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Research paper

Targeting the K_v11.1 (hERG) channel with allosteric modulators. Synthesis and biological evaluation of three novel series of LUF7346 derivatives

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ABSTRACT

We synthesized and evaluated three novel series of substituted benzophenones for their allosteric modulation of the human K_v11.1 (hERG) channel. We compared their effects with reference compound LUF7346 previously shown to shorten the action potential of cardiomyocytes derived from human stem cells. Most compounds behaved as negative allosteric modulators (NAMs) of [^{3}H]dofetilide binding to the channel. Compound **9i** was the most potent amongst all ligands, remarkably reducing the affinity of dofetilide in competitive displacement assays. One of the other derivatives (**6k**) tested in a second radioligand binding set-up, displayed unusual displacement characteristics with a pseudo-Hill coefficient significantly distinct from unity, further indicative of its allosteric effects on the channel. Some compounds were evaluated in a more physiologically relevant context in beating cardiomyocytes derived from human induced pluripotent stem cells. Surprisingly, the compounds tested showed effects quite different from the reference NAM LUF7346. For instance, compound **5e** prolonged, rather than shortened, the field potential duration, while it did not influence this parameter when the field potential was already prolonged by dofetilide. In subsequent patch clamp studies on HEK293 cells expressing the hERG channel the compounds behaved as channel blockers. In conclusion, we successfully synthesized and identified new allosteric modulators of the hERG channel. Unexpectedly, their effects differed from the reference compound in functional assays on hERG-HEK293 cells and human cardiomyocytes, to the extent that the compounds behaved as stand-alone channel blockers.

1. Introduction

The voltage-gated potassium ion channel K_v11.1 (hERG) has a pore that accommodates many drugs and drug-like molecules. The (blocking) interactions of these drugs with the channel often lead to cardiovascular side effects, such as prolongation of the QT-interval and, although rarely, potentially lethal Torsade de Pointes [1]. This has raised serious concerns among “drug hunters” and regulatory authorities alike since it has led to the market withdrawal of multiple drugs [2,3]. This is costly and demonstrates that present assays do not always detect risk in patients. As an example, the antibiotic grepafloxacin was taken off the market worldwide in 1999 because of sudden cardiac deaths, illustrating that potentially life-saving drugs can become unavailable due to hERG liabilities [4]. Other examples include histamine H_1 receptor antagonist astemizole, and sertindole used in psychiatric disorders [5,6].

Strategies to avoid these liabilities include efforts to synthesize hERG affinity “out” of new chemical entities, but, again, this may result in shelving of otherwise clinically valuable molecules that solve unmet medical needs. An alternative approach is to try and reduce a compound’s affinity for the channel by altering the

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conformation of the channel. This could increase the therapeutic window between the desired effect at another target and hERG blocking activity. A pharmacological approach to achieve this would be to allosterically modulate the K_v11.1 channel by small molecules leading to conformational changes in the pore with a concomitant reduction in affinity of (other) drugs for the channel [7]. Such negative allosteric modulators (NAMs) or, alternatively, channel activators have been the subject of a number of recent studies, showing that this principle can work ([8] and references therein [9–13]).

In this respect we have recently synthesized and studied substituted benzophenones as NAMs, including two representative examples, LUF7244 and LUF7346 [14], which have been analyzed in more detail previously [15–18]. In the present study we aimed at exploring the structure-activity relationships (SAR) around LUF7346 (Fig. 1) further by synthesizing three different series with over 25 new derivatives. Some of these molecules were evaluated in more detail in electrophysiological experiments on HEK293 cells expressing the channel and cardiomyocytes derived from human induced pluripotent stem cells (hiPSCs).

2. Materials and methods

2.1. Materials

Dofetilide was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Tritium-labeled dofetilide (specific activity 82.5 Ci/mmol) was purchased from PerkinElmer (Groningen, The Netherlands). HEK293 cells stably expressing the K_v11.1 channel (HEK293Kv11.1) were kindly provided by Dr. Eckhard Ficker (University of Cleveland, USA). Bicinchoninic acid (BCA) and BCA protein assay reagent were obtained from Pierce Chemical Company (Rockford, IL, USA). All other chemicals were of analytical grade and obtained from standard commercial sources.

2.2. Cell culture

HEK293K_v11.1 cells were cultured in a humidified atmosphere at 37 °C and 7% CO_2 in DMEM, containing 10% fetal calf serum, 100 IU/mL penicillin, 100 μg/mL streptomycin and 500 μg/mL G418 for selection. Cells were subcultured twice a week at a ratio 1:6. Then the cells were transferred to large 15-cm diameter plates for membrane preparation. For patch clamp experiments, HEK293K_v11.1 cells were cultured on gelatine-coated cell culture plates in DMEM with 10% FBS, 1:200 penicillin/streptomycin, 1:100 Glutamax (Thermofisher Scientifics) and passaged twice a week.

HiPSC-derived cardiomyocytes (hiPSC-CMs) were differentiated from the wild type hiPSC line LUMC0020iCTRL06 [19] as previously described [20]. hiPSC-CMs at day 18 of differentiation were frozen as stocks. Seven days before the measurements, hiPSC-CMs were thawed and 50,000 cells plated in an 8 μL drop of low-insulin, bovine serum albumin, polyvinylalcohol, essential lipids (LI-BPEL) medium [21] onto the center of a multielectrode array (MEA) well, coated with 40 μg/mL fetal bovine fibronectin (Sigma-Aldrich), and allowed to attach for at least 4 h at 37 °C in a humidified incubator before topping up the medium to 500 μL [22]. After 24 h half of the medium was refreshed. hiPSC-CMs were kept in culture at 37 °C in 5% CO_2 until measurements.

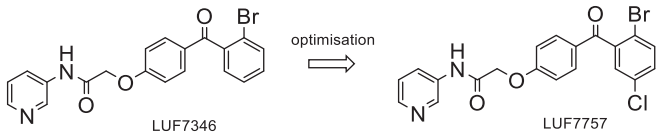


Fig. 1. Optimisation of the substituted benzophenone moiety.

2.3. Membrane preparation

HEK293K_v11.1 cells were grown to 80–90% confluence and detached from the plates by scraping them into 5 mL of PBS. The detached cells were collected and centrifuged at 250 g for 10 min. The cell pellets were resuspended in 50 mM ice-cold Tris–HCl buffer supplemented with 2 mM $MgCl_2$, pH 7.4. An UltraTurrax (Heidolph Instruments, Schwabach, Germany) was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by centrifugation at 100,000 g in an Optima LE-80 K ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) at 4 °C for 20 min. The pellets were resuspended in the Tris–HCl buffer and the homogenization and centrifugation steps were repeated. The resulting pellets were resuspended in ice-cold incubation buffer without BSA (10 mM HEPES, 130 mM NaCl, 60 mM KCl, 0.8 mM MgCl2, 1 mM EGTA, 10 mM glucose, pH 7.4) using the UltraTurrax. Aliquots (250 or 500 μl) were stored at −80 °C. The protein concentration of the membranes was measured using the BCA method [23].

2.4. Radioligand displacement assay

Membrane aliquots containing 40 μg protein were incubated with 5 nM [^{3}H]dofetilide in the absence (control) and presence of test compounds in a total volume of 100 μl assay buffer (10 mM HEPES, 130 mM NaCl, 60 mM KCl, 0.8 mM MgCl2, 1 mM EGTA, 10 mM glucose, 0.1% BSA, pH 7.4) at 25 °C for 60 min. At this [^{3}H]dofetilide concentration, total radioligand binding did not exceed 10% of the radioligand added to prevent ligand depletion. Non-specific binding was determined in the presence of 10 μM astemizole and represented approximately 15% of the total binding. [^{3}H]Dofetilide did not bind to membranes prepared from empty HEK293 cells lacking the K_v11.1 channel (data not shown). First, all compounds were tested at one concentration of 10 μM. When radioligand displacement by the compound was greater than approx. 75%, full curves of dofetilide displacement were recorded in the absence (control) or presence of 10 μM compound unless otherwise noted to obtain the shift in IC_{50} values of dofetilide induced by the compounds. For the full displacement curves 7 increasing concentrations of dofetilide were used, which were added by an HP D300 digital dispenser (Tecan, Giessen, The Netherlands). Increasing concentrations of compound **6k** alone were also tested in the [^{3}H]dofetilide displacement assay. Incubations were terminated by dilution with ice-cold wash buffer. Separation of bound from free radioligand was performed by rapid filtration through a 96-well GF/B filter plate using a Perkin Elmer Filtermate-harvester (Perkin Elmer, Groningen, The Netherlands). Filters were subsequently washed 12 times with ice-cold wash buffer (25 mM Tris, 130 mM NaCl, 60 mM KCl, 0.8 mM MgCl2, 0.05 mM CaCl2, 0.05% BSA, pH 7.4). The filter-bound radioactivity was determined by scintillation spectrometry using the P-E 2450 Microbeta^2 counter (Perkin Elmer) after addition of 25 μL Microscint fluid and 3 h extraction.

2.5. Electrophysiology

MEA experiments using hiPSC-CMs were carried out at 37 °C in LI-BPEL medium using 24-well 8-electrodes MEA plates in the Multiwell-MEA system (Multichannel Systems, Reutlingen, Germany). Data were recorded and analyzed with the Multiwell-Screen software (Multichannel Systems) to calculate the average field potential duration (FPD) and inter-beat (RR) interval for each

well. hERG allosteric modulators were dissolved in DMSO to a stock concentration of 100 mM and cumulative doses of a compound were added to the same well every 10 min, recording each time for 90 s. The maximum ratio of DMSO:medium volumes was 1:3333. One well per experiment was used as negative control by adding equivalent volumes of DMSO and no effects were observed. Dofetilide was dissolved in DMSO at a stock solution of 50 mM and used at 10 nM; cells were incubated with dofetilide for 10 min before addition of the compounds. Concentration-effect curves were generated in which variation (in percentage) of FPD or RR interval with respect to the baseline was expressed on the Y-axis. In experiments where dofetilide was added, the effect of dofetilide was taken as baseline.

Patch clamp experiments were carried out on isolated HEK293K_v11.1 cells plated on gelatine-coated glass coverslips at 37 °C. hERG current was recorded using a voltage clamp technique in whole-cell configuration with Axonpatch 200B amplifier, Digidata 1440A and pClamp10.7 software (Molecular Devices). Cells were superfused with Tyrode's solution containing (in mM): 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES-NaOH, 5.5 D-glucose, pH 7.4. Glass pipettes had a resistance of 1.5–2.5 MΩ and were filled with an intracellular-like solution containing (in mM): 125 K-gluconate, 20 KCl, 10 NaCl, 10 HEPES, 5 K_2-ATP, pH 7.2. hERG current was activated from a holding potential of −60 mV with 4 s voltage steps to the range −50/+40 mV every 10 mV, followed by 16 s at −60 mV. Current densities were obtained normalizing the peak tail current at −60 mV for the cell capacitance. Cell capacitance and series resistance were compensated for at least 70%. For each cell, hERG current was measured in Tyrodes solution and after superfusion for at least 5 min with Tyrode's solution supplemented with 5 μM of one of the compounds. Reversibility of compound effects was tested by recording hERG current on the same cell 10 min after Tyrode wash-out.

2.6. Data analysis

All data of radioligand binding were analyzed using the non-linear regression curve fitting program Prism 8 (GraphPad, San Diego, CA, USA). Total binding was determined in the presence of incubation buffer and was set at 100% in all experiments, whereas nonspecific binding was set at 0%. IC_{50} values in displacement assays were directly obtained from non-linear regression analysis of concentration-effect curves. The fold shifts in dofetilide's IC_{50} values induced by the compounds tested were calculated by dividing the IC_{50} value of dofetilide measured in the presence of test compound divided by the IC_{50} value of dofetilide in the absence of test compound. RStudio and OriginPro 2016 (Origin Lab, Northampton, MA, USA) were used for statistical analysis of the data from the electrophysiological experiments on cardiomyocytes; data from the patch clamp studies were analyzed using ClampFit 10.7 (Molecular Devices). For graphical representation of these functional data Prism 8 was used.

2.7. Chemistry

All solvents and reagents used were of analytical grade and from commercial sources. Demineralized water was used in all cases, unless stated otherwise, and is simply referred to as H_2O. All reactions were monitored by thin-layer chromatography (TLC) using aluminum plates coated with silica gel 60 F254 (Merck), and compounds were visualized under ultraviolet light at 254 nm or via $KMnO_4$ staining. Column chromatography for compound purification was performed using silica gel (Merck Millipore) with particle size 0.04–0.63 mm. Chemical identity of final compounds was established using ^{1}H NMR and liquid chromatography–mass spectrometry (LC–MS). ^{1}H NMR spectra were recorded on a Bruker AV 400 liquid spectrometer (^{1}H NMR, 400 MHz) at room temperature. Chemical shifts (δ) are reported in parts per million (ppm), and coupling constants (J) in Hz. Liquid chromatography–mass spectrometry (LC–MS) and analytical purity of final compounds was determined using a Shimadzu high pressure liquid chromatography (HPLC) system and LCMS2020 equipment with a Phenomenex Gemini column (C18 110A column, 50 mm × 3 mm, 3 μm). A flow rate of 0.55 mL/min and an elution gradient of 10–90% MeCN/H_2O (0.1% CHCOOH) were used. The absorbance of the UV spectrophotometer was set at 254 nm. All compounds tested in biological assays showed a single peak at the designated retention time and were ≥95% pure. Sample preparations for HPLC and LC–MS were as follows unless stated otherwise: 0.3 mg/mL of compound was dissolved in a 1:1:1 mixture of H_2O:MeOH:tBuOH.

Ethyl 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetate (**3**). A mixture of 2-bromo-5-chlorobenzoic acid (12.0 g, 51.0 mmol, 1.00 eq.) in thionyl chloride (55 mL, 764 mmol, 15.0 eq) was refluxed under a nitrogen atmosphere for 2 h. Thionyl chloride was distilled off and the residue redissolved and evaporated twice with 50 mL of toluene. The obtained crude 2-bromo-5-chlorobenzoyl chloride (**2**) was dissolved in CH_2Cl_2 (80 mL), ethyl 2-phenoxyacetate (**1**) (9.18 g, 51.0 mmol, 1.00 eq.) was added and the mixture was cooled to 0 °C on ice. Aluminium chloride (13.59 g, 102 mmol, 2.00 eq.) was added portion-wise and the mixture was stirred overnight at RT. As revealed from TLC (EtOAc/Pe 1:5) full conversion of the benzoyl chloride (**2**) and ethyl 2-phenoxyacetate (**1**) into the product (Rf = 0.5) was reached. The mixture was poured on ice, extracted with CH_2Cl_2 (3 × 80 mL), backwashed with 2 M HCl solution (aq.), brine, dried over $MgSO_4$ and concentrated *in vacuo*. The resulting 19.52 g of crude orange oil was purified by column chromatography using a gradient of EtOAc/PE 1:5–1:2 yielding ethyl 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetate (13.24 g, 33.3 mmol, 65% yield) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78 (d, J = 8.8 Hz, 2H), 7.57 (dd, J = 8.0, 0.8 Hz, 1H), 7.33–7.30 (m, 2H), 6.96 (d, J = 9.2 Hz, 2H), 4.70 (s, 2H), 4.29 (q, J = 7.2 Hz, 2H), 1.30 (t, J = 7.2 Hz, 3H) ppm.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (4). To a mixture of ethyl 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetate (**3**) (13.24 g, 33.3 mmol, 1.00 eq.) dissolved in equal amounts of 60 mL THF and 60 mL of methanol was added 66 mL of a 1 M aqueous solution of LiOH. After stirring for 1 h at room temperature the ester was fully hydrolysed as shown by TLC (EtOAc/PE 1:3). THF and methanol were distilled off and the pH was adjusted to pH = 1 using an aqueous 1 M HCl solution. A precipitate was formed, collected by filtration and dried *in vacuo*, yielding 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) as a white solid (12.04 g, 32.6 mmol, 98% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.19 (s br, 1H), 7.78 (d, J = 8.4 Hz, 1H), 7.68 (d, J = 8.8 Hz, 2H), 7.61 (d, J = 2.4 Hz, 1H), 7.55 (dd, J = 8.8, 2.4 Hz, 1H), 7.07 (d, J = 8.8 Hz, 2H), 4.82 (s, 2H) ppm.

General peptide coupling procedure for 4-pyridine compounds 5a-f. To a mixture of the appropriately substituted 4-aminopyridine (1.00 eq) and 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (1.10 eq) dissolved in DMF (0.10 M) were added DIPEA (2.00 mmol, 2.00 eq.) and bromotripyrrolidinophosphonium hexafluorophosphate (PyBroP) (1.20 eq.) for compounds **5b-f**. In the case of **5a** triethylamine (1.50 eq.) and 1-[bis(dimethylamino) methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU) (1.10 eq.) was used. The mixture was stirred under a nitrogen atmosphere at RT overnight. TLC showed full conversion of the starting materials into the desired product. DMF was evaporated *in vacuo* after which the mixture was dissolved in ethyl acetate, backwashed with $NaHCO_3$ solution (aq.) and twice with brine. The organic layers were dried over $MgSO_4$ and evaporated *in vacuo*. Purification by column chromatography (0.5%–3%

methanol in dichloromethane) resulted in the pure desired products **5a-f**.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(pyridin-4-yl) acetamide (5a). Compound was synthesized according to general procedure, using the following reagents: 4-aminopyridine (46 mg, 0.492 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy) acetic acid (**4**) (200 mg, 0.541 mmol, 1.10 eq.), triethylamine (103 μL, 0.738 mmol, 1.50 eq.) and HATU (206 mg, 0.541 mmol, 1.10 eq.). White solid, 164 mg, 75% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.67 (s br, 1H), 8.54 (dd, J = 4.8, 1.6 Hz, 1H), 7.82 (d, J = 9.2 Hz, 2H), 7.60–7.55 (m, 3H), 7.33 (dd, J = 8.4, 2.4 Hz, 1H), 7.30–7.27 (m, 1H), 7.04 (d, J = 8.8 Hz, 2H), 4.71 (s, 2H) ppm; LC-MS calculated for $C_{20}H_{14}BrClN_2O_3$ $[M+H]^+$: 444.99, found 445.14. LC t_R = 6.36 min, purity: 98%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(2-methoxypyridin-4-yl)acetamide (5b). Compound was synthesized according to general procedure, using the following reagents: 4-amino-2-methoxypyridine (45 mg, 0.361 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (147 mg, 0.397 mmol, 1.10 eq.), DIPEA (126 μL, 0.722 mmol, 2.00 eq.) and PyBroP (185 mg, 0.397 mmol, 1.20 eq.). White solid, 69 mg, 40% yield. ^{1}H NMR (400 MHz $CDCl_3$): δ 8.33 (s br, 1H), 8.10 (d, J = 5.6 Hz, 1H), 7.83 (d, J = 8.8 Hz, 2H), 7.57 (d, J = 8.4 Hz, 1H), 7.33 (dd, J = 8.4, 2.8 Hz, 1H), 7.30 (d, J = 2.4 Hz, 1H), 7.10 (d, J = 2.0 Hz, 1H), 7.09–7.02 (m, 3H), 4.68 (s, 2H), 3.93 (s, 3H) ppm; LC-MS calculated for $C_{21}H_{16}BrClN_2O_4$ $[M+H]^+$: 475.00, found 475.05. LC t_R = 9.57 min, purity: 98%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(2-chloropyridin-4-yl)acetamide (5c). Compound was synthesized according to general procedure, using the following reagents: 4-amino-2-chloropyridine (66 mg, 0.515 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (209 mg, 0.566 mmol, 1.10 eq.), DIPEA (180 μL, 1.03 mmol, 2.00 eq.) and PyBroP (288 mg, 0.618 mmol, 1.20 eq.). White solid, 16 mg, 6% yield. ^{1}H NMR (400 MHz $CDCl_3$): δ 8.50 (s br, 1H), 8.31 (d, J = 5.6 Hz, 1H), 7.83 (d, J = 8.8 Hz, 2H), 7.72 (d, J = 2.0 Hz, 1H), 7.57 (d, J = 8.4 Hz, 1H), 7.48 (dd, J = 5.6, 2.0 Hz, 1H), 7.34 (dd, J = 8.8, 2.4 Hz, 1H), 7.30 (d, J = 2.4 Hz, 1H),7.05 (d, J = 8.8 Hz, 2H), 4.72 (s, 2H); LC-MS calculated for $C_{20}H_{13}BrCl_2N_2O_3$ $[M+H]^+$: 478.95, found 479.00. LC t_R = 11.40 min, purity: 98%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(2-fluoropyridin-4-yl)acetamide (5d). Compound was synthesized according to general procedure, using the following reagents: 4-amino-2-fluoropyridine (58 mg, 0.515 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (209 mg, 0.566 mmol, 1.10 eq.), DIPEA (180 μL, 1.03 mmol, 2.00 eq.) and PyBroP (288 mg, 0.618 mmol, 1.20 eq.). White solid, 22 mg, 9% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.54 (s br, 1H), 8.15 (d, J = 5.6 Hz, 1H), 7.84 (d, J = 8.8 Hz, 2H), 7.58 (d, J = 8.8 Hz, 2H), 7.42 (d, J = 1.6 Hz, 1H), 7.36–7.30 (m, 3H), 7.06 (d, J = 8.8 Hz, 2H), 4.72 (s, 2H) ppm; LC-MS calculated for $C_{20}H_{13}BrClFN_2O_3$ $[M+H]^+$: 462.98, found 463.05. LC t_R = 11.20 min, purity: 99%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(2-methylpyridin-4-yl)acetamide (5e). Compound was synthesized according to general procedure, using the following reagents: 4-amino-2-methylpyridine (56 mg, 0.515 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (209 mg, 0.566 mmol, 1.10 eq.), DIPEA (180 μL, 1.03 mmol, 2.00 eq.) and PyBroP (288 mg, 0.618 mmol, 1.20 eq.). White solid, 126 mg, 53% yield. ^{1}H NMR (400 MHz $CDCl_3$): δ 8.45 (d, J = 6.0 Hz, 1H), 8.28 (s br, 1H), 7.85 (d, J = 8.8 Hz, 2H), 7.58 (d, J = 8.4 Hz, 1H), 7.48 (d, J = 2.0 Hz, 1H), 7.39–7.33 (m, 2H), 7.32 (d, J = 2.4 Hz, 1H), 7.07 (d, J = 8.8 Hz, 2H), 4.71 (s, 2H), 2.58 (s, 3H) ppm; LC-MS calculated for $C_{21}H_{16}BrClN_2O_3$ $[M+H]^+$: 459.00, found 459.05. LC t_R = 8.92 min, purity: 99%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(3-methylpyridin-4-yl)acetamide (5f). Compound was synthesized according to general procedure, using the following reagents: 4-amino-3-methylpyridine (56 mg, 0.515 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (209 mg, 0.566 mmol, 1.10 eq.), DIPEA (180 μL, 1.03 mmol, 2.00 eq.) and PyBroP (288 mg, 0.618 mmol, 1.20 eq.). White solid, 70 mg, 30% yield. ^{1}H NMR (400 MHz $CDCl_3$): δ 8.45 (d, J = 6.4 Hz, 1H), 8.41 (s, 1H), 8.37 (s br, 1H), 8.21 (d, J = 5.6 Hz, 1H), 7.86 (d, J = 8.8 Hz, 2H), 7.58 (d, J = 8.4 Hz, 1H), 7.34 (dd, J = 8.4, 2.8 Hz, 1H), 7.32 (d, J = 2.4 Hz, 1H), 7.06 (d, J = 9.2 Hz, 2H), 4.75 (s, 2H), 2.26 (s, 3H) ppm; LC-MS calculated for $C_{21}H_{16}BrClN_2O_3$ $[M+H]^+$: 459.00, found 459.00. LC t_R = 7.95 min, purity: 98%.

*2.8. General peptide coupling procedure for 3-cyanoaryl compounds **6a-k***

To a mixture of the appropriately substituted 3-aminobenzonitrile (1.00 eq.) dissolved in DMF (0.10 M) were added 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (1.05 eq.), triethylamine (1.50 eq.) and 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo [4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU) (1.10 eq.), except for compound **6k** which was synthesized using DIPEA and PyBroP. The mixture was stirred under a nitrogen atmosphere at RT overnight, after which TLC showed full conversion of the starting materials. DMF was evaporated *in vacuo*, after which the mixture was dissolved in ethyl acetate, backwashed with $NaHCO_3$ (aq.) followed by brine twice. The resulting organic layer was dried over $MgSO_4$ and evaporated to dryness *in vacuo*. Purification by column chromatography (0 %–3% methanol in chloroform) resulted in the pure desired products **6a-k**.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(3-cyanophenyl)acetamide (6a). Compound was synthesized according to general procedure, using the following reagents: 3-aminobenzonitrile (30 mg, 0.258 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (100 mg, 0.271 mmol, 1.05 eq.), Et_3N (54 μL, 0.387 mmol, 1.50 eq.) and HATU (108 mg, 0.283 mmol, 1.10 eq.). White solid, 73 mg, 60% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.32 (s br, 1H), 8.05–8.02 (m, 1H), 7.85 (d, J = 8.8 Hz, 2H), 7.80 (dt, J = 6.8, 2.0 Hz, 1H), 7.58 (d, J = 8.4 Hz, 1H), 7.51–7.44 (m, 2H), 7.34 (dd, J = 8.4, 2.4 Hz, 1H), 7.31 (d, J = 2.4 Hz, 1H), 7.07 (d, J = 9.2 Hz, 2H), 4.72 (s, 2H) ppm; LC-MS calculated for $C_{22}H_{14}BrClN_2O_3$ $[M-H]^-$: 466.99, found 466.98. LC t_R = 8.79 min, purity: 100%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(3-cyano-4-methoxyphenyl)acetamide (6b). Compound was synthesized according to general procedure, using the following reagents: 5-amino-2-methoxybenzonitrile (74 mg, 0.500 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (194 mg, 0.525 mmol, 1.05 eq.), Et_3N (104 μL, 0.75 mmol, 1.50 eq.) and HATU (209 mg, 0.550 mmol, 1.10 eq.). Off-white solid, 240 mg, 96% yield. ^{1}H NMR (400 MHz CDCl3): δ 8.22 (s br, 1H), 7.85–7.77 (m, 4H), 7.58 (d, J = 8.4 Hz, 1H), 7.35–7.31 (m, 2H), 7.07 (d, J = 8.8 Hz, 2H), 6.97 (d, J = 8.8 Hz, 1H), 4.70 (s, 2H), 3.94 (s, 3H) ppm. LC-MS calculated for $C_{23}H_{16}BrClN_2O_4$ $[M-H]^-$: 497.00, found 496.99. LC t_R = 11.44 min, purity: 99%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(4-chloro-3-cyanophenyl)acetamide (6c). Compound was synthesized according to general procedure, using the following reagents: 5-amino-2-methoxybenzonitrile (77 mg, 0.500 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (194 mg, 0.525 mmol, 1.05 eq.), Et_3N (104 μL, 0.75 mmol, 1.50 eq.) and HATU (209 mg, 0.550 mmol, 1.10 eq.). White solid, 214 mg, 85% yield. ^{1}H NMR (400 MHz DMSO): δ 10.62 (s br, 1H), 8.20 (d, J = 2.4 Hz, 1H), 7.90 (dd, J = 8.8, 2.8 Hz, 1H), 7.77 (d, J = 8.4 Hz, 1H), 7.74–7.70 (m,

3H), 7.61 (d, J = 2.4 Hz, 1H), 7.55 (dd, J = 8.8, 2.4 Hz, 1H), 7.15 (d, J = 9.2 Hz, 2H), 4.89 (s, 2H) ppm; LC-MS calculated for $C_{22}H_{13}BrCl_2N_2O_3$ $[M-H]^-$: 500.95, found 500.94. LC t_R = 11.98 min, purity: 100%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(3-cyano-4-fluorophenyl)acetamide (6d). Compound was synthesized according to general procedure, using the following reagents: 5-amino-2-fluorobenzonitrile (68 mg, 0.500 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (194 mg, 0.525 mmol, 1.05 eq.), Et_3N (104 μL, 0.75 mmol, 1.50 eq.) and HATU (209 mg, 0.550 mmol, 1.10 eq.). Off-white solid, 210 mg, 86% yield. ^{1}H NMR (400 MHz DMSO): δ 10.55 (s, 1H), 8.13 (dd, J = 6.0, 2.4 Hz, 1H), 7.92 (ddd, J = 9.2, 4.8, 2.8 Hz, 1H), 7.77 (d, J = 8.4 Hz, 1H), 7.72 (d, J = 9.2 Hz, 2H), 7.61 (d, J = 2.4 Hz, 1H), 7.57−7.51 (m, 2H), 7.15 (d, J = 9.2 Hz, 2H), 4.88 (s, 2H) ppm; LC-MS calculated for $C_{22}H_{13}BrClFN_2O_3$ $[M-H]^-$: 484.98, found 484.97. LC t_R = 11.66 min, purity: 97%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(3-cyano-4-methylphenyl)acetamide (6e). Compound was synthesized according to general procedure, using the following reagents: 5-amino-2-methylbenzonitrile (68 mg, 0.515 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (200 mg, 0.541 mmol, 1.05 eq.), Et_3N (108 μL, 0.773 mmol, 1.50 eq.) and HATU (216 mg, 0.567 mmol, 1.10 eq.). White solid, 217 mg, 87% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (s br, 1H), 7.94 (d, J = 2.4 Hz, 1H), 7.84 (d, J = 9.2 Hz, 2H), 7.68 (dd, J = 8.4, 2.4 Hz, 1H), 7.57 (d, J = 8.4 Hz, 1H), 7.36−7.28 (m, 3H), 7.07 (d, J = 8.8 Hz, 2H), 4.71 (s, 2H), 2.53 (s, 3H) ppm; LC-MS calculated for $C_{23}H_{16}BrClN_2O_3$ $[M-H]^-$: 481.00, found 481.00. LC t_R = 9.00 min, purity: 99%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(5-cyano-2-methoxyphenyl)acetamide (**6f**). Compound was synthesized according to general procedure, using the following reagents: 3-amino-4-methoxybenzonitrile (58 mg, 0.397 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (154 mg, 0.417 mmol, 1.05 eq.), Et_3N (83 μL, 0.596 mmol, 1.50 eq.) and HATU (166 mg, 0.437 mmol, 1.10 eq.). White solid, 28 mg, 14% yield. ^{1}H NMR (400 MHz $CDCl_3$): δ 8.92 (s br, 1H), 8.77 (d, J = 2.0 Hz, 1H), 7.85 (d, J = 9.2 Hz, 2H), 7.58 (d, J = 8.4 Hz, 1H), 7.43 (dd, J = 8.4, 2.0 Hz, 1H), 7.36−7.30 (m, 2H), 7.06 (d, J = 8.8 Hz, 2H), 6.96 (d, J = 8.4 Hz, 1H), 4.72 (s, 2H), 3.96 (s, 3H) ppm; LC-MS calculated for $C_{23}H_{16}BrClN_2O_4$ $[M+H]^+$: 499.00, found 499.00. LC t_R = 11.83 min, purity: 97%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(2-chloro-5-cyanophenyl)acetamide (6g). Compound was synthesized according to general procedure, using the following reagents: 3-amino-4-chlorobenzonitrile (61 mg, 0.397 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (154 mg, 0.417 mmol, 1.05 eq.), Et_3N (83 μL, 0.596 mmol, 1.50 eq.) and HATU (166 mg, 0.437 mmol, 1.10 eq.). White solid, 30 mg, 19% yield. ^{1}H NMR (400 MHz $CDCl_3$): δ 9.04 (s br, 1H), 8.87 (d, J = 2.0 Hz, 1H), 7.85 (d, J = 8.8 Hz, 2H), 7.58 (d, J = 8.4 Hz, 1H), 7.53 (d, J = 8.0 Hz, 1H), 7.39 (dd, J = 8.4, 2.0 Hz, 1H), 7.36−7.30 (m, 2H), 7.07 (d, J = 9.2 Hz, 2H), 4.77 (s, 2H) ppm; LC-MS calculated for $C_{22}H_{13}BrCl_2N_2O_3$ $[M+H]^+$: 502.95, found 503.00. LC t_R = 12.05 min, purity: 100%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(5-cyano-2-methylphenyl)acetamide (6h). Compound was synthesized according to general procedure, using the following reagents: 3-amino-4-methylbenzonitrile (54 mg, 0.397 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (154 mg, 0.417 mmol, 1.05 eq.), Et_3N (83 μL, 0.596 mmol, 1.50 eq.) and HATU (166 mg, 0.437 mmol, 1.10 eq.). White solid, 28 mg, 14% yield. ^{1}H NMR (400 MHz $CDCl_3$): δ 8.43 (d, J = 1.6 Hz, 1H), 8.25 (s br, 1H), 7.86 (d, J = 8.8 Hz, 2H), 7.58 (d, J = 8.4 Hz, 1H), 7.40 (dd, J = 7.6, 2.0 Hz, 1H), 7.36−7.29 (m, 3H), 7.06 (d, J = 8.8 Hz, 2H), 4.76 (s, 2H), 2.32 (s, 3H) ppm; LC-MS calculated for $C_{23}H_{16}BrClN_2O_3$ $[M+H]^+$: 483.00, found 483.00. LC t_R = 11.54 min, purity: 100%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(3-cyano-2-fluorophenyl)acetamide (6i). Compound was synthesized according to general procedure, using the following reagents: 3-amino-2-fluorobenzonitrile (54 mg, 0.397 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (154 mg, 0.417 mmol, 1.05 eq.), Et_3N (83 μL, 0.596 mmol, 1.50 eq.) and HATU (166 mg, 0.437 mmol, 1.10 eq.). White solid, 79 mg, 40% yield. ^{1}H NMR (400 MHz $CDCl_3$): δ 8.68 (td, J = 8.0, 1.6 Hz, 1H), 8.59 (s br, 1H), 7.85 (d, J = 8.8 Hz, 2H), 7.58 (d, J = 8.4 Hz, 1H), 7.40 (ddd, J = 8.0, 6.0, 2.0 Hz, 1H), 7.36−7.38 (m, 3H), 7.07 (d, J = 9.2 Hz, 2H), 4.75 (s, 2H) ppm; LC-MS calculated for $C_{22}H_{13}BrClFN_2O_3$ $[M+H]^+$: 486.98, found 487.00. LC t_R = 11.58 min, purity: 99%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(3-chloro-5-cyanophenyl)acetamide (6j). Compound was synthesized according to general procedure, using the following reagents: 3-amino-5-chlorobenzonitrile (61 mg, 0.397 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (154 mg, 0.417 mmol, 1.05 eq.), Et_3N (83 μL, 0.596 mmol, 1.50 eq.) and HATU (166 mg, 0.437 mmol, 1.10 eq.). White solid, 49 mg, 25% yield. ^{1}H NMR (400 MHz $CDCl_3$): δ 8.33 (s br, 1H, NH), 7.92 (t, J = 2.0 Hz, 1H), 7.91 (t, J = 2.0 Hz, 1H), 7.86 (d, J = 9.2 Hz, 2H), 7.58 (d, J = 8.4 Hz, 1H), 7.44 (t, J = 1.6 Hz, 1H), 7.35 (dd, J = 8.8, 2.4 Hz, 1H), 7.32 (d, J = 2.4 Hz, 1H), 7.06 (d, J = 8.8 Hz, 2H), 4.72 (s, 2H) ppm; LC-MS calculated for $C_{22}H_{13}BrCl_2N_2O_3$ $[M+H]^+$: 502.95, found 503.15. LC t_R = 12.16 min, purity: 100%.

2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)-*N*-(5-cyanopyridin-3-yl)acetamide (6k). Compound was synthesized according to general procedure, using the following reagents: 5-aminonicotinonitrile (65 mg, 0.546 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (212 mg, 0.573 mmol, 1.05 eq.), DIPEA (141 μL, 1.09 mmol, 2.00 eq.) and PyBroP (305 mg, 0.655 mmol, 1.20 eq.). White solid, 111 mg, 43% yield. ^{1}H NMR (400 MHz $CDCl_3$): δ 8.88 (s br, 1H), 8.73 (s, 1H), 8.68−8.62 (m, 2H), 7.82 (d, J = 8.8 Hz, 2H), 7.57 (d, J = 8.4 Hz, 1H), 7.33 (dd, J = 8.4, 2.4 Hz, 1H), 7.28 (t, J = 2.4 Hz, 1H), 7.07 (d, J = 8.8 Hz, 2H), 4.76 (s, 2H) ppm; LC-MS calculated for $C_{21}H_{13}BrClN_3O_3$ $[M+H]^+$: 469.98, found 470.00. LC t_R = 10.95 min, purity: 99%.

*2.9. General peptide coupling procedure for 3-amino-benzamides **8b-k***

To a solution of the primary amine (**7b-e**) (1.3 eq) and the respective (substituted) 3-aminobenzoic acid (2.0 mmol, 1.0 eq) in DMF were added DIPEA (2.0 eq), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC*HCl) (2.0 eq) and 1-hydroxybenzotriazole (HOBt) (0.15−0.30 eq.) for the preparation of **8b,c,e-i,k**. HATU or PyBrop was used as coupling reagent in the case of products **8d** and **8j** respectively, which required purification by column chromatography afterwards. The mixture was stirred overnight at RT. TLC showed full conversion of the starting materials. DMF was evaporated *in vacuo* where after the mixture was dissolved in ethyl acetate, washed with $NaHCO_3$ solution (aq.) once and with brine twice. The organic layer was dried over $MgSO_4$ and the solvent evaporated *in vacuo*. The resulting compounds **8b-k** were used without further purification in the next reaction. Compound **8a** was obtained from a commercial source.

3-amino-*N*-isobutylbenzamide (8b) [24]. Compound was synthesized according to general procedure, using the following reagents: 2-methylpropan-1-amine (**7b**) (400 μL, 4.00 mmol, 2.00 eq.), 3-aminobenzoic acid (274 mg, 2.00 mmol, 1.00 eq.), DIPEA (700 μL, 4.00 mmol, 2.00 eq.), EDC*HCl (767 mg, 4.00 mm mol, 2.00 eq.), HOBt (81 mg, 0.60 mmol, 0.3 eq.) and DMF (7 mL). Yellow oil, 274 mg, 100% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17 (t, J = 7.6 Hz, 1H), 7.12 (t, J = 1.8 Hz, 1H), 7.05 (dd, J = 7.6, 1.6, 0.4 Hz, 1H), 6.77

(ddd, J = 8.0, 2.4, 1.2 Hz, 1H), 6.31 (s br, 1H), 3.83 (s br, 2H), 3.24 (dd, J = 6.0, 0.8 Hz, 2H), 1.88 (septet, J = 6.8 Hz, 1H), 0.96 (d, J = 6.8 Hz, 6H) ppm. LC-MS calculated for $C_{11}H_{16}N_2O$ $[M+H]^+$: 193.13, found 193.05.

3-amino-*N*-(cyclopropylmethyl)benzamide (8c). Compound was synthesized according to general procedure, using the following reagents: cyclopropylmethanamine (**7c**) (173 μL, 2.00 mmol, 1.33 eq.), 3-aminobenzoic acid (205 mg, 1.50 mmol, 1.00 eq.), DIPEA (609 μL, 3.50 mmol, 2.30 eq.), EDC*HCl (575 mg, 3.00 mm mol, 2.00 eq.), HOBt (27 mg, 0.20 mmol, 0.13 eq.) and DMF (10 mL). Yellow oil, 210 mg, 70% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17 (t, J = 7.6 Hz, 1H), 7.14 (t, J = 2.0 Hz, 1H), 7.08 (dt, J = 7.6, 1.2 Hz, 1H), 6.78 (ddd, J = 8.0, 2.4, 1.0 Hz, 1H), 6.40 (s br, 1H), 3.86 (s br, 2H), 3.27 (dd, J = 5.6, 1.6 Hz, 2H), 1.10–1.00 (m, 1H), 0.56–0.51 (m, 2H), 0.28–0.24 (m, 2H) ppm.

3-amino-*N*-(cyclobutylmethyl)benzamide (8d). Compound was synthesized according to general procedure, using the following reagents: cyclobutylmethanamine (**7d**) (365 μL, 3.00 mmol, 1.50 eq.), 3-aminobenzoic acid (274 mg, 2.00 mmol, 1.00 eq.), DIPEA (700 μL, 4.00 mmol, 2.00 eq.), HATU (1.14 g, 3.00 mm mol, 1.50 eq.) and DMF (7 mL). Yellow oil, 300 mg, 72% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.13 (t, J = 7.6 Hz, 1H), 7.09 (t, J = 2.0 Hz, 1H), 7.06 (dt, J = 7.8, 1.4 Hz, 1H), 6.75 (ddd, J = 7.8, 2.4, 1.0 Hz, 1H), 6.56 (t br, J = 1.6 Hz, 1H), 3.90 (s br, 2H), 3.41 (dd, J = 5.6, 1.6 Hz, 2H), 2.55 (septet, J = 7.6 Hz, 1H), 2.09–2.01 (m, 2H), 1.92–1.84 (m, 2H), 1.76–1.69 (m, 2H) ppm.

3-amino-*N*-cyclopropylbenzamide (8e). [25] Compound was synthesized according to general procedure, using the following reagents: cyclopropanamine (**7e**) (277 μL, 4.00 mmol, 2.00 eq.), 3-aminobenzoic acid (274 mg, 2.00 mmol, 1.00 eq.), DIPEA (700 μL, 4.00 mmol, 2.00 eq.), EDC*HCl (767 mg, 4.00 mm mol, 2.00 eq.), HOBt (81 mg, 0.60 mmol, 0.30 eq.) and DMF (7 mL). Yellow oil, 196 mg, 56% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.17 (t, J = 7.8 Hz, 1H), 7.12 (t, J = 2.0 Hz, 1H), 7.00 (ddd, J = 8.0, 2.0, 0.8 Hz, 1H), 6.78 (ddd, J = 8.0, 2.4, 0.8 Hz, 1H), 6.20 (s br, 1H), 3.79 (s br, 2H), 2.91–2.85 (m, 1H), 0.88–0.82 (m, 2H), 0.62–0.58 (m, 2H) ppm. LC-MS calculated for $C_{10}H_{12}N_2O$ $[M+H]^+$: 177.09, found 177.05.

3-amino-4-chloro-*N*-(cyclopropylmethyl)benzamide (8f). Compound was synthesized according to general procedure, using the following reagents: cyclopropylmethanamine (**7c**) (173 μL, 2.00 mmol, 1.33 eq.), 3-amino-4-chlorobenzoic acid (275 mg, 2.00 mmol, 1.00 eq.), DIPEA (523 μL, 3.00 mmol, 2.00 eq.), EDC*HCl (575 mg, 3.00 mmol, 2.00 eq.), HOBt (30 mg, 0.23 mmol, 0.15 eq.) and DMF (7 mL). Yellow oil, 318 mg, 95% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28–7.25 (m, 2H), 7.00 (dd, J = 8.0, 2.0 Hz, 1H), 6.31 (s br, 1H), 4.24 (s br, 2H), 3.27 (dd, J = 5.6, 1.6 Hz, 2H), 1.09–0.99 (m, 1H), 0.57–0.52 (m, 2H), 0.28–0.24 (m, 2H) ppm.

3-amino-5-chloro-*N*-(cyclopropylmethyl)benzamide (8g). Compound was synthesized according to general procedure, using the following reagents: cyclopropylmethanamine (**7c**) (173 μL, 2.00 mmol, 1.33 eq.), 3-amino-5-chlorobenzoic acid (275 mg, 2.00 mmol, 1.00 eq.), DIPEA (523 μL, 3.00 mmol, 2.00 eq.), EDC*HCl (575 mg, 3.00 mmol, 2.00 eq.), HOBt (30 mg, 0.23 mmol, 0.15 eq.) and DMF (7 mL). Yellow oil, 337 mg, 100% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.02 (t, J = 1.6 Hz, 1H), 6.99 (t, J = 1.6 Hz, 1H), 6.74 (t br, J = 2.0 Hz, 1H), 6.45 (s br, 1H), 3.89 (s br, 2H), 3.26 (dd, J = 5.6, 1.6 Hz, 2H), 1.08–0.95 (m, 1H), 0.56–0.52 (m, 2H), 0.27–0.23 (m, 2H) ppm.

5-amino-2-chloro-*N*-(cyclopropylmethyl)benzamide (8h). Compound was synthesized according to general procedure, using the following reagents: cyclopropylmethanamine (**7c**) (173 μL, 2.00 mmol, 1.33 eq.), 5-amino-2-chlorobenzoic acid (275 mg, 2.00 mmol, 1.00 eq.), DIPEA (523 μL, 3.00 mmol, 2.00 eq.), EDC*HCl (575 mg, 3.00 mmol, 2.00 eq.), HOBt (40 mg, 0.30 mmol, 0.20 eq.) and DMF (7 mL). Yellow oil, 337 mg, 100% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.10 (d, J = 8.8 Hz, 1H), 6.91 (d, J = 2.8 Hz, 1H), 6.62 (dd, J = 8.8, 2.8 Hz, 1H), 6.52 (s br, 1H), 3.94 (s br, 2H), 3.28 (dd, J = 5.6, 1.6 Hz, 2H), 1.10–1.00 (m, 1H), 0.56–0.52 (m, 2H), 0.28–0.24 (m, 2H) ppm.

3-amino-2-chloro-*N*-(cyclopropylmethyl)benzamide (8i). Compound was synthesized according to general procedure, using the following reagents: cyclopropylmethanamine (**7c**) (173 μL, 2.00 mmol, 1.33 eq.), 3-amino-2-chlorobenzoic acid (257 mg, 1.50 mmol, 1.00 eq.), DIPEA (523 μL, 3.00 mmol, 2.00 eq.), EDC*HCl (575 mg, 3.00 mm mol, 2.00 eq.), HOBt (30 mg, 0.23 mmol, 0.15 eq.) and DMF (7 mL). Yellow solid, 342 mg, 100% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.03 (t, J = 8.0 Hz, 1H), 6.81 (dd, J = 7.6, 1.6 Hz, 1H), 6.77 (dd, J = 8.0, 1.2 Hz, 1H), 6.28 (s br, 1H), 4.29 (s br, 2H), 3.26 (dd, J = 5.6, 1.6 Hz, 2H), 1.08–0.95 (m, 1H), 0.54–0.49 (m, 2H), 0.26–0.22 (m, 2H) ppm.

4-amino-*N*-(cyclopropylmethyl)picolinamide (8j). Compound was synthesized according to general procedure, using the following reagents: cyclopropylmethanamine (**7c**) (260 μL, 3.00 mmol, 2.00 eq.), 4-aminopicolinic acid (262 mg, 1.50 mmol, 1.00 eq.), DIPEA (697 μL, 4.00 mmol, 2.70 eq.), PyBroP (1.39 g, 3.00 mmol, 2.00 eq.) and DMF (7 mL). Purified by column chromatography using EtOAc/PE 3:1 as eluent. Yellow oil, 162 mg, 57% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (t br, J = 5.2 Hz, 1H), 8.12 (d, J = 5.6 Hz, 1H), 7.46 (d, J = 2.4 Hz, 1H), 6.63 (dd, J = 5.6, 3.2 Hz, 1H), 4.18 (s br, 2H), 3.29 (dd, J = 6.4, 1.2 Hz, 2H), 1.11–1.01 (m, 1H), 0.57–0.52 (m, 2H), 0.30–0.26 (m, 2H) ppm.

5-amino-*N*-(cyclopropylmethyl)nicotinamide (8k). Compound was synthesized according to general procedure, using the following reagents: cyclopropylmethanamine (**7c**) (173 μL, 2.00 mmol, 1.33 eq.), 5-aminonicotinic acid (207 mg, 1.50 mmol, 1.00 eq.), DIPEA (523 μL, 3.00 mmol, 2.00 eq.), EDC*HCl (575 mg, 3.00 mmol, 2.00 eq.), HOBt (30 mg, 0.23 mmol, 0.15 eq.) and DMF (7 mL). Yellow oil, 125 mg, 43% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.31 (d, J = 2.0 Hz, 1H), 8.13 (d, J = 2.8 Hz, 1H), 7.42 (dd, J = 6.8, 1.2 Hz, 1H), 6.78 (s br, 1H), 4.09 (br s, 2H), 3.29 (dd, J = 5.6, 1.6 Hz, 2H), 1.11–1.01 (m, 1H), 0.57–0.52 (m, 2H), 0.28–0.25 (m, 2H) ppm.

2.10. General procedure of 3-amido-benzamides ***9a-k***

To a solution of arylamides **8a-k** (1.0 eq.) in DMF (7 mL) was added DIPEA (2.00 eq.) and one of the couplings reagents HATU (1.5 eq.) (**9,b,d-f,h,k**) or PyBroP (1.5 eq.) (**9c,j**) or ECD*HCl (2.0 eq.) and HOBt (0.2 eq.) (**9g,i**). The mixture was stirred overnight at RT. TLC showed full conversion of the starting materials into the desired product. DMF was evaporated *in vacuo* where after the mixture was dissolved in ethyl acetate, washed with $NaHCO_3$ once and with brine twice, dried over $MgSO_4$ and evaporated *in vacuo*. The final compounds were purified by column chromatography using an eluens mixture of 0.5%–2% methanol in dichloromethane and subsequent recrystallization from 2-propanol.

3-(2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetamido)-*N*-methylbenzamide (9a). Compound was synthesized according to general procedure, using the following reagents: 3-amino-*N*-methylbenzamide (**8a**) (77 mg, 0.515 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (200 mg, 0.541 mmol, 1.05 eq.), DIPEA (179 μL, 1.03 mmol, 2.00 eq.) and HATU (216 mg, 0.567 mmol, 1.10 eq.). White solid, 207 mg, 80% yield. ^{1}H NMR (400 MHz $CDCl_3$): δ 8.72 (s br, 1H), 7.80 (t, J = 1.6 Hz, 1H), 7.80–7.75 (m, 3H), 7.55 (d, J = 8.8 Hz, 1H), 7.51 (d, J = 8.0 Hz, 1H), 7.36–7.30 (m, 2H), 7.28 (d, J = 2.4 Hz, 1H), 7.01 (d, J = 8.8 Hz, 2H), 6.74 (q, J = 4.4 Hz, 1H), 4.67 (s, 2H), 2.95 (d, J = 4.8 Hz, 3H) ppm; LC-MS calculated for $C_{23}H_{18}BrClN_2O_4$ $[M+H]^+$: 501.01, found 501.00. LC t_R = 7.93 min, purity: 98%.

3-(2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetamido)-*N*-isobutylbenzamide (9b). Compound was synthesized according to general procedure, using the following reagents: 3-amino-*N*-

isobutylbenzamide (**8b**) (274 mg, 2.00 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (813 mg, 2.20 mmol, 1.1 eq.), DIPEA (700 μL, 4.00 mmol, 2.00 eq.), HATU (1.14 g, 3.00 mmol, 1.50 eq.) and DMF 8 mL. White solid, 91 mg, 8% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.44 (s, 1H), 7.95 (s, 1H), 7.87–7.82 (m, 3H), 7.58 (d, J = 8.4 Hz, 1H), 7.53 (dt, J = 8.0, 1.2 Hz, 1H), 7.41 (t, J = 8.0 Hz, 1H), 7.35–7.31 (m, 2H), 7.06 (d, J = 8.8 Hz, 2H), 6.35 (t, J = 6.0 Hz, 1H), 4.70 (s, 2H), 3.28 (t, J = 6.6 Hz, 2H), 1.90 (nonet, J = 6.8 Hz, 1H), 0.97 (d, J = 6.8 Hz, 6H) ppm; LC-MS calculated for $C_{26}H_{24}BrClN_2O_4$ $[M+H]^+$: 543.06, found 543.10. LC t_R = 11.50 min, purity: 100%.

3-(2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetamido)-*N*-(cyclopropylmethyl)benzamide (9c). Compound was synthesized according to general procedure, using the following reagents: 3-amino-*N*-(cyclopropylmethyl)benzamide (**8c**) (201 mg, 1.06 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy) acetic acid (**4**) (431 mg, 1.17 mmol, 1.1 eq.), DIPEA (369 μL, 2.12 mmol, 2.00 eq.), PyBroP (741 mg, 1.59 mmol, 1.50 eq.) and DMF 8 mL. White solid, 455 mg, 79% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (s br, 1H), 7.94 (t, J = 2.0 Hz, 1H), 7.90–7.87 (m, 1H), 7.85 (d, J = 8.8 Hz, 2H), 7.59–7.54 (m, 2H), 7.44 (t, J = 8.0 Hz, 1H), 7.35–7.31 (m, 2H), 7.07 (J = 8.8 Hz, 2H), 6.30 (s br, 1H), 4.71 (s, 2H), 3.32 (dd, J = 5.6, 1.6 Hz, 2H), 1.12–1.02 (m, 1H), 0.59–0.54 (m, 2H), 0.30–0.26 (m, 2H) ppm; LC-MS calculated for $C_{26}H_{22}BrClN_2O_4$ $[M+H]^+$: 541.05, found 541.20. LC t_R = 11.26 min, purity: 100%.

3-(2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetamido)-*N*-(cyclobutylmethyl)benzamide (9d). Compound was synthesized according to general procedure, using the following reagents: 3-amino-*N*-(cyclobutylmethyl)benzamide (**8d**) (300 mg, 1.47 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (598 mg, 1.62 mmol, 1.10 eq.), DIPEA (512 μL, 2.94 mmol, 2.00 eq.), HATU (838 mg, 2.21 mmol, 1.50 eq.) and DMF (8 mL). White solid, 112 mg, 14% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (s br, 1H), 7.93 (t, J = 1.8 Hz, 1H), 7.88–7.82 (m, 3H), 7.58 (d, 8.4 Hz, 1H), 7.51 (dt, J = 8.0, 1.2 Hz, 1H), 7.41 (t, J = 8.0 Hz, 1H), 7.35–7.31 (m, 2H), 7.06 (d, J = 8.8 Hz, 2H), 6.24 (t, J = 1.8 Hz, 1H), 4.70 (s, 2H), 3.50–3.46 (m, 2H), 2.58 (septet, J = 7.6 Hz, 1H), 2.14–2.06 (m, 2H), 1.97–1.86 (m, 2H), 1.80–1.70 (m, 2H) ppm; HPLC t_R = 11.67 min, purity: 99%; LC-MS calculated for $C_{27}H_{24}BrClN_2O_4$ $[M+H]^+$: 555.06, found 555.15. LC t_R = 11.67 min, purity: 99%.

3-(2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetamido)-*N*-cyclopropylbenzamide (9e). Compound was synthesized according to general procedure, using the following reagents: 3-amino-*N*-cyclopropylbenzamide (**8e**) (196 mg, 1.11 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetic acid (**4**) (492 mg, 1.33 mmol, 1.20 eq.), DIPEA (387 μL, 2.22 mmol, 2.00 eq.), HATU (635 mg, 1.67 mmol, 1.50 eq.) and DMF (8 mL). White solid, 65 mg, 11% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (s br, 1H), 7.92 (t, J = 2.0 Hz, 1H), 7.87–7.83 (m, 3H), 7.58 (d, J = 8.4 Hz, 1H), 7.51 (dt, J = 7.6, 1.4 Hz, 1H), 7.43 (t, J = 8.0 Hz, 1H), 7.36–7.32 (m, 2H), 7.07 (d, J = 8.8 Hz, 2H), 6.30 (s br, 1H), 4.70 (s, 2H), 2.94–2.87 (m, 1H), 0.91–0.86 (m, 2H), 0.66–0.62 (m, 2H) ppm; LC-MS calculated for $C_{25}H_{20}BrClN_2O_4$ $[M+H]^+$: 527.03, found 527.05. LC t_R = 10.87 min, purity: 97%.

3-(2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetamido)-4-chloro-*N*-(cyclopropylmethyl)benzamide (9f). Compound was synthesized according to general procedure, using the following reagents: 3-amino-4-chloro-*N*-cyclopropylbenzamide (**8f**) (318 mg, 1.42 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy) acetic acid (**4**) (523 mg, 1.42 mmol, 1.00 eq.), DIPEA (500 μL, 2.84 mmol, 2.00 eq.), HATU (1.08 g, 2.84 mmol, 2.00 eq.) and DMF (7 mL). White solid, 96 mg, 12% yield. ^{1}H NMR (400 MHz, DMSO) δ 9.94 (s, 1H), 8.68 (t, J = 5.6 Hz, 1H), 8.20 (d, J = 2.0 Hz, 1H), 7.78 (d, J = 8.4 Hz, 1H), 7.75–7.70 (m, 3H), 7.64–7.62 (m, 2H), 7.56 (dd, J = 8.4, 2.4 Hz, 1H), 7.18 (d, J = 9.2 Hz, 2H), 4.95 (s, 2H), 3.12 (t, J = 6.0 Hz, 2H), 1.04–0.98 (m, 1H), 0.44–0.40 (m, 2H), 0.23–0.20 (m, 2H) ppm; LC-MS calculated for $C_{26}H_{21}BrCl_2N_2O_4$ $[M+H]^+$: 575.01, found 575.05. LC t_R = 11.87 min, purity: 99%.

3-(2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetamido)-5-chloro-*N*-(cyclopropylmethyl)benzamide (9g). Compound was synthesized according to general procedure, using the following reagents: 3-amino-5-chloro-*N*-cyclopropylbenzamide (**8g**) (337 mg, 1.50 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl) phenoxy)acetic acid (**4**) (554 mg, 1.50 mmol, 1.00 eq.), DIPEA (523 μL, 3.00 mmol, 2.00 eq.), EDC*HCl (575 mg, 3.00 mmol, 2.00 eq.), HOBt (41 mg, 0.30, 0.20 eq.) and DMF (7 mL). White solid, 93 mg, 11% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.68 (s, 1H), 7.94 (t, J = 2.0 Hz, 1H), 7.82–7.78 (m, 3H), 7.56 (d, J = 8.8 Hz, 1H), 7.48 (t, J = 1.6 Hz, 1H), 7.34–7.28 (m, 2H), 7.03 (d, J = 8.8 Hz, 2H), 6.58 (t, J = 5.4 Hz, 1H), 4.69 (s, 2H), 3.29–3.26 (m, 2H), 1.09–0.99 (m, 1H), 0.58–0.50 (m, 2H), 0.28–0.24 (m, 2H) ppm; LC-MS calculated for $C_{26}H_{21}BrCl_2N_2O_4$ $[M+H]^+$: 575.01, found 575.05. LC t_R = 11.94 min, purity: 95%.

5-(2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetamido)-2-chloro-*N*-(cyclopropylmethyl)benzamide (9h). Compound was synthesized according to general procedure, using the following reagents: 5-amino-2-chloro-*N*-cyclopropylbenzamide (**8h**) (337 mg, 1.50 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl) phenoxy)acetic acid (**4**) (554 mg, 1.50 mmol, 1.00 eq.), DIPEA (523 μL, 3.00 mmol, 2.00 eq.), HATU (1.14 g, 3.00 mmol, 2.00 eq.) and DMF (7 mL). White solid, 175 mg, 20% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.35 (s, 1H), 7.90 (dd, J = 8.8, 2.8 Hz, 1H), 7.85 (d, J = 8.8 Hz, 2H), 7.75 (d, J = 2.8 Hz, 1H), 7.58 (d, J = 8.4 Hz, 1H), 7.40 (d, J = 8.8 Hz, 1H), 7.36–7.32 (m, 2H), 7.06 (d, J = 8.8 Hz, 2H), 6.46 (t, J = 1.8 Hz, 1H), 4.69 (s, 2H), 3.36–3.33 (m, 2H), 1.12–1.04 (m, 1H), 0.60–0.55 (m, 2H), 0.31–0.27 (m, 2H) ppm; LC-MS calculated for $C_{26}H_{21}BrCl_2N_2O_4$ $[M+H]^+$: 575.01, found 575.05. LC t_R = 11.52 min, purity: 99%.

3-(2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetamido)-2-chloro-*N*-(cyclopropylmethyl)benzamide (9i). Compound was synthesized according to general procedure, using the following reagents: 3-amino-2-chloro-*N*-cyclopropylbenzamide (**8i**) (109 mg, 0.48 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy) acetic acid (**4**) (214 mg, 0.58 mmol, 1.20 eq.), DIPEA (167 μL, 0.96 mmol, 2.00 eq.), EDC*HCl (184 mg, 0.96 mmol, 2.00 eq.), HOBt (13 mg, 0.096 mmol, 0.20 eq.) and DMF (7 mL). White solid, 38 mg, 14% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.02 (s br, 1H), 8.51 (dd, J = 8.2, 1.8 Hz, 1H), 7.85 (d, J = 8.8 Hz, 2H), 7.58 (d, J = 8.0 Hz, 1H), 7.38–7.29 (m, 4H), 7.07 (d, J = 8.8 Hz, 2H), 5.99 (t, J = 5.4 Hz, 1H), 4.75 (s, 2H), 3.35–3.32 (m, 2H), 1.11–1.02 (m, 1H), 0.60–0.55 (m, 2H), 0.31–0.27 (m, 2H) ppm; LC-MS calculated for $C_{26}H_{21}BrCl_2N_2O_4$ $[M+H]^+$: 575.01, found 575.05. LC t_R = 11.43 min, purity: 98%.

4-(2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetamido)-*N*-(cyclopropylmethyl)picolinamide (9j). Compound was synthesized according to general procedure, using the following reagents: 4-amino-*N*-(cyclopropylmethyl)picolinamide (**8j**) (162 mg, 0.85 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy) acetic acid (**4**) (377 mg, 1.02 mmol, 1.20 eq.), DIPEA (296 μL, 1.70 mmol, 2.00 eq.), PyBroP (600 mg, 1.28 mmol, 1.50 eq.) and DMF (7 mL). Yellow solid, 9.5 mg, 2% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (s br, 1H), 8.53 (d, J = 5.6 Hz, 1H), 8.26 (dd, J = 5.6, 2.4 Hz, 1H), 8.14 (t br, J = 5.6 Hz, 1H), 7.94 (d, J = 2.0 Hz, 1H), 7.86 (d, J = 8.8 Hz, 2H), 7.58 (d, J = 8.4 Hz, 1H), 7.36–7.32 (m, 2H), 7.07 (d, J = 8.8 Hz, 2H), 4.73 (s, 2H), 3.36–3.32 (m, 2H), 1.11–1.04 (m, 1H), 0.59–0.55 (m, 2H), 0.32–0.28 (m, 2H) ppm; LC-MS calculated for $C_{25}H_{21}BrClN_3O_4$ $[M+H]^+$: 542.04, found 542.10. LC t_R = 11.56 min, purity: 98%.

5-(2-(4-(2-bromo-5-chlorobenzoyl)phenoxy)acetamido)-*N*-(cyclopropylmethyl)nicotinamide (9k). Compound was synthesized according to general procedure, using the following reagents:

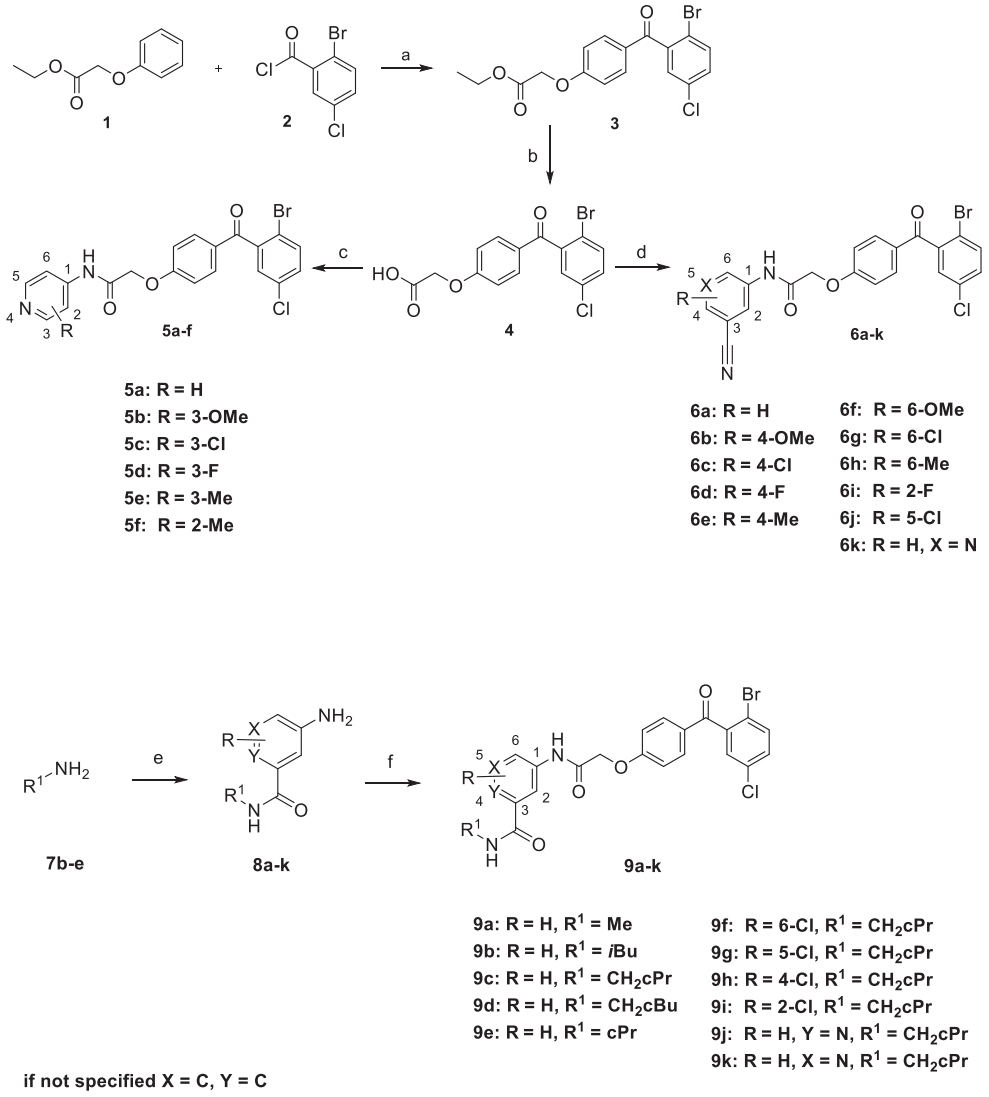
5-amino-*N*-(cyclopropylmethyl)nicotinamide (**8k**) (125 mg, 0.65 mmol, 1.00 eq.), 2-(4-(2-bromo-5-chlorobenzoyl)phenoxy) acetic acid (**4**) (240 mg, 0.65 mmol, 1.00 eq.), DIPEA (227 μL, 1.30 mmol, 2.00 eq.), HATU (371 mg, 0.98 mmol, 1.50 eq.) and DMF (7 mL). White solid precipitated from CH_2Cl_2, 170 mg, 48% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.96 (d, J = 2.4 Hz, 1H), 8.81 (d, J = 1.6 Hz, 1H), 8.48 (t, J = 2.2 Hz, 1H), 8.44 (s, 1H), 7.86 (d, J = 9.2 Hz, 2H), 7.58 (dd, J = 8.4, 0.8 Hz, 1H), 7.36–7.32 (m, 3H), 7.08 (d, J = 9.2 Hz, 2H), 6.37 (s br, 1H), 4.75 (s, 2H), 3.36–3.33 (m, 2H), 1.13–1.03 (m, 1H), 0.61–0.57 (m, 2H), 0.32–0.28 (m, 2H) ppm; LC-MS calculated for $C_{25}H_{21}BrClN_3O_4$ $[M+H]^+$: 542.04, found 542.10. LC t_R = 10.69 min, purity: 99%.

3. Results

In two earlier papers we developed a series of substituted benzophenones, in which LUF7346 (Fig. 1) stood out in terms of affinity for the K_v11.1 channel and potency in reducing both genetic and drug-induced long QT syndrome [14,18]. In subsequent experiments, we learned that a further improvement could be obtained by replacing the 2-bromophenyl substituent in the benzophenone moiety by a 2-bromo, 5-chlorophenyl substituent (LUF7757, Fig. 1). Therefore, we used the latter compound as the starting point for our current study, in which we aimed at altering the 3-pyridine moiety of both LUF7346 and LUF7757 to charter new chemical space.

3.1. Chemistry

The synthetic route towards final compounds **5a-f**, **6a-k** and **9a-k** is depicted in Scheme 1. A similar approach as in the synthesis of LUF7346 was used, but the number of reaction steps was reduced by starting from the commercially available ethyl 2-phenoxyacetate (**1**) instead of anisol [14]. A Friedel-Crafts acylation of ethyl 2-phenoxyacetate (**1**) and 2-bromo-5-chlorobenzoyl chloride (**2**), obtained by refluxing 2-bromo-5-chlorobenzoic acid in thionyl



Scheme 1. Synthesis of negative allosteric modulators; series 5a-f, 6a-k and 9a-k.
Reagents and conditions: (a) (i) 2-bromo-5-chlorobenzoic acid, $SOCl_2$, reflux, 2 h; (ii) **1**, $AlCl_3$, CH_2Cl_2, rt, overnight; (b) LiOH, THF/MeOH/H_2O, rt, 1 h; (c) substituted 4-aminopyridine, DIPEA, PyBroP, DMF, rt, overnight or substituted 4-aminopyridine, Et_3N, HATU, DMF, rt, overnight; (d) substituted 3-aminobenzonitrile, Et_3N, HATU, DMF, rt, overnight; (e) substituted 3-aminobenzoic acid, DIPEA, EDC*HCl, HOBt, DMF, rt, overnight; (f) **4**, DIPEA, HATU or PyBroP, DMF, rt, overnight or **4**, DIPEA, EDC*HCl, HOBt DMF, rt, overnight.

chloride, resulted in ester **3** in 65% yield. Hydrolysis of ester **3** with an 1 M aqueous LiOH solution at room temperature gave carboxylic acid **4** as a precipitate in a high yield after acidic aqueous work-up. Starting from carboxylic acid **4** three series of final compounds **6a-k, 5a-f** and **9a-k** were obtained via peptide coupling reactions using various conditions. Compounds **6a-k** were synthesized using 3-amino-(substituted) benzonitriles, HATU and Et_3N conditions with yields between 14 and 96%. However, the 4-pyridine series (**5a-f**) could not be synthesized using HATU as the coupling reagent, except for the unsubstituted 4-pyridine derivative **5a** in 75% yield. Nonetheless the desired products **5a-f** were obtained in 6–53% yield using PyBroP and DIPEA as the base. As for the third series, amide formation between 3-aminobenzoic acid and the respective alkylamines **7b-e**, using EDC*HCl as coupling reagent, resulted in intermediate 3-amino-*N*-alkylbenzamides (**8b-k**). These were coupled to carboxylic acid **4,** resulting in the final benzamides **9a-k**. HATU (**9a,b,d-f,h,k**), PyBroP (**9c,j**) or EDC*HCl (**9g,i**) were used as coupling reagents with yields in the 10–20% range.

3.2. Biology

3.2.1. Radioligand binding studies

All compounds were initially tested in a single point radioligand binding assay. HEK293 cell membranes expressing the $K_v11.1$ channel were incubated with the radiolabeled hERG blocker [^{3}H] dofetilide (approx. 5 nM) in the absence or presence of each of the newly synthesized compounds (**5a-f**, **6a-k**, **9a-k**) at a concentration of 10 μM. In this assay reference compound LUF7346, and LUF7757 displaced approx. 70% of [^{3}H]dofetilide binding (Table 1, last column), while quite a few of the newly synthesized derivatives were more effective in doing so.

Those compounds that displaced more than 75% of the radioligand (SB approx. ≤25% in Table 1) were subjected to a more comprehensive radioligand displacement assay in which the IC_{50} value of unlabeled dofetilide in the absence or presence of a compound was determined (Fig. 2).

Some of the compounds were tested at a concentration of 3, 1 or 0.5 μM rather than 10 μM, as the latter concentration reduced specific [^{3}H]dofetilide binding to such low values that a displacement curve could not be recorded, an indication of the higher efficacy of these compounds. In all three series, highly active chemical entities were identified, such as **5a**, **5e**, **5f** (4-pyridines), **6a**, **6d**, **6h**, **6i**, **6k** (3-cyano derivatives) and **9e**, **9h**, **9i**, **9k** (3-amido derivatives). In particular, compounds **5e**, **6a/k** and **9i/k** reduced the affinity of dofetilide for the $K_v11.1$ channel more prominently than reference LUF7346 (3.9-fold at 10 μM).

A concentration range of **6k** was also tested in a [^{3}H]dofetilide displacement assay (Fig. 3). The IC_{50} value of **6k** was 2.7 ± 0.3 μM, while the steepness of the displacement curve was characterized by a pseudo-Hill coefficient (n_H) of 1.7; this value was significantly different from unlabeled dofetilide ($n_H = 1.0$).

3.2.2. Compound effects on cardiomyocytes derived from hiPSCs

Fig. 4 illustrates the concentration-effect curves for each compound, in the absence or presence of the hERG channel blocker dofetilide. While reference compound LUF7346 caused a concentration-dependent shortening of FPD, both **5e** and **9k** caused FPD prolongation and **6k** had no effect (Fig. 4A, top). Except for **5e,**

Table 1
Reduction of dofetilide's hERG channel affinity by compound classes **5**, **6** and **9**.

Compound	$IC_{50,dofetilide}$ (nM)	fold[b]	SB at 10 μM (%)[c]
Control (dofetilide)	11.7 ± 1.2 (A) or 9.1 ± 0.1 (B)[a]	1.0	(100)
+ **LUF7346**	35.6 ± 3.2	3.9 (B, 10 μM)	29.8 ± 0.1
+ **LUF7757**	34.7 ± 6.9	3.8 (B, 3 μM)	28.2 ± 1.6
+ **5a**	43.7 ± 2.6	3.7 (A, 10 μM)	26.1 ± 1.5
+ **5b**	–	–	90 (84, 95)
+ **5c**	–	–	52 (51, 53)
+ **5d**	17.6 ± 2.5	1.5 (A, 10 μM)	56.1 ± 1.3
+ **5e**	59.7 ± 1.6	6.6 (B, 3 μM)	9 (7, 11)
+ **5f**	14.1 ± 0.8	1.6 (B, 3 μM)	15 (15, 15)
+ **6a**	28.3 ± 6.9	2.4 (A, 1 μM)	20 (20,19)
+ **6b**	–	–	60 (49, 71)
+ **6c**	–	–	86 (81, 92)
+ **6d**	69.5 ± 6.2	5.9 (A, 10 μM)	22.7 ± 0.6
+ **6e**	–	–	87 (81, 92)
+ **6f**	–	–	28 (28, 27)
+ **6g**	–	–	44 (46, 42)
+ **6h**	12.8 ± 1.0	1.4 (B, 3 μM)	23 (23, 24)
+ **6i**	12.7 ± 1.7	1.4 (B, 3 μM)	21 (26, 16)
+ **6j**	–	–	36 (38, 34)
+ **6k**	18.9 ± 0.4	2.1 (B, 3 μM)	8 (8, 9)
+ **9a**	22.0 ± 2.8	1.9 (A, 10 μM)	39.8 ± 2.2
+ **9b**	–	–	46 (42, 49)
+ **9c**	26.0 ± 8.4	2.2 (A, 10 μM)	36.6 ± 3.3
+ **9d**	–	–	38 (32, 44)
+ **9e**	11.2 ± 0.6	1.2 (B, 3 μM)	27 (28, 25)
.+ **9f**	–	–	89 (86, 91)
+ **9g**	–	–	36 (35, 37)
+ **9h**	17.8 ± 1.3	2.0 (B, 3 μM)	20 (19, 21)
+ **9i**	17.5 ± 3.7	1.9 (B, 0.5 μM)	−2 (−2, −2)
+ **9j**	–	–	53 (55, 50)
+ **9k**	19.5 ± 1.6	2.1 (B, 1 μM)	2 (3, 2)

[a] Two rounds of experiments (A and B) were performed, with slightly different IC_{50} values for dofetilide.
[b] For every compound tested A or B is indicated, together with the concentration of the test compound used; fold = IC_{50} (dofetilide + test compound)/IC_{50} (dofetilide).
[c] Specific radioligand binding (SB) remaining (%) at a concentration of 10 μM of each test compound. In some cases only two experiments were done (in duplicate), yielding a mean value with the two independent values in parentheses. Otherwise: n = 3.

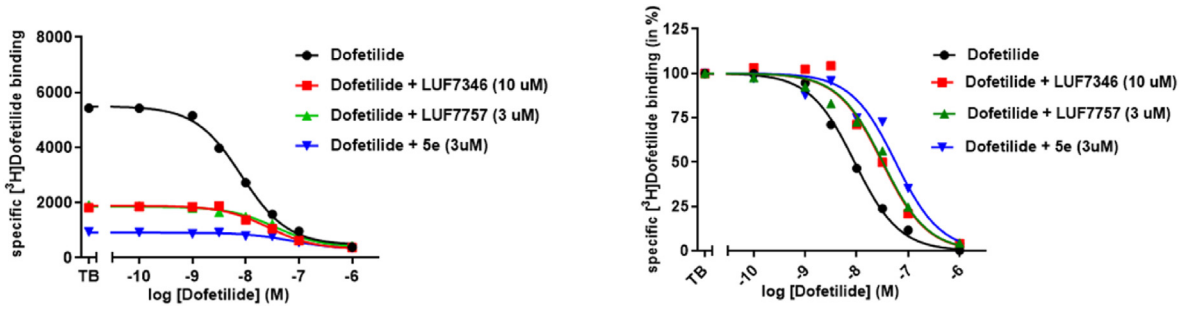


Fig. 2. Displacement curves ([^{3}H]dofetilide/dofetilide) in the absence and presence of LUF7346 and two novel allosteric modulators. left: Y-axis: disintegrations per minute (dpm); right: Y-axis: percentage displacement of normalized specific [^{3}H]dofetilide binding. Data from one representative experiment.

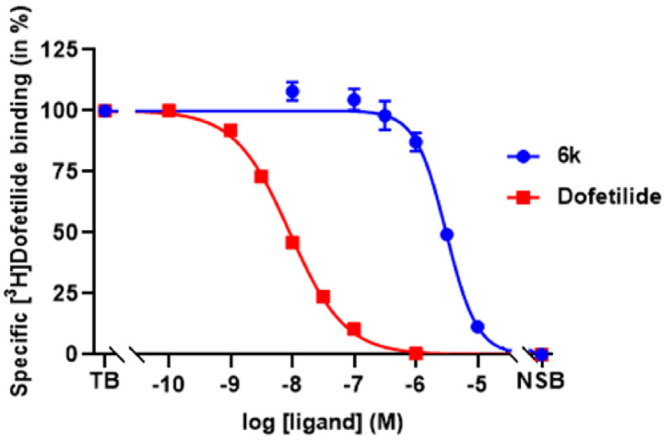


Fig. 3. Displacement of specific [^{3}H]dofetilide binding by dofetilide and 6k (n=3). NSB: non-specific binding.

high concentrations of all compounds slightly reduced the hiPSC-CM beating rate, shown as RR interval variation (Fig. 4A, bottom). Compound 9k at 30 μM caused hiPSC-CMs to completely stop beating. The reference compound at 10, 20 and 30 μM was able to revert the effect of dofetilide by shortening the FPD without affecting RR interval, while addition of **5e** or **6k** in the presence of dofetilide did not further modify FPD nor RR interval (Fig. 4B).

3.2.3. Compound effects on hERG current

Intrigued by the results on hiPSC-CMs, we performed patch clamp studies on HEK293 cells stably expressing the K_v11.1 channel to investigate the effects of the compounds (5 μM) on hERG current density in particular. Fig. 5A shows representative traces of normalized hERG tail currents before and after addition of the compounds. While application of reference compound LUF7346 resulted in an increase in current density when compared to baseline as previously shown [18], the new compounds caused a decrease in hERG current density (Fig. 5B).

4. Discussion and conclusions

Over the last several years a number of drugs have been withdrawn from the market due to cardiovascular safety concerns. Their pro-arrhythmogenic characteristics, potentially resulting in lethal Torsade de Pointes (TdP) in the worst case, have been the reason for these market actions instigated by both regulatory agencies and the pharmaceutical industry [26]. The main molecular mechanism behind these serious side effects is the interaction of a wide range of drugs and clinical candidates with the so-called ether-a-go-go-related gene (hERG) channel, better defined as the voltage-gated channel (K_v11.1) conducting the rapid component of the cardiac repolarizing potassium current (I_{Kr}) [2,6]. Although it is now realized that hERG blockade is not necessarily the only reason for cardiotoxic effects, this drug anti-target became the focus of early regulatory guidelines such as ICH S7B, requiring a mandatory test for hERG activity of every new chemical entity (NCE) to proceed into clinical studies. It has been estimated that, as a consequence of this and later guidelines (ICH E14) [27], development of up to 30% of NCEs is often halted because of blockade, albeit modest, of the hERG channel [26]. Torsade de Pointes is a very rare side effect and therefore it has been questioned whether excluding compounds "flagged" for hERG activity is not too rigorous. In that context, counteracting the hERG activity of otherwise useful and potentially life-saving drug molecules may be worthwhile. We and others have shown that negative allosteric modulators or, alternatively termed, activators of the hERG channel may be such compounds, the first of which, RPR260243 and PD-118057, were reported in 2005 [28,29]. Two later compounds, ML-T531 [30] and VU0405601 [12], fueled our interest, and constituted the starting point for our medicinal chemistry efforts. As a result, two molecules emerging from our synthetic program, LUF7244 [15,16] and LUF7346 [18], were further profiled for their anti-arrhythmic activity in dogs and on human stem cell-derived cardiomyocytes, either in the absence or presence of reference hERG blockers dofetilide or astemizole. Both NAMs appeared to reduce the affinity of the hERG blockers by increasing their rate of dissociation from the hERG channel, a clear proof of their allosteric modulation. As an example, LUF7346 at a concentration of 10 μM caused a 6.3-fold decrease in astemizole's hERG affinity [14].

4.1. Radioligand binding studies

With this benchmark in mind, we aimed at synthesizing and evaluating further LUF7346 derivatives that would act similarly but at lower concentrations. Our starting point for the synthetic endeavors was LUF7757, the slightly more potent, close analog of LUF7346 (Fig. 1, Table 1). It displayed similar activity as LUF7346 but at 3 μM rather than at 10 μM. By focusing on the "left hand" side of the molecule, we replaced the 3-pyridyl moiety by other scaffolds, such as a 3-cyanophenyl, 3-carboxamidophenyl and 4-pyridyl. It turned out that quite a few of the novel derivatives were more potent than both LUF7346 and LUF7757 in allosterically modulating the affinity of dofetilide. Some of the derivatives were active at 1 μM (**6a**, **9k**) or even at 0.5 μM (**9i**). The allosteric nature of one of the more potent compounds, **6k**, was further assessed by examining its displacement of [^{3}H]dofetilide binding in more detail (Fig. 3). The increased steepness of the displacement curve compared to the one of dofetilide (i.e. a regular hERG blocker) is yet

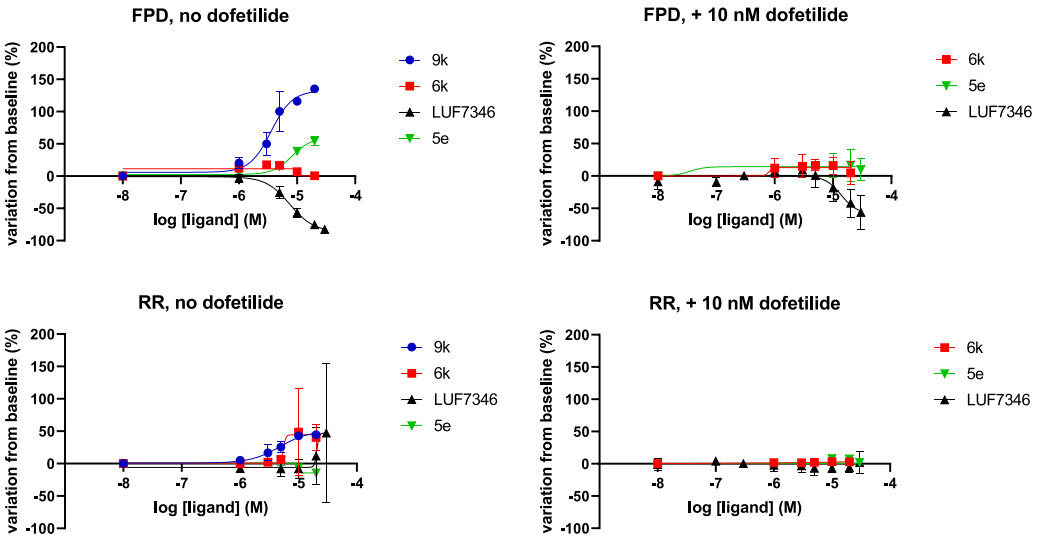


Fig. 4. Compound effects in hiPSC-CMs. (A) Concentration-effect curves showing field potential duration (FPD, top) and RR interval (RR, bottom) variations in hiPSC-CMs after administration of increasing cumulative doses of the compounds. (B) Concentration-effect curves as in panel A, but after pre-incubation of hiPSC-CMs with 10 nM dofetilide. Dofetilide (10 nM) alone caused a slight reduction in RR (−14.0 ± 3.7%) and an increase in FPD (33.4 ± 5.8%) compared to baseline. Data are shown as mean ± SEM, n ≥ 3.

another indication of the allosteric characteristics of **6k**. Also the chemical nature of the novel compounds, lacking a protonated amino group at physiological pH, points to a binding site on the hERG channel that is different from the common hERG blockers. A similar conclusion for similar compounds was drawn in a recent review article [31] addressing the structural insights obtained from the recent elucidation of the cryo-EM structure of the hERG channel [32].

4.2. Electrophysiology

These data encouraged us to test a number of these molecules in a more physiologically relevant cellular context. For this purpose, we employed hiPSC-CMs and studied their electrophysiological properties using MEAs. As mentioned before, molecules that alter hERG channel activity have an impact on the QT-interval of the electrocardiogram (ECG) in humans [33], which is reflected *in vitro* in the FPD. As a reference we used LUF7346, which had previously been demonstrated to shorten the FPD in both healthy and diseased (Long QT syndrome) hiPSC-CMs and to counteract the FPD prolongation due to hERG channel block [18]. First, we learned that solubility issues prevented the analysis of some of the new derivatives in this experimental setup, including LUF7757. Secondly, and to our surprise, many molecules behaved very differently from LUF7346, when representatives of the three different classes were examined in more detail (**5e**, **6k**, and **9k**) for their effects on FPD and RR variations. The reasons for

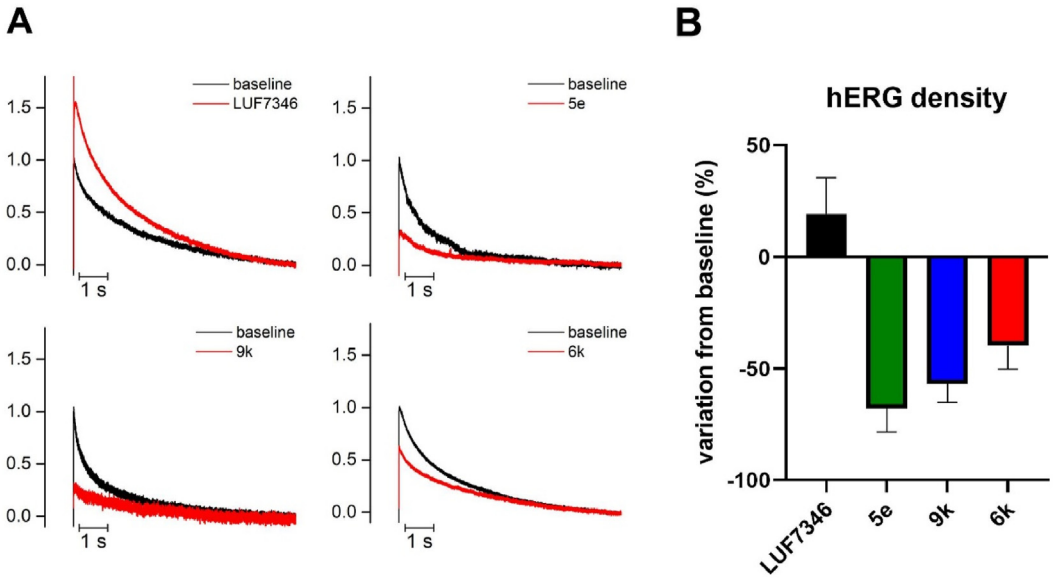


Fig. 5. Compound effects on hERG current. (A) Representative traces recorded in HEK293Kv11.1 cells of hERG peak tail currents at −60 mV, normalized to baseline peak current intensity. Black traces were recorded in Tyrode (baseline) and red traces in Tyrode + 5 μM compound, as indicated. (B) Bar graph showing hERG peak tail current density variation at −60 mV compared to the baseline after administration of the different compounds (5 μM). Data shown as mean ± SEM, n = 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the different behavior compared to LUF7346 are unknown. One possibility is that the novel molecules induce yet another conformational change in the hERG channel, leading to the differential effects. Alternatively, the molecules interact with other cardiac ion channels that influence cardiomyocyte action and field potential parameters. As an example, the hERG channel activator/NAM NS1643 also interacted with calcium and other ion channels [8]. These findings also led us to reflect further and examine the compounds in a patch clamp setup of HEK293 cells expressing the hERG channel. The three novel compounds all blocked channel activity by reducing current density, while LUF7346 acted as a potentiator of current density (Fig. 5A and B). The former effect, i.e. a reduced tail current in the voltage protocol for hERG activation, is typical of standard hERG blockers such as dofetilide [34].

In conclusion, new derivatives of LUF7346 were synthesized to charter unexplored chemical space. In *in vitro* radioligand binding experiments on the hERG channel, many of these proved superior to LUF7346. Surprisingly, in a system better recapitulating the cardiac cellular electrophysiology of human cardiomyocytes in which some of the new molecules were examined, divergent behavior was observed, reflecting a block of the hERG channel, which was subsequently verified in a patch clamp study on HEK293 cells expressing the hERG channel. This precludes the compounds' potential application in symptoms such as long QT syndrome. Altogether, our results demonstrate that chemical modifications of known molecular entities with allosteric modulator activity on the hERG channel represent a realistic approach for identifying more potent molecules. However, it is important to test their biological significance in cardiac-relevant physiological systems *in vitro* and, ultimately, *in vivo*.

The search for more active molecules that reduce QT-prolongation induced by hERG blockers but are "silent" when applied alone, is still open.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Christine Mummery is a cofounder of Pluriomics B.V. (now NCardia B.V.).

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