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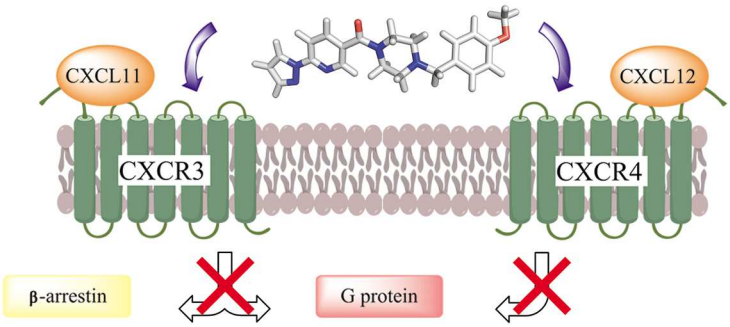


Insight into Structural Requirements for Selective and/or Dual CXCR3 and CXCR4 Allosteric Modulators

Anja Kolarič[a,b], Urban Švajger[c], Tihomir Tomašič[a], Regine Brox[d], Theresa Frank[d], Nikola Minovski[b], Nuska Tschammer[d,1] and Marko Anderluh[a,*]

[a]Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, 1000 Ljubljana, Slovenia

[b]National Institute of Chemistry, Ljubljana, Slovenia

[c]Blood Transfusion Center of Slovenia, Šlajmerjeva 6, 1000 Ljubljana, Slovenia

[d]Department of Chemistry and Pharmacy, Medicinal Chemistry, Emil Fischer Center, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany

Corresponding author:

* Prof. Dr. Marko Anderluh, University of Ljubljana, Faculty of Pharmacy, Aškerčeva 7, SI – 1000 Ljubljana, Slovenia; Tel.: +386 1 476 96 39; fax: +386 1 425 80 31.

[1] NanoTemper Technologies, Flößergasse 4, 81369 München, Germany.

Abbreviations: CXCR3, CXC chemokine receptor 3; CXCR4, CXC chemokine receptor 4; CXCL9, CXC chemokine ligand 9; CXCL10, CXC chemokine ligand 10; CXCL11, CXC chemokine ligand 11; CXCL12, CXC chemokine ligand 12; CD4, immune cells (cluster of differentiation 4); Boc, *tert*-butyloxycarbonyl; IFN-γ, interferon gamma; Th, helper T cell; CD25, interleukin 2 receptor alpha; FITC, Fluorescein isothiocyanate; SDF-1, stromal cell-derived factor 1.

ABSTRACT

Based on the previously published pyrazolopyridine-based hit compound for which negative allosteric modulation of both CXCR3 and CXCR4 receptors was disclosed, we designed, synthesized and biologically evaluated a set of novel, not only negative, but also positive allosteric modulators with preserved pyrazolopyridine core. Compound **9e** is a dual negative modulator, inhibiting G protein activity of both receptors. For CXCR4 receptor *para*-substituted aromatic group of compounds distinguishes between negative and positive modulation. *Para*-methoxy substitution leads to functional antagonism, while *para*-chloro triggers agonism. Additionally, we discovered that chemotaxis is not completely correlated with G protein pathways. This is the first work in which we have on a series of compounds successfully demonstrated that it is possible to produce selective as well as dual-acting modulators of chemokine receptors, which is very promising for future research in the field of discovery of selective or dual modulators of chemokine receptors.

Keywords: chemokine receptors, CXCR3, CXCR4, allosteric modulators

1. Introduction

G protein – coupled receptors (GPCRs) orchestrate diverse biological functions. Numerous diseases and disorders are associated with the malfunction in their signaling, which makes them interesting and promising drug targets [1]. Approximately 30% of marketed drugs target GPCRs [2]. Historically, drug discovery efforts in the field of GPCRs were oriented in targeting GPCRs orthosteric binding site and thus competing with the endogenous ligands. Today the focus shifted to the targeting of allosteric sites of GPCRs, which are distinct from the orthosteric site where the endogenous ligands bind. Addressing allosteric sites has several advantages over targeting orthosteric sites: a) they are less conserved among related GPCRs and thus offer the potential for the design of highly selective synthetic ligands and b) because they do not compete with the orthosteric ligand, the effects like probe dependence and insurmountable effects are observed. In general, allosteric ligands can act in three different ways; positive allosteric modulators enhance the action, while negative allosteric modulators decrease the affinity and/or efficacy of the orthosteric agonist. Thirdly, and neutral allosteric ligands do not affect affinity and/or efficacy of the orthosteric agonist, but prevent binding of other allosteric modulators [3,4]. Additionally, the phenomenon of biased signaling complicates the outcome of GPCRs modulation; biased signaling is the ability of ligands to stabilize different conformations of GPCRs, resulting in the stimulation of selected (but not all) signaling pathways that can be activated by the receptor and thus evoking different functional outcomes [5,6]. Therefore, allosteric modulators provide a range of novel opportunities in modulation of GPCR function. Especially in the case of chemokine receptors that are the key regulators of our immune

response, the development of allosteric drugs like maraviroc (allosteric inhibitor of the CCR5 receptor) indicates the power of this approach [7].

Chemokine receptors are representatives of family A of GPCRs [8]. Endogenous ligands for these receptors are chemokines (chemoattractant cytokines), small proteins playing essential role in the development, homeostasis, and function of the immune system [9]. Among chemokine receptors, CXCR3 is mostly expressed on activated Th1 cells, but is also located on circulating blood T cells, B cells and natural killer cells [10-12]. Three distinct CXCR3 isoforms have been identified, including CXCR3-A, CXCR3-B and less known alternative CXCR3-alt [13, 14]. Receptor's endogenous ligands are CXCL9, CXCL10 and CXCL11, of which CXCL11 is the dominant ligand followed by CXCL10 [15]. Additionally, endogenous CXCL4 was discovered as a high affinity ligand for CXCR3-B isoform. [13].On the other hand, CXCR4 receptor is expressed on several immune cells like monocytes, B cells and naive T cells, and binds endogenous chemokine CXCL12 only [15,16]. Overexpression of CXCR3 and its endogenous ligands has been found to play an important role in a variety of inflammatory diseases including autoimmune diseases (e.g., multiple sclerosis, psoriasis, and rheumatoid arthritis) and transplant rejection [15]. It is also responsible for the promotion of melanoma, osteosarcoma and for metastasis of colon, breast, lymph nodes and lung metastasis. Likewise, CXCR4 is often overexpressed in various cancers (e.g., breast cancer, prostate cancer, ovarian cancer and melanoma) [17] and serves as a co-entry receptor for HIV virus [18]. CXCR3 and CXCR4 co-involvement in metastasis of colorectal cancer into other tissues, especially lymph nodes, liver and lungs has been discovered and accordingly higher pharmacological effect obtained by blocking both receptors instead of antagonism of each individual receptor was

proven for treatment of colorectal cancer [19]. The two receptors act synergistically, meaning that activation of CXCR3 strengthens binding of CXCL12 to CXCR4 [19]. All these findings lead to the conclusion that compounds that would bind and inhibit the function of both receptors thereby acting as non-selective dual CXCR3 and CXCR4 antagonists/negative allosteric modulators would have interesting potential in treatment of the aforementioned diseases.

Structurally diverse small-molecule ligands with ability to individually modulate the function of either CXCR3 or CXCR4 have been disclosed including (aza)quinazolinones (AMG487, Figure 1), piperazinylpiperidines (VUF11211, Figure 1), pyrido[1,2-*a*]pyrimidin-4-ones, 1-aryl-3-piperidin-4-yl-ureas, 4-*N*-aryl[1,4]diazepanylureas, 2-iminobenzimidazoles, bispiperidines and ergolines for CXCR3 [20,21]. These ligands are presumed to be allosteric modulators [22]. AMG487 (Figure 1), the most studied member of azaquinazolines, was the only reported CXCR3 small molecule antagonist that progressed to Phase II clinical trials for psoriasis, but failed due to lack of efficacy [23]. Different structural classes of CXCR4 small-molecule antagonists have been designed, but only a few of them have been studied in detail: tetrahydroquinolines (AMD070, Figure 1), *N*-substituted indoles, 1,4-phenylenebis(methylene) derivatives and *N*-containing heterocycles are among them [24]. AMD070 (Figure 1) is one of the most known representatives of tetrahydroquinolines, an orally active CXCR4 negative allosteric modulator for the prevention of T-tropic HIV infection [25-27].

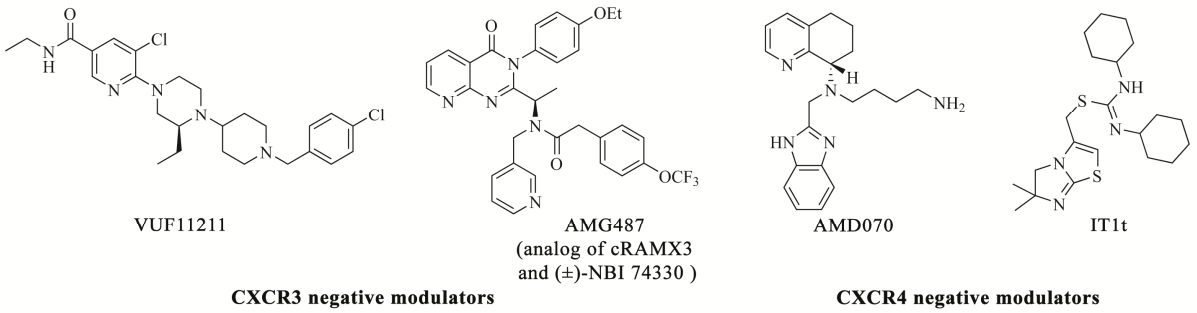


Figure 1. Known small molecule CXCR3 and CXCR4 antagonists/allosteric modulators.

Despite of sharing 36.7% and 63.9% sequence identity and similarity, respectively, small molecule ligand chemical spaces of CXCR3 and CXCR4 differ markedly. Using ChEMBL22 (version 17), 858 and 484 tested ligands were found for CXCR3 and CXCR4, respectively, but unfortunately no of it seems to be active on the opposite receptor at a threshold of 10 mM [28]. Schmidt et al. described the hit compound **7h** (re-synthesized, Figure 2) as a dual negative allosteric modulator, potently inhibiting the activation of CXCR3 and CXCR4 with the pK_B value of 8.82 and 8.72, respectively, as determined in the [^{35}S]GTPγS assay [28]. The cooperativity factor αβ of the hit compound **7h** was 0.12 and 0.04 for CXCR3 and CXCR4, respectively, clearly demonstrating that this compound acts as a negative allosteric modulator.

Starting from this dual negative allosteric modulator, our goal was to design and synthesize a series of novel potent dual allosteric modulators of CXCR3 and CXCR4. With the intention to avoid the limitations of [^{35}S]GTPγS assay, which is performed with isolated membrane preparations of cells expressing a given receptor, we decided to characterize new series of compound in a whole cell-based *in vitro* assays using transiently transfected HEK293 and primary peripheral blood mononuclear cells (PBMCs) or CD4+ T cells. Predicted binding poses and interactions identified as crucial for negative allosteric modulation studied by

molecular docking gave us a new insight into structure-activity relationship (SAR) providing progress in the on-going design and optimization of novel small molecule dual CXCR3 and CXCR4 negative allosteric modulators.

2. Results and discussion

2.1. Design

By examining the structural features of known small molecule CXCR3 and CXCR4 antagonists (Figure 1), we can conclude that in general, small molecule ligands consist of aromatic heterocyclic system connected through a cyclic or linear alkyl linker to a lipophilic moiety of one or two substituted or un-substituted aromatic/heterocyclic rings. Our hit compound (**7h**) [28] is in accordance with these findings, where pyrazolopyridine scaffold represents aromatic heterocyclic system connected with 1,3-diaminopropane linker to lipophilic moiety represented by benzene and ethyl group as depicted in Figure 2.

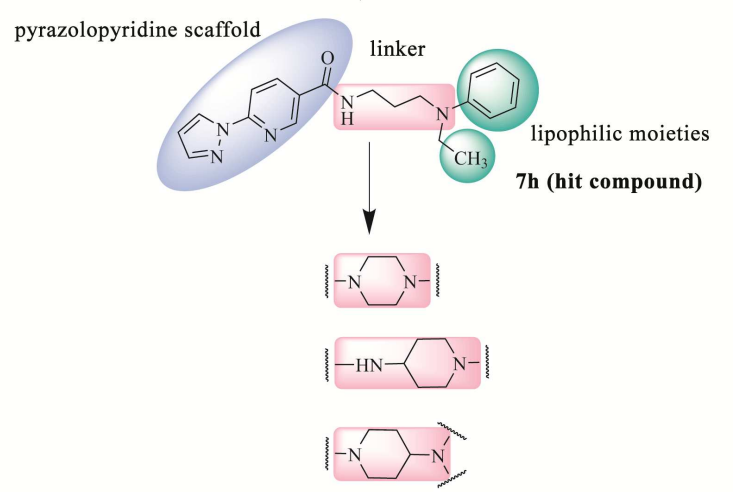


Figure 2. Investigation of structural features of our hit compound (**7h)** discovered by Schmidt et al. [28] and design of novel compounds.

We have designed a series of compounds with preserved pyrazolopyridine scaffold, connected with different (cyclic and linear) linkers to lipophilic moieties. A previous study of Schmidt et al. [28] anticipated optimal interactions of pyrazolopyridine scaffold with receptor,

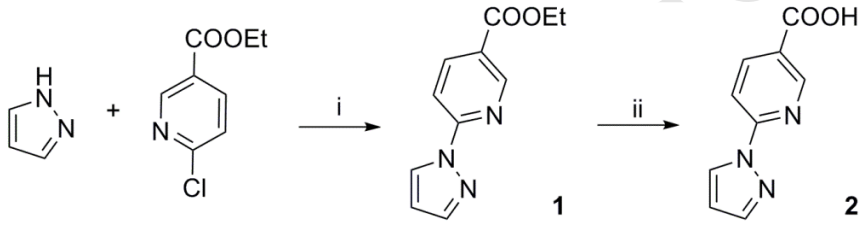
which was the reason for its preservation. As presented in Figure 2, we have introduced four different linkers including linear 1,3-diaminopropane, cyclic 4-aminopiperidine (in two different orientations) and piperazine linker, in which we varied flexibility, length and influence of the cationic center. A comparison of the properties of ligands between CXCR3 and CXCR4 shows that CXCR4 tends to bind ligands with a stronger positive charge compared to CXCR3 [28]. Also, these ligands differ in the distribution and the number of hydrogen bond donors and the predicted lipophilicity. Our prediction was that modification of the linker drives selectivity and influences biased signaling of individual or even both receptors. According to the structure of known small molecule ligands (Figure 1), it seems that those acting on CXCR3 have more linear shape, while those acting on CXCR4 have two lipophilic parts that point in different directions. Therefore, we connected linker to one or two aromatic lipophilic moieties. To provide stronger interactions with receptor, the influence of substitution pattern was determined by introduction of one or two substituents with different electronic effects.

Complete novel series of compounds was characterized in detail in the whole-cell based assays, including their ability to elicit biased signaling at the receptors of interest. The PathHunter β-arrestin 2 recruitment assay (DiscoverX) detects the recruitment of β-arrestin 2 to activated receptor by complementing two β-galactosidase (β-gal) enzyme fragments, while BRET (bioluminescence resonance energy transfer) based cAMP assay measures the concentration of cAMP. Moreover, the transwell migration assay using PBMC or CD4+ T cells was employed to measure chemokine induced cell migration.

2.2. Chemistry

Synthetic strategy for construction of the pyrazolopyridine scaffold **2** is described in Scheme 1. Commercially available pyrazole and ethyl 6-chloronicotinate reacted in the presence of sodium hydride in anhydrous *N*,*N*-dimethylformamide (DMF) under inert atmosphere at room temperature to give ester **1**, which was hydrolyzed to carboxylic acid **2** upon treatment with 3 M sodium hydroxide in ethanol.

Scheme 1. Synthesis of pyrazolopyridine scaffold.[a]

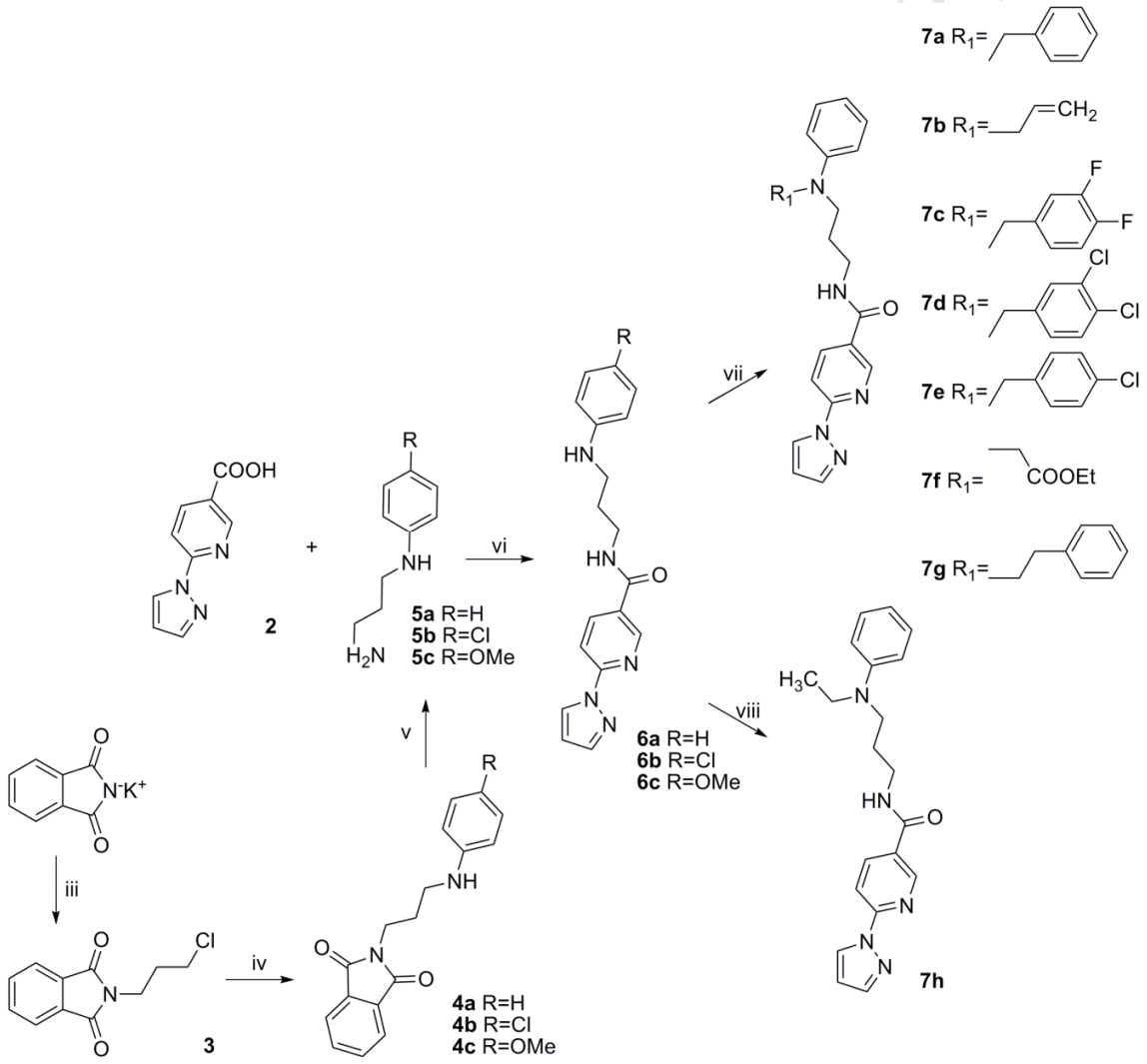


[a]Reagents and conditions: (i) NaH (60% in mineral oil), DMF, rt; (ii) 3M NaOH, EtOH, rt.

Synthesis of the derivatives with 1,3-diaminopropane linker (Scheme 2) started with the formation of compound **3** from phthalimide potassium salt and 1-bromo-3-chloropropane in anhydrous DMF at 70 °C. *N*-Alkylation with various aniline derivatives (aniline, 4-chloroaniline and 4-methoxyaniline) in DMF at 70 °C afforded compounds **4a**, **4b** and **4c** respectively. Following deprotection of the phthalimide group with hydrazine hydrate in DMF at 90 °C gave the desired amines **5a**, **5b** and **5c**. In the next step coupling of **2** with **5a**, **5b** or **5c** using 1-ethyl-3-(3-dimethylaminoisopropyl) carbodiimide (EDC), 1-hydroxybenzotriazole (HOBt) and triethylamine (Et_3N) in dry DMF at 0 °C provided **6a**, **6b** and **6c**. Only compound **6a** was used for further synthesis of the derivatives **7a-7h** with substituted 1,3-diaminopropane linker. Various reagents were used for *N*-alkylation with catalytic amount of benzyltriethylammonium

chloride (BTEAC) and potassium iodide in acetonitrile under inert atmosphere at 60 °C to form final compounds **7a**-**7g**. Ester of **7f** was hydrolyzed to carboxylic acid **7i** with 3 M sodium hydroxide in ethanol. Compound **7h** was synthesized in microwave reactor (100 °C, 15 bar, 20 min) using BTEAC and potassium carbonate.

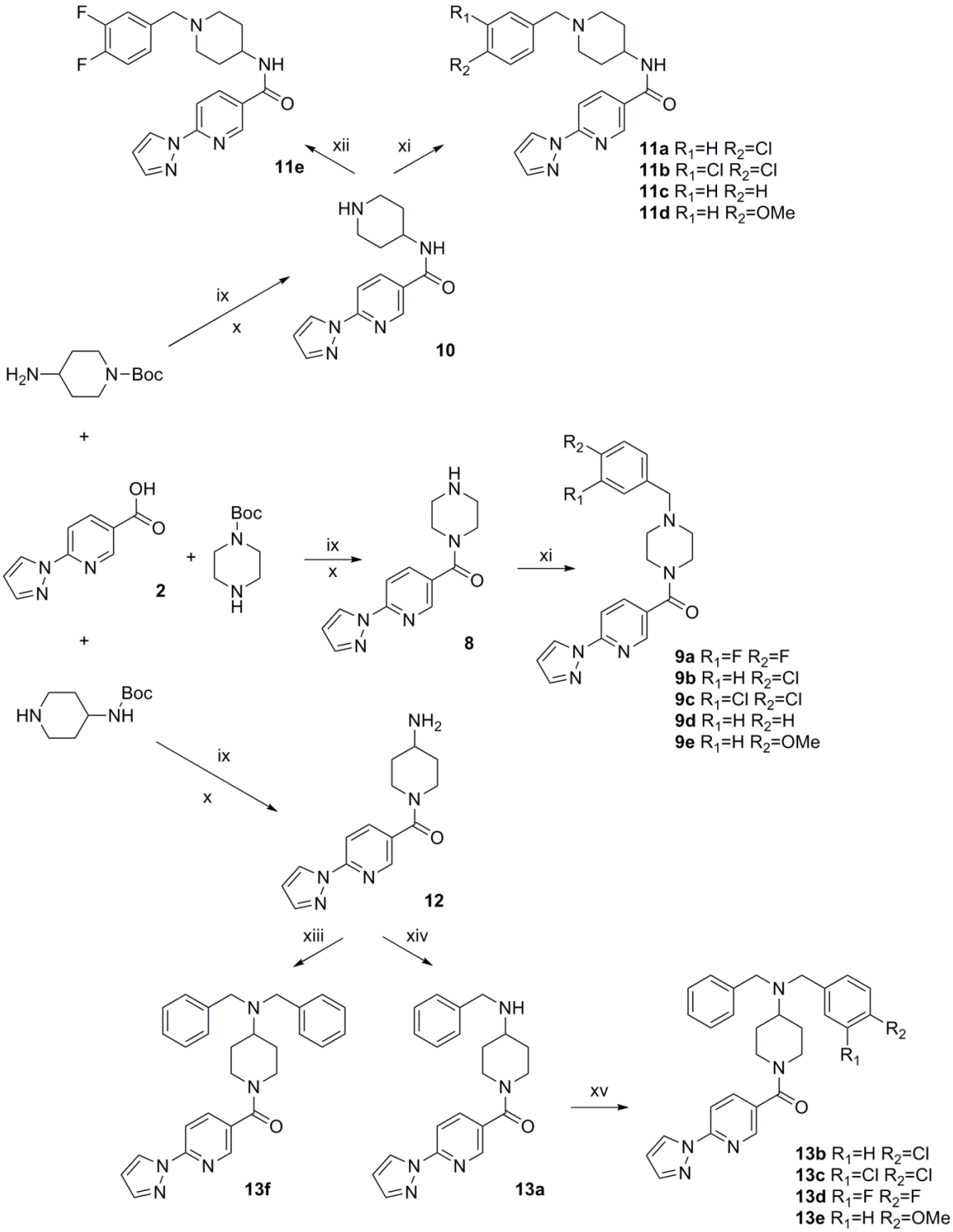
Scheme 2. Synthesis of the compounds with 1,3-diaminopropane linker.[a]



[a]Reagents and conditions: (iii) 1-bromo-3-chloropropane, DMF, 70 °C; (iv) K_2CO_3, DMF, 60 °C; (v) $H_2NNH_2 \times H_2O$, EtOH, 90 °C; (vi) HOBt, NMM, EDC × HCl, DMF, 0 °C; (vii) BTEAC, K_2CO_3, KI, CH_3CN, 60 °C; (viii) bromoethane, BTEAC, K_2CO_3, CH_3CN, MW: 100 °C, 20 min.

The synthetic strategy for compounds with piperazine (**8**-**9e**) and 4-aminopiperidine (**10**-**13f**) linker is shown in Scheme 3. The first step was the same for all three series of compounds; coupling of **2** with three different linkers (1-Boc piperazine, 4-amino-1-Boc-piperidine and 4-(*N*-Boc-amino)piperidine) using *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) in the mixture of DCM and DMF (1:1) in the presence of *N*-methylmorpholine (NMM) under inert atmosphere at room temperature. Removal of Boc protection by TFA was performed in dry DCM at 40 °C in inert atmosphere to give the desired amines **8**, **10** and **12**. Final compounds **9a**-**9e** and **11a**-**11d** were formed by *N*-alkylation in acetonitrile with various benzyl halides and potassium carbonate at 55 °C. The problem of reaction selectivity has been encountered in the synthesis of **13a**, where we first tried to achieve monosubstitution with *N*-alkylation in the presence of BTEAC, potassium carbonate, potassium iodide in acetonitrile at 55 °C, but the major final product was always disubstituted **13f**, independent of equivalents of aryl halide used in the reaction. Reductive amination using sodium cyanoborohydride gave monosubstituted **13a**.

Scheme 3. Synthesis of the compounds with 4-aminopiperidine and piperazine linker.[a]


xii
xi
11e
11a R1=H R2=Cl
11b R1=Cl R2=Cl
11c R1=H R2=H
11d R1=H R2=OMe
ix
x
10
2
8
xi
9a R1=F R2=F
9b R1=H R2=Cl
9c R1=Cl R2=Cl
9d R1=H R2=H
9e R1=H R2=OMe
12
xiii
xiv
13f
13a
xv
13b R1=H R2=Cl
13c R1=Cl R2=Cl
13d R1=F R2=F
13e R1=H R2=OMe

[a]Reagents and conditions: (ix) TBTU, NMM, DMF, DKM, r.t; (x) 1. TFA, DKM, 40 °C; (xi) various benzyl halides, K_2CO_3, CH_3CN, 55 °C; (xii) 3,4-difluorobenzaldehyde, CH_3COOH, $NaCNBH_3$, MeOH, r.t.; (xiii) benzyl chloride, BTEAC, K_2CO_3, KI, CH_3CN, 55 °C; (xiv) benzaldehyde, CH_3COOH, $NaCNBH_3$, MeOH, r.t; (xv) various benzyl halides, BTEAC, K_2CO_3, KI, CH_3CN, 80 °C.

2.3. Functional characterization

Upon binding of the endogenous chemokines to CXCR3 and CXCR4, the G-protein dependent and independent signaling pathways are activated. Both receptors are the $G_{\alpha i}$-coupled receptors, thus the G protein-dependent signaling can be monitored as a decrease in the intracellular cAMP level [29]. In the G protein-independent signaling, β-arrestin 2 is involved [30]. The activation of these pathways leads to the chemotaxis of the cells expressing given receptors.

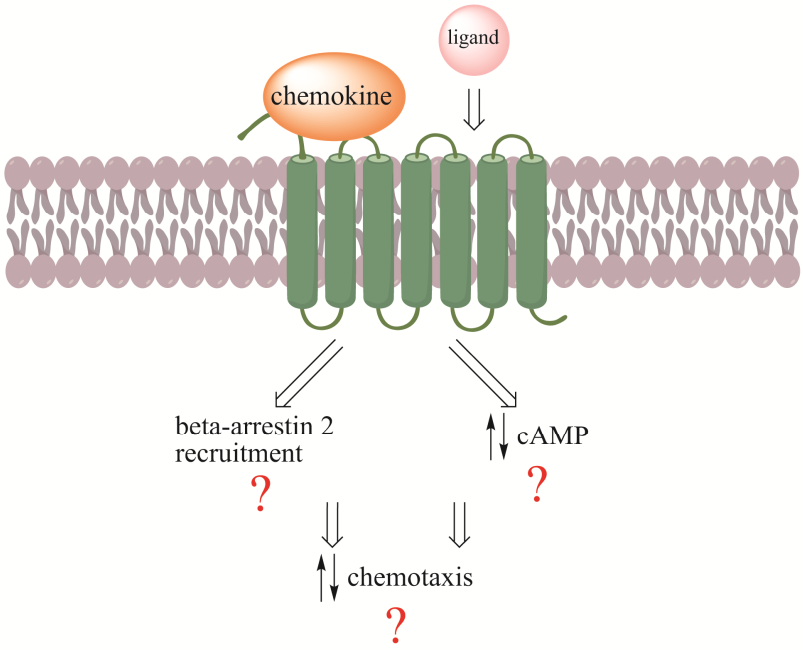


Figure 3. Graphical illustration of functional assays used to study the binding of novel compounds and their ability to modulate chemokine mediated activation of CXCR3 and CXCR4.

In β-arrestin 2 recruitment assay, all novel compounds were assessed for their ability to suppress chemokine mediated β-arrestin 2 recruitment to activated CXCR3 receptor. The compounds **7a**, **7h**, **9e**, **13b**, **13d**, **13e** and **13f** were able to suppress CXCL11-mediated β-arrestin recruitment for at least 20%, which was chosen as a limit (Figure 4). Known antagonist (±)-NBI-74330 (for structure see Figure S1 in Supplementary data) [31], which served as a control compound, could fully inhibit β-arrestin 2 recruitment at 10 μM. Concise determination of the IC_{50} values for the novel compounds was unfortunately not possible due to small assay windows, resulting only up to 30% of inhibition of CXCL11-mediated β-arrestin 2 recruitment.

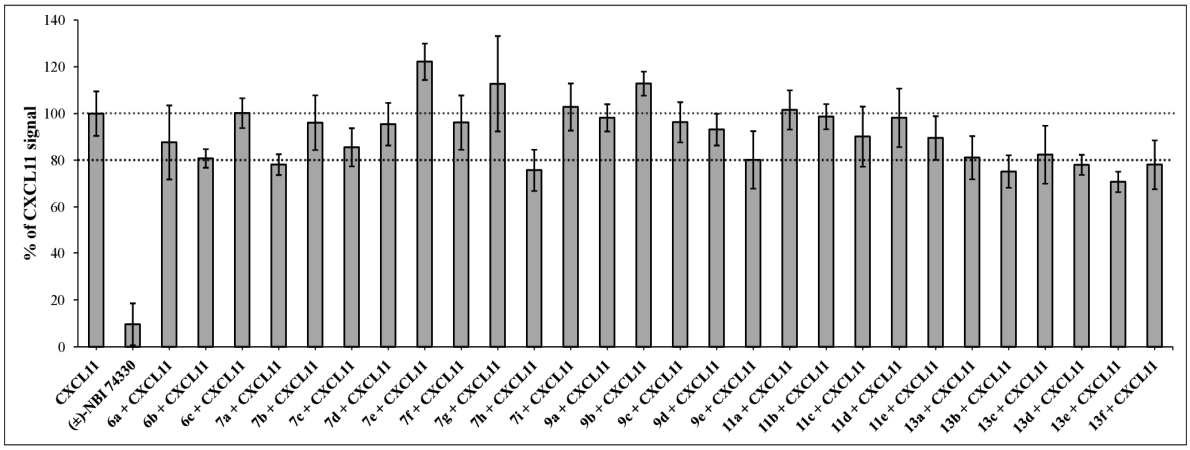


Figure 4. The ability of the tested compounds at 10 μM to inhibit the β-arrestin 2 recruitment, representing the percents of chemiluminescence in comparison with CXCL11 (100% activation) at 50 nM and (±)-NBI-74330 at 10 μM (full inhibition) [31] in single point β-arrestin 2 recruitment assay. The values represent the mean ± S.E.M. of three experiments performed in triplicate.

All the compounds were also screened for agonism, the ability to activate CXCR3 and cause β-arrestin 2 recruitment in the absence of the parent chemokine, and none was found to possess direct agonistic activity (Figure S3 in Supplementary data).

Our efforts to detect the CXCL12-mediated recruitment of the β-arrestin2 to CXCR4 failed for unknown reasons, although the expression of receptor was adequate (Figure S2 in Supplementary data). The detailed testing of various CXCR4 constructs containing either ProLink 1 (PK1) or ProLink 2 (PK2) (PathHunter assay, DiscoverX) remained without success, however we believe that the literature underscores the importance of β-arrestin 2 in CXCR4 signaling and further efforts will be needed to elucidate it [32,33].

Next, we investigated the ability of our novel compounds to reverse the chemokine mediated modulation of adenylate cyclases, which are the main effectors of CXCR3 and CXCR4 coupled G_i-proteins [34]. Because coupling of CXCR3 and CXCR4 receptor to $G_{\alpha i}$ leads to a negative regulation of the adenylate cyclase, which decreases cAMP levels, the adenylate cyclase has to be pre-stimulated with forskolin. Therefore, the measured activity actually represents inhibition of forskolin-stimulated cAMP production. All of the compounds were first screened in a single point BRET based cAMP experiment. Compounds **9b**, **9e**, **11a**, **11e** and **13c** inhibited the CXCL11 induced CXCR3 activation for more than 50% (Figure 5).

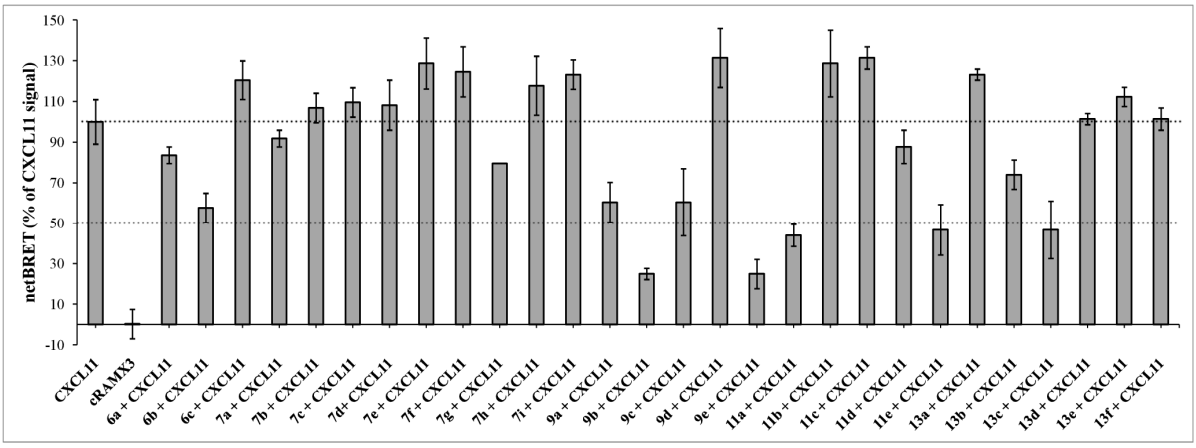
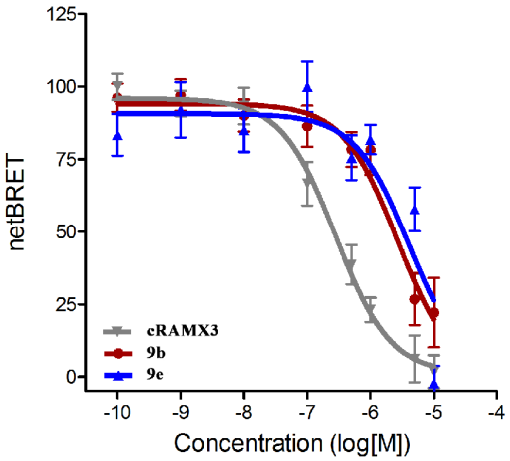


Figure 5. The chart of the tested compounds at 10 μM for CXCR3 antagonism in BRET based cAMP assay, representing the percents of netBRET signal in comparison with CXCL11 at 50 nM (100%) and cRAMX3 at 10 μM (full inhibition, receptor activation reduced to 0%) [35]. The values represent the mean ± S.E.M. of three experiments performed in triplicate.

Surprisingly, the only compound that appeared active in both assays (β-arrestin 2 recruitment and BRET based cAMP assay) on CXCR3 is compound **9e**. Compounds **9b** and **9e** with best efficacy of more than 60%, were further tested in dose-response assay (Figure 6). For

further characterization of allosteric profile for these novel compounds, the data of dose-response assays were analyzed with a ternary complex model of allosterism, using the algorithms in Prism 5.0 (GraphPad Software, San Diego, CA, USA) to determine pK_B and α. pK_B measures the affinity of allosteric modulator for its allosteric binding site and α denotes the cooperativity between allosteric and orthosteric ligand and it characterizes the affinity of orthosteric ligand in the presence of allosteric modulator [5]. Values of $\alpha > 1$ denote positive cooperativity (increase in orthosteric ligand binding), whereas $\alpha < 1$ denotes negative cooperativity (reduction of orthosteric ligand binding). $\alpha = 0$ cannot be distinguishable from competitive antagonism and K_B value approaches K_i value. If $\alpha = 1$, the result is unaltered ligand affinity [5].



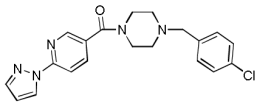
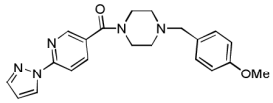
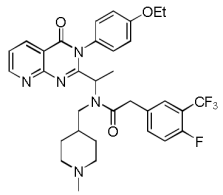
Compound		$pK_B \pm SEM$	$\alpha \pm SEM$
9b		6.35 ± 0.10	~ 0.0
9e		6.15 ± 0.15	~ 0.0
cRAMX3		7.33 ± 0.11	~ 0.0

Figure 6. The ability of novel negative allosteric modulators **9b** and **9e** to inhibit the CXCL11 (50 nM) mediated CXCR3 G protein activation in BRET based cAMP assay with representative dose-effect curves. The dose-dependent receptor activity is plotted for known allosteric antagonist (cRAMX3) as a reference [35]. The values represent the mean ± S.E.M. of three experiments performed in triplicate.

pK_B values of both compounds are comparable (pK_B = 6.35 ± 0.10 for **9b** and pK_B = 6.15 ± 0.15 for **9e**) and are one order of magnitude weaker than the known allosteric antagonist cRAMX3 (Figure 6) [35]. We assume that both **9b** and **9e** act as negative allosteric modulators,

with the α value equaling zero marring their behavior is undistinguishable from competitive antagonism. While compound **9b** was not identified with β-arrestin 2 recruitment assay as a promising inhibitor of recruitment, but it was significant negative allosteric modulator of CXCR3 in the G protein-dependent pathway, we assume that it is capable of biased CXCR3 signaling. Comparing the structures of both compounds, the only difference lies in the substitution of benzyl moiety; *para*-chloro for **9b** and *para*-methoxy for **9e**. The substituents differ in electronic effects, but mostly in H-bonding acceptor ability of the methoxy group, which indicates that a single H-bond could be the determining factor for biased signaling. To confirm biased CXCR3 signaling of compound **9b**, Student t-test was performed. Unfortunatelly no significant difference was observed between compounds **9b** and **9e**, therefore **9b** can not be considered as biased. Dose-effect curves also indicate that compounds could fully inhibit activation of G_i proteins. None of the compounds demonstrated intrinsic agonist properties when tested alone (Figure S4 in Supplementary data).

On CXCR4, the compounds showed significantly different profile. For CXCR4, the ability of novel compounds to inhibit CXCL12-mediated modulation of cAMP production was monitored in a single point experiment with the known inhibitor IT1t (Figure 1) as a control [36]. Compounds **7a**, **7g**, **9c**, **9e** and **13e** inhibited CXCL12 induced CXCR4 activation for more than 50% (Figure 7B). Strikingly, several compounds (**13a** being the most potent) enhanced the efficacy of CXCL12, which suggests that these compounds act as positive modulators of CXCR4 (Figure 7B).

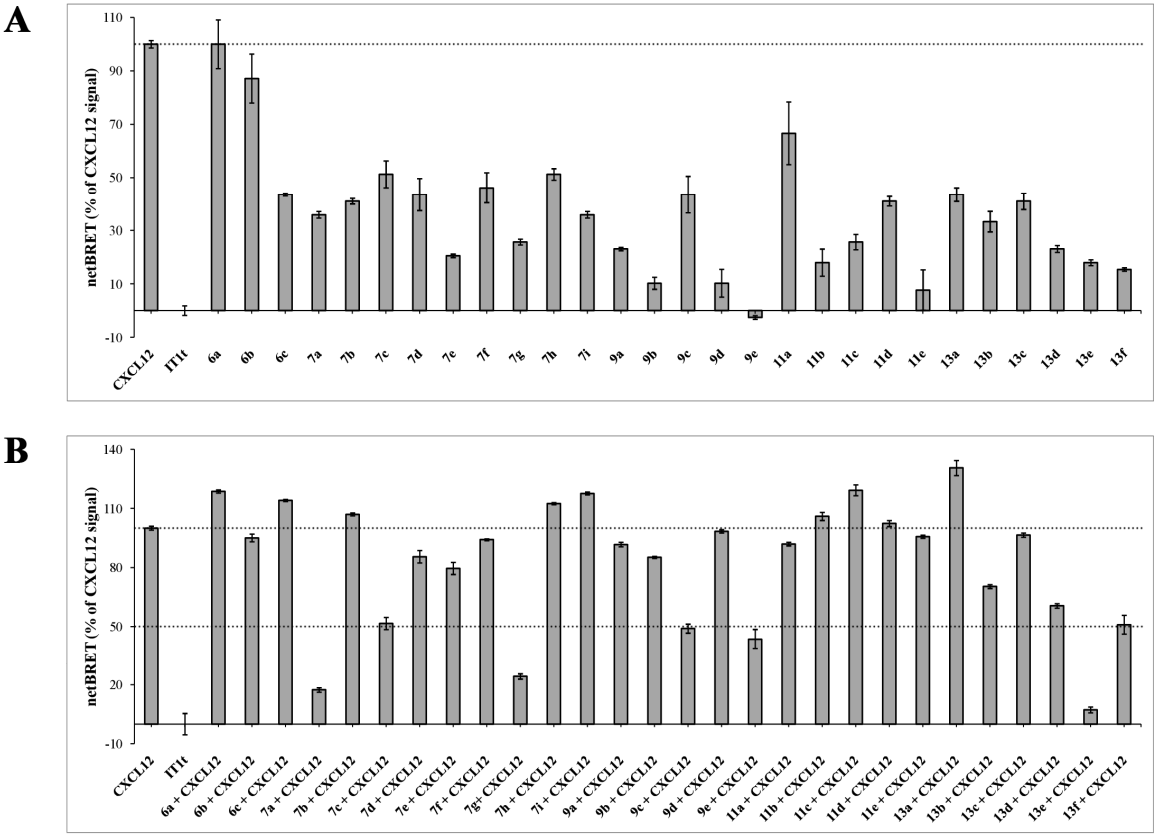
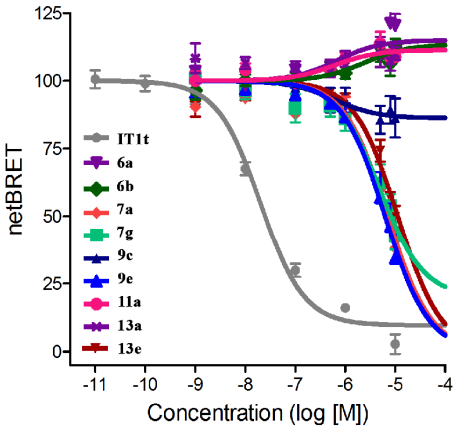


Figure 7. (A) The results of CXCR4 agonism screening of compounds at 10 μM with CXCL12 at 20 nM as a positive control. (B) The chart of the tested compounds at 10 μM for CXCR4 antagonism in BRET based cAMP assay, representing the percents of netBRET signal in comparison with CXCL12 at 20 nM (100%) and IT1t at 10 μM (full inhibition, receptor activation reduced to 0%) [36]. The values represent the mean ± S.E.M. of three experiments performed in triplicate.

To determine potential intrinsic agonist properties of these compounds, the compounds were tested for direct agonism in the cAMP assay. The compounds **6a**, **6b** and **11a** clearly acted as agonists, meaning they activated the receptor in the absence of the chemokine (Figure 7A).

For the further characterization of allosteric profile for these promising compounds, the activity profile of these compounds was characterized in the presence of CXCL12 and the data were analyzed with a ternary complex model of allosterism [5].



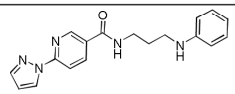
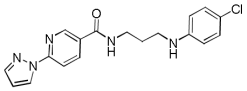
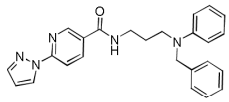
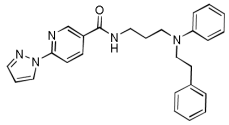
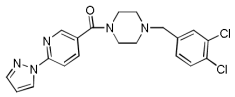
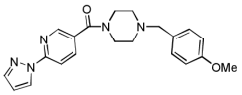
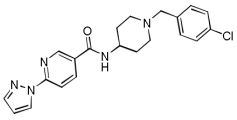
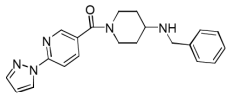
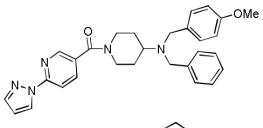
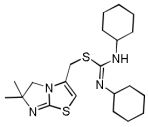
Compound		$pK_B \pm SEM$	$\alpha \pm SEM$	pEC_{50}
6a		/	/	5.02
6b		4.64 ± 1.67	10.38 ± 0.05	/
7a		6.04 ± 0.04	~ 0.0	/
7g		6.13 ± 0.17	0.04 ± 0.03	/
9c		6.50 ± 0.48	0.45 ± 0.08	/
9e		6.09 ± 0.06	~ 0.0	/
11a		5.60 ± 0.59	4.66 ± 0.54	/
13a		/	/	5.23
13e		5.83 ± 0.03	~ 0.0	/
IT1t		8.54 ± 0.06	0.01 ± 0.07	/

Figure 8. The ability of novel negative modulators **7a**, **7g**, **9c**, **9e** and **13e** to inhibit and positive modulator **13a** to activate the CXCL12 (20 nM) mediated CXCR4 protein activation in BRET based cAMP assay with representative dose-effect curves. Compounds **6a**, **6b** and **11a**, showing direct agonistic effect were also tested for allosteric profile. The dose-dependent receptor activity is plotted for known antagonist (IT1t) as a reference [36]. The values represent the mean ± S.E.M. of three experiments performed in triplicate.

Compounds **7a**, **7g**, **9c** and **9e** have comparable affinities (Figure 8). Closer look into the structure-activity relationship revealed that cationic center is not relevant for activity. Two types of compounds seem to favor inhibition of chemokine induced activation of G protein; compounds with longer, flexible linker, connected to two unsubstituted aromatic rings, such as **7a** and **7g**, and compounds with short, rigid linker and substituted aromatic group, such as **9c** and **9e**. This is supported by **13e** where additional substituted aromatic group on more rigid linker decreased the activity. Compounds **6a**, **6b**, **11a** and **13a** with one aromatic group act as agonists, regardless of linker's properties. Mono-substitution pattern seems to play the major role as a molecular switch between agonistic and antagonistic behavior having in mind that compounds most likely act as modulators and not direct agonists/antagonists. *Para*-Chloro substitution seems to favor agonism (**6b** and **11a**), while *para*-methoxy favors antagonism (**9e** and **13e**). Disubstitution seems to exhibit weak negative cooperativity, by which the affinity of chemokine is poorly decreased and results in the lack of efficacy as in **9c**. Only **7g** displays strong negative cooperativity, while the cooperativity of **7a**, **9e** and **13e** cannot be distinguished from competitive antagonism. Therefore, only **9c** and **7g** can be determined as allosteric modulators, while for the rest we can only assume allosteric binding. Compounds **6a**, **6b** and **11a** could

partially enhance the activity of CXCL12 on CXCR4 and thus, positive allosteric agonists with intrinsic agonist activity, which is observable from positive cooperativity value α of compounds **6b** and **11a**. In terms of agonism, none of the compounds acted as allosteric agonist on CXCR3, but only on CXCR4. From dose-effect curves of compounds **7a**, **9e** and **13e,** we can conclude that the compounds would be able to fully inhibit activation of G_i protein and act as good negative allosteric modulators of chemokine mediated activation. Due to poor signal-to-noise ratio, the data obtained for compounds **6a** and **13a** could be fitted only by non-linear regression curve fixing the Hills slope at 1 to obtain approximate pEC_{50} values.

To confirm negative modulatory effect of novel compounds in an *ex vivo* assay, we examined the influence of compounds **9b**, **9e**, **11a**, **11e** and **13c** for CXCR3 and compounds **7a**, **7g**, **9c**, **9e** and **13e** for CXCR4 mediated migration of PBMC or CD4+ T with transwell migration assay. The activation of CXCR3 has been shown to induce migration by activation of $G_{\alpha i}$ through multiple pathways, including phosphoinositide-3 kinase (PI3K) [37]. For CXCR4 it was published that $G_{\beta\gamma}$ subunit interacting with ion channels is responsible for activation of PI3K, which leads to chemotaxis [38]. Till now no direct studies on CXCR3 migration induced by β-arrestin 2 have been published [39]. However, Fong and co-workers pointed out the involvement of β-arrestin 2 in chemotaxis regulation [40].

Two types of cells were used for evaluation of compounds influence on chemokine induced migration; PBMCs in the case of CXCR3 receptor [41] and CD4+ T cells for CXCR4 [42]. Firstly, phenotypic characterization was done, using flow cytometric analysis in order to confirm sufficient expression of CXCR3 and CXCR4 receptors on selected cells. Moreover, the cells were pre-treated with selected compounds afterwards to determine the negative modulatory

effect. All investigated compounds (**9b**, **9e**, **11a**, **11e** and **13c**) were able to decrease CXCL11 induced migration of cells expressing CXCR3 (Figure 9A). At lower concentration (30 μM) the decrease was more than 10% and at higher concentration (150 μM) more than 20% compared to control. With approximately 80% decreased migration, **11a** was the most potent migration inhibitor.

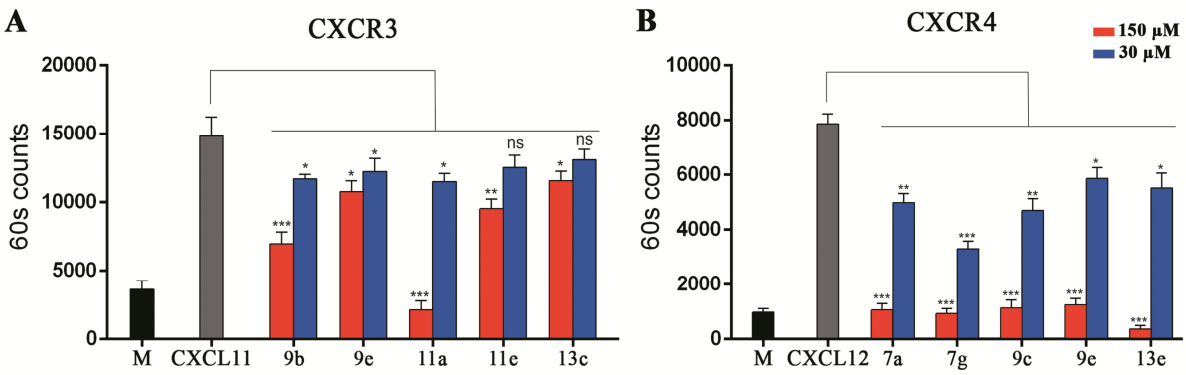


Figure 9. Inhibition of cell migration using transwell assay. Selected compounds were tested for their capacity to inhibit migration of human PBMCs from upper to lower chamber in a transwell system, as described in Experimental. Briefly, PBMCs (A) or isolated $CD4^+$ T cells (B) were either left untreated (black) or pre-treated with CXCR3 (A) or CXCR4 (B) antagonists and their migratory capacity toward endogenous chemokine ligands for CXCR3 (CXCL11) or CXCR4 (CXCL12) was evaluated. After the incubation period, the migrated cells from the lower chamber were thoroughly collected and counted on a flow cytometer using 60s counts. Using bar graphs, mean ± SD is shown from four independent experiments. Statistical significance between individual pairs (migration of untreated vs. treated with all individual compounds) was

calculated using Student's unpaired t test (ns – non-significant; $p<0.05$ - *; $p<0.01$ - **; $p<0.001$ - ***).

The compounds **7a**, **7g**, **9c**, **9e** and **13e** were tested for their ability to suppress the CXCL12 induced migration of CXCR4-expressing cells (Figure 9B). All of the compounds were able to decrease migration for more than 80% at higher (150 μM) concentration in comparison to control, with compound **13e** being able to inhibit migration almost completely. Interestingly, compounds **11a** (for CXCR3) and **13e** (for CXCR4) were identified as modest negative allosteric modulators in the BRET based cAMP assay, but did cause the most potent inhibition of migration. This suggests that quantitative effect on inhibition of chemotaxis is not necessarily in a strong correlation with the inhibition of G protein-dependent pathways. Other signaling pathway(s) may be involved in chemotaxis, which alone, or in combination with cAMP-pathway triggers the full chemotaxis. The very same observation was also confirmed by Milanos et al. [39].

Surprisingly, compound **7h**, which was our starting hit showed only weak negative allosteric effect on CXCR3 in β-arrestin 2 recruitment assay. Furthermore, it did not have any effect on G protein activation in BRET based cAMP assay, neither on CXCR3 nor on CXCR4 receptor. This discrepancy between the published and our observations might be explained by different assay used for the initial characterization of compounds from the virtual screening, which detects GDP/GTP exchange rather than cAMP concentration as the final consequence of GPCR activation [28]. According to Kleemann et al. [43], CXCR4 couples to various isoforms of G_i proteins and to G_o. The activation of G_o does not lead to the inhibition of adenylyl cyclase [44]. For example, the activation of G_o in the dopamine D2 receptor does not inhibit adenylyl

cyclase [45]. Thus, it is possible that in the [^{35}S]GTPγS assay the compound **7h** successfully inhibits the activation of G_o proteins, but to lesser extend than that of G_i. Accordingly, the lack of efficacy of the compound **7h** to prevent chemokine-induced inhibition of cAMP production by the G_i proteins results in no detectable effect of this compound in the cAMP assay. The same applies to CXCR3. Since β-arrestin 2 and cAMP signaling pathways are truly important for functional agonism/antagonism, we believe that the assays used in this work offer more relevant information to decipher compounds' role in CXCR activation.

Overall in our work, we have synthesized and characterized novel negative and positive allosteric modulators of the chemokine receptors CXCR3 and CXCR4, which are both prominent drug targets. Importantly, we identified the compound **9e** as dual negative allosteric modulator with comparable functional affinity to both receptors. The compound **9e** was able to suppress the migration of CXCR4 expressing CD4+ T cells.

2.4. Proposed binding modes of most potent negative allosteric modulators

To propose the binding mode of the most promising negative allosteric modulators as confirmed by the cAMP BRET assay, a series of molecular docking calculations were performed using the crystal structure of CXCR4 (PDB code: 3ODU) and its derived CXCR3 homology model (Supplementary data). The proposed binding modes are shown in Figures 10 and 11 by representatives **9b** and **9e** for CXCR3 and **7a** and **9e** for CXCR4.

Figure 10 shows that methoxy substituted benzyl moiety of **9e** forms H-bond with $Y60^{1.39}$ (superscript numbers denote Ballesteros-Weinstein numbering) [46], while chloro substituent cannot. This is in line with previous reports where $Y60^{1.39}$ has been reported as H-bond interaction partner important for binding of known CXCR3 ligands [22]. Furthermore, $D112^{2.63}$

and W109$^{2.60}$ have been found to be involved in chemokine binding and receptor activation, which leads to signal transduction [47,48]. Of particular importance is W109$^{2.60}$, crucial for regulation of G protein activation, by which interactions with negative allosteric modulators stop G protein activation. Therefore, hydrophobic interactions with aromatic groups of **9b** and **9e** seem to be mainly responsible for G protein inhibition [48].

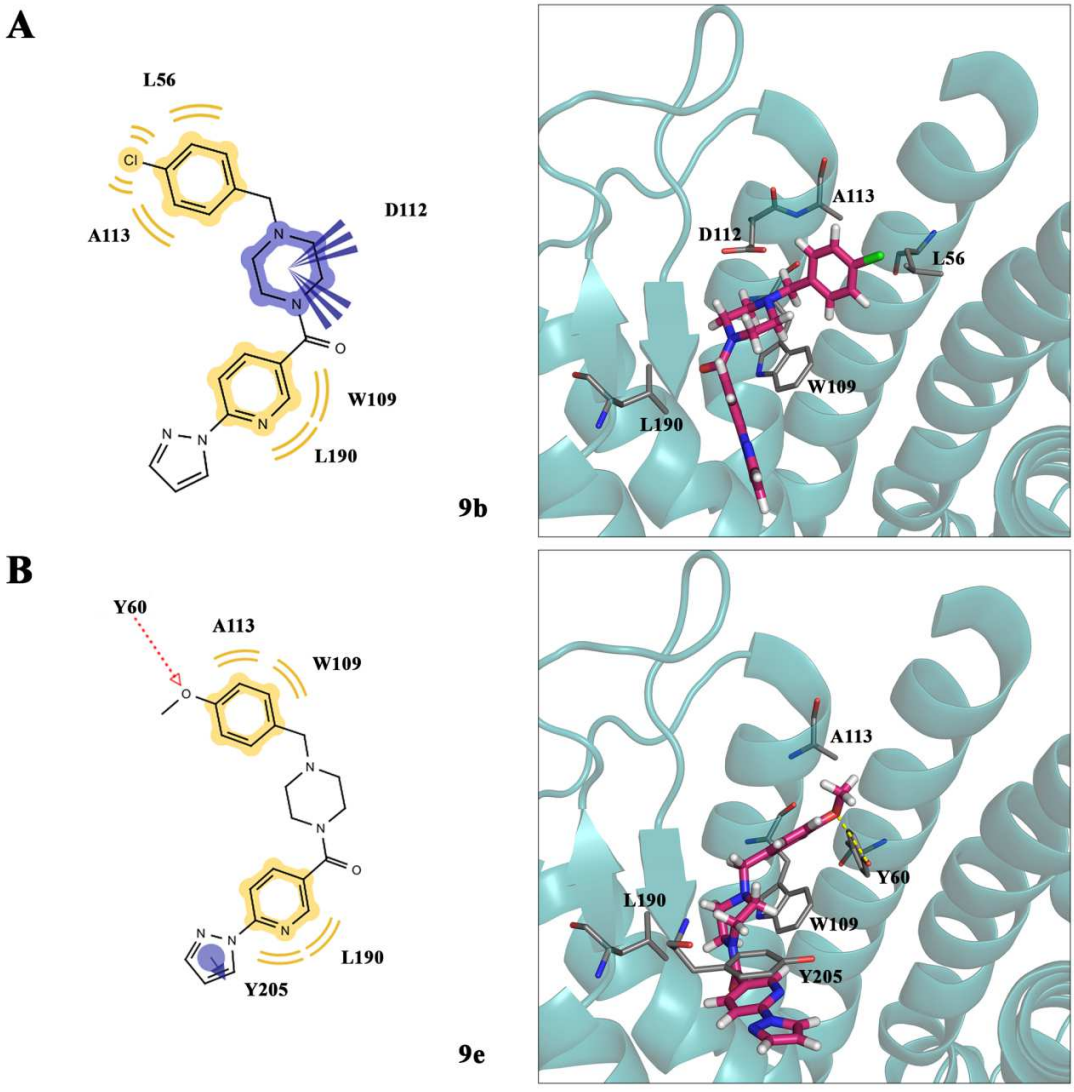


Figure 10. Ligand interaction diagrams and predicted binding modes of **9b** (**A**) and **9e** (**B**), respectively, obtained by molecular docking in the CXCR3 model's binding site. In diagrams,

red arrows represent H-bond acceptors, yellow regions hydrophobic areas, magenta circle and vector heteroaromatic moiety capable to establish π- π interactions and purple regions positive ionizable moieties.

For CXCR4, D97$^{2.63}$ was reported as of key importance for CXCL12 binding to receptor [49], while Y45$^{1.39}$ was identified as signal initiator upon chemokine binding [50]. Compounds **7a** and **9e** (Figure 11) show favorable interactions with these residues by which they can weaken not only the binding of chemokine, but also signal transduction and thus, decrease the activation of G protein as determined by cAMP assay. Moreover, H-bond with S285$^{7.36}$ contributes to binding of the modulators, which was firmly corroborated with the previous investigations [51].

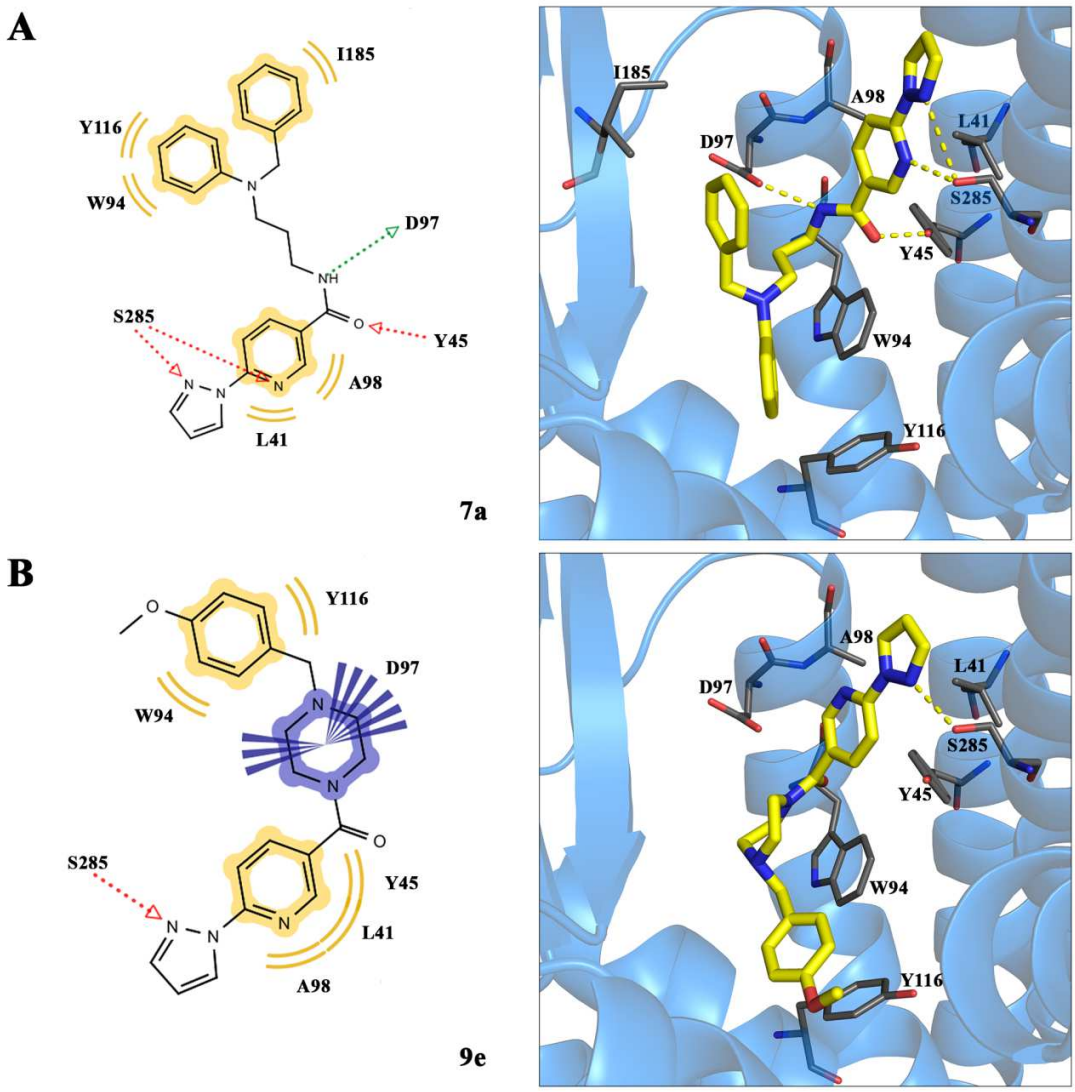


Figure 11. Ligand interaction diagrams and predicted binding modes of **7a** (**A**) and **9e** (**B**), respectively, obtained by molecular docking within CXCR4 model's binding site. In diagrams, red arrows represent H-bond acceptors, green arrows H-bond donors, yellow regions hydrophobic areas and purple regions positive ionizable moieties.

It should be stressed however that some of the CXCR4 residues identified to bind ligands herein (Figure 11) were not only reported previously as crucial for binding of small molecules

with confirmed allosteric behavior (including our hit compound **7h**), but also involved in the chemokine binding [22,27,52]. Therefore, we believe that our compounds bind to different, allosteric pocket, which is partially overlapping with chemokine binding site. This would perfectly explain our experimental observation that cooperativity is undistinguishable from competitive antagonism.

3. Conclusion

In this work we report the synthesis and detailed functional characterization of novel compounds that act as positive or negative allosteric modulators of CXCR3 and CXCR4. Novel ligands are characterized by pyrazolopiridine scaffold, connected through carbonyl group with 1,3-diaminopropane, 4-aminopiperidine and piperazine linkers to anilino and/or benzylamino group.

All synthesized compounds were tested in the β-arrestin 2 recruitment and the BRET based cAMP assay to assess the ability of novel compounds to modulate the activity of CXCR3 and CXCR4. Furthermore, transwell migration assay was used for further confirmation of compounds' functional antagonism measured by the influence on chemokine-induced migration of PBMCs or CD4+ cells and its connection to negative modulation.

Compounds **9b** and **9e** were identified as the most potent negative modulators of CXCR3, and compounds **7a**, **7g**, **9c**, **9e** and **13e** the most potent negative modulators of CXCR4, with compound **9e** acting as dual CXCR3/CXCR4 negative modulator. We were also able to identify modest positive modulator **13a** on receptor CXCR4, while **6a**, **6b** and **11a** act as weak allosteric agonists on the same receptor.

We postulate that for the CXCR3 negative allosteric modulation, piperazine linker seems to be the best choice with one mono-substituted lipophilic end functionalities regardless of their substitution pattern. For targeting CXCR4, 1,3-diaminopropane linker with two unsubstituted aromatic groups seems more suitable for functional antagonism, while for piperazine linker *para*-substitution on aromatic group guides negative and/or positive modulation. *Para*-Methoxy substitution triggers antagonism, while *para*-chloro substituted compounds were proven to act as allosteric agonists of CXCR4. Moreover, dichlorophenyl substituted compounds exhibited weakened negative cooperativity toward chemokine CXCL11 on CXCR4. Furthermore, *para*-methoxy substitution in **9e** was shown to be important for dual binding and negative modulation on both receptors. Comparable K_B values (6.15 and 6.09) on both CXCR3 and CXCR4 respectively, make **9e** the first in class dual negative modulator of CXCR3/CXCR4 with a very well-balanced activity.

We believe that our study enabled initial insight in the SAR of these ligands and provided valuable information for the future development of modulators targeting CXCR3 and CXCR4.

4. Experimental

4.1. Chemistry

All reagents from commercial sources were without further purification. All reactions requiring anhydrous conditions were carried out under inert atmosphere using anhydrous solvents. The reactions were monitored by TLC using Merck silica gel (60 F254) plates (0.20 mm). For detection we used UV light (λ=254 nm) and ninhydrine staining reagent. Column

chromatography was used for compound purification on silica gel 60, particle size 0.040–0.063 mm.

HPLC purity analyses were assessed on Thermo Scientific DIONEX UltiMate 3000 instrument equipped with diode array detector using Agilent Eclipse Plus C18 column (5 μm, 4.6 mm × 150 mm). The solvent system that was used includes acetonitrile (ACN) and water with 0.1% trifluoroacetic acid (TFA) in gradient 10% ACN to 90% ACN in 20 min with flow rate 1.0 mL/min. All tested compounds were ≥95% pure by HPLC. Melting points were determined on Leica hot stage microscope. NMR spectra (^{1}H and ^{13}C) were recorded on a Bruker AVANCE III spectrometer at 400 and 100 MHz at 298K in DMSO-d_6 or $CDCl_3$ solution with TMS as an internal standard. Spectra can be found in Supplementary data. Mass spectra were determined using a VG-Analytical Autospec Q mass spectrometer with ESI and HRMS method. Microwave reaction was done in septum-sealed glass vials (10 mL) which enable high-pressure reaction conditions (max 20 bar) using microwave reactor Discover™, CEM Corporation, Matthews, NC. The temperature of the reaction mixture was monitored with a calibrated infrared temperature controller under the reaction vessel.

*4.1.1. 6-(1H-Pyrazol-1-yl)nicotinic acid (**2**).* Compound **1** (8.66 g, 40.43 mmol, 1 equiv) was dissolved in ethanol (80 mL). 3 M $NaOH_{(aq)}$ (40.40 mL, 121.3 mmol, 3 equiv) was then added and the mixture stirred overnight. The solvent was evaporated under reduced pressure and the residue was dissolved in water (60 mL) and extracted with diethyl ether (40 mL) two times. pH of the water phase was adjusted with 2 M HCl till 3 where white precipitate was formed (7.42 g, 97%). ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 6.65 (dd, J_1 = 2.7 Hz, J_2 = 1.6 Hz, 1H, Ar-H), 7.92 (dd, J_1 = 1.6 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.04 (dd, J_1 = 8.6 Hz, J_2 = 0.8

Hz, 1H, Ar-H), 8.44 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, 1H, Ar-H), 8.70 (dd, J_1 = 2.7 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.96 (dd, J_1 = 2.3 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 13.44 (s, 1H, COOH).

*4.1.2. 2-(3-Chloropropyl)isoindoline-1,3-dione (**3**).* Phthalimide potassium salt (15.61 g, 84.26 mmol, 1 equiv) was dissolved in anhydrous DMF (80 mL), 1-bromo-3-chloropropane (10.00 mL, 101.12 mmol, 1.2 equiv) was added and the reaction was stirred at 70 °C overnight, protected from the moist with chlorcalcium tube. The reaction mixture was poured into ice cold water (150 mL), which yielded white precipitate. The precipitate was filtered with vacuum and dried on air or under reduced pressure. The compound was taken into the next step without further purification and characterization.

*4.1.3. General procedure **A**. Synthesis of compounds **4a-4c**.* Compound **3** (1 equiv) was dissolved in anhydrous DMF and K_2CO_3 (2 equiv) was added. Aniline derivatives (1 equiv) were added and the reaction mixture was stirred at 120 °C overnight, equipped with chlorcalcium tube. The reaction mixture was poured into ice cold water, where precipitate of compound **4b** formed. For compounds **4a** and **4c**, water phase was extracted three times with DCM. The organic phase was dried over sodium sulphate, filtered and the solvent evaporated under reduced pressure. Compounds were purified by column chromatography.

*4.1.3.1. 2-(3-(Phenylamino)propyl)isoindoline-1,3-dione (**4a**).* Compound **4a** was prepared according to general procedure **A** from **3** (20.93 g, 93.55 mmol) and aniline (8.53 mL, 93.55 mmol). Column chromatography (toluene/acetone: 10/1) was used to afford the compound **4a** (5.89 g, 23%) as yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.87 (p, J = 7.0 Hz, 2H, $CH_2CH_2CH_2$), 3.04 (m, J_1 = 7.0 Hz, J_2 = 5.8 Hz, 2H, $CH_2CH_2CH_2$-NH-Ar), 3.65-3.72 (m,

2H, C<u>H</u>$_2$CH$_2$CH$_2$-NH-Ar), 5.55 (t, *J* = 5.8 Hz, 1H, N<u>H</u>), 6.48-6.57 (m, 3H, Ar-<u>H</u>), 7.02-7.10 (m, 2H, Ar-<u>H</u>), 7-81-7.91 (m, 4H, Ar-<u>H</u>).

*4.1.3.2. 2-(3-((4-Chlorophenyl)amino)propyl)isoindoline-1,3-dione (**4b**).* Compound **4b** was prepared according to general procedure **A** from **3** (2.20 g, 9.84 mmol) and 4-chloroaniline (1.25 g, 9.84 mmol). Column chromatography (EtOAc/hexane: 1/3) was used to afford the compound **4b** (0.12 g, 4%) as white solid. ^{1}H NMR (400 MHz, DMSO-d$_6$) δ(ppm) = 1.82-1.93 (m, 2H, CH$_2$C<u>H</u>$_2$CH$_2$), 2.97-3.10 (m, 2H, CH$_2$CH$_2$C<u>H</u>$_2$-NH-Ar), 3.68 (t, *J* = 7.1 Hz, 2H, C<u>H</u>$_2$CH$_2$CH$_2$-NH-Ar), 5.73-5.85 (m, 1H, N<u>H</u>), 6.54 (d, *J* = 8.9 Hz, 2H, Ar-<u>H</u>), 7.07 (d, *J* = 8.9 Hz, 2H, Ar-<u>H</u>), 7.80-7.91 (m, 4H, Ar-<u>H</u>).

*4.1.3.3. 2-(3-((4-Methoxyphenyl)amino)propyl)isoindoline-1,3-dione (**4c**).* Compound **4c** was prepared according to general procedure **A** from **3** (0.50 g, 2.24 mmol) and 4-methoxyaniline (0.28 g, 2.24 mmol). Column chromatography (EtOAc/hexane: 1/1) was used to afford the compound **4c** (0.27 g, 38%) as brown solid. ^{1}H NMR (400 MHz, DMSO-d$_6$) δ(ppm) = 1.86 (q, *J* = 6.9 Hz, 2H, CH$_2$C<u>H</u>$_2$CH$_2$), 2.99 (q, *J* = 6.9 Hz, 2H, CH$_2$CH$_2$C<u>H</u>$_2$-NH-Ar), 3.65-3.73 (m, 2H, C<u>H</u>$_2$CH$_2$CH$_2$-NH-Ar), 5.12 (d, *J* = 5.8 Hz, 1H, N<u>H</u>), 6.48-6.56 (m, 2H, Ar-<u>H</u>), 6.67-6.76 (m, 2H, Ar-<u>H</u>), 7.81-7.93 (m, 4H, Ar-<u>H</u>).

*4.1.4. General procedure **B**. Synthesis of compounds **5a-5c**.* Compounds **4a**, **4b** and **4c** were dissolved in ethanol, hydrazine hydrate (5 equiv) was added and the reaction was stirred overnight at 90 °C. Ethanol was evaporated under reduced pressure. DCM and water were added to the residue, pH of the water phase was adjusted with 1 M NaOH to 12, and the phases were separated. Aqueous phase was extracted with DCM two times, combined organic phases were

dried over sodium sulphate, filtered and the solvent evaporated under reduced pressure. The compound was used in the next reaction without purification.

*4.1.4.1. N1-Phenylpropane-1,3-diamine (**5a**).* Compound **5a** was prepared according to general procedure **B** from **4a** (1.99 g, 7.08 mmol) and was afforded as brown oil (0.76 g, 71%). ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.61 (p, *J* = 6.7 Hz, 2H, $CH_2C\underline{H_2}CH_2$), 2.64 (t, *J* = 6.7 Hz, 2H, $CH_2CH_2C\underline{H_2}$-NH_2), 3.02 (q, *J* = 6.7 Hz, 2H, $C\underline{H_2}CH_2CH_2$-NH_2), 5.54 (s, 1H, N<u>H</u>), 6.46-6.52 (m, 1H, Ar-<u>H</u>), 6.52-6.58 (m, 2H, Ar-<u>H</u>), 6.97-7.09 (m, 2H, Ar-<u>H</u>). A peak for NH_2 is missing due to H-bonding with the solvent and/or H_2O.

*4.1.4.2. N1-(4-Chlorophenyl)propane-1,3-diamine (**5b**).* Compound **5b** was prepared according to general procedure **B** from **4b** (0.30 g, 0.94 mmol) and was used as a grey solid (0.51 g, 30%) in the next reaction without purification and characterization.

*4.1.4.3. N1-(4-Methoxyphenyl)propane-1,3-diamine (**5c**).* Compound **5c** was prepared according to general procedure **B** from **4c** (0.47 g, 1.52 mmol) and was used as a brown solid (0.23 g, 85%) in the next reaction without purification and characterization.

*4.1.5. General procedure **C**. Synthesis of compounds **6a-6c**.* Compounds **2** (1 equiv) and **5a-5c** (1.05 equiv) were dissolved in anhydrous DMF and stirred at 0 °C under argon atmosphere. First HOBt (1.3 equiv), than NMM (2.2 equiv) and after 10 minutes EDC × HCl (1.4 equiv) were added and the reaction mixture was stirred overnight while allowing to warm up to the room temperature. Most of the DMF was evaporated under reduced pressure and to the residue EtOAc was added and extracted three times with distilled water, three times with saturated $NaHCO_3$(aq) and once with saturated NaCl(aq). The organic phase was dried over

sodium sulphate, filtered and the solvent evaporated under reduced pressure. The compounds were purified by column chromatography.

*4.1.5.1. N-(3-(Phenylamino)propyl)-6-(1H-pyrazol-1-yl)nicotinamide (**6a**).* Compound **6a** was prepared according to general procedure **C** from **2** (0.23 g, 1.19 mmol) and **5a** (0.19 g, 1.25 mmol). Column chromatography (EtOAc/hexane: 1/1) was used to afford the compound **6a** (0.18 g, 47%) as white solid. Melting point = 135-138°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.79-1.88 (m, 2H, $CH_2C\underline{H_2}CH_2$), 3.09 (q, *J* = 6.7 Hz, 2H, CO-NH-$C\underline{H_2}CH_2CH_2$), 3.38-3.44 (m, 2H, CO-NH-$CH_2CH_2C\underline{H_2}$), 5.59 (t, *J* = 5.7 Hz, 1H, Ar-N<u>H</u>), 6.49-6.54 (m, 1H, Ar-<u>H</u>), 6.56-6.59 (m, 2H, Ar-<u>H</u>), 6.63 (dd, J_1 = 2.7 Hz, J_2 =1.7 Hz, 1H, Ar-<u>H</u>), 7.04-7.10 (m, 2H, Ar-<u>H</u>), 7.89 (dd, J_1 = 1.7 Hz, J_2 =0.8 Hz, 1H, Ar-<u>H</u>), 8.01 (dd, J_1 = 8.6 Hz, J_2 =0.8 Hz, 1H, Ar-<u>H</u>), 8.40 (dd, J_1 = 8.6 Hz, J_2 =2.3 Hz, 1H, Ar-<u>H</u>), 8.69 (dd, J_1 = 2.7 Hz, J_2 =0.8 Hz, 1H, Ar-<u>H</u>), 8.75 (t, *J* = 5.5 Hz, 1H, Ar-CO-N<u>H</u>), 8.90 (dd, J_1 = 2.3 Hz, J_2 =0.8 Hz, 1H, Ar-<u>H</u>). ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm) = 28.7 ($CH_2\underline{C}H_2CH_2$), 38.2 (CO-NH-$\underline{C}H_2CH_2CH_2$), 42.7 (CO-NH-$CH_2CH_2\underline{C}H_2$), 108.5 (Ar-<u>C</u>), 111.9 (2×Ar-<u>C</u>), 114.1 (2×Ar-<u>C</u>), 119.1 (Ar-<u>C</u>), 127.4 (Ar-<u>C</u>), 127.6 (Ar-<u>C</u>), 129.5 (2×Ar-<u>C</u>), 137.6 (Ar-<u>C</u>), 142.9 (Ar-<u>C</u>), 147.3 (Ar-<u>C</u>), 153.2 (Ar-<u>C</u>), 165.3 (Ar-<u>C</u>O). MS (ES+) m/z = 322.2 (MH+). HRMS (ES+) m/z for $C_{18}H_{19}N_5O$ calculated: 322.1668, found: 322.1661.

*4.1.5.2. N-(3-((4-Chlorophenyl)amino)propyl)-4-(1H-pyrazol-1-yl)benzamide (**6b**).* Compound **6b** was prepared according to general procedure **C** from **2** (0.05 g, 0.26 mmol) and **5b** (0.05 g, 0.28 mmol). Column chromatography (EtOAc/hexane: 1/1) was used to afford the compound **6b** (0.06 g, 59%) as white solid. Melting point = 170-173°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.78-1.86 (m, 2H, $CH_2C\underline{H_2}CH_2$), 3.07 (q, *J* = 4.9 Hz, 2H,CO-NH-$C\underline{H_2}CH_2CH_2$), 3.39 (t, *J* = 4.9 Hz, 2H, CO-NH-$CH_2CH_2C\underline{H_2}$), 5.84 (t, *J* = 5.7 Hz, 1H, Ar-N<u>H</u>),

6.56-6.60 (m, 2H, Ar-H), 6.63 (dd, J_1 = 2.6 Hz, J_2 =1.7, Hz, 2H, Ar-H), 7.07-7.11 (m, 2H, Ar-H), 7.89 (dd, J_1 = 1.7 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.01 (dd, J_1 = 8.6 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.39 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, 1H, Ar-H), 8.69 (dd, J_1 = 2.6 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.74 (t, J = 5.6 Hz, 1H, Ar-CO-NH), 8.90 (dd, J_1 = 2.3 Hz, J_2 = 0.8 Hz, 1H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm) = 28.8 ($CH_2CH_2CH_2$), 37.9 (COO-NH-$CH_2CH_2CH_2$ and COO-NH-$CH_2CH_2CH_2$), 108.5 (Ar-C), 111.9 (2×Ar-C), 114.8 (3×Ar-C), 127.3 (Ar-C), 127.6 (Ar-C), 129.3 (2×Ar-C), 137.6 (Ar-C), 142.9 (Ar-C), 147.1 (Ar-C), 153.2 (Ar-C), 165.4 (Ar-CO). MS (ES+) m/z = 356.1 (MH+). HRMS (ES+) m/z for $C_{19}H_{19}ClN_5O$ calculated: 356.1278, found: 356.1273.

*4.1.5.3. N-(3-((4-Methoxyphenyl)amino)propyl)-4-(1H-pyrazol-1-yl)benzamide (**6c**).* Compound **6c** was prepared according to general procedure **C** from **2** (0.23 g, 1.23 mmol) and **5c** (0.23 g, 1.29 mmol)**.** Column chromatography (EtOAc/hexane: 2/1) was used to afford the compound **6c** (0.30 g, 69%) as beige solid. Melting point = 140-143°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.82 (p, J = 6.8 Hz, 2H, $CH_2CH_2CH_2$), 3.04 (q, J = 6.8 Hz, 2H, CO-NH-$CH_2CH_2CH_2$), 3.40 (q, J = 6.8 Hz, 2H, CO-NH-$CH_2CH_2CH_2$), 3.63 (s, 3H, Ar-OCH_3), 5.12-5.20 (m, 1H, Ar-NH), 6.51-6.57 (m, 2H, Ar-H), 6.63 (dd, J_1 = 2.7 Hz, J_2 = 1.7, Hz, 1H, Ar-H), 6.69-6.75 (m, 2H, Ar-H), 7.89 (dd, J_1 = 1.7 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.01 (dd, J_1 = 8.6 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.40 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, 1H, Ar-H), 8.69 (dd, J_1 = 2.7 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.74 (t, J = 5.5 Hz, 1H, Ar-CO-NH), 8.91 (dd, J_1 = 2.3 Hz, J_2 = 0.8 Hz, 1H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 28.7 ($CH_2CH_2CH_2$), 37.4 (CO-NH-$CH_2CH_2CH_2$), 41.3 (CO-NH-$CH_2CH_2CH_2$), 55.3 (OCH_3), 108.8 (Ar-C), 111.3 (Ar-C), 113.1 (2×Ar-C), 114.6 (2×Ar-C), 127.5 (Ar-C), 127.9 (Ar-C), 138.5 (Ar-C), 142.9 (Ar-C), 143.2 (Ar-C), 147.7 (Ar-C),

150.6 (Ar-C), 152.1 (Ar-C), 163.9 (Ar-CO). MS (ES+) m/z = 352.2 (MH+). HRMS (ES+) m/z for $C_{19}H_{21}N_5O_2$ calculated: 352.1774, found: 352.1777.

*4.1.6. General procedure **D**. Synthesis of compounds **7a-7g**.* Compound **6a** (0.15 g, 0.47 mmol, 1 equiv), BTEAC (0.01 g, 0.05 mmol, 0.1 equiv), KI (0.01 g, 0.05 mmol, 0.1 equiv) and K_2CO_3 (0.39 g, 2.80 mmol, 6 equiv) were dissolved in acetonitrile (10 mL). Various reagents (benzyl bromide (4 equiv), allyl bromide (4 equiv), 3,4-difluorobenzyl bromide (4 equiv), 3,4-dichlorobenzyl bromide (2 equiv), 4-chlorobenzyl chloride (2 equiv), ethyl 2-bromoacetate (3 equiv) and 2-bromoethyl benzene (2 equiv)) were added and stirred under argon atmosphere at 60 °C overnight. The reaction mixture was filtered to remove K_2CO_3, the solvent was evaporated under reduced pressure and the compounds were purified by column chromatography.

*4.1.6.1. N-(3-(Benzyl(phenyl)amino)propyl)-6-(1H-pyrazol-1-yl)nicotinamide (**7a**).* Compound **7a** was prepared according to general procedure **D** from **6a** and benzyl bromide (0.22 mL, 1.87 mmol, 4 equiv)**.** Column chromatography (EtOAc/hexane: 1/1) was used to afford the compound **7a** (0.18 g, 92%) as yellow-green solid. Melting point = 121-124°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.84-1.94 (m, 2H, $CH_2C\underline{H}_2CH_2$), 3.38-3.42 (m, 2H, CO-NH-$C\underline{H}_2CH_2CH_2$), 3.47-3.54 (m, 2H, CO-NH-$CH_2CH_2C\underline{H}_2$), 4.58 (s, 2H, Ar-$C\underline{H}_2$), 6.55-6.59 (m, 1H, Ar-H), 6.63 (dd, J_1 = 2.7 Hz, J_2 = 1.7 Hz, 1H, Ar-H), 6.65-6.70 (m, 2H, Ar-H), 7.06-7.14 (m, 2H, Ar-H), 7.18-7.26 (m, 3H, Ar-H), 7.26-7.34 (m, 3H, Ar-H), 7.89 (dd, J_1 = 1.7 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.01 (dd, J_1 = 8.6 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.39 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, 1H, Ar-H), 8.69 (dd, J_1 = 2.7 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.76 (t, J = 5.6 Hz, 1H, Ar-CO-NH), 8.90 (dd, J_1 = 2.3 Hz, J_2 = 0.8 Hz, 1H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 26.8 ($CH_2\underline{C}H_2CH_2$), 37.2 (CO-NH-$\underline{C}H_2CH_2CH_2$), 48.6 (CO-NH-$CH_2CH_2\underline{C}H_2$), 53.6 (Ar-$\underline{C}H_2$), 108.8

(Ar-C), 111.3 (Ar-C), 111.9 (2×Ar-C), 115.5 (Ar-C), 126.4 (2×Ar-C), 126.6 (Ar-C), 127.5 (Ar-C), 127.5 (Ar-C), 128.0 (Ar-C), 128.4 (2×Ar-C), 129.0 (2×Ar-C), 138.5 (Ar-C), 139.2 (Ar-C), 142.9 (Ar-C), 147.7 (Ar-C), 147.8 (Ar-C), 152.1 (Ar-C), 164.0 (Ar-CO). MS (ES+) m/z = 412.2 (MH+). HRMS (ES+) m/z for $C_{25}H_{25}N_5O$ calculated: 412.2137, found: 412.2126.

*4.1.6.2. N-(3-(Allyl(phenyl)amino)propyl)-6-(1H-pyrazol-1-yl)nicotinamide (**7b**).* Compound **7b** was prepared according to general procedure **D** from **6a** and allyl bromide (0.16 mL, 1.89 mmol, 4 equiv)**.** Column chromatography (EtOAc/hexane: 1/1) was used to afford the compound **7b** (0.16 g, 89%) as light-pink solid. Melting point = 111-112°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.78-1.89 (m, 2H, $CH_2CH_2CH_2$), 3.36 (s, 4H, CO-NH-$CH_2CH_2CH_2$ and CO-NH-$CH_2CH_2CH_2$)*, 3.93-9.95 (m, 2H, N-CH_2-$CH=CH_2$), 5.07-5.15 (m, 2H, N-CH_2-$CH=CH_2$), 5.77-5.88 (m, 1H, N-CH_2-$CH=CH_2$), 6.55-6.59 (m, 1H, Ar-H), 6.63 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-H), 6.65-6.71 (m, 2H, Ar-H), 7.08-7.16 (m, 2H, Ar-H), 7.89 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 8.02 (dd, J_1 = 8.6 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.40 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, 1H, Ar-H), 8.69 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 8.76 (t, J = 5.5 Hz, 1H, Ar-CO-NH), 8.91 (dd, J_1 = 2.3 Hz, J_2 = 0.8 Hz, 1H, Ar-H). *Signal partially overlaps with that of H_2O in DMSO. ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm) = 27.1 ($CH_2CH_2CH_2$), 38.6 (CO-NH-$CH_2CH_2CH_2$), 48.8 (CO-NH-$CH_2CH_2CH_2$), 54.1 (N-CH_2-$CH=CH_2$), 108.5 (Ar-C), 111.8 (Ar-C), 117.7 (N-CH_2-$CH=CH_2$), 113.2 (3×Ar-C), 127.6 (Ar-C), 129.5 (3×Ar-C), 137.6 (Ar-C), 133.8 (N-CH_2-$CH=CH_2$), 142.9 (Ar-C), 147.2 (2×Ar-C), 153.1 (Ar-C), 165.0 (Ar-CO). MS (ES+) m/z = 362.2 (MH+). HRMS (ES+) m/z for $C_{21}H_{23}N_5O$ calculated: 362.1981, found: 362.1980.

*4.1.6.3. N-(3-((3,4-Difluorobenzyl)(phenyl)amino)propyl)-6-(1H-pyrazol-1-yl)nicotinamide (**7c**).* Compound **7c** was prepared according to general procedure **D** from **6a** and

3,4-difluorobenzyl bromide (0.24 mL, 1.87 mmol, 4 equiv)**.** Column chromatography (DCM/methanol: 20/1) was used to afford the compound **7c** (0.14 g, 66%) as white solid. Melting point = 123-124°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.88 (p, *J* = 7.3 Hz, 2H, $CH_2C\underline{H_2}CH_2$), 3.36-3.41 (m, 2H, CO-NH-$C\underline{H_2}CH_2CH_2$), 3.51 (t, *J* = 7.3 Hz, 2H, CO-NH-$CH_2CH_2C\underline{H_2}$), 4.56 (s, 2H, Ar-$C\underline{H_2}$), 6.57-6.62 (m, 1H, Ar-<u>H</u>), 6.63 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-<u>H</u>), 6.65-6.71 (m, 2H, Ar-<u>H</u>), 7.02-7.08 (m, 1H, Ar-<u>H</u>), 7.08-7.15 (m, 2H, Ar-<u>H</u>), 7.20-7.25 (m, 1H, Ar-<u>H</u>), 7.33-7.40 (m, 1H, Ar-<u>H</u>), 7.89 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-<u>H</u>), 8.01 (dd, J_1 = 8.6 Hz, J_2 = 0.8 Hz, 1H, Ar-<u>H</u>), 8.38 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, 1H, Ar-<u>H</u>), 8.69 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-<u>H</u>), 8.75 (t, *J* = 5.5 Hz, 1H, Ar-CO-N<u>H</u>), 8.90 (dd, J_1 = 2.3 Hz, J_2 = 0.8 Hz, 1H, Ar-<u>H</u>). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 26.8 ($CH_2\underline{C}H_2CH_2$), 37.1 (CO-NH-$\underline{C}H_2CH_2CH_2$), 48.6 (CO-NH-$CH_2CH_2\underline{C}H_2$), 52.8 (Ar-$\underline{C}H_2$), 108.8 (Ar-<u>C</u>), 111.3 (Ar-<u>C</u>), 112.1 (2×Ar-<u>C</u>), 115.3 (Ar-<u>C</u>), 115.9 (Ar-<u>C</u>), 117.4 (Ar-<u>C</u>), 123.0 (Ar-<u>C</u>), 127.5 (2×Ar-<u>C</u>), 128.0 (Ar-<u>C</u>), 129.1 (2×Ar-<u>C</u>), 137.9 (2×Ar-<u>C</u>), 142.9 (Ar-<u>C</u>), 148.1 (Ar-C-F), 148.9 (2×Ar-<u>C</u>), 149.4 (Ar-C-F), 164.0 (Ar-<u>C</u>O). MS (ES+) m/z = 448.2 (MH+). HRMS (ES+) m/z for $C_{25}H_{23}F_2N_5O$ calculated: 448.1949, found: 448.1953.

*4.1.6.4. N-(3-((3,4-Dichlorobenzyl)(phenyl)amino)propyl)-6-(1H-pyrazol-1-yl)nicotinamide (**7d**).* Compound **7d** was prepared according to general procedure **D** from **6a** and 3,4-dichlorobenzyl bromide (0.22 g, 0.93 mmol, 2 equiv)**.** Column chromatography (EtOAc/hexane: 1/1) was used to afford the compound **7d** (0.11 g, 46%) as yellow-green solid. Melting point = 149-152°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.88 (p, *J* = 7.3 Hz, 2H, $CH_2C\underline{H_2}CH_2$), 3.38-3.42 (m, 2H, CO-NH-$C\underline{H_2}CH_2CH_2$), 3.52 (t, *J* = 7.3 Hz, 2H, CO-NH-$CH_2CH_2C\underline{H_2}$), 4.59 (s, 2H, Ar-$C\underline{H_2}$), 6.58-6.60 (m, 1H, Ar-<u>H</u>), 6.63 (dd, J_1 = 2.7 Hz, J_2 = 1.7 Hz,

1H, Ar-H), 6.65-6.71 (m, 2H, Ar-H), 7.09-7.16 (m, 2H, Ar-H), 7.19 (dd, J_1 = 8.3 Hz, J_2 = 2.0 Hz, 1H, Ar-H), 7.44 (d, J = 2.0 Hz, 1H, Ar-H), 7.57 (d, J = 8.3 Hz, 1H, Ar-H), 7.89 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 8.01 (dd, J_1 = 8.6 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.38 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, 1H, Ar-H), 8.69 (dd, J_1 = 2.7 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 8.75 (t, J = 5.5 Hz, 1H, Ar-CO-NH), 8.89 (dd, J_1 = 2.3 Hz, J_2 = 0.8 Hz, 1H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm) = 27.3 ($CH_2CH_2CH_2$), 38.2 (CO-NH-$CH_2CH_2CH_2$), 48.6 (CO-NH-$CH_2CH_2CH_2$), 53.9 (Ar-CH_2), 108.5 (Ar-C), 111.9 (Ar-C), 113.3 (2×Ar-C), 117.8 (Ar-C), 126.1 (Ar-C), 127.6 (2×Ar-C), 128.6 (Ar-C), 129.6 (2×Ar-C), 130.7 (2×Ar-C), 132.8 (Ar-C), 137.6 (2×Ar-C), 142.9 (2×Ar-C), 147.1 (Ar-C), 147.9 (Ar-C). Ar-CO signal was not detected. MS (ES+) m/z = 480.1 (MH+). HRMS (ES+) m/z for $C_{25}H_{23}Cl_2N_5O$ calculated: 480.1358, found: 480.1349.

*4.1.6.5. N-(2-((4-Chlorobenzyl)(phenyl)amino)ethyl)-6-(1H-pyrazol-1-yl)nicotinamide (**7e**).* Compound **7e** was prepared according to general procedure **D** from **6a** and 4-chlorobenzyl chloride (0.15 g, 0.93 mmol, 2 equiv)**.** Column chromatography (EtOAc/hexane: 1/1) was used to afford the compound **7e** (0.11 g, 52%) as white solid. Melting point = 80-84°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.87 (q, J = 7.4 Hz, 2H, $CH_2CH_2CH_2$), 3.36-3.41 (m, 2H, CO-NH-$CH_2CH_2CH_2$), 3.50 (t, J = 7.4 Hz, 2H, CO-NH-$CH_2CH_2CH_2$), 4.57 (s, 2H, Ar-CH_2), 6.56-6.60 (m, 1H, Ar-H), 6.63 (dd, J_1 = 2.6 Hz, J_2 = 1.6 Hz, 1H, Ar-H), 6.64-6.69 (m, 2H, Ar-H), 7.07-7.14 (m, 2H, Ar-H), 7.19-7.25 (m, 2H, Ar-H), 7.33-7.39 (m, 2H, Ar-H), 7.89 (dd, J_1 = 1.6 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 8.01 (dd, J_1 = 8.6 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.38 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, 1H, Ar-H), 8.69 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 8.75 (t, J = 5.5 Hz, 1H, Ar-CO-NH), 8.89 (dd, J_1 = 2.3 Hz, J_2 = 0.8 Hz, 1H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm) = 27.2 ($CH_2CH_2CH_2$), 38.3 (CO-NH-$CH_2CH_2CH_2$), 40.9 (CO-NH-$CH_2CH_2CH_2$), 48.8 (Ar-CH_2),

108.5 (Ar-C), 111.8 (Ar-C), 113.4 (2×Ar-C), 117.6 (Ar-C), 127.6 (2×Ar-C), 128.2 (2×Ar-C), 128.8 (2×Ar-C), 129.5 (2×Ar-C), 137.5 (Ar-C), 142.9 (Ar-C), 147.2 (Ar-C), 148.2 (Ar-C). Three Ar-C and Ar-CO signals were not detected. MS (ES+) m/z = 446.2 (MH+). HRMS (ES+) m/z for $C_{25}H_{24}ClN_5O$ calculated: 446.1748, found: 446.1759.

*4.1.6.6. Ethyl 2-((3-(6-(1H-pyrazol-1-yl)nicotinamido)propyl)(phenyl)amino)acetate (**7f**).* Compound **7f** was prepared according to general procedure **D** from **6a** and ethyl 2-bromoacetate (0.16 mL, 1.40 mmol, 3 equiv)**.** Column chromatography (EtOAc/hexane: 1/1) was used to afford the compound **7f** (0.35 g, 91%) as white solid. Melting point = 123-126°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.19 (t, *J* = 7.1 Hz, 3H, CO-CH_2-CH_3), 1.86 (p, *J* = 7.2 Hz, 2H, $CH_2CH_2CH_2$), 3.37-3.40 (m, 2H, CO-NH-$CH_2CH_2CH_2$), 3.44 (t, *J* = 7.2 Hz, 2H, CO-NH-$CH_2CH_2CH_2$), 4.11 (q, *J* = 7.1 Hz, 2H, COO-CH_2-CH_3), 4.18 (s, 2H, Ar-CH_2), 6.58-6.65 (m, 4H, Ar-H), 7.11-7.18 (m, 2H, Ar-H), 7.89 (dd, J_1 = 1.6 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 8.02 (dd, J^1 = 8.6 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.39 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, 1H, Ar-H), 8.69 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 8.75 (t, *J* = 5.5 Hz, 1H, Ar-CO-NH), 8.91 (dd, J_1 = 2.3 Hz, J_2 = 0.8 Hz, 1H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm) = 14.2 (CH_2CH_3), 27.0 ($CH_2CH_2CH_2$), 39.0 (CO-NH-$CH_2CH_2CH_2$), 51.1 (CO-NH-$CH_2CH_2CH_2$), 55.6 (CH_3CH_2), 61.7 (COO-CH_2), 108.5 (Ar-C), 111.4 (2×Ar-C), 113.6 (Ar-C), 118.7 (Ar-C), 127.5 (Ar-C), 127.8 (Ar-C), 129.4 (2×Ar-C), 137.6 (Ar-C), 142.6 (Ar-C), 147.6 (2×Ar-C), 152.7 (Ar-C), 165.1 (Ar-C), 172.6 (COO). MS (ES+) m/z = 408.2 (MH+). HRMS (ES+) m/z for $C_{22}H_{25}N_5O_3$ calculated: 408.2036, found: 408.2031.

*4.1.6.7. N-(3-(Phenethyl(phenyl)amino)propyl)-6-(1H-pyrazol-1-yl)nicotinamide (**7g**).* Compound **7g** was prepared according to general procedure **D** from **6a** and 2-bromoethyl

benzene (0.13 mL, 0.93 mmol, 2 equiv)**.** Column chromatography (EtOAc/hexane: 1/1) was used to afford the compound **7g** (0.14 g, 70%) as yellow-brown solid. Melting point = 95-98°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.81 (p, *J* = 7.1 Hz, 2H, C$\underline{H_2}$CH$_2$CH$_2$), 2.77-2.83 (m, 2H, Ar-C$\underline{H_2}$-CH$_2$), 3.31-3.35 (m, 4H, CO-NH-C$\underline{H_2}$CH$_2$CH$_2$ and Ar-CH$_2$-C$\underline{H_2}$), 3.47-3.54 (m, 2H, CO-NH-CH$_2$CH$_2$C$\underline{H_2}$), 6.57-6.61 (m, 1H, Ar-$\underline{H}$), 6.63 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, Ar-$\underline{H}$), 6.72-6.76 (m, 2H, Ar-$\underline{H}$), 7.14-7.23 (m, 4H, Ar-$\underline{H}$), 7.25-7.32 (m, 5H, Ar-$\underline{H}$), 7.90 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-$\underline{H}$), 8.02 (dd, J_1 = 8.6 Hz, J_2 = 0.8 Hz, 1H, Ar-$\underline{H}$), 8.39 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, 1H, Ar-$\underline{H}$), 8.70 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-$\underline{H}$), 8.75 (t, *J* = 5.5 Hz, 1H, Ar-CO-N$\underline{H}$), 8.91 (dd, J_1 = 2.3 Hz, J_2 = 0.8 Hz, 1H, Ar-$\underline{H}$). ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm) = 27.2 (CH$_2$$\underline{C}H_2CH_2$), 33.2 (Ar-$\underline{C}H_2CH_2$), 38.6 (CO-NH-$\underline{C}H_2CH_2CH_2$), 49.4 (CO-NH-CH$_2CH_2$$\underline{C}H_2$), 53.7 (Ar-CH$_2$$\underline{C}H_2$), 108.4 (Ar-$\underline{C}$), 111.8 (2×Ar-$\underline{C}$), 113.1 (Ar-$\underline{C}$), 117.1 (Ar-$\underline{C}$), 126.3 (Ar-$\underline{C}$), 127.6 (Ar-$\underline{C}$), 128.6 (2×Ar-$\underline{C}$), 128.8 (3×Ar-$\underline{C}$), 129.1 (Ar-$\underline{C}$), 129.7 (2×Ar-$\underline{C}$), 137.5 (Ar-$\underline{C}$), 142.9 (Ar-$\underline{C}$), 147.2 (Ar-$\underline{C}$), 147.5 (Ar-$\underline{C}$), 153.1 (Ar-$\underline{C}$), 164.9 (Ar-$\underline{C}$O). MS (ES+) m/z = 426.2 (MH+). HRMS (ES+) m/z for $C_{26}H_{27}N_5O$ calculated: 426.2294, found: 426.2288.

*4.1.6.8. N-(3-(Ethyl(phenyl)amino)propyl)-6-(1H-pyrazol-1-yl)nicotinamide (**7h**).* In a 10 mL vial compound **6a** (0.15 g, 0.47 mmol, 1 equiv), K_2CO_3 (0.19 g, 1.40 mmol, 3 equiv), BTEAC (0.01 g, 0.05 mmol, 0.1 equiv), and bromoethane (0.11 mL, 1.40 mmol, 3 equiv) were dissolved in AcCN (3 mL). The reaction was carried out in microwave reactor under the following conditions: 100°C, 15 bar for 20 minutes. Because there was still a lot of compound **6a**, bromoethane (0.35 mL, 4.67 mmol, 10 equiv) and BTEAC (0.01 g, 0.05 mmol, 0.1 equiv) were added and the reaction was carried out again in microwave reactor for 20 minutes under the

same conditions. After that the reaction mixture was filtered to remove K_2CO_3, the solvent was evaporated under reduced pressure and the compound was purified by column chromatography (EtOAc/hexane: 1/1) as white solid (0.10 g, 60%). Melting point = 105-106°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.08 (t, *J* = 7.0 Hz, 3H, $CH_2C\underline{H}_3$), 1.77-1.86 (m, 2H, CO-NH-$CH_2C\underline{H}_2CH_2$), 2.30-2.47(m, 2H, CO-NH-$C\underline{H}_2CH_2CH_2$), 3.35-3.41 (m, 4H, $C\underline{H}_2CH_3$ and CO-NH-$CH_2CH_2C\underline{H}_2$), 6.53-6.58 (m, 2H, Ar-H), 6.63 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-H), 6.67 (dd, J_1 = 8.8 Hz, J_2 = 0.8 Hz, 2H, Ar-H), 7.09-7.16 (m, 2H, Ar-H), 7.90 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 8.02 (dd, J_1 = 8.6 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.40 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, 1H, Ar-H), 8.69 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 8.76 (t, *J* = 5.4 Hz, 1H, Ar-CO-NH), 8.91 (dd, J_1 = 2.3 Hz, J_2 = 0.8 Hz, 1H, Ar-H). ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm) = 11.9 ($\underline{C}H_3CH_2$), 27.1 ($CH_2\underline{C}H_2CH_2$), 38.9 (CO-NH-$\underline{C}H_2CH_2CH_2$), 45.9 ($CH_3\underline{C}H_2$), 48.8 (CO-NH-$CH_2CH_2\underline{C}H_2$), 108.4 (Ar-C), 111.8 (2×Ar-C), 113.5 (Ar-C), 117.2 (Ar-C), 127.6 (2×Ar-C), 129.6 (2×Ar-C), 137.5 (Ar-C), 142.9 (2×Ar-C), 147.3 (Ar-C), 153.1 (Ar-C), 164.9 (Ar-CO). MS (ES+) m/z = 350.2 (MH+). HRMS (ES+) m/z for $C_{20}H_{23}N_5O$ calculated: 350.1981, found: 350.1982.

*4.1.6.9. 2-((3-(6-(1H-Pyrazol-1-yl)nicotinamido)propyl)(phenily)amino) acetic acid (**7i**).* Compound **7f** (0.18 g, 0.43 mmol, 1 equiv) was dissolved in ethanol (5 mL), 3 M $NaOH_{(aq)}$ (0.43 mL, 1.29 mmol, 3 equiv) was added, and the mixture stirred for 2 hours at room temperature. Ethanol was evaporated under reduced pressure; 20 mL of distillated water was added to the residue and extracted with 10 mL of diethyl ether two times. pH of the water phase was adjusted with 2M HCl to 3 to obtain white precipitate. The precipitate was filtered with vacuum and dried at room temperature to afford white-gray solid (0.14 g, 84%). Melting point = 127-130°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.86 (p, *J* = 7.2 Hz, 2H, $CH_2C\underline{H}_2CH_2$), 3.36-6.41 (m, 2H,

CO-NH-C$\underline{H_2}$CH$_2$CH$_2$), 3.44 (t, *J* = 7.2 Hz, 2H, CO-NH-CH$_2$CH$_2$C$\underline{H_2}$), 4.08 (s, 2H, C$\underline{H_2}$-COOH), 6.60 (m, 3H, Ar-$\underline{H}$), 6.63 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-$\underline{H}$), 7.11-7.17 (m, 2H, Ar-$\underline{H}$), 7.90 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-$\underline{H}$), 8.01 (dd, J_1 = 8.6 Hz, J_2 = 0.8 Hz, 1H, Ar-$\underline{H}$), 8.39 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, 1H, Ar-$\underline{H}$), 8.69 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-$\underline{H}$), 8.76 (t, *J* = 5.5 Hz, 1H, Ar-CO-N$\underline{H}$), 8.90 (dd, J_1 = 2.3 Hz, J_2 = 0.8 Hz, 1H, Ar-$\underline{H}$), 12.59 (s, 1H, COO$\underline{H}$). ^{13}C NMR (100 MHz, DMSO-d$_6$) δ(ppm) = 27.0 (CH$_2$$\underline{C}H_2CH_2$), 37.2 (CO-NH-$\underline{C}H_2CH_2CH_2$), 49.0 (CO-NH-CH$_2CH_2$$\underline{C}H_2$), 52.1 ($\underline{C}H_2$-COOH) 108.8 (Ar-$\underline{C}$), 111.3 (Ar-$\underline{C}$), 111.4 (2×Ar-$\underline{C}$), 115.7 (Ar-$\underline{C}$), 127.5 (Ar-$\underline{C}$), 128.0 (Ar-$\underline{C}$), 129.0 (2×Ar-$\underline{C}$), 138.5 (Ar-$\underline{C}$), 142.9 (Ar-$\underline{C}$), 147.7 (Ar-$\underline{C}$), 147.8 (Ar-$\underline{C}$), 152.1 (Ar-$\underline{C}$), 164.0 (Ar-$\underline{C}$O), 172.4 ($\underline{C}$OOH). MS (ES+) m/z = 378.2 (MH-). HRMS (ES+) m/z for $C_{20}H_{21}N_5O_3$ calculated: 378.1566, found: 378.1560.

*4.1.7. General procedure **E**. Synthesis of compounds **8**, **10** and **12**.* Compound **2** (1 equiv) and TBTU (1.3 equiv) were added into equal mixture of DMF and DCM, and stirried under the argon atmosphere at room temperature. After few minutes, NMM (3 equiv) was added, leaving the reaction for 40 minutes. Different Boc protected amines (1-Boc piperazine, 4-Amino-1-Boc-piperidine and 4-(*N*-Boc-amino)piperidine, 1 equiv) were disolved in DCM and added dropwise. In the end NMM (1 equiv) was added and the reaction was left stirring overnight. The next day the reaction mixture was evaporated and diluted in EtOAc and then extracted three times with distilled water, three times with saturated $NaHCO_{3(aq)}$ and once with saturated $NaCl_{(aq)}$. The organic phase was dried over sodium sulphate, filtered and the solvent evaporated under reduced pressure. The compound was taken to the next step without further characterization. It was diluted in redistilled DCM. To that trifluoroacetic acid (10 equiv) was added and stirred at 40 °C under the argon atmosphere overnight. DCM was added and extracted with saturated $NaHCO_{3(aq)}$

three times. Because the substance was still in aqueous phase, it was extracted few times with DCM and EtOAc. Organic phase was then washed with saturated $NaCl_{(aq)}$, dried over sodium sulphate, filtered and evaporated under reduced pressure.

*4.1.7.1. (6-(1H-Pyrazol-1-yl)pyridin-3-yl)(piperazin-1-yl)methanone (**8**).* Compound **8** was prepared according to general procedure **E** from **2** (0.50 g, 2.64 mmol) and 1-Boc piperazine (0.49 g, 2.64 mmol) as white solid (0.49 g, 85%). Melting point = 122°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 2.70 (s, 4H, $N(CH_2C\underline{H_2})_2NH$), 3.34-3.58 (2×s, 5H, $N(C\underline{H_2}CH_2)_2NH$ and $N(CH_2CH_2)_2N\underline{H}$)), 6.62 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-H), 7.88 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 7.98 (dd, J_1 = 8.4 Hz, J_2 = 0.9, Hz, 1H, Ar-H), 8.03 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, 1H, Ar-H), 8.51 (dd, J_1 = 2.2 Hz, J_2 = 0.9 Hz, 1H, Ar-H), 8.66 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-H). MS (ES+) m/z = 258.1 (MH+). HRMS (ES+) m/z for $C_{13}H_{15}N_5O$ calculated: 258.1355, found: 258.1354.

*4.1.7.2. N-(Piperidin-4-yl)-6-(1H-pyrazol-1-yl)nicotinamide (**10**).* Compound **10** was prepared according to general procedure **E** from **2** (0.94 g, 4.99 mmol) and 4-amino-1-Boc-piperidine (1.00 g, 4.99 mmol) as white solid (1.16 g, 93.9%). Melting point = 195-198°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.47 (m, 2H, $NHCH(C\underline{H_2}CH_2)_2NH$), 1.72-1.88 (m, 2H, $NHCH(C\underline{H_2}CH_2)_2NH$), 2.58 (q, J_1 = 14.3 Hz, J_2 = 13.1 Hz, 2H, $NHCH(CH_2C\underline{H_2})_2NH$), 3.02 (d, J = 12.3 Hz, 2H, $NHCH(CH_2C\underline{H_2})_2NH$), 3.15-3.78 (m, 1H, $NHCH(CH_2CH_2)_2N\underline{H}$)*, 3.88 (s, 1H, $NHC\underline{H}(CH_2CH_2)_2NH$), 6.62 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-H), 7.89 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 7.99 (dd, J_1 = 8.6 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.40 (dd, J_1 = 8.6 Hz, J_2 = 2.4 Hz, 1H, Ar-H), 8.53 (d, J = 7.7 Hz, 1H, Ar-H), 8.68 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 8.89 (dd, J_1 = 2.4 Hz, J_2 = 0.8 Hz, 1H, Ar-H). *Signal partially overlaps with that of H_2O in

DMSO. MS (ES+) m/z = 272.1 (MH+). HRMS (ES+) m/z for $C_{14}H_{17}N_5O$ calculated: 272.1511, found: 272.1515.

*4.1.7.3. (6-(1H-Pyrazol-1-yl)pyridin-3-yl)(4-aminopiperidin-1-yl)methanone (**12**).* Compound **12** was prepared according to general procedure **E** from **2** (0.94 g, 4.99 mmol) and 4-(*N*-Boc-amino)piperidine (1.00 g, 4.99 mmol) as white solid (1.44 g, 100%).

*4.1.8. General procedure **F**. Synthesis of compounds **9a-9e**.* Compound **8** (1 equiv) and K_2CO_3 (2.5 equiv) were diluted in acetonitrile. To that various benzyl halydes (1.2 equiv) were added and stirred overnight at 55°C, equipped with chlorcalcium tube. K_2CO_3 was removed with filtration and the compounds were purified by column chromatography or recrystallization.

*4.1.8.1. (6-(1H-Pyrazol-1-yl)pyridin-3-yl)(4-(3,4-difluorobenzyl)piperazin-1-yl)methanone (**9a**).* Compound **9a** was prepared according to general procedure **F** from **8** (0.25 g, 0.97 mmol) and 3,4-difluorobenzyl bromide (0.15 mL, 1.17 mmol) and it was purified by recrystallization, using EtOAc and hexane to afford white-silver solid (0.25 g, 67%). Melting point = 122-124°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 2.36-2.47 (m , 4H, N($CH_2C\underline{H}_2$)$_2$N-CH_2), 3.41 (d, J = 13.3 Hz, 2H, N($C\underline{H}_2CH_2$)$_2$N-CH_2), 3.52 (s, 2H, N(CH_2CH_2)$_2$N-$C\underline{H}_2$), 3.66 (s, 2H, N-$C\underline{H}_2$-Ar), 6.62 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-$\underline{H}$), 7.16-7.20 (m, 1H, Ar-$\underline{H}$), 7.34-7.43 (m, 2H, Ar-$\underline{H}$), 7.88 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-$\underline{H}$), 7.97 (dd, J_1 = 8.5 Hz, J_2 = 0.9 Hz, 1H, Ar-$\underline{H}$), 8.03 (dd, J_1 = 8.5 Hz, J_2 = 2.3 Hz, 1H, Ar-$\underline{H}$), 8.51 (dd, J_1 = 2.3 Hz, J_2 = 0.9 Hz, 1H, Ar-$\underline{H}$), 8.65 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-$\underline{H}$). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 60.3 (N(CH_2CH_2)$_2$N-$\underline{C}H_2$), 108.7 (Ar-$\underline{C}$), 111.5 (Ar-$\underline{C}$), 117.3 (3×Ar-$\underline{C}$), 125.4 (Ar-$\underline{C}$), 127.3 (Ar-$\underline{C}$), 129.5 (Ar-$\underline{C}$), 135.8 (Ar-$\underline{C}$), 138.6 (Ar-$\underline{C}$), 142.8 (Ar-$\underline{C}$), 146.9 (Ar-$\underline{C}$), 147.3 (Ar-$\underline{C}$), 151.1 (Ar-$\underline{C}$), 166.1 (Ar-$\underline{C}$O). N($\underline{C}H_2CH_2$)$_2$N-CH_2 and N($CH_2\underline{C}H_2$)$_2$N-CH_2 signals were not

detected. MS (ES+) m/z = 384.2 (MH+). HRMS (ES+) m/z for $C_{20}H_{19}F_2N_5O$ calculated: 384.1636, found: 384.1639.

*4.1.8.2. (6-(1H-Pyrazol-1-yl)pyridin-3-yl)(4-(4-chlorobenzyl)piperazin-1-yl)methanone (**9b**)*. Compound **9b** was prepared according to general procedure **F** from **8** (0.20 g, 0.78 mmol) and 4-chlorobenzyl chloride (0.15 g, 0.93 mmol)**.** Column chromatography (EtOAc/hexane: 2/1) was used to afford the compound **9b** (0.24 g, 81%) as white solid. Melting point = 135-138°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 2.31-2.48 (m, 4H, N($CH_2C\underline{H_2}$)$_2$N-CH2), 3.42 and 3.64 (2×s, 4H, N($C\underline{H_2}CH_2$)$_2$N-CH2), 3.51 (s, 2H, N-C$\underline{H_2}$-Ar), 6.62 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-$\underline{H}$), 7.31-7.42 (m, 4H, Ar-$\underline{H}$), 7.88 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-$\underline{H}$), 7.97 (dd, J_1 = 8.5 Hz, J_2 = 0.9 Hz, 1H, Ar-$\underline{H}$), 8.03 (dd, J_1 = 8.5 Hz, J_2 = 2.2 Hz, 1H, Ar-$\underline{H}$), 8.51 (dd, J_1 = 2.2 Hz, J_2 = 0.9 Hz, 1H, Ar-$\underline{H}$), 8.65 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-$\underline{H}$). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 38.8-40.1 (N($\underline{C}H_2CH_2$)$_2$N-CH_2 and N($CH_2\underline{C}H_2$)$_2$N-CH_2)*, 60.8 (N(CH_2CH_2)$_2$N-$\underline{C}H_2$), 108.7 (Ar-$\underline{C}$), 111.5 (Ar-$\underline{C}$), 127.3 (Ar-$\underline{C}$), 128.2 (2×Ar-$\underline{C}$), 129.5 (Ar-$\underline{C}$), 130.6 (2×Ar-$\underline{C}$), 131.5 (Ar-$\underline{C}$), 136.9 (Ar-$\underline{C}$), 138.6 (Ar-$\underline{C}$), 142.8 (Ar-$\underline{C}$), 146.9 (Ar-$\underline{C}$), 151.1 (Ar-$\underline{C}$), 166.1 (Ar-$\underline{C}$O). N($\underline{C}H_2CH_2$)$_2$N-CH_2 and N($CH_2\underline{C}H_2$)$_2$N-CH_2 signals were not detected. *Signal partially overlaps with that of DMSO. MS (ES+) m/z = 382.1 (MH+). HRMS (ES+) m/z for $C_{20}H_{20}ClN_5O$ calculated: 382.1435, found: 382.1428.

*4.1.8.3. (6-(1H-Pyrazol-1-yl)pyridin-3-yl)(4-(3,4-dichlorobenzyl)piperazin-1-yl)methanone (**9c**)*. Compound **9c** was prepared according to general procedure **F** from **8** (0.20 g, 0.78 mmol) and 3,4-dichlorobenzyl bromide (0.19 g, 0.93 mmol)**.** Column chromatography (EtOAc/hexane: 2/1) was used to afford the compound **9c** (0.24 g, 73%) as white-yellow solid. Melting point = 129-132°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 2.32-2.48 (m, 4H,

N($CH_2C\underline{H_2}$)$_2$N-CH_2), 3.43 and 3.65 (2×s, 4H, N($C\underline{H_2}CH_2$)$_2$N-CH_2), 3.53 (s, 2H, N-$C\underline{H_2}$-Ar), 6.62 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-H), 7.33 (dd, J_1 = 8.2 Hz, J_2 = 2.0 Hz, 1H, Ar-H), 7.57-7.62 (m, 2H, Ar-H), 7.88 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 7.97 (dd, J_1 = 8.5 Hz, J_2 = 0.9 Hz, 1H, Ar-H), 8.03 (dd, J_1 = 8.5 Hz, J_2 = 2.2 Hz, 1H, Ar-H), 8.51 (dd, J_1 = 2.2 Hz, J_2 = 0.9 Hz, 1H, Ar-H), 8.65 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 41.7 and 47.3 (N($\underline{C}H_2CH_2$)$_2$N-CH_2), 52.0 and 52.5 (N($CH_2\underline{C}H_2$)$_2$N-CH_2), 60.1 (N(CH_2CH_2)$_2$N-$\underline{C}H_2$), 108.7 (Ar-C), 111.5 (Ar-C), 127.3 (Ar-C), 129.1 (Ar-C), 129.5 (Ar-C), 130.4 (2×Ar-C), 130.6 (2×Ar-C), 130.9 (Ar-C), 138.6 (Ar-C), 139.3 (Ar-C), 142.8 (Ar-C), 146.9 (Ar-C), 151.1 (Ar-C), 166.1 (Ar-CO). MS (ES+) m/z = 416.1 (MH+). HRMS (ES+) m/z for $C_{20}H_{19}Cl_2N_5O$ calculated: 416.1045, found: 416.1041.

*4.1.8.4. (6-(1H-Pyrazol-1-yl)pyridin-3-yl)(4-benzylpiperazin-1-yl)methanone (**9d**).* Compound **9d** was prepared according to general procedure **F** from **8** (0.12 g, 0.47 mmol) and Benzyl chloride (0.07 mL, 0.56 mmol)**.** Column chromatography (EtOAc/hexane: 2/1) was used to afford the compound **9d** (0.12 g, 73%) as white solid. Melting point = 108-110°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 2.41 (d, J = 14.6 Hz, 4H, N($CH_2C\underline{H_2}$)$_2$N-CH_2), 3.42 and 3.65 (2×s, 4H, N($C\underline{H_2}CH_2$)$_2$N-CH_2), 3.52 (s, 2H, N-$C\underline{H_2}$-Ar), 6.61 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-H), 7.23-7.29 (m, 1H, Ar-H), 7.29-7.37 (m, 4H, Ar-H), 7.87 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-H) 7.97 (dd, J_1 = 8.5 Hz, J_2 = 0.9 Hz, 1H, Ar-H), 8.03 (dd, J_1 = 8.5 Hz, J_2 = 2.2 Hz, 1H, Ar-H), 8.51 (dd, J_1 = 2.2 Hz, J_2 = 0.9 Hz, 1H, Ar-H), 8.65 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 41.7 and 47.3 (N($\underline{C}H_2CH_2$)$_2$N-CH_2), 52.1 and 52.1 (N($CH_2\underline{C}H_2$)$_2$N-CH_2), 61.8 (N(CH_2CH_2)$_2$N-$\underline{C}H_2$), 108.7 (Ar-C), 111.5 (Ar-C), 127.0 (Ar-C), 127.3 (Ar-C), 128.2 (2×Ar-C), 128.9 (2×Ar-C), 129.5 (Ar-C), 137.8 (Ar-C), 138.6 (Ar-C),

142.8 (Ar-C), 146.9 (Ar-C), 151.0 (Ar-C), 166.1 (Ar-CO). MS (ES+) m/z = 348.2 (MH+). HRMS (ES+) m/z for $C_{20}H_{21}N_5O$ calculated: 348.1824, found: 348.1818.

*4.1.8.5. (6-(1H-Pyrazol-1-yl)pyridin-3-yl)(4-(4-methoxybenzyl)piperazin-1-yl)methanone (**9e**).* Compound **9e** was prepared according to general procedure **F** from **8** (0.20 g, 0.78 mmol) and 4-methoxybenzyl chloride (0.27 g, 1.95 mmol)**.** Column chromatography (EtOAc/hexane: 2/1) was used to afford the compound **9e** (0.22 g, 76%) as yellow solid. Melting point = 104-105°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 2.30-2.46 (m, 4H, N(CH_2CH_2)$_2$N-CH_2), 3.40 and 3.63 (2×s, 4H, N(CH_2CH_2)$_2$N-CH_2), 3.44 (s, 2H, N-CH_2-Ar), 3.73 (s, 3H, Ar-OCH_3), 6.61 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-H), 6.86-6.91 (m, 2H, Ar-H), 7.19-7.25 (m, 2H, Ar-H), 7.87 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-H), 7.97 (dd, J_1 = 8.5 Hz, J_2 = 0.9 Hz, 1H, Ar-H), 8.02 (dd, J_1 = 8.5 Hz, J_2 = 2.2 Hz, 1H, Ar-H), 8.51 (dd, J_1 = 2.2 Hz, J_2 = 0.9 Hz, 1H, Ar-H), 8.65 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 55.0 (OCH_3), 61.2 (N(CH_2CH_2)$_2$N-CH_2), 108.7 (Ar-C), 111.5 (Ar-C), 113.6 (Ar-C), 127.3 (Ar-C), 129.5 (2×Ar-C), 129.5 (Ar-C), 130.1 (3×Ar-C), 138.6 (Ar-C), 142.8 (Ar-C), 146.9 (Ar-C), 151.0 (Ar-C), 158.3 (Ar-C), 166.1 (Ar-CO). N(CH_2CH_2)$_2$N-CH_2 and N(CH_2CH_2)$_2$N-CH_2 signals were not detected. MS (ES+) m/z = 378.2 (MH+). HRMS (ES+) m/z for $C_{21}H_{23}N_5O_2$ calculated: 378.1930, found: 378.1926.

*4.1.9. General procedure **G**. Synthesis of compounds **11a-11d**.* Compound **10** (0.20 g, 0.74 mmol, 1 equiv) and K_2CO_3 (0.26 g, 1.84 mmol, 2.5 equiv) were diluted in acetonitrile (15 mL). To that various benzyl bromides/chlorides (0.89 mmol, 1.2 equiv) were added and stirred overnight at 55 °C, equipped with chlorcalcium tube. Next day, after evaporating the solvent, 70 mL of DCM was added and extracted with 3 × 20 mL of distillated water. Organic phase was

dried over sodium sulphate, filtered and evaporated under reduced pressure. The compounds were purified by recrystallization, using ethanol.

*4.1.9.1. N-(1-(4-Chlorobenzyl)piperidin-4-yl)-6-(1H-pyrazol-1-yl)nicotinamide (**11a**).* Compound **11a** was prepared according to general procedure **G** from **10** and 4-chlorobenzyl chloride as white solid (0.27 g, 92%). Melting point = 235-139°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.53-1.63 (m, 2H, NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 1.79-1.84 (m, 2H, NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 2.01-2.08 (m, 2H, NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 2.75-2.86 (m, 2H, NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 3.47 (s, 2H, Ar-C$\underline{H_2}$-N), 3.77-3.81 (m, 1H, NHC$\underline{H}$(CH$_2$CH$_2$)$_2$N), 6.62 (dd, J_1 = 2.6 Hz, J_2 = 1.6 Hz, 1H, Ar-$\underline{H}$), 7.30-7.42 (m, 4H, Ar-$\underline{H}$), 7.89 (d, J = 1.6 Hz, 1H, Ar-$\underline{H}$), 7.99 (dd, J_1 = 8.6 Hz, J_2 = 0.8 Hz, 1H, Ar-$\underline{H}$), 8.39 (dd, J_1 = 8.6 Hz, J_2 = 2.4 Hz, 1H, Ar-$\underline{H}$), 8.49 (d, J = 7.6 Hz, 1H, Ar-CO-N$\underline{H}$), 8.66-8.70 (m, 1H, Ar-$\underline{H}$), 8.88 (dd, J_1 = 2.4 Hz, J_2 = 0.8 Hz, 1H, Ar-$\underline{H}$). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 31.4 (NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 47.0 (NHCH(CH$_2$CH$_2$)$_2$N), 52.1 (NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 61.1 (Ar-C$\underline{H_2}$), 108.8 (Ar-$\underline{C}$), 111.2 (Ar-$\underline{C}$), 127.5 (Ar-$\underline{C}$), 128.0 (Ar-$\underline{C}$), 128.1 (2×Ar-$\underline{C}$), 130.4 (2×Ar-$\underline{C}$), 131.3 (Ar-$\underline{C}$), 137.7 (Ar-$\underline{C}$), 138.6 (Ar-$\underline{C}$), 142.9 (Ar-$\underline{C}$), 147.8 (Ar-$\underline{C}$), 152.0 (Ar-$\underline{C}$), 163.3 (Ar-$\underline{C}$O). MS (ES+) m/z = 396.2 (MH+). HRMS (ES+) m/z for $C_{21}H_{22}ClN_5O$ calculated: 396.1591, found: 396.1602.

*4.1.9.2. N-(1-(3,4-Dichlorobenzyl)piperidin-4-yl)-6-(1H-pyrazol-1-yl)nicotinamide (**11b**).* Compound **11b** was prepared according to general procedure **G** from **10** and 3,4-dichlorobenzyl bromide as white solid (0.19 g, 59%). Melting point = 245-247°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.55-1.64 (m, 2H, NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 1.77-1.87 (m,2 H, NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 2.04-2.11 (m, 2H, NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 2.81 (d, J = 11.4 Hz, 2H, NHCH(CH$_2$C$\underline{H_2}$)$_2$N) 3.49 (s, 2H, Ar-C$\underline{H_2}$-N), 3.78-3.82 (m, 1H, NHC$\underline{H}$(CH$_2$CH$_2$)$_2$N), 6.62 (dd, J_1 = 2.7 Hz, J_2 = 1.7 Hz, 1H,

Ar-H), 7.32 (dd, J_1 = 8.2 Hz, J_2 = 2.0 Hz, 4H, Ar-H), 7.54-7.63 (m, 2H, Ar-H), 7.89 (d, J = 1.7 Hz, 1H, Ar-H), 8.00 (d, J = 8.6 Hz, 1H, Ar-H), 8.39 (dd, J_1 = 8.6 Hz, J_2 = 2.3 Hz, 1H, Ar-H), 8.49 (d, J = 7.6 Hz, 1H, Ar-CO-NH), 8.68 (d, J = 2.7 Hz, 1H, Ar-H), 8.88 (d, J = 2.3 Hz, 1H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 31.4 (NHCH(CH$_2$CH$_2$)$_2$N), 46.8 (NHCH(CH$_2$CH$_2$)$_2$N), 52.0 (NHCH(CH$_2$CH$_2$)$_2$N), 60.4 (Ar-CH$_2$), 103.6 (Ar-C), 108.8 (Ar-C), 111.2 (Ar-C), 127.5 (Ar-C), 128.0 (Ar-C), 128.9 (Ar-C), 129.2 (Ar-C), 130.3 (Ar-C), 130.8 (Ar-C) 138.6 (Ar-C), 140.2 (Ar-C), 142.9 (Ar-C), 147.8 (Ar-C), 152.0 (Ar-C), 163.3 (Ar-CO). MS (ES+) m/z = 430.1 (MH+). HRMS (ES+) m/z for $C_{21}H_{21}Cl_2N_5O$ calculated: 430.1201, found: 430.1209.

*4.1.9.3. N-(1-Benzylpiperidin-4-yl)-6-(1H-pyrazol-1-yl)nicotinamide (**11c**).* Compound **11c** was prepared according to general procedure **G** from **10** and benzyl chloride as white solid (0.16 g, 60%). Melting point = 218-222°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.57-1.64 (m, 2H, NHCH(CH$_2$CH$_2$)$_2$N), 1.80-1.83 (m, 2H, NHCH(CH$_2$CH$_2$)$_2$N), 1.99-2.09 (m, 2H, NHCH(CH$_2$CH$_2$)$_2$N), 2.84 (d, J = 11.6 Hz, 2H, NHCH(CH$_2$CH$_2$)$_2$N), 3.48 (s, 2H, Ar-CH$_2$-N), 3.75-3.85 (m, 1H, NHCH(CH$_2$CH$_2$)$_2$N), 6.63 (dd, J_1 = 2.7 Hz, J_2 = 1.7 Hz, 1H, Ar-H), 7.23-7.28 (m, 1H, Ar-H), 7.29-7.37 (m, 4H, Ar-H), 7.89 (dd, J_1 = 1.7 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.00 (dd, J_1 = 8.6 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.39 (dd, J_1 = 8.6 Hz, J_2 = 2.4 Hz, 1H, Ar-H), 8.50 (d, J = 7.6 Hz, 1H, Ar-CO-NH), 8.68 (dd, J_1 = 2.7 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 8.88 (dd, J_1 = 2.4 Hz, J_2 = 0.8 Hz, 1H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 31.5 (NHCH(CH$_2$CH$_2$)$_2$N), 47.0 (NHCH(CH$_2$CH$_2$)$_2$N), 52.1 (NHCH(CH$_2$CH$_2$)$_2$N), 62.1 (Ar-CH$_2$), 108.8 (Ar-C), 111.2 (Ar-C), 126.8 (Ar-C), 127.5 (Ar-C), 128.0 (Ar-C), 128.1 (2×Ar-C), 128.7 (2×Ar-C), 138.6 (Ar-C),

138.6 (Ar-C), 142.9 (Ar-C), 147.8 (Ar-C), 152.0 (Ar-C), 163.3 (Ar-CO). MS (ES+) m/z = 362.2 (MH+). HRMS (ES+) m/z for $C_{21}H_{23}N_5O$ calculated: 362.1981, found: 362.1986.

*4.1.9.4. N-(1-(4-Methoxybenzyl)piperidin-4-yl)-6-(1H-pyrazol-1-yl)nicotinamide (**11d**).* Compound **11d** was prepared according to general procedure **G** from **10** and 4-methoxybenzyl chloride as white-silver solid (0.16 g, 55%). Melting point = 207-211°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.42-2.20 (m, 6H, NHCH(CH_2CH_2)$_2$N and NHCH(CH_2CH_2)$_2$N), 2.93 (d, *J* = 84.5 Hz, 2H, NHCH(CH_2CH_2)$_2$N), 3.77 (s, 5H, Ar-CH_2-N and Ar-OCH_3), 4.20 (m, 1H, NHCH(CH_2CH_2)$_2$N), 6.63 (dd, J_1 = 2.7 Hz, J_2 = 1.6 Hz, 1H, Ar-H), 6.81-7.60 (m, 4H, Ar-H), 7.29-7.37 (m, 4H, Ar-H), 7.89 (d, *J* = 1.6 Hz, 1H, Ar-H), 8.00 (d, *J* = 8.6 Hz, 1H, Ar-H), 8.35-8.44 (m, 1H, Ar-CO-NH), 8.68 (d, *J* = 2.7 Hz, 1H, Ar-H), 8.90 (s, 1H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 55.1 (OCH_3), 108.8 (Ar-C), 111.2 (Ar-C), 113.9 (3×Ar-C), 127.5 (4×Ar-C), 138.7 (Ar-C), 142.9 (Ar-C), 147.9 (Ar-C), 152.1 (Ar-C). NHCH(CH_2CH_2)$_2$N, NHCH(CH_2CH_2)$_2$N, NHCH(CH_2CH_2)$_2$N, Ar-CH_2, Ar-C and Ar-CO signals were not detected. MS (ES+) m/z = 392.2 (MH+). HRMS (ES+) m/z for $C_{22}H_{25}N_5O_2$ calculated: 392.2087, found: 392.2082.

*4.1.9.5. N-(1-(3,4-Difluorobenzyl)piperidin-4-yl)-6-(1H-pyrazol-1-yl)nicotinamide (**11e**).* Compound **10** (0.20 g, 0.74 mmol, 1 equiv) was dissolved in 10 mL of anhydrous methanol, where acetic acid ≥ 99 % (0.04 mL, 0.74 mmol, 1 equiv) was added. After 15 minutes of stirring at room temperature under argon atmosphere, NaCNBH3 (0.10 g, 1.55 mmol, 2.1 equiv) was dissolved in 5 mL of methanol and added dropwise. After 10 minutes 3,4-difluorobenzaldehide (0.08 mL, 0.74 mmol, 1 equiv) was added and the reaction was left stirring overnight. 20 mL of DCM and 10 mL of saturated $NaHCO_3$(aq) was added in the reaction mixture, and the pH of the

water phase was adjusted to 11 with 4M NaOH. Water phase was than extracted with 2 × 10 mL of DCM, combined organic phases were dried over sodium sulphate, filtered and evaporated under reduced pressure. The crude product was purified by column chromatography (EtOAc/hexane: 2/1) as white-silver solid (0.10 g, 36%). Melting point = 225-229°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.54-1.64 (m, 2H, NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 1.80-1.84 (m, 2H, NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 2.03-2.09 (m, 2H, NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 2.79-2.84 (m, 2H, NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 3.47 (s, 2H, Ar-C$\underline{H_2}$-N), 3.76-3.83 (m, 1H, NHC$\underline{H}$(CH$_2$CH$_2$)$_2$N), 6.62 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-$\underline{H}$), 7.14-7.18 (m, 1H, Ar-$\underline{H}$), 7.31-7.43 (m, 2H, Ar-$\underline{H}$), 7.89 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-$\underline{H}$), 8.00 (dd, J_1 = 8.7 Hz, J_2 = 0.8 Hz, 1H, Ar-$\underline{H}$), 8.39 (dd, J_1 = 8.7 Hz, J_2 = 2.3 Hz, 1H, Ar-$\underline{H}$), 8.50 (d, J = 7.6 Hz, 1H, Ar-CO-N$\underline{H}$), 8.68 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-$\underline{H}$), 8.88 (dd, J_1 = 2.3 Hz, J_2 = 0.8 Hz, 1H, Ar-$\underline{H}$). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 31.4 (NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 46.9 (NHC$\underline{H}$(CH$_2$CH$_2$)$_2$N), 52.0 (NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 60.6 (Ar-C$\underline{H_2}$), 108.8 (Ar-$\underline{C}$), 111.2 (Ar-$\underline{C}$), 117.1 (3×Ar-$\underline{C}$), 127.5 (Ar-$\underline{C}$), 128.0 (Ar-$\underline{C}$), 137.6 (3×Ar-$\underline{C}$), 142.9 (2×Ar-$\underline{C}$), 147.8 (Ar-$\underline{C}$), 152.0 (Ar-$\underline{C}$), 163.3 (Ar-$\underline{C}$O). MS (ES+) m/z = 398.2 (MH+). HRMS (ES+) m/z for $C_{21}H_{21}F_2N_5O$ calculated: 398.1792, found: 398.1782.

*4.1.9.6. (6-(1H-Pyrazol-1-yl)pyridin-3-yl)(4-(benzylamino)piperidin-1-yl)methanone (**13a**).* Compound **13a** (0.70 g, 2.58 mmol, 1 equiv), was dissolved in 35 mL of anhydrous methanol, acetic acid (≥ 99 %, 0.15 mL, 2.58 mmol, 1 equiv) was added to the solution, and after 30 minutes of stirring at room temperature under the argon atmosphere, $NaCNBH_3$ (0.34 g, 5.42 mmol, 2.1 equiv) dissolved in 5 mL of MeOH was added dropwise. After 10 minutes benzaldehyde (0.26 mL, 2.58 mmol, 1 equiv) was added and the reaction was left stirring

overnight. Into the reaction mixture 70 mL of DCM and 30 mL of saturated $NaHCO_3$ (aq) was added, and pH of the water phase was adjusted to 11 with 4 M NaOH. Organic phase was than extracted with 2× 20 mL of DCM, dried over sodium sulphate, filtered and evaporated under reduced pressure. The compound was cleaned by column chromatography (EtOAc/methanol: 9/1) to afford white solid (0.53 g, 57%). Melting point = 100-101°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.21-1.39 (m, 2H, N($CH_2C\underline{H_2}$)$_2$CH), 1.74-1.90 (m, 2H, N($CH_2C\underline{H_2}$)$_2$CH), 2.16 (s, 1H, N<u>H</u>), 2.65-2.71 (m, 1H, N(CH_2CH_2)$_2$C<u>H</u>), 3.03-3.12 (m, 2H, N($C\underline{H_2}CH_2$)$_2$CH), 3.60 (s, 1H, N($C\underline{H_2}CH_2$)$_2$CH) , 3.74 (s, 2H, NH-C<u>H$_2$</u>-Ar), 4.24 (s, 1H, N($C\underline{H_2}CH_2$)$_2$CH), 6.62 (dd, J_1 = 2.6 Hz, J_2 = 1.6 Hz, 1H, Ar-<u>H</u>), 7.18-7.24 (m, 1H, Ar-<u>H</u>), 7.27-7.37 (m, 4H, Ar-<u>H</u>), 7.88 (dd, J_1 = 1.6 Hz, J_2 = 0.8 Hz, 1H, Ar-<u>H</u>), 7.97 (dd, J_1 = 8.4 Hz, J_2 = 0.9 Hz, 1H, Ar-<u>H</u>), 8.02 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, 1H, Ar-<u>H</u>), 8.50 (dd, J_1 = 2.2 Hz, J_2 = 0.9 Hz, Ar-<u>H</u>), 8.65 (dd, J_1 = 2.6 Hz, J_2 = 0.8 Hz, 1H, Ar-<u>H</u>). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 31.5 and 32.2 (N($CH_2\underline{C}H_2$)$_2$CH), 45.8 (N($\underline{C}H_2CH_2$)$_2$CH), 49.7 (Ar-$\underline{C}H_2$), 52.9 (N(CH_2CH_2)$_2\underline{C}$H), 108.6 (Ar-<u>C</u>), 111.5 (Ar-<u>C</u>), 126.4 (Ar-<u>C</u>), 127.3 (Ar-<u>C</u>), 127.8 (2×Ar-<u>C</u>), 128.0 (2×Ar-<u>C</u>), 130.0 (Ar-<u>C</u>), 138.3 (Ar-<u>C</u>), 141.2 (Ar-<u>C</u>), 142.7 (Ar-<u>C</u>), 146.6 (Ar-<u>C</u>), 151.0 (Ar-<u>C</u>), 166.0 (Ar-<u>C</u>O). MS (ES+) m/z = 362.2 (MH+). HRMS (ES+) m/z for $C_{21}H_{23}N_5O$ calculated: 362.1981, found: 362.1986.

*4.1.10.General procedure **H**. Synthesis of compounds **13b-13e***. Compound **13a**, K_2CO_3 (4 equiv), BTEAC (0.1 equiv) and KI (0.1 equiv) were diluted in acetonitrile (15 mL). To that various benzyl derivatives (2 equiv) were added and stirred overnight at 80 °C, equipped with chlorcalcium tube. The K_2CO_3 was removed by filtration, the solvent was evaporated under reduced pressure and the compounds were cleaned by column chromatography.

*4.1.10.1. (6-(1H-Pyrazol-1-yl)pyridin-3-yl)(4-(benzyl(4-chlorobenzyl)amino)piperidin-1-yl) methanone (**13b**).* Compound **13b** was prepared according to general procedure **H** from **13a** (0.11 g, 0.29 mmol) and 4-chlorobenzyl chloride (0.06 g, 0.35 mmol)**.** Column chromatography (EtOAc/hexane: 1/1) was used to afford the compound **13b** (0.09 g, 65%) as white-yellow solid. Melting point = 111-114°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.65-1.98 (m, 4H, N($CH_2C\underline{H}_2$)$_2$CH), 2.63-2.77 (m, 2H, N($C\underline{H}_2CH_2$)$_2$CH), 2.99 (s, 1H, N(CH_2CH_2)$_2C\underline{H}$), 3.63 (s, 5H, 2×Ar-C$\underline{H}_2$-N and N($C\underline{H}_2CH_2$)$_2$CH), 4.56 (s, 1H, N($C\underline{H}_2CH_2$)$_2$CH), 6.62 (dd, J_1 = 2.6 Hz, J_2 = 1.6 Hz, 1H, Ar-$\underline{H}$), 7.19-7.24 (m, 1H, Ar-$\underline{H}$), 7.28-7.40 (m, 9H, Ar-$\underline{H}$), 7.87 (dd, J_1 = 1.8 Hz, J_2 = 0.7 Hz, 1H, Ar-$\underline{H}$), 7.96 (dd, J_1 = 8.5 Hz, J_2 = 0.8 Hz, 1H, Ar-$\underline{H}$), 8.04 (dd, J_1 = 8.5 Hz, J_2 = 2.2 Hz, 1H, Ar-$\underline{H}$), 8.53 (dd, J_1 = 2.2 Hz, J_2 = 0.9 Hz, 1H, Ar-$\underline{H}$), 8.65 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-$\underline{H}$). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 52.5 (Ar-$\underline{C}H_2$), 53.3 (Ar-$\underline{C}H_2$), 56.2 (N(CH_2CH_2)$_2\underline{C}$H), 108.6 (Ar-$\underline{C}$), 111.4 (Ar-$\underline{C}$), 126.7 (Ar-$\underline{C}$), 127.3 (Ar-$\underline{C}$), 128.1 (2×Ar-$\underline{C}$), 128.1 (2×Ar-$\underline{C}$), 128.2 (2×Ar-$\underline{C}$), 129.4 (2×Ar-$\underline{C}$), 130.0 (Ar-$\underline{C}$), 131.0 (Ar-$\underline{C}$), 138.3 (Ar-$\underline{C}$), 139.6 (Ar-$\underline{C}$), 140.3 (Ar-$\underline{C}$), 142.7 (Ar-$\underline{C}$), 146.7 (Ar-$\underline{C}$), 151.0 (Ar-$\underline{C}$), 165.9 (Ar-$\underline{C}$O). N($\underline{C}H_2CH_2$)$_2$CH and N($CH_2\underline{C}H_2$)$_2$CH signals were not detected. MS (ES+) m/z = 486.2 (MH+). HRMS (ES+) m/z for $C_{28}H_{28}ClN_5O$ calculated: 486.2061, found: 486.2053.

*4.1.10.2. (6-(1H-Pyrazol-1-yl)pyridin-3-yl)(4-(benzyl(3,4-dichlorobenzyl)amino)piperidin-1-yl)methanone (**13c**).* Compound **13c** was prepared according to general procedure **H** from **13a** (0.15 g, 0.42 mmol) and 3,4-dichlorobenzyl bromide (0.20 g, 0.83 mmol)**.** Column chromatography (EtOAc/hexane: 1/1) was used to afford the compound **13c** (0.18 g, 81%) as white solid. Melting point = 48-51°C. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.64-1.92 (m, 4H, N($CH_2C\underline{H}_2$)$_2$CH), 2.58-2.79 (m, 2H, N($C\underline{H}_2CH_2$)$_2$CH and

N(CH_2CH_2)$_2$C<u>H</u>), 3.01 (s, 1H, N(C<u>H$_2$</u>CH_2)$_2$CH), 3.65 (s, 5H, 2×Ar-C<u>H$_2$</u>-N and N(C<u>H$_2$</u>CH_2)$_2$CH), 4.54 (s, 1H, N(C<u>H$_2$</u>CH_2)$_2$CH), 6.62 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-<u>H</u>), 7.19-7.25 (m, 1H, Ar-<u>H</u>), 7.28-7.38 (m, 5H, Ar-<u>H</u>), 7.54-7.58 (m, 1H, Ar-<u>H</u>), 7.88 (dd, J_1 = 1.7 Hz, J_2 = 0.8 Hz, 1H, Ar-<u>H</u>), 7.97 (dd, J_1 = 8.4 Hz, J_2 = 0.8 Hz, 1H, Ar-<u>H</u>), 8.05 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, 1H, Ar-<u>H</u>), 8.53 (dd, J_1 = 2.2 Hz, J_2 = 0.8 Hz, 1H, Ar-<u>H</u>), 8.65 (dd, J_1 = 2.6 Hz, J_2 = 0.8 Hz, 1H, Ar-<u>H</u>). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 52.1 (Ar-<u>C</u>H_2), 53.6 (Ar-<u>C</u>H_2), 56.6 (N(CH_2CH_2)$_2$<u>C</u>H), 108.6 (Ar-<u>C</u>), 111.4 (Ar-<u>C</u>), 126.8 (Ar-<u>C</u>), 127.3 (Ar-<u>C</u>), 128.1 (2×Ar-<u>C</u>), 128.2 (2×Ar-<u>C</u>), 128.2 (Ar-<u>C</u>), 128.9 (Ar-<u>C</u>), 129.8 (Ar-<u>C</u>), 130.0 (Ar-<u>C</u>), 130.3 (Ar-<u>C</u>), 130.7 (Ar-<u>C</u>), 138.3 (Ar-<u>C</u>), 140.1 (Ar-<u>C</u>), 142.2 (Ar-<u>C</u>), 142.7 (Ar-<u>C</u>), 146.7 (Ar-<u>C</u>), 151.0 (Ar-<u>C</u>), 165.9 (Ar-<u>C</u>O). N(<u>C</u>H_2CH_2)$_2$CH and N(CH_2<u>C</u>H_2)$_2$CH signals were not detected. MS (ES+) m/z = 520.2 (MH+). HRMS (ES+) m/z for $C_{28}H_{27}Cl_2N_5O$ calculated: 520.1671, found: 520.1676.

*4.1.10.3. (6-(1H-Pyrazol-1-yl)pyridin-3-yl)(4-(benzyl(3,4-difluorobenzyl)amino)piperidin-1-yl)methanone (**13d**).* Compound **13d** was prepared according to general procedure **H** from **13a** (0.13 g, 0.36 mmol) and 3,4-difluorobenzyl bromide (0.09 mL, 0.72 mmol)**.** Column chromatography (EtOAc/hexane: 1/1) was used to afford the compound **13d** (0.15 g, 84%) as yellow oil. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.50-2.00 (m, 4H, N(CH_2C<u>H$_2$</u>)$_2$CH), 2.57-2.77 (m, 2H, N(C<u>H$_2$</u>CH_2)$_2$CH), 3.00 (s, 1H, N(CH_2CH_2)$_2$C<u>H</u>) 3.63 (d, J = 3.7 Hz, 5H, 2×Ar-C<u>H$_2$</u>-N and N(C<u>H$_2$</u>CH_2)$_2$CH), 4.57 (s, 1H, N(C<u>H$_2$</u>CH_2)$_2$CH), 6.62 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-<u>H</u>), 7.17-7.23 (m, 2H, Ar-<u>H</u>), 7.27-7.41 (m, 6H, Ar-<u>H</u>), 7.88 (d, J = 1.6 Hz, 1H, Ar-<u>H</u>), 7.94-8.00 (m, 1H, Ar-<u>H</u>), 8.04 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, 1H, Ar-<u>H</u>), 8.53 (dd, J_1 = 2.2 Hz, J_2 = 0.8 Hz, 1H, Ar-<u>H</u>), 8.65 (d, J = 2.6 Hz, 1H, Ar-<u>H</u>). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 52.2 (Ar-<u>C</u>H_2), 53.4 (Ar-<u>C</u>H_2), 56.4 (N(CH_2CH_2)$_2$<u>C</u>H), 99.5 (Ar-<u>C</u>), 108.6

(Ar-C), 111.4 (Ar-C), 116.6 (Ar-C), 117.0 (Ar-C), 124.4 (Ar-C), 126.7 (Ar-C), 127.3 (Ar-C), 128.1 (2×Ar-C), 128.2 (2×Ar-C), 130.0 (Ar-C), 138.6 (Ar-C), 140.2 (Ar-C), 142.7 (Ar-C), 146.7 (Ar-C), 151.0 (Ar-C), 165.9 (Ar-CO). $N(CH_2CH_2)_2CH$, $N(CH_2CH_2)_2CH$ and two Ar-C signals were not detected. MS (ES+) m/z = 488.2 (MH+). HRMS (ES+) m/z for $C_{28}H_{27}F_2N_5O$ calculated: 488.2262, found: 488.2267.

*4.1.10.4. (6-(1H-Pyrazol-1-yl)pyridin-3-yl)(4-(benzyl(4-methoxybenzyl)amino)piperidin-1-yl)methanone (**13e**).* Compound **13e** was prepared according to general procedure **H** from **13a** (0.15 g, 0.42 mmol) and 3,4-methoxybenzyl chloride (0.11 mL, 0.83 mmol)**.** Column chromatography (EtOAc/hexane: 1/1) was used to afford the compound **13e** (0.17 g, 83%) as orange oil. ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.53-1.95 (m, 4H, $N(CH_2CH_2)_2CH$), 2.55-2.76 (m, 2H, $N(CH_2CH_2)_2CH$), 2.97 (s, 1H, $N(CH_2CH_2)_2CH$), 3.58 (d, J = 25.2 Hz, 5H, 2×Ar-CH_2-N and $N(CH_2CH_2)_2CH$), 3.72 (s, 3H, Ar-OCH_3), 4.56 (s, 1H, $N(CH_2CH_2)_2CH$), 6.61 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-H), 6.84-6.90 (m, 2H, Ar-H), 7.17-7.38 (m, 7H, Ar-H), 7.87 (dd, J_1 = 1.7 Hz, J_2 = 0.8 Hz, 1H, Ar-H), 7.96 (dd, J_1 = 8.4 Hz, J_2 = 0.9 Hz, 1H, Ar-H), 8.04 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, 1H, Ar-H), 8.52 (dd, J_1 = 2.2 Hz, J_2 = 0.9 Hz, 1H, Ar-H), 8.65 (dd, J_1 = 2.6 Hz, J_2 = 0.8 Hz, 1H, Ar-H). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 52.5 (Ar-CH_2), 53.0 (Ar-CH_2), 54.9 (OCH_3), 55.9 ($N(CH_2CH_2)_2CH$), 108.6 (Ar-C), 111.4 (Ar-C), 113.6 (2×Ar-C), 126.6 (Ar-C), 127.3 (Ar-C), 128.0 (2×Ar-C), 128.1 (2×Ar-C), 129.2 (2×Ar-C), 130.0 (Ar-C), 132.1 (Ar-C), 138.3 (Ar-C), 140.6 (Ar-C), 142.7 (Ar-C), 146.7 (Ar-C), 151.0 (Ar-C), 158.0 (Ar-C), 165.9 (Ar-CO). $N(CH_2CH_2)_2CH$ and $N(CH_2CH_2)_2CH$ signals were not detected. MS (ES+) m/z = 482.2 (MH+). HRMS (ES+) m/z for $C_{29}H_{31}N_5O_2$ calculated: 482.2556, found: 482.2553.

*4.1.10.5. (6-(1H-Pyrazol-1-yl)pyridin-3-yl)(4-(dibenzylamino)piperidin-1-yl)methanone (**13f**).* Compound **12** (0.99 g, 3.65 mmol, 1 equiv), K_2CO_3 (3.03 g, 0.37 mmol, 0.1 equiv), BTEAC (0.08 g, 0.37 mmol, 0.1 equiv) and KI (0.06 g, 0.37 mmol, 0.1 equiv) were dissolved/dispersed in acetonitrile (15 mL). To that benzyl chloride was added and stirred overnight at 55 °C under argon atmosphere. The K_2CO_3 was removed by filtration, the solvent was evaporated under reduced pressure and the compound was purified by column chromatography (EtOAc/hexane: 2/1) to afford compound as brown oil (1.46 g, 89%). ^{1}H NMR (400 MHz, DMSO-d_6) δ(ppm) = 1.65-2.00 (m, 4H, N($CH_2C\underline{H}_2$)$_2$CH), 2.61-2.75 (m, 2H, N($C\underline{H}_2CH_2$)$_2$CH), 2.98 (s, 1H, N(CH_2CH_2)$_2C\underline{H}$), 3.64 (s, 5H, 2×Ar-C$\underline{H}_2$-N and N($C\underline{H}_2CH_2$)$_2$CH), 4.58 (s, 1H, N($C\underline{H}_2CH_2$)$_2$CH), 6.62 (dd, J_1 = 2.6 Hz, J_2 = 1.7 Hz, 1H, Ar-$\underline{H}$), 7.17-7.26 (m, 2H, Ar-$\underline{H}$), 7.28-7.43 (m, 8H, Ar-$\underline{H}$), 7.88 (dd, J_1 = 1.7 Hz, J_2 = 0.7 Hz, 1H, Ar-$\underline{H}$), 7.97 (dd, J_1 = 8.4 Hz, J_2 = 0.8 Hz, 1H, Ar-$\underline{H}$), 8.05 (dd, J_1 = 8.4 Hz, J_2 = 2.2 Hz, 1H, Ar-$\underline{H}$), 8.53 (dd, J_1 = 2.2 Hz, J_2 = 0.8 Hz, 1H, Ar-$\underline{H}$), 8.65 (dd, J_1 = 2.6 Hz, J_2 = 0.7 Hz, 1H, Ar-$\underline{H}$). ^{13}C NMR (100 MHz, DMSO-d_6) δ(ppm) = 53.2 (2×Ar-$\underline{C}H_2$), 56.1 (N(CH_2CH_2)$_2\underline{C}$H), 108.6 (Ar-$\underline{C}$), 111.4 (Ar-$\underline{C}$), 126.6 (2×Ar-$\underline{C}$), 127.3 (Ar-$\underline{C}$), 128.0 (4×Ar-$\underline{C}$), 128.2 (4×Ar-$\underline{C}$), 130.0 (Ar-$\underline{C}$), 138.3 (2×Ar-$\underline{C}$), 140.5 (Ar-$\underline{C}$), 142.7 (Ar-$\underline{C}$), 146.7 (Ar-$\underline{C}$), 151.0 (Ar-$\underline{C}$), 158.0 (Ar-$\underline{C}$), 165.9 (Ar-$\underline{C}$O). N($\underline{C}H_2CH_2$)$_2$CH and N($CH_2\underline{C}H_2$)$_2$CH signals were not detected. MS (ES+) m/z = 452.2 (MH+). HRMS (ES+) m/z for $C_{28}H_{29}N_5O$ calculated: 452.2450, found: 452.2443.

4.2. Biological characterization. Cell culture and transfection

The human embryonic kidney (HEK) 293T cells were cultured in a petri dish in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin-

streptomycin and incubated at 37 °C in a humid atmosphere with 5% CO_2. The cells for cAMP accumulation assay were cultured in 10 cm petri dish and transfected with 2 μg of the wild type CXCR4/CXCR3 and 2 μg of biosensor, after they reached 50-70% of the maximum occupancy of the petri dish. Cells for β-arrestin 2 assay were transfected with 1μg of ProLink (PK1) tagged wild-type CXCR3 at the same occupancy in 4 cm petri dish. Transfection was performed using TransIT-293 transfection reagent from Mirus (purchased from MoBiTec, Göttingen, Germany) according manufacturers' instructions. A day after transfection, the cell were seeded in a microtiter plate as described below.

4.3. β-Arrestin 2 recruitment assay

The PathHunter assay from DiscoverX (DiscoverX, Birmingham, U.K.) was used to measure β-arrestin recruitment according to the manufacturer's protocol. HEK293T cells stably expressing β-arrestin 2/incomplete β-gal enzyme chimera were transfected with the CXCR3-PK1 receptor, where PK1 is annotating the second half of the β-gal enzyme. 24 hours after transfection, the cells were seeded in the 384-well plate at the density of 10.000 cells per well and in the volume of 20 μL. After incubation overnight at 37 °C and 5% CO_2, EC_{80} of chemokine CXCL11 and various concentrations of test compounds, dissolved in phosphate buffered saline (PBS) containing 0.2% bovine serum albumin (BSA), were added and the cells incubated for additional 4 hours. Afterwards the detection mix was added and the plate incubated for additional 60 minutes at room temperature. The activation or inhibition of the β-arrestin 2 recruitment was determined by measuring the chemiluminescence using BMG Clariostar (BMG LabTech, Ortenberg, Germany).

4.3.1. Statistics

In vitro CXCR3 β-arestin assay was performed in three independent experiments, with average values expressed as means of triplicates ± S.E.M. (the data was analyzed using descriptive statistics). Statistical significance was determined by Student t-test (two-tailed; unpaired). Differences were considered significant for $p<0.05$. Analyses were performed using Microsoft Excel 2013 for Windows.

4.4. BRET based cAMP assay

HEK293T cells were transfected with wild type CXCR4 or CXCR3 receptor and biosensor CAMYEL (purchased at ATCC). 24 hours after transfection, the cells were seeded in the 96-well plate at the density of 20.000 cells per well and in the volume of 100 µL. After incubation overnight at 37 °C and 5% CO_2 the medium was replaced with 30 µL of Dulbecco's phosphate-buffered saline (dPBS) and incubated for 1 hour. After substrate 10 µL of 25 µM Coelenterazin h (Promega, Mannheim, Germany) was added to a final concentration 5 µM and incubated for additional 5 minutes. Concomitantly EC_{80} of chemokines CXCL12/CXCL11 and various concentrations of test compounds, dissolved in PBS containing 0.2% BSA and forskoline (final concentration 10 µM), were added and incubated for 20 minutes. The emission from Renilla luciferase (RL) and YFP were simultaneously measured at 475-30 nm/535-30 nm with BMG Clariostar. The netBRET signal calculated as the ratio between the light emitted by YFP (505 to 555 nm) and the light emitted by RLuc (465 to 505 nm). Three experiments were performed per compound, with each concentration in triplicate.

4.5. Calculation of inhibitory constants

The data obtained from functional assays were analized with ternary complex model of allosterisem to characterise allosteric profile of novel ligands by fitting them to the following equations using Prism 5.0 (GraphPad Software, San Diego, CA, USA):

$$K_{app} = \frac{K_A \left(1 + \frac{[B]}{K_B}\right)}{\left(1 + \frac{\alpha[B]}{K_B}\right)}$$

$$Y = \frac{Y_0 \left(1 + K_A\right)}{\left([c] + K_{app}\right)}$$

K_{app} describes the occupancy of the orthosteric site; K_A is the EC_{50} value of CXCL11 or CXCL12. Y_0 is the basal value of luminescence in the absence of modulator, Y is the value of luminescence, [c] is the concentration (EC_{80}) of CXCL11/CXCL12 used, [B] is the concentration of test compounds, K_B is the equilibrium dissociation constant of modulator binding and α is the ternary complex constant, cooperativity factor. The K_A value for CXCL11 was set to 0.9 nM, the concentration of CXCL11 was set to 5 nM and compounds were tested on CXCR3 in concentrations 10^{-12}- 10^{-5} M. The K_A value for CXCL12 was set to 3 nM, concentration of the CXCL12 was set to 20 nM and compounds were tested on CXCR4 in concentrations 10^{-9}-10^{-5} M.

4.6. Functional antagonism studies.

4.6.1. Cell isolation and culture

Buffy coats from venous blood of normal healthy volunteers were obtained by the Blood Transfusion Centre of Slovenia, according to institutional guidelines. PBMCs were isolated using Lympholyte®-H (Cedarlane laboratories, Ontario, Canada). After isolation, the PBMCs were washed 3 times at 300g for 10 minutes to remove the majority of platelets. When needed, whole CD4+ T cells were isolated from PBMCs via immunomagnetic selection using CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of CD4+ cells was

always > 95% as determined by flow cytometry. All cells were cultured using 1640 Roswell Park Memorial Institute (RPMI) medium substituted with 10% FBS (complete medium).

4.6.2. Solubility and cytotoxicity studies

All aliquots of tested compounds were prepared in USP-grade DMSO (CryoSure-DMSO, WAK-Chemie Medical GmbH, Germany). Since we did not want to use more than 1% DMSO in cell culture medium, we first tested the maximum solubility of compounds in relation to their DMSO aliquot concentrations. Briefly, in the beginning the compounds were prepared at 50 mM DMSO aliquots and their maximum solubility in cell culture media was tested (max 10% DMSO or 1µL / 1mL cell medium). The solubility was determined in detail by inverted light microscopy (Nikon Eclipse Ti-S, Tokyo, Japan). In case of insolubility, the respective compound's aliquot was diluted further by factor of 2 until solubility was satisfactory. After the maximum solubility was determined for each compound, cytotoxicity of maximum concentrations (1% DMSO in cell culture medium) was tested. For this purpose, PBMCs were incubated in cell culture medium with the compounds for 6 hours. Afterwards, the cells were washed and stained with 7-aminoactinomycin D (7-AAD). The percentage of dead cells was analyzed by flow cytometry (FACSCalibur, Beckton Dickinson, San Diego, CA).

4.6.3. Phenotypic characterization and intracellular staining

For flow cytometric analysis of PBMCs and CD4+ T cells the following monoclonal antibodies were used: PE-labeled anti-CXCR3, anti-CXCR4, anti-CD25, anti-T-bet and FITC-labeled anti-CD4 (all from Biolegend, CA, USA). FITC-IgG1 and R-PE-IgG2a cocktail was used for isotype control (Biolegend). Either PBMCs or CD4+ T cells were incubated with selected antibodies for 15 minutes in the dark at room temperature. Afterwards the cells were

washed twice and resuspended in 2% paraformaldehyde. For characterization of T-bet transcription factor, PBMCs were collected and first stained with anti-CD4-FITC. Afterwards the cells were fixed using 4% paraformaldehyde for 30 minutes. In the next step the cells were permeabilized using water-free, ice-cold methanol for 10 minutes. The cells were then washed and incubated in dPBS containing 3% FBS to avoid unspecific staining. Finally, anti-T-bet antibody was added for minimum of 45 minutes. All samples were analyzed on a FACSCalibur system (Beckton Dickinson). Data was analyzed with the Cell Quest software (BD biosciences).

4.6.4. Quantification of cytokine production

PBMCs were stimulated using T cell activation/expansion macrobeads (Miltenyi Biotec) which stimulate the T cell receptor, according to manufacturer's protocol. Briefly, 3 × 105 PBMCs/well were cultured in 96 wells in four replicates. After 48 hours the cell culture supernatants were collected. The level of IFN-γ was measured using BD cytometric bead array Th1/Th2/Th17 kit (BD biosciences).

4.6.5. Migration assay

Transwells (Corning Costar) with 5-µm polycarbonate filters (5-µm pore size) were used. PBMCs or CD4+ T cells were (5 × 105) were added to the upper compartments in 100 µL of complete medium. The lower compartments were filled with 500 µL of complete medium containing either CXCL12 (SDF-1, 200 ng/mL) or CXCL10 (200 ng/mL) (both form Peprotech, London, UK) for CXCR4 or CXCR3 migration studies, respectively. To determine the antagonistic effect, the cells in the upper compartments were pre-treated for 1 hour with various compounds beforehand. In this manner, the transwell cultures were incubated at 37 ºC, 5% CO2

for 3 hours. At the end of migration assay, upper compartments were removed and cells from the lower compartment collected and counted by flow cytometry using 60 second counts.

4.7. Homology modeling

Homology model of CXCR3 receptor was built based on the crystal structure of the CXCR4 receptor (PDB code: 3ODU) in complex with small molecule antagonist. CXCR3 amino acid sequence (UniProt accession number P49682) was retrieved from the UniProt archive [53]. Sequence alignment was performed using the T-COFFEE server [54] and the obtained alignment (Figure S5 in Supplementary data) was used as an input for homology modeling software MODELLER 9.13 [55] to build a CXCR3 homology model. Ligand information from the crystal structure was used as an additional restraint in the homology modeling procedure. Ligand was kept rigid during model building and water molecules were ignored. Fifty models were generated and the best model for further studies was selected based on the highest discrete optimized protein energy (DOPE) score and stereochemical quality of the model as evaluated by PROCHECK [56] and ProSA-web [57,58] server. For the selected model Ramachandran plot (Figure S6 in Supplementary data) showed that the backbone dihedral angles of 100% of the amino acid residues were found in allowed regions (91.2% in the core region, 7.7% in additional allowed regions and 1.1% in generously allowed regions) and 0% of the residues located in disallowed regions. Steric clashes of the initial model were removed using Chiron server, which rapidly minimizes steric clashes in proteins using short discrete molecular dynamics simulations [59,60]. Next, the overall quality of non-bonded atomic interactions was assessed by ERRAT [61]. The ERRAT score (Figure S7 in Supplementary data) of the energy minimized CXCR3 homology model was 89.46, which is well within the range of a high quality model, considering

the normally accepted range of >50 for high quality models. Finally, ProSA-web evaluation of the model reported a Z-score value of -3.84 (Figure S8 in Supplementary data), which is within the range of native conformations of the crystal structures.

4.8. Molecular docking

Structure-based (molecular docking) calculations of most potent negative allosteric modulators identified by cAMP BRET assay within the binding pocket of CXCR4 and its derived CXCR3 homology model were performed by GOLD docking suite [62]. The experimental coordinates of the co-crystallized small molecule antagonist IT1t [63] were used to define the binding site (cavity radius of 10Å and 8.5Å for CXCR4 and CXCR3, respectively). The same settings and technical parameters of the GOLD genetic algorithm (population size = 100, selection pressure = 1.1, number of operations = 100.000, number of islands = 5, niche size = 2, migrate = 10, mutate = 95, cross-over = 95) were used for all calculations by docking each molecule 10 times into the binding site. The quality of calculated docking poses was determined based on the GOLDScore Fitness function, which was used as a main scoring function for evaluation of the ligands binding affinity [62]. Molecular figures of poses were generated with PyMol [64].

4.9. Interference compounds screening

All tested compounds were also subjected to Pan-assay interference compounds (PAINS) filter [65], and only compound **6c** was filtered out due to 4-methoxy-*N*-methylaniline as problematic structure feature. Fortunately this compound did not show any activity on CXCR3 and CXCR4 receptors.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Appendix A. Supplementary data

Figures showing agonist single point screening on CXCR3; spectral characterization and purity data of final compounds; figures showing homology model sequence alignment and structure quality assessment (PDF)

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- CXCR3/CXCR4 allosteric modulators were synthesised and functionally characterized
- **9e** was identified as dual CXCR3/CXCR4 negative modulator
- **13a** acts as positive modulator and **6a**, **6b** and **11a** as allosteric agonists on CXCR4