 1 h (60%).

Receptor binding assay

A P2 membrane fraction was prepared from whole brains of adult Wistar rats (200–300 g). The tissue was homogenized in 10× (w/v) 10% sucrose solution, and the suspension was centrifuged at 1000×g for 10 min (4℃). The supernatant was decanted and retained on ice. The pellet was resuspended in 50 mmol/L Tris-HCl buffer (pH=7.4) at 1:5 (w/v). Then, the Lowry method was employed to determine the protein concentration. All the solutions were diluted to 2 mg/mL and stored at -80℃.

In the D₁ receptor competitive assay, compound samples, the D₁ receptor antagonist, [³H]-SCH23390 (0.5 nmol/L) and 200 μg of rat cortex membrane protein mixture were added to each tube. All of the above operations were performed in an ice bath. The sequence in which the samples were added and their volumes are listed in Table 1. The final volume was adjusted to 200 μL with Tris-HCl buffer (Tris-HCl 50 mmol/L, NaCl 120 mmol/L, KCl 5 mmol/L, MgCl₂ 1 mmol/L, CaCl₂ 2 mmol/L, pH 7.4). The tubes were kept in a 37℃ water bath for 30 min. Twenty microliters of Tris-HCl buffer was used to replace the sample in the total binding tube, and 20 μL of the specific ligand, DPCPX (10 μmol/L) was used to replace the sample in the nonspecific binding tube.

When the water bath was finished, the reaction buffer was immediately filtered using a Brandel 24-well cell collector and GF/B filter to stop the reaction. The filter was washed three times with ice-cold 50 mmol/L Tris-HCl buffer (pH=7.4). After the filter was dry and transferred to a 0.5 mL EP, the filter was dried and transferred to a 0.5 mL EP. Flashing liquid was added and a MicroBeta2 porous plate was used to measure the radiation activity. The equation for calculating the inhibition rate was as follows:

\[
\text{Inhibition rate (\%) = \frac{100 - (cpm_{sample} - cpm_{non-specific\ binding})}{(cpm_{total\ binding} - cpm_{non-specific\ binding})} \times 100}
\]

Non-linear regression implemented using GraphPad Prism 4.0 (GraphPad Software Inc) was used to calculate the IC₅₀.

Measurement of FRET efficiency in HEK293 cells

FRET technology detects protein-protein interactions in live cells with high sensitivity. FRET occurs when a donor chromophore, initially in its electronic excited state, transfers energy to a nearby acceptor chromophore (10–100 Å) through non-radiative dipole-dipole coupling. The adenosine A₁ receptor and dopaminergic D₁ receptor form heterodimers when co-localized. This experiment employs FRET technology to screen for compounds that promote the formation of A₁R and D₁R heterodimers.

HEK293 cells were transiently transfected with plasmid DNA corresponding to the D₁-CFP (acceptor) and A₁-YFP (donor), with a ratio of donor to acceptor DNA of 1:1. GFP-YFP plasmids were used as a control. A mixture of cells separately transfected with either A₁ YFP or D₁ CFP (A₁YFP+D₁CFP) were used as negative controls. HEK293 cells were transfected with the plasmids using the Trans Messenger Transfection Reagent (QIAGEN) for 48 h before collection with cold D-Hanks’ buffer. The cells were washed twice, resuspended and seeded into 96-well plates at a density of 1×10⁵ cells/well for fluorescence detection. Fluorescence intensity...
was measured with a double-channel Novo star fluorescent spectrophotometer (channel 1: excitation wavelength 440 nm, emission wavelength 520 nm; channel 2: excitation wavelength 440 nm, emission wavelength 470 nm). The energy transfer efficiency (FRET efficiency) was calculated using the following formula:

\[
\text{FRET efficiency} = \frac{F_{440/520}}{F_{440/470}}
\]

where \(F_{440/520}\) was the fluorescence intensity detected through channel 1, and \(F_{440/470}\) was the fluorescence intensity detected through channel 2. The excitation wavelength for CFP was 440 nm and for YFP was 520 nm, and 470 nm was the emission wavelength for CFP.

### Ligand docking studies

Homology models of the adenosine A1 receptor and dopamine D1 receptor were built using Modeller 9.10. The model of the A1 receptor was constructed using the crystal structure of the adenosine A2A receptor (PDB code: 3EML) as a template. The model of the D1 receptor was constructed using multiple templates from the crystal structures of Rhodopsin (PDB code: 1U19), the \(\beta_2\) adrenergic receptor (PDB code: 2RH1), the histamine H1 receptor (PDB code: 3RZE), the S1P1 receptor (PDB code: 3V2Y), and the dopamine D3 receptor (PDB code: 3PBL). All the alignments were generated using Modeller 9.10, using default settings that were manually modified afterwards with careful attention to the alignment of all the conserved residues. The 'Biopolymer structure preparation' protocol of Sybyl 7.5 was used to prepare the models, and the minimization tool of Sybyl 7.5 was used to minimize all the models stepwise from hydrogen side-chains and main-chains to whole molecule minimization. The dopamine D1 receptor models were later subjected to molecular dynamics (MD) simulation using Gromacs 4.5 for further refinement of the long ECL2. The MD simulation was performed for 40 ns using the CHARMM27 force field, and all the heavy atoms, except those on ECL2, were restrained by a constant force of 100000 (kJ mol\(^{-1}\) nm\(^{-2}\)). The production runs were conducted using NTP ensemble at 300K, with a 1 fs time-step integration. The Particle Mesh Ewald method (PME) was used to calculate the electrostatic contribution to non-bonded interactions, with a cutoff of 9 Å and a time step of 1 fs. The cutoff distance of the van der Waals interaction and coulomb interaction were 14 Å and 9 Å, respectively. The LINCS algorithm was applied to the system. All the models were evaluated and validated using PROCHECK and the Pro-SA web server.

Autodock 4.2 was used to search for the binding conformation of compound 1 and SKF38393 in the A1 receptor and D1 receptor, respectively. A binding box of 19×19×22 Å, centered at the binding cavity, was used, and 100 conformations were generated for each ligand using genetic algorithm. The binding conformation was chosen from the largest cluster (cluster tolerance was determined using default settings) and validated with the available mutation experiment data.

The ZDOCK protocol in Discovery studio 2.5 suite was used to search the heterodimer interface. Default parameters were used, and the results were selected manually. The PEG linker was added to the heterodimer and minimized using Sybyl 7.5.

### Results

#### Chemistry

The bivalent ligands, 20a–20b, and monovalent ligands, 21a–21b, were designed and synthesized through the route outlined in Scheme 1. All the compounds were purified by silica gel thin-layer chromatography (>95%), and their structures were determined by nuclear magnetic resonance spectra and low-resolution mass spectra. Details of their structures are shown in Table 2.

#### Receptor binding assay

The binding affinity assay was performed using the bivalent ligands, 20a–20b, with 4 and 6 polyethylene glycol units, respectively, along with their monovalent ligands, 21a–21b. As expected, the bivalent ligands, 20a–20b, showed moderate binding for the A1 receptor and D1 receptor, while the monovalent control ligands, 21a–21b, showed moderate binding for the A1 receptor. However, there was no correlation between linker length and binding affinity for the bivalent ligands, 20a–20b. It is possible that the high flexibility of the PEG linker could explain this phenomenon. The affinities for A1R and D1R were 10–100 times higher for bivalent ligands compared with their monovalent controls. These results are summarized in Table 2.

#### Measurement of FRET efficiency in HEK293 cells

FRET technology detects protein-protein interactions in live cells with high sensitivity. The adenosine A1 receptor and dopaminergic D1 receptor form heterodimers when co-localized. This experiment employed FRET technology to screen for compounds that promote the formation of A1R and D1R heterodimers.

As shown in Figure 2, A1YFP+D1CFP represents the cells transfected with either A1YFP or D1CFP, set as the negative control group. CFPPYFP represents the positive control. A1YFP/D1CFP indicates the cells co-transfected with both D1-CFP and...
A<sub>1</sub>-YFP. Plates were read 48 h after the transfection was finished. Parameters of the spectrophotometer were set according to the manual.

FRET efficiency values were obtained from the positive control, in which the cells co-transfected with A<sub>1</sub>YFP/D<sub>1</sub>CFP differed significantly compared to the negative control group (cells transfected with either A<sub>1</sub>YFP or D<sub>1</sub>CFP, P<0.01). Similar significant differences in the FRET efficiency values were also detected in cells treated with 100 nmol/L 20<sub>a</sub>, 1 μmol/L 20<sub>a</sub> or 100 nmol/L 20<sub>b</sub> compared to the negative control cells. These results indicate that the bivalent ligands, 20<sub>a</sub>--20<sub>b</sub>, increased the heterodimerization of the A<sub>1</sub> receptor and D<sub>1</sub> receptor to a higher extent than the monovalent control ligands, 21<sub>a</sub>--21<sub>b</sub>. It was consistent with that of the receptor binding assay.

**Ligand docking studies**

A docking experiment was performed to confirm the length of the linker and the bivalent ligand binding mode. The A<sub>1</sub>–D<sub>1</sub> heterodimer interface was searched by ZDOCK and checked manually based on the ZDOCK score and associated bioinformatics data. Tarakanov and Fuxe deduced a set of triplets that may be responsible for receptor-receptor interactions<sup>[28, 29]</sup>. Leucine-rich motifs, one type of these triplets, are charged residues that may serve as an ‘adhesive guide’ for the formation of strong bonds between the amino acids of two receptors. As seen in Figure 3, the homology triplets, ILS and AAV, exist in helix 4 of A<sub>1</sub> and helix 1 of D<sub>1</sub>. Combined with the docking rank, we established a heterodimer model with the interface of helices 3, 4, and 5 of the A<sub>1</sub> receptor and helices 1, 6, and 7 from the D<sub>1</sub> receptor (Figure 4). The distance between the two ligand binding sites in this heterodimer model is approximately 48.4 Å, which is shorter than the length of the bivalent ligands. The linker of 20<sub>b</sub> was manually added to the docked ligands in the heterodimer model and minimized with Sybyl tools. As shown in Figure 4, the linker tightly interacts with ECL2 and ECL1 of the A<sub>1</sub> receptor and D<sub>1</sub> receptor. Moreover,
the oxygen atoms on the PEG linker form polar interactions with the polar residues on the extracellular loops of the heterodimer, which enhances the binding ability of the bivalent ligand to the heterodimer. The ligand docking studies verify that the bivalent ligand binds to both binding sites in the heterodimer.

**Discussion**

According to the binding assay results shown in Table 2, bivalent ligands \(20a-20b\) showed moderate binding for the A\(_1\) and D\(_1\) receptors, while monovalent control ligands \(21a-21b\) only showed moderate binding for the A\(_1\) receptor. The affinity for A\(_1R\) was 10–100 times higher for bivalent ligands compared with their monovalent controls. One possible reason for this phenomenon is conformational crosstalk within one receptor protomer or modulation of the interaction between two protomers of a receptor dimer induced by positive cooperativity. Positive cooperativity can also be induced when bivalent ligands bind two adjacent binding sites of A\(_1\)--D\(_1\) receptor heteromers because binding of the second pharmacophore is significantly accelerated due to the vicinity of ligand, thus facilitating local concentration enrichment. As a result, a bivalent ligand that binds to D\(_1\)R may subsequently have a higher affinity with A\(_1\)R located in the near vicinity compared to the corresponding A\(_1\)R monovalent ligand\([9, 30]\). These data indicate the specific interaction of these bivalent ligands with A\(_1\)--D\(_1\) receptor heteromers and suggest that these ligands could be useful as pharmacological tools to detect receptor heteromers in native tissue.

FRET technology has been decisive for demonstrating heteromer formation in heterologous expression systems. This study employed FRET technology to screen for bivalent ligands that promote the formation of A\(_1\)R and D\(_1\)R heterodimers. FRET efficiency values obtained from cells co-transfected with A\(_1\)YFP/D\(_1\)CFP differed significantly compared to those of the negative control group. Similar significant differences were also observed in the FRET efficiency values of cells treated with bivalent ligands compared to the negative control cells. These results confirm that bivalent ligands can detect the presence of heterodimers of A\(_1\)R and D\(_1\)R.

The docking experiments showed that the linker of the bivalent ligands is long enough to allow for the two pharmacophoric moieties to bind an A\(_1\)--D\(_1\) heterodimer. The minimum distance between the two ligand binding sites in this heterodimer model was 48.4 Å. This length is similar to the one proposed for other GPCR dimers, such as A\(_{2a}\)--D\(_2\) receptor heterodimers. As shown in Figure 4, the linker has a tight interaction with ECL2 and ECL1 of the A\(_1\) and D\(_1\) receptors. Moreover, the oxygen atoms on the PEG linker form polar interactions with the polar residues on the extracellular loops of the heterodimer, which enhances the binding ability of the bivalent ligand to the heterodimer. Ligand docking studies verify that bivalent ligands bind to both binding sites in the heterodimer.

**Conclusion**

This report provides evidence for the existence of A\(_1\)R and D\(_1\)R heteromers. However, in native tissues, there is no direct evidence to prove the existence of A\(_1\)--D\(_1\) receptor heterodimerization. In the present study, we designed and synthesized a
set of the bivalent ligands, differing in length, that link two different pharmacophores. The binding affinities for bivalent ligands for A1R were 10-100 times higher compared with their monovalent controls. The results of the binding assays revealed the specific interaction of these bivalent ligands with A1-D1 receptor heteromers. FRET efficiency values obtained from cells co-transfected with 100 nmol/L 20a, 1 μmol/L 20a or 100 nmol/L 20b differed significantly compared to those of the negative control group. These results confirmed that bivalent ligands can detect the presence of A1R and D1R heterodimers. The docking experiments also verified that bivalent ligands act on both binding sites of the heterodimer and indicated the possible length of the linker. In summary, we designed and synthesized bivalent ligands that act as molecular probes for A1–D1 receptor heteromers. A1–D1 bivalent ligands for A1R were 10–100 times higher compared with different pharmacophores. The binding affinities for bivalent ligands revealed the specific interaction of these bivalent ligands with A1–D1 receptor heteromers. A1–D1 bivalent ligands acted on both binding sites of the heterodimer and explored further biological functions of A1–D1 receptor heterodimers. Further studies are in progress.

Appendix

The chemical reagents (chemicals) were purchased from Lancaster, Acros, and Shanghai Chemical Reagent Company which were used without further purification. 3H- and 13C-NMR spectra were recorded in DMSO-d6 or CDCl3 on Varian Mercury-300 or Varian Mercury-400 instruments. The ESI-MS were carried out on Thermo Finnigan LCQ DECAXP. TLC was carried out with glass pre-coated silica gel GF254 plates.

4.7,10,13,16-pentaoxanonadecane-1,19-dinitrile (2a)

In a RB flask, was added acrylonitrile (50 mL, 0.76 mol), 2,2‘-oxybis(ethane-2,1-diyl)bis(oxy))diethanol (6.53 g, 33.67 mmol) and KOH (28.8 mg, 0.51 mmol), the reaction mixture was stirred at rt, was added 20% NaOH (11 mL). The reaction mixture was stirred at 60 oC for 3 h. After cooling the reaction, the solvent was removed under vacuo, the residue was dissolved in DCM, filter through celite, the filtrate was evaporated to dryness to give the title compound as colorless oil without further purification (7.75 g, 99%); LRMS (ESI) m/z 301 [M+H]+.

4.7,10,13,16,19,22-Heptaoxapentacosane-1,25-diamine (2b)

Compound 2b (yield 98%) was prepared according to the method described for the preparation of compound 2a; LRMS (ESI) m/z 389 [M+H]+.

4.7,10,13,16-pentaoxanonadecane-1,19-dinitrile (3a)

To a solution of compound 3a (4 g, 13.3 mmol) in dry THF (30 mL) stirred under N2 at 0°C, was added 1 mol/L BH3-THF (100 mL, 100 mmol) dropwise during 30 min. The reaction mixture was heated to reflux for 3.5 h. Then the reaction was quenched with MeOH (30 mL) and HCl (7 mL) at 0°C. After removing of the solvent, the residue was dissolved in 2 mol/L NaOH (50 mL), extracted with DCM (25 mL×15), dried over Na2SO4 filtered and concentrated to give the title compound as colorless oil without further purification (4.01 g, 97%); LRMS (ESI) m/z 309 [M+H]+.

4.7,10,13,16,19,22-Heptaoxapentacosane-1,25-diamine (3b)

Compound 3b (yield 98%) was prepared according to the method described for the preparation of compound 3a; LRMS (ESI) m/z 397 [M+H]+.

N-(6-amino-1-(3-hydroxypropyl)-2,4-dioxo-3-propyl-1,2,3,4-tetrahydropyrimidin-5-yl)-2-naphthamide (5)

To a solution of Compound 4 (1.2 g, 3.55 mmol) in dry DMF (50 mL) stirred under N2 at rt, were added ethyl 4-bromobutanoate (2.03 mL, 14.2 mmol), DBU (0.72 mL, 7.1 mmol). The reaction mixture was stirred at 60°C for 48 h. After cooling reaction, the solvent was removed under vacuo, the residue was dissolved in EtOAc (50 mL), washed with water and brine, dried over Na2SO4 filtered and concentrated. The crude product was purified by chromatography (2:1 petroleum ether-EtOAc) to give the title compound as an oil (700 mg, 44%); 1H-NMR (300 MHz, DMSO-d6, DMSO-d6): δ 9.08 (s, 1H), 8.62 (s, 1H), 8.07-7.98 (m, 4H), 7.63-7.60 (m, 2H), 6.79 (s, 2H), 4.10-4.05 (m, 2H), 3.92 (t, J=5.7 Hz, 2H), 3.73 (t, J=5.1 Hz, 2H), 2.38 (t, J=5.7 Hz, 2H), 1.84-1.80 (m, 2H), 1.55-1.49 (m, 2H), 1.19 (t, J=5.7 Hz, 3H), 0.84 (t, J=5.7 Hz, 3H).

3-(3-hydroxypropyl)-8-(naphthalen-2-yl)-1-propyl-1H-purine-2,6(3H,7H)-dione (6)

To a solution of Compound 5 (600 mg, 1.32 mmol) in methanol (120 mL) stirred at rt, was added 20% NaOH (11 mL). The reaction mixture was stirred at 60°C for 3 h. After cooling the reaction, the solution was acidified by 2 mol/L HCl to adjust pH<3, the solid was collected by filter, dried to give the title compound as white solid (350 mg, 61%); 1H-NMR (400 MHz, DMSO-d6): δ 8.69 (s, 1H), 8.22 (d, J=8.8 Hz, 1H), 8.02-7.93 (m, 3H), 7.58-7.55 (m, 2H), 4.12 (t, J=6.4 Hz, 2H), 3.85 (t, J=7.2 Hz, 2H), 2.32-2.29 (t, J=7.2, 2H), 2.20-1.59 (m, 2H), 1.61-1.55 (m, 2H), 0.87 (t, J=7.6 Hz, 3H); LRMS (ESI) m/z 379 [M+H]+; mp 190–194°C.

N-(19-amino-4,7,10,13,16-pentaoxanonadecyl)-4-(8-naphthalen-2-yl)-2,6-dioxo-1-propyl-1H-purin-3(2H,6H,7H)-ylbutanamide (7a)

To a solution of compound 6 (100 mg, 0.25 mmol) and 3a (60 mg, 0.28 mmol) in dry DMF (10 mL) stirred at rt, were added PyBOP (143 mg, 0.28 mmol), DIPEA (88 μL, 0.5 mmol), the reaction mixture was stirred at rt overnight. The solvent was evaporated, the residue was purified by chromatography (1:1 DCM-MeOH) to afford the title compound as a white solid (96 mg, 60%). 1H-NMR (400 MHz, DMSO-d6): δ 8.70 (s, 1H), 8.22-8.21 (m, 1H), 8.05-7.95 (m, 3H), 7.79 (m, 1H), 7.60-7.57 (m, 1H), 4.10 (t, J=8 Hz, 2H), 3.87 (t, J=9.1 Hz, 2H), 3.48-3.43 (m, 18H), 3.36-3.32 (m, 2H), 3.06-3.04 (m, 2H), 2.83 (t, J=9.6 Hz, 2H), 2.15 (m, 2H), 2.01-1.99 (m, 2H), 1.78-1.74 (m, 2H), 1.61-1.55 (m, 4H), 0.89 (t, J=9.6 Hz, 3H); LRMS (ESI) m/z 697 [M+H]+.

N-(25-amino-4,7,10,13,16,19,22-heptaoxapentacosyl)-4-(8-naphthalen-2-yl)-2,6-dioxo-1-propyl-1H-purin-3(2H,6H,7H)-ylbutanamide (7b)

Compound 7b (yield 50%) was prepared according to the methodology described for the preparation of compound 7a. 1H-NMR (400 MHz, DMSO-d6): δ 8.70 (s, 1H), 8.04-8.01 (m, 1H), 7.97-7.96 (m, 3H), 7.60-7.59 (m, 1H), 7.68-7.57 (m, 1H), 4.10 (t, J=8 Hz, 2H), 3.87 (t, J=9.1 Hz, 2H), 3.50-3.42 (m, 26 H), 3.34 (t, J=8.4 Hz, 2H), 3.06-3.04 (m, 2H), 2.82 (t, J=10.4 Hz, 2H), 2.16 (m, 2H), 2.03-1.96
washed with brine, dried over Na₂SO₄, filtered and concentrated. The combined organic phase was washed with water and brine, dried over Na₂SO₄, filtered and concentrated to give the title compound as a yellow solid (20 g, 69%); 1H-NMR (300 MHz, DMSO-d₆): δ 7.96–7.92 (m, 3H), 7.90–7.83 (m, 1H), 7.65–7.63 (m, 2H), 7.60–7.57 (m, 1H), 7.14 (s, 4H), 6.82 (s, 1H), 6.44 (s, 1H), 4.48–4.42 (m, 1H), 4.02–4.00 (m, 3H), 3.81–3.78 (m, 1H), 3.72 (s, 3H), 3.61–3.58 (m, 2H), 3.56 (s, 3H), 3.40–3.35 (m, 1H), 3.36–3.33 (m, 1H), 2.96–2.81 (m, 2H), 2.21–2.11 (m, 1H), 1.37 (s, 2H), 1.35 (s, 2H), 1.20–1.16 (m, 1H), 1.14–1.11 (m, 3H); LRMS (ESI) m/z 749 [M+H]+.

N-[4-(7,8-dimethoxy-3-(2-nitrophenylsulfonyl)-2,3,4,5-tetrahydro-1H-benzo[d]azepin-1-yl)phenyl]-2-nitrobenzenesulfonamide (13)

To a solution of 12 (4.5 g, 15.1 mmol), triethylamine (4.62 mL, 33.2 mmol) in dry DCM (150 mL) stirred under N₂ at 0°C, was added 2-nitrobenzenesulfonyl chloride (7.03 g, 31.7 mmol) dropwise during 10 min, the reaction mixture was stirred at rt overnight. The mixture was washed with H₂O (20 mL) and brine (15 mL), dried over Na₂SO₄, filtered and concentrated. The crude product was then purified by chromatography (200:1 DCM-MeOH) to give the title compound as an amorphous yellow solid (4.5 g, 80%); 1H-NMR (300 MHz, CDCl₃): δ 7.96–7.92 (m, 8H), 7.90–7.83 (m, 4H), 6.78 (s, 1H), 6.34 (s, 1H), 4.40–4.27 (m, 1H), 4.00–3.85 (m, 1H), 3.70 (s, 3H), 3.68–3.61 (m, 1H), 3.47 (s, 3H), 3.40–3.31 (m, 2H), 2.91–2.96 (m, 2H); LRMS (ESI) m/z 669 [M+H]+.

N-[4-(7,8-dimethoxy-3-(2-nitrophenylsulfonyl)-2,3,4,5-tetrahydro-1H-benzo[d]azepin-1-yl)phenyl]-2-nitro-N-(pent-4-ynyl)benzenesulfonamide (14)

To a solution of 13 (5 g, 7.48 mmol), triphenyl phosphine (4.56 g, 14.9 mmol) and hex-5-yn-1-ol (1.65 mL, 14.9 mmol) in dry DCM (30 mL) stirred under N₂ at 0°C, was added DIAD (2.95 mL, 14.9 mmol) dropwise during 10 min, the reaction mixture was stirred at rt overnight. The mixture was washed with H₂O (20 mL) and brine (15 mL), dried over Na₂SO₄, filtered and concentrated. The crude product was then purified by chromatography (200:1 DCM-MeOH) to give the title compound as an amorphous white solid (4.5 g, 80%); 1H-NMR (300 MHz, CDCl₃): δ 7.96–7.92 (m, 3H), 7.90–7.83 (m, 2H), 7.65–7.63 (m, 2H), 7.60–7.57 (m, 1H), 7.14 (s, 4H), 6.82 (s, 1H), 6.44 (s, 1H), 4.48–4.42 (m, 1H), 4.02–4.00 (m, 3H), 3.81–3.78 (m, 1H), 3.72 (s, 3H), 3.61–3.58 (m, 2H), 3.56 (s, 3H), 3.40–3.35 (m, 1H), 3.36–3.33 (m, 1H), 2.96–2.81 (m, 2H), 2.21–2.11 (m, 1H), 1.37 (s, 2H), 1.35 (s, 2H), 1.20–1.16 (m, 1H), 1.14–1.11 (m, 3H); LRMS (ESI) m/z 794 [M+H]+.

The solvent was removed under vacuo to give the title compound as brown solid without further purification; LRMS (ESI) m/z 783.

(11) Compound 10 (10 g, 0.029 mmol) was treated with PPA (80 mL) and the reaction was heated to 100°C for 1.5 h. After cooling the reaction, the solvent was poured into ice water, basified by ammonia to adjust the pH to 9, extracted with DCM (200 mL) and the combined organic phase was washed with water and brine, dried over Na₂SO₄, filtered and concentrated to give the title compound as a red solid (11.3 g, 75%); 1H-NMR (300 MHz, CDCl₃): δ 8.16–8.14 (m, 2H), 7.38–7.35 (m, 2H), 6.79 (s, 1H), 6.58 (s, 1H), 4.34–4.20 (m, 1H), 3.73 (s, 3H), 3.60 (s, 3H), 3.60–3.51 (m, 1H), 3.10–3.00 (m, 1H), 2.90–2.80 (m, 1H), 2.71–2.58 (m, 3H); LRMS (ESI) m/z 347 [M+H]+; mp 90–93°C.

7.8-dimethoxy-1-(4-nitrophenyl)-2,3,4,5-tetrahydro-1H-benzo[d]azepine (11)

Compound 10 (10 g, 0.029 mmol) was treated with PPA (80 mL) and the reaction was heated to 100°C for 1.5 h. After cooling the reaction, the solvent was poured into ice water, basified by ammonia to adjust the pH to 9, extracted with DCM (200 mL) and the combined organic phase was washed with water and brine, dried over Na₂SO₄, filtered and concentrated to give the title compound as a red solid (11.3 g, 75%); 1H-NMR (300 MHz, CDCl₃): δ 8.16–8.14 (m, 2H), 7.38–7.35 (m, 2H), 6.79 (s, 1H), 6.58 (s, 1H), 4.34–4.20 (m, 1H), 3.73 (s, 3H), 3.60 (s, 3H), 3.60–3.51 (m, 1H), 3.10–3.00 (m, 1H), 2.90–2.80 (m, 1H), 2.71–2.58 (m, 3H); LRMS (ESI) m/z 347 [M+H]+; mp 90–93°C.
Ethyl 6-[(47,8-bis(methoxymethoxy)-3-(2-nitrophenylsulfonyl)-
2,3,4,5-tetrahydro-1H-benzo[d]azepin-1-yl)phenyl]-2-nitrophenyl-
sulfonamido) hexanoate (16)
To a solution of compound 15 (100 mg, 0.13 mmol) in dry DCM
(10 mL) stirred at 0°C, was added MOMBr (22 μL, 0.27 mmol),
DIPEA (48 μL, 0.28 mmol), the reaction mixture was stirred at rt
and stirred overnight. The solution was diluted with DCM (20
mL), washed with water and brine, dried over Na₂SO₄, filtered
and concentrated. The residue was purified by chromatography
with CH₂Cl₂ to afford the title compound as colorless oil without
further purification; LRMS (ESI) m/z 888 [M+NH₄]⁺.

6-(47,8-bis(methoxymethoxy)-3-(2-nitrophenylsulfonyl)-
2,3,4,5-tetrahydro-1H-benzo[d]azepin-1-yl)phenyl)-2-nitrophenyl-
sulfonamido) hexanoic acid (17)
To a solution of compound 16 (30 mg, 0.03 mmol) in dry THF
(10 mL) stirred at rt, was added TFA (1 mL), the reaction mixture was
stirred at rt, was added TFA (1 mL), the reaction mixture was
stirred at rt overnight. The solution was evaporated under vacuo, the residue was purified by silica gel thin-layer chromatography
(9:1 DCM-MeOH) to afford the title compound as a yellow oil (20 mg, 70%); 1H-NMR (400 MHz, DMSO-d₆): δ 8.66 (s, 1H), 3.75–3.72
(m, 1H), 3.68–3.62 (m, 1H), 3.53 (s, 3H), 3.44–3.41 (m, 1H), 3.00–2.90
(m, 2H), 2.32 (t, J=7.6 Hz, 2H), 1.70–1.58 (m, 2H), 1.55–1.38 (m,
4H), LRMS (ESI) m/z 843 [M+H]⁺.

6-(47,8-bis(methoxymethoxy)-3-(2-nitrophenylsulfonyl)-
2,3,4,5-tetrahydro-1H-benzo[d]azepin-1-yl)phenyl)-2-nitrophenyl-
sulfonamido) N-(24-(8-(naphthalen-2-yl)-2,6-dioxo-1-propyl-1H-
purin-3(2H,6H,7H)-yl)-21-oxo-4,7,10,13,16-pentaoxa-20-
azatetracosyl)hexanamide (18a)
To a solution of compound 17 (110 mg, 0.13 mmol) and 7a (91 mg,
0.13 mmol) in dry DCM (10 mL) stirred at rt, was added EDCI
(31 mg, 0.16 mmol), HOBt (22 mg, 0.16 mmol) and DIPEA (68 μL,
0.38 mmol), the reaction mixture was stirred at rt overnight. The solvent was evaporated, and the residue was purified by chromato-
graphy (10:1 DCM-MeOH) to afford the title compound as an oil
(80 mg, 40%); 1H-NMR (400 MHz, DMSO-d₆): δ 8.85 (s, 1H), 8.36–8.34
(m, 1H), 8.06–8.03 (m, 1H), 7.95–7.92 (m, 1H), 7.87–7.85 (m, 1H), 7.55–7.52
(m, 2H), 7.22–7.21 (m, 1H), 7.00–6.99 (m, 1H), 6.80–6.77 (m, 4H), 6.39–6.36
(m, 2H), 5.25–5.24 (m, 2H), 5.11–5.09 (m, 2H), 4.32–4.30 (m, 1H),
4.28 (t, J=16Hz, 2H), 3.85–3.77 (m, 2H), 3.63–3.62 (m, 1H), 3.16–3.52
(m, 3H), 3.45 (s, 3H), 3.34–3.31 (m, 2H), 2.89–2.86 (m, 4H), 2.32–2.30
(m, 2H), 2.20–2.16 (m, 5H), 1.88–1.73 (m, 5H), 1.54–1.51 (m, 4H),
0.88–0.84 (m, 3H); LRMS (ESI) m/z 1151 [M+H]⁺.

6-(47,8-bis(methoxymethoxy)-2,3,4,5-tetrahydro-1H-benzo[d]
azepin-1-yl)phenylamino)-N-(24-(8-(naphthalen-2-yl)-2,6-dioxo-1-
propyl-1H-purin-3(2H,6H,7H)-yl)-21-oxo-4,7,10,13,16-pentaoxa-20-
azatetracosyl)hexanamide (18b)
To a solution of compound 18a (45 mg, 0.03 mmol) and K₂CO₃ (40
mg, 0.3 mmol) in dry DCM (10 mL) stirred at rt, was added PhSH
(30 μL, 0.3 mmol), the reaction mixture was stirred at rt overnight.
The solvent was evaporated under vacuo, the residue was purified
by silica gel thin-layer chromatography (9:1 DCM-MeOH) to afford the title compound as a yellow solid (20 mg, 60%); 1H-NMR
(400 MHz, DMSO-d₆): δ 8.79 (s, 1H), 8.32 (d, J=8.8 Hz, 1H), 8.02–
8.00 (m, 4H), 7.92–7.90 (m, 1H), 7.88–7.83 (m, 1H), 7.70–7.69 (m,
1H), 7.52–7.50 (m, 2H), 7.21–7.19 (m, 1H), 6.99 (s, 1H), 6.83–6.81
(m, 2H), 6.74 (d, J=7.6 Hz, 2H), 6.29 (d, J=8 Hz, 2H), 5.24–5.23 (m,
2H), 5.12–5.11 (m, 2H), 4.28–4.24 (m, 3H), 3.94–3.89 (m, 1H), 3.79–
3.75 (m, 1H), 3.61–3.60 (m, 12H), 3.45–3.52 (m, 14H), 3.39 (s, 3H),
3.35–3.32 (m, 5H), 3.14–3.08 (m, 2H), 2.89–2.86 (m, 4H), 2.32–2.30
(m, 2H), 2.20–2.16 (m, 5H), 1.88–1.73 (m, 5H), 1.54–1.51 (m, 4H),
0.88–0.84 (m, 3H); LRMS (ESI) m/z 1239 [M+H]⁺.

6-(47,8-bis(methoxymethoxy)-2,3,4,5-tetrahydro-1H-benzo[d]
azepin-1-yl)phenylamino)-N-(30-(8-(naphthalen-2-yl)-2,6-dioxo-
1-propyl-1H-purin-3(2H,6H,7H)-yl)-21-oxo-4,7,10,13,16-pentaoxa-20-
azatetracontyl)hexanamide (20a)
To a solution of compound 19a (12 mg, 0.01 mmol) in DCM (4
mL) stirred at rt, was added TFA (1 mL), the reaction mixture was
stirred at rt for 30 min. The solvent was evaporated under vacuo, the residue was purified by silica gel thin-layer chromatography
(9:1 DCM-MeOH) to afford the title compound as an amorphous
white solid (6.5 mg, 60%); 1H-NMR (400 MHz, DMSO-d₆): δ 8.81
(brs, 1H), 8.71 (s, 1H), 8.66 (brs, 1H), 8.26–8.23 (m, 1H), 8.04–7.93
Hz), 3.35–3.33 (m, 16H), 3.07–3.01 (m, 4H), 2.96–2.91 (m, 2H), 2.85–2.84 (m, 1H), 4.10 (t, J = 8.4 Hz, 2H), 6.61–6.54 (m, 3H), 6.05 (s, 1H), 5.61–5.54 (m, 1H), 4.33–4.31 (m, 2H), 3.87 (t, J = 8.8 Hz, 2H), 3.40–3.35 (m, 8H), 3.38–3.36 (m, 8H), 3.07–3.00 (m, 4H), 2.15 (t, J = 8.4 Hz, 2H), 2.03–1.98 (m, 4H), 1.50–1.54 (m, 6H), 1.46–1.45 (m, 2H), 0.86 (t, J = 8 Hz, 3H), 0.83–0.84 (m, 3H); LRMS (ESI) m/z 883 [M+H]+.

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Author contribution
Jing-kang SHEN, Lin-yin FENG, and Ye-chun XU designed the research; Jian SHEN, Tao MENG, Lei ZHANG, and Wan-ling SONG performed the research; Xin WANG and Lin CHEN contributed analytical tools and reagents; Jian SHEN and Tao MENG analyzed the compound and Jian SHEN wrote the paper.

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