Supporting Information

Supplementary Methods

Recombinant expression and purification of calmodulin

Rat calmodulin (CaM) (GenBank accession: AAD55398.1) was sub-cloned between 5′ NcoI and 3′ HindIII sites into a pETGQ vector (1), devoid of an N-terminal hexa-histidine affinity tag and a tobacco etch virus protease (TEV) cleavage site. The T79D mutation was introduced into this construct using the QuickChange approach. CaM expression and purification were performed following a published procedure with modifications (2). Briefly, proteins were expressed in T7 Express E. coli cells (New England Biolabs, Ipswich, MA, USA) cultured in terrific broth (Formedium, Norfolk, UK) by induction with 0.25 mM isopropyl β-D-1-thiogalactopyranoside (Formedium, Norfolk, UK) overnight at 16 °C. Cells were harvested by centrifugation (6500 g, 10 minutes) and cell pellets were kept at -80 °C until use. For protein purification, pellets were resuspended in buffer A (2 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris-HCl pH 7.5), supplemented with 1 mM phenylmethane sulfonyl fluoride (Sigma-Aldrich, St. Louis, Missouri, United States) and 10 mg lysozyme (Fisher Scientific, Hampton, NH, USA). Cells were lysed with an EmulsiFlex C-3 homogenizer (Avestin, Ottawa, ON, Canada) and the lysate was cleared by centrifugation at 40,000 g for 45 min at 4 °C. Next, the clarified lysate was supplemented with 5 mM CaCl2 and loaded onto a phenyl Sepharose 6 fast flow (high sub) column (GE Healthcare, Chicago, IL, USA), followed by low salt (buffer A supplemented with 0.1 mM CaCl2) and high salt (buffer A supplemented with 0.1 mM CaCl2 and 500 mM NaCl) wash steps. CaM was eluted using buffer A supplemented with 1 mM EDTA. Finally, pooled elution fractions were concentrated to ~2300 μM, using a 10-kDa molecular weight cut-off concentrator (Millipore, Burlington, MA, USA), flash-frozen in liquid N2 and stored at -80 °C until use.

Electrophysiology

Whole-cell patch-clamp recording

Voltage-clamp recordings were performed using the whole-cell configuration of the patch-clamp technique. Signals were amplified using an Multiclamp 700B patch-clamp amplifier (Molecular Devices), sampled at 10 kHz and filtered at 4 kHz via a four pole Bessel low pass filter. Data were acquired using pClamp 10.5 software in conjunction with a DigiData 1440A interface (Molecular Devices). The patch pipettes were pulled from borosilicate glass (Harvard Apparatus) with a resistance of 3–5 megaohms. The intracellular pipette solution contained 130 mM KCl, 5 mM EGTA , 10 mM Hepes, pH 7.3 (adjusted with KOH), and $CaCl_2$ calculated for a final concentration of 1µM free Ca^{2+}, as calculated by MAXCHELATOR (WEBMAXC STANDARD) software; https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/webmaxc/webmaxcS.htm), with sucrose added to adjust osmolarity to 290 mosM. The external solution (310 mosM) contained 140 mM NaCl, 4 mM KCl, 1.8 mM $CaCl^2$, 1.2 mM $MgCl^2$, 11 mM glucose, and 5.5 mM Hepes adjusted with NaOH to pH 7.3. CHO cells were held at -90 mV and SK4 K^+ currents were activated using a voltage ramp protocol from -100mV to +60mV for 150 ms. Electrophysiological data analysis was performed using the Clampfit program (pClamp 10.5; Molecular Devices).

Inside-out macro-patch recording

To measure the Ca^{2+} concentration dependence for SK4 channel activation, CHO cells were co-transfected with plasmids encoding WT SK4 1µg and WT CaM 1µg using the Lipofectamine reagent at a ratio of 1:2. The cells were transfected 48h prior to recordings. The patch pipettes were pulled and fire-polished with a MF-900 micro-forge (Narashige) to reach an internal diameter of ~3-5µM. The resistance of the patch electrodes ranged from 2-3MΩ. The pipette solution contained 135 mM KCl, 1 mM MgSO4, 0.91mM $CaCl_2$ and 10 mM HEPES at pH 7.3. The bath solution contained 135 mM KCl, 5 mM EGTA and 10 mM HEPES at pH 7.3. EGTA was used to titer the different Ca^{2+}concentration solutions, calculated using the software by C. Patton of Stanford University (http://maxchelator.stanford.edu/).

Currents were recorded by 10 repetitive 1s duration voltage ramps from -100mV to +100mV from a holding potential of 0 mV. The current amplitudes in response to increasing Ca^{2+} concentrations were normalized to those obtained at a saturating Ca^{2+} concentration (3 µM). Furthermore, to determine the Ca^{2+} concentration dependence for activation in the presence of BA6b9 (10 µM), the current amplitudes were normalized to those obtained at large maximal Ca^{2+} concentration of 10µM. For PIP2-dependent activation, PIP2 affinity to the SK4 channel was also examined in the inside-out configuration. The dose-response curve for PIP2 was measured with increasing concentrations of diC8-PIP2 in the presence of 1µM Ca^{2+}. The diC8-PIP2 effect on the channel current was measured after complete depletion of the native PIP2 using sonicated poly L-Lysine 50µg/ml (PLL) and a subsequent 2-min washout. The current amplitudes in response to increasing diC8-PIP2 concentrations were normalized to those obtained at maximal diC8-PIP2 concentration of 10µM in the presence of 1µM Ca^{2+}. Apparent EC_{50} values for Ca^{2+} or diC8-PIP2 were determined by fitting the data points to a standard dose-response curve Y=100/(1+10^((LogEC50-X)*HillSlope)).

Electrophysiological data analyses

Data analysis was performed using the Clampfit program (pClamp 10.5; Axon Instruments), Microsoft Excel (Microsoft,Redmond, WA), and Prism 9.0 (GraphPad Software, Inc., San Diego, CA). Leak subtraction was performed off-line, using the Clampfit program of the pClamp 10.5 software. All data were expressed as mean ± S.E.M. Statistically significant differences were assessed by paired, unpaired two-tailed Student's t test or one-way ANOVA, as indicated in figure legends.

Immunostaining

Cellular immunofluorescence

To probe the specificity of the anti-SK4 channel antibodies, we performed immunofluorescence experiments on non-transfected and SK4 channel-transfected CHO cells. Cells were fixed with 4% paraformaldehyde approximately 48 hours post-transfection; followed by permeabilization and blocking

in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, 5% normal goat serum and 0.1% triton. The cells were incubated in the same buffer for 1h at room temperature with the primary antibody against the SK4 channel (IK1 (D-5): sc-365265, SANTA CRUZ, mouse monoclonal antibody against the C-terminus of human origin SK4, 1:50), washed three times for 5 min each in PBS, then incubated for an additional 1 h at room temperature in the same buffer with the secondary antibody (Alexa Fluor 488, Jackson ImmunoResearch, polyclonal donkey anti-mouse antibody, 1:500), and washed again three times for 5 min each in PBS. The staining was visualized with a laser confocal microscope (Leica SP8. Leica Biosystems, Buffalo Grove, Illinois, 60089, USA) and the images were processed using Adobe illustrator (Adobe Systems, Inc., Mountain View, CA).

Immunohistochemistry of heart slices

Healthy adult male Sprague-Dawley rat hearts were excised into cold PBS solution. Left atrial (LA) and left ventricular (LV) tissues were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned in short-axis slices (4 μm thickness) and stained on positively charged glass slides (Histobond+, Marienfeld). Human slices (4 μm thickness) were obtained from Pantomics Inc. (Fairfield, CA USA). For staining, an automated immunohistochemistry (IHC) system (Multistainer Leica ST5020, Leica Biosystems, Buffalo Grove, Illinois, 60089, USA) was used. IHC antigen retrieval was performed using BOND Epitope Retrieval Solution 2 (Tris-EDTA, prediluted, pH 8.0) for 20 min at 100 °C. For detection and localization of sarcomeric α-actinin in the working myocardium of the healthy rat heart, we incubated the specimens with the primary antibody anti-sarcomeric alpha actinin antibody (ab137346, abcam, rabbit polyclonal, 1:2000) for 30min and stained with 3,3′-Diaminobenzidine (DAB) for 90 s (Bond Polymer refine detection kit, Leica biosystems). For detection and co-localization of the SK4 channel in the working myocardium we have incubated the specimens with the primary antibody (IK1 D-5: sc-365265, SANTA CRUZ, mouse monoclonal antibody, 1:50) for 90min followed by staining with Congo RED for 10min (Bond Polymer Refine Red detection kit, Leica biosystems). The specimens were then

counterstained with hematoxylin and cover-slipped. Each IHC run contained four groups: α-actinin stained with DAB for LA and LV sections originated from the same animal, SK4 stained with Congo RED for LA and LV originated from the same animal, double staining for co-localization of α-actinin and SK4 for LA and LV originated from the same animal and negative control with secondary antibody only for LA and LV. The stained sections were left to dry for 24h and scanned the following day via slide scanner Aperio (Aperio Versa 200 DM6000 B, Leica Biosystems) and analyzed using Qupath, a quantitative pathology and bioimage analysis software (3).

Computational Methods

System preparation

Molecular Dynamics were performed for the human SK4/calmodulin complex cryo-EM structure in its open conformation (PDB code: 6CNN) (4). Since the cryo-EM map was recorded in the absence of PIP2, PIP2 was docked to the interface of calmodulin and the SK4 proximal C-terminus with calmodulin T79 serving as an anchor and using the Glide docking algorithm, as implemented in Maestro version 11.2 (https://www.schrodinger.com/). Next, the Orientations of Proteins in Membranes (OPM; https://opm.phar.umich.edu/) and Membrane Builder in CHARMM-GUI (https://www.charmm-gui.org/) web-servers were used to build a system of SK4/calmodulin/PIP2 in a POPC lipid bilayer. Then the system was solvated in TIP3P water molecules (5) to form a 162 X 162 X 142 angstroms simulation box. Finally, potassium and chloride ions were added to the water phase in order to neutralize the system and to obtain a salt concentration of 0.15 M.

Molecular Dynamics (MD) Simulation

MD simulation was performed with Gromacs 2018.2 software (http://www.gromacs.org/) with CHARMM36 force field (6). The simulation was conducted using periodic boundary conditions (PBC) with particle-mesh Ewald (PME) electrostatics with 12 Å cutoff for long range interactions.

The simulation was composed of three steps: (1) energy minimization with the steepest descent minimization algorithm; (2) six equilibration steps with restraints that were applied on protein and membrane atoms. The restrains were gradually reduced to zero during these steps; (3) production simulation for 200 ns with a constant temperature of 310 K and a constant pressure of 1 atm (under Nosé-Hoover and Parrinello-Rahman coupling algorithms, respectively), and with an integration time step of 2 fs.

Molecular Data Analysis

The resulting trajectories were visually inspected using VMD 1.9.3 software (https://www.ks.uiuc.edu/Research/vmd/). The stability of the resulting trajectories was tested based on the root mean square deviation (RMSD), which was calculated using the rms utility of Gromacs 2018.2 package. Next, in order to find the most prevalent conformations, the simulation trajectory was clustered using the Gromos clustering algorithm and a cut-off of 0.2 nm. Based on the clustering analysis, centers of four largest clusters, which cover 90% of the conformational space of the trajectory simulation, were picked for further calculations.

Docking of small molecules

The designed molecules were docked into the protein structures. Prior to docking, the protein structures were prepared using the Protein Preparation wizard in Schrodinger's Maestro. Docking calculations were preformed using Glide, as implemented in Maestro version 11.2 (Schrodinger). Glide's grid was centered on Arginine 180, with box size of 10 angstroms. The docking calculations were performed in the presence of calmodulin, as well as in its absence, in order to identify molecules that may interfere calmodulin binding. Next, Prime MM-GBSA module in Maestro version 11.2 (Schrodinger) was used to optimize the complexes obtained from docking calculations in the presence of implicit membrane.

Medicinal Chemistry

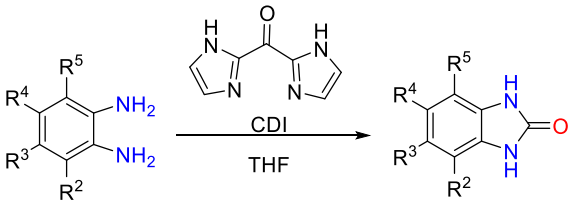
Materials

All chemicals were purchased from Tzamal D-Chem Laboratories Ltd. Petah-Tikva, Israel. All solvents were purchased from BioLab Ltd. (Israel).

Equipment

- Discover® SP: Microwave irradiation experiments were performed using CEM Discover SP machine.
- LC-MS system: Waters Autopurification system analytical module equipped with SQD2 MS detector at the following conditions: a. LC: Waters XSelect Peptide CSH C18 column (5µm, 4.6mm x 100mm) using a 10-minute gradient from 95:5 Water: acetonitrile (both with 0.1% formic acid) to acetonitrile; b. MS: scan mode 100-1000.
- Biotage® Isolera™ One flash chromatography system.
- ^{1}H and ^{13}C NMR spectra were measured on Bruker 400 (400 MHz ^{1}H, 100 MHz ^{13}C). Chemical shifts values (δ) are reported in ppm (calibration of spectra to the residual peak of TMS: δ = 0.0 ppm (s) for ^{1}H NMR; δ = 0.0 ppm for ^{13}C NMR if not mentioned otherwise). All the proton spectra reported as following: δ value (multiplicity, J coupling constant (in Hz), number of nuclei). Multiplicity contractions used: (s) – singlet, (d) – doublet, (dd) – doublet of doublet, (t) – triplet, (q) – quartet, (m) – multiplet, and (br) – broad signal.
- V-10 automatic Evaporation System for 20 ml vails.

General Procedure 1



R^2=H
R^3=H, CH_3, Cl
R^4=H,CH_3, OMe, CN, NO_2, Cl, Br, F
R^5=H, Me, NO_2

In a reaction vehicle equipped with magnetic stirrer and addition funnel, diamine derivative was dissolved in anhydrous THF. CDI was added dropwise through the addition funnel at 0 ^oC, and the resulting mixture was stirred at room temperature overnight. Alternatively, in a reaction vehicle equipped with stirring bar, diamine derivative, CDI, and THF were added. The vial was sealed and the reaction mixture irradiated for 20 min. at 180 ^oC (ramp = 2 min., P_{max} = 150 W). The reaction was monitored by LCMS. After completion of the reaction, the solid was filtered, and dried under vacuum. Purification of the crude by chromatography yielded the corresponding product.

Purification procedure 1

Compounds were submitted to a reverse phase flash purification procedure using a Biotage SNAP Ultra C18 cartridge. The Biotage SNAP Ultra C18 60 g cartridge (HP-Sphere, 25 µm particle size) was mounted on a fully automated flash chromatography instrument (BIOTAGE ISOLERA ONE). The system was equipped with an expanded fraction collector bed and dual wave length UV-V detector. For the purification, the crude powder was dissolved in the DMSO. Then, 1 mL of the resulted solution were loaded onto the cartridge. The elution process was done at a flow rate of 50 mL/min and 20 mL of fraction were collected per tube by UV absorbance at 254 nm. All the chromatographic procedure was performed

using a linear gradient solvent system. The elution started by equilibrating the column with 95% of water (solvent A) and 2 CV of 5% of acetonitrile (solvent B). Then, the cartridge was eluted with 10 CV of the mobile phase starting from 5% to 100% of solvent B.

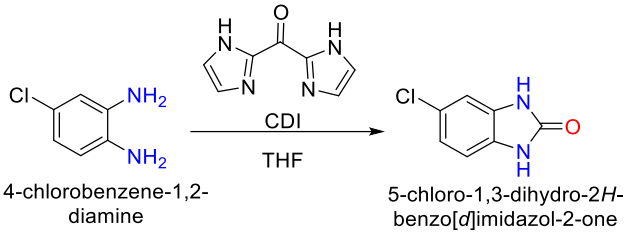
Purification procedure 2

Compounds were submitted to a normal phase flash purification procedure using a silica Biotage SNAP Ultra cartridge. The Biotage SNAP Ultra C18 12 g cartridge (HP-Sphere, 25 µm particle size) was mounted on a fully automated flash chromatography instrument (BIOTAGE ISOLERA ONE). The system was equipped with an expanded fraction collector bed and dual wave length UV-V detector. For the purification, the crude powder absorbed on silica, and transferred to empty Biotage DVL column and equipment with DLV Plunger. The elution process was done at a flow rate of 36 mL/min and 20 mL of fraction were collected per tube by UV absorbance at 254 nm. All the chromatographic procedure was performed using a linear gradient solvent system. The elution started by equilibrating the column with 95% of hexane (solvent A) and 2 CV of 5% of EtOAc (solvent B). Then, the cartridge was eluted with 10 CV of the mobile phase starting from 5% to 100% of solvent B.

Purification procedure 3

Compounds were submitted to a reverse phase HPLC purification procedure using a Waters Autopurification system. The preparative module equipped with SQD2 MS detector at the following conditions: Waters XSelect CSH130 C18 5µm 19x250mm OBD column using a 20 min. gradient from 95:5 Water:acetonitrile (both with 0.1% formic acid) to 100% acetonitrile (0.1% formic acid).

*5-chloro-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA10**)*



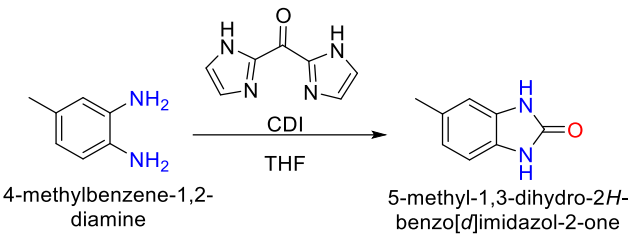
4-chlorobenzene-1,2-diamine

5-chloro-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

BA10

Into a 10 ml process vial equipped with stirring bar, commercial 4-chlorobenzene-1,2-diamine (1 equiv., 3 mmol, 428 mg), CDI (1.5 equiv., 4.5 mmol, 730 mg) and THF (4 ml) were added. The vial was sealed and the reaction mixture irradiated for 20 min. at 180 °C. The reaction was monitored by LCMS. After completion of the reaction, the solvent was evaporated under reduced pressure, and the residue was neutralized with aq. HCl (10 ml, 2N), the solution was decanted, washed again with water (10 ml) and dried under vacuum. Purification of the crude was performed by purification procedure 1, yielding **BA10** as a white solid (83% yield, >95% HPLC purity at diode array). ^{1}H NMR (500 MHz, DMSO) δ 10.77 (s, 2H), 7.04 – 6.85 (m, 3H). ^{13}C NMR (126 MHz, DMSO) δ 155.25, 130.86, 128.64, 124.52, 120.12, 109.53, 108.41.

*5-methyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA20**)*



4-methylbenzene-1,2-diamine

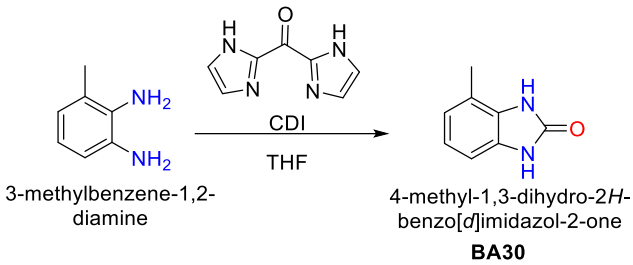
5-methyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

BA20

Into a 10 ml process vial equipped with stirring bar, commercial 4-methylbenzene-1,2-diamine (1 equiv., 3 mmol, 366 mg), CDI (1.5 equiv., 4.5 mmol, 730 mg) and THF (4 ml) were added. The vial was sealed and the reaction mixture irradiated for 20 min. at 180 °C. The reaction was monitored by LCMS. After completion of the reaction, the solvent was evaporated under reduced pressure, and the residue was neutralized with aq. HCl (10 ml, 2N), the solution was decanted, washed again with water (10 ml) and dried under vacuum. Purification of the crude was performed by purification procedure 1, yielding **BA20**

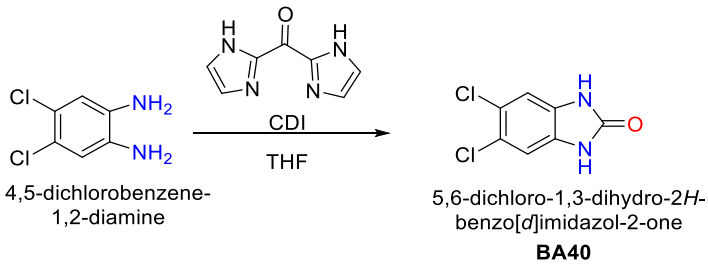
as a white solid (79% yield, >95% HPLC purity at diode array). ^{1}H NMR (500 MHz, DMSO) δ 10.47 (s, 1H), 10.43 (s, 1H), 6.79 (dd, *J* = 7.7, 2.5 Hz, 1H), 6.75 – 6.70 (m, 2H), 2.27 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 155.32, 129.74, 129.23, 127.33, 120.76, 108.91, 108.87, 108.05, 20.92.

*4-methyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA30**)*



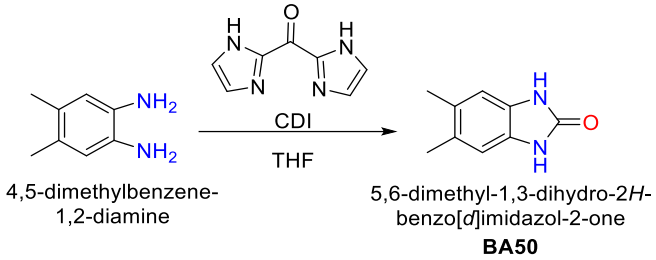
Into a 10 ml process vial equipped with stirring bar, commercial 3-methylbenzene-1,2-diamine (1 equiv., 3 mmol, 366 mg), CDI (1.5 equiv., 4.5 mmol, 730 mg) and THF (4 ml) were added. The vial was sealed and the reaction mixture irradiated for 20 min. at 180 °C. The reaction was monitored by LCMS. After completion of the reaction, the solvent was evaporated under reduced pressure, and the residue was neutralized with aq. HCl (10 ml, 2N), the solution was decanted, washed again with water (10 ml) and dried under vacuum. Purification of the crude was performed by purification procedure 1, yielding **BA30** as a white solid (84% yield, >95% HPLC purity at diode array). ^{1}H NMR (500 MHz, DMSO) δ 10.64 (s, 1H), 10.52 (s, 1H), 6.82 (t, *J* = 7.7 Hz, 1H), 6.77 – 6.70 (m, 2H), 2.25 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 155.38, 129.15, 128.47, 121.47, 120.25, 118.03, 105.96, 16.07.

*5-bromo-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA40**)*



In a reaction vehicle equipped with magnetic stirrer and addition funnel, commercial 4,5-dichlorobenzene-1,2-diamine (1 equiv., 3 mmol, 531 mg) was dissolved in anhydrous THF (20 ml). CDI (1.5 equiv., 4.5 mmol, 730 mg, 0.45 M in THF) was added dropwise through the addition funnel at 0 °C, and the resulting mixture was stirred at room temperature overnight. The reaction was monitored by LCMS. After completion of the reaction, the solid was filtered, and dried under vacuum. Purification of the crude was performed by purification procedure 1, yielding **BA40** as a white solid (42% yield, >95% HPLC purity at diode array). ^{1}H NMR (500 MHz, DMSO) δ 10.92 (s, 2H), 7.12 (s, 2H). ^{13}C NMR (126 MHz, DMSO) δ 155.12, 129.78, 122.29, 109.67.

*5,6-dimethyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA50**)*



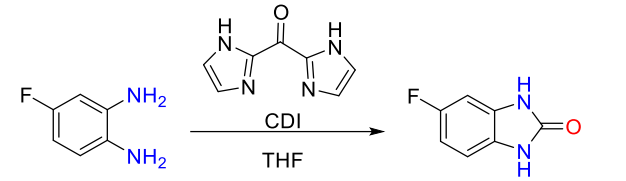
4,5-dimethylbenzene-1,2-diamine

5,6-dimethyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

BA50

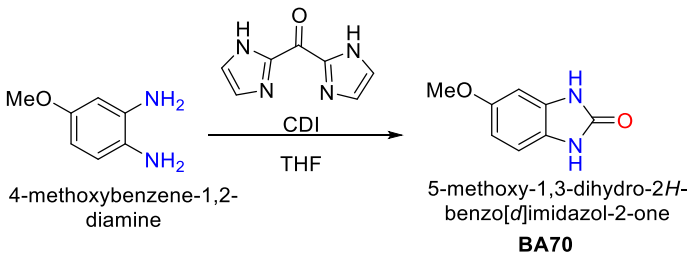
In a reaction vehicle equipped with magnetic stirrer and addition funnel, commercial 4,5-dimethylbenzene-1,2-diamine (1 equiv., 3 mmol, 408 mg) was dissolved in anhydrous THF (5 ml). CDI (1.5 equiv., 4.5 mmol, 730 mg, 0.45 M in THF) was added dropwise through the addition funnel at 0 °C, and the resulting mixture was stirred at room temperature overnight. The reaction was monitored by LCMS. After completion of the reaction, the solid was filtered, and dried under vacuum. Purification of the crude was performed by purification procedure 1, yielding **BA50** as a white solid (49.3% yield, >95% HPLC purity at diode array). ^{1}H NMR (500 MHz, DMSO) δ 10.37 (s, 2H), 6.72 (s, 2H), 2.18 (s, 6H). ^{13}C NMR (126 MHz, DMSO) δ 155.33, 127.64, 109.45, 19.27.

*5-fluoro-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA60**)*



Into a 10 ml process vial equipped with stirring bar, commercial 4-fluorobenzene-1,2-diamine (1 equiv., 3 mmol, 379 mg), CDI (1.5 equiv., 4.5 mmol, 730 mg) and THF (4 ml) were added. The vial was sealed and the reaction mixture irradiated for 20 min. at 180 ^{o}C. The reaction was monitored by LCMS. After completion of the reaction, the solvent was evaporated under reduced pressure, and the residue was neutralized with aq. HCl (10 ml, 2N), the solution was decanted, washed again with water (10 ml) and dried under vacuum. Purification of the crude was performed by purification procedure 1, yielding **BA60** as a white solid (57% yield, >95% HPLC purity at diode array). ^{1}H NMR (500 MHz, DMSO) δ 10.74 (s, 1H), 10.63 (s, 1H), 6.91 – 6.84 (m, 1H), 6.81 – 6.69 (m, 2H). ^{13}C NMR (126 MHz, DMSO) δ 158.30, 156.44, 155.57, 130.31, 130.21, 125.96, 108.67, 108.60, 106.48, 106.29, 96.49, 96.26.

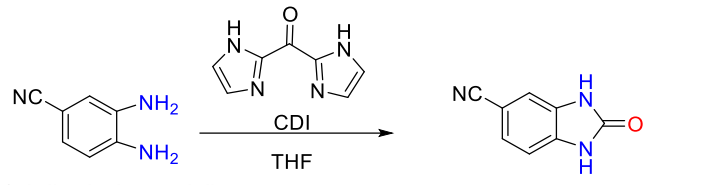
*5-methoxy-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA70**)*



Into a 10 ml process vial equipped with stirring bar, commercial 4-methoxybenzene-1,2-diamine (1 equiv., 3 mmol, 415 mg), CDI (3 equiv., 6 mmol, 973 mg) and THF (4 ml) were added. The vial was sealed and the reaction mixture irradiated for 20 min. at 180 ^{o}C. The reaction was monitored by LCMS. After completion of the reaction, the solvent was evaporated under reduced pressure, and the residue was neutralized with aq. HCl (10 ml, 2N), the solution was decanted, washed again with water (10 ml) and dried under vacuum. Purification of the crude was performed by purification procedure 1, yielding **BA70**

as a white solid (31% yield, >95% HPLC purity at diode array). ^{1}H NMR (500 MHz, DMSO) δ 10.51 (s, 1H), 10.37 (s, 1H), 6.84 – 6.78 (m, 1H), 6.54 – 6.48 (m, 2H), 3.70 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 155.57, 154.27, 130.42, 123.49, 108.64, 105.99, 95.21, 55.34.

*2-oxo-2,3-dihydro-1H-benzo[d]imidazole-5-carbonitrile (**BA80**)*



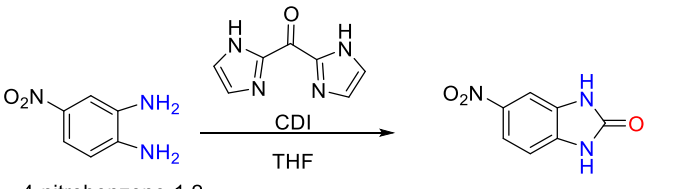
3,4-diaminobenzonitrile

2-oxo-2,3-dihydro-1*H*-benzo[*d*]imidazole-5-carbonitrile

BA80

Into a 10 ml process vial equipped with stirring bar, commercial 3,4-diaminobenzonitrile (1 equiv., 3 mmol, 399 mg), CDI (6 equiv., 6 mmol, 973 mg) and THF (4 ml) were added. The vial was sealed and the reaction mixture irradiated for 20 min. at 180 °C. The reaction was monitored by LCMS. After completion of the reaction, the solvent was evaporated under reduced pressure, and the residue was neutralized with aq. HCl (10 ml, 2N), the solution was decanted, washed again with water (10 ml) and dried under vacuum. Purification of the crude was performed by purification procedure 1, yielding **BA80** as a white solid (37% yield, >95% HPLC purity at diode array). ^{1}H NMR (500 MHz, DMSO) δ 11.19 (s, 1H), 11.06 (s, 1H), 7.40 (d, *J* = 8.1 Hz, 1H), 7.32 (s, 1H), 7.08 (d, *J* = 8.4 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 155.06, 133.62, 129.87, 125.77, 119.75, 111.30, 109.07, 102.19.

*5-nitro-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA90**)*



4-nitrobenzene-1,2-diamine

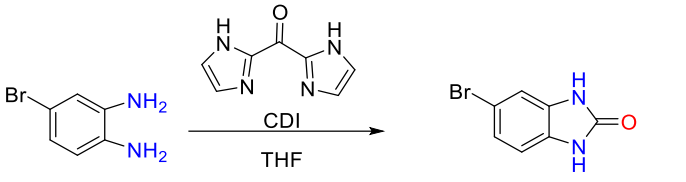
5-nitro-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

BA90

Into a 10 ml process vial equipped with stirring bar, commercial 4-nitrobenzene-1,2-diamine (1 equiv., 3 mmol, 399 mg), CDI (6 equiv., 6 mmol, 973 mg) and THF (4 ml) were added. The vial was sealed and

the reaction mixture irradiated for 20 min. at 180 °C. The reaction was monitored by LCMS. After completion of the reaction, the solvent was evaporated under reduced pressure, and the residue was neutralized with aq. HCl (10 ml, 2N), the solution was decanted, washed again with water (10 ml) and dried under vacuum. Purification of the crude was performed by purification procedure 1, yielding **BA90** as a white solid (54% yield, >95% HPLC purity at diode array). ^{1}H NMR (500 MHz, DMSO) δ 11.41 (s, 1H), 11.18 (s, 1H), 7.94 (dd, *J* = 8.9, 2.3 Hz, 1H), 7.71 (d, *J* = 2.3 Hz, 1H), 7.11 (d, *J* = 8.9 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 155.38, 141.18, 135.63, 129.65, 117.70, 108.00, 103.56.

*5-bromo-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA100**)*



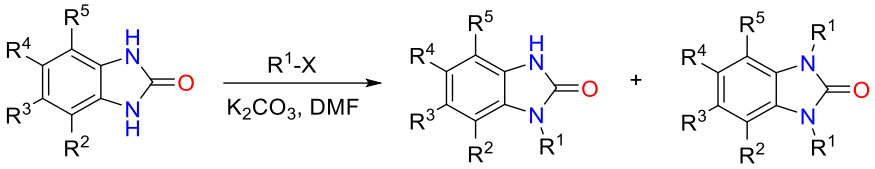
4-bromobenzene-1,2-diamine

5-bromo-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

BA100

Into a 10 ml process vial equipped with stirring bar, commercial 4-bromobenzene-1,2-diamine (1 equiv., 3 mmol, 561 mg), CDI (6 equiv., 6 mmol, 973 mg) and THF (4 ml) were added. The vial was sealed and the reaction mixture irradiated for 20 min. at 180 °C. The reaction was monitored by LCMS. After completion of the reaction, the solvent was evaporated under reduced pressure, and the residue was neutralized with aq. HCl (10 ml, 2N), the solution was decanted, washed again with water (10 ml) and dried under vacuum. Purification of the crude was performed by purification procedure 1, yielding **BA100** as a white solid (46% yield, >95% HPLC purity at diode array). ^{1}H NMR (500 MHz, DMSO) δ 10.76 (s, 2H), 7.08 (dd, *J* = 8.2, 1.0 Hz, 1H), 7.05 (d, *J* = 1.0 Hz, 1H), 6.87 (d, *J* = 8.3 Hz, 1H). ^{13}C NMR (126 MHz, DMSO) δ 154.91, 131.09, 128.87, 122.77, 111.86, 110.90, 109.94.

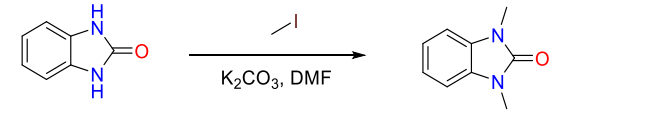
General Procedure 2



R^1=H,alkyl
R^2, R^5=H, NO_2
R^3=H, CH_3, Cl
R^4=H,CH_3, Cl, F

To a solution of benzoimidazol in DMF, potassium carbonate was added. The reaction mixture was cooled to 0 °C, then alkyl halide was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The resulting suspension was filtered and the collected solids were washed with water and dried over vacuum. Alternatively, the solvents evaporated and the crude dissolved in DMSO. The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum.

*<u>1,3-dimethyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA2A**)</u>*



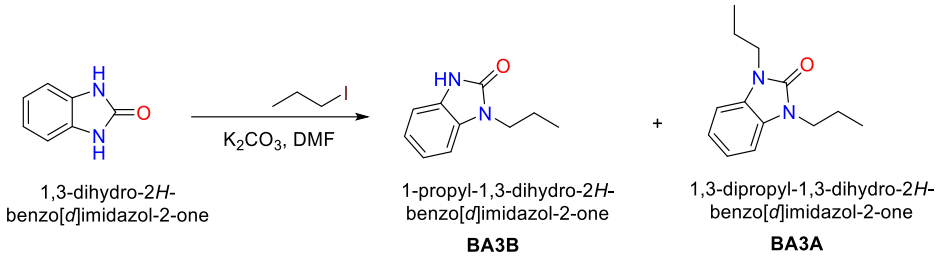
1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

1,3-dimethyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

BA2A

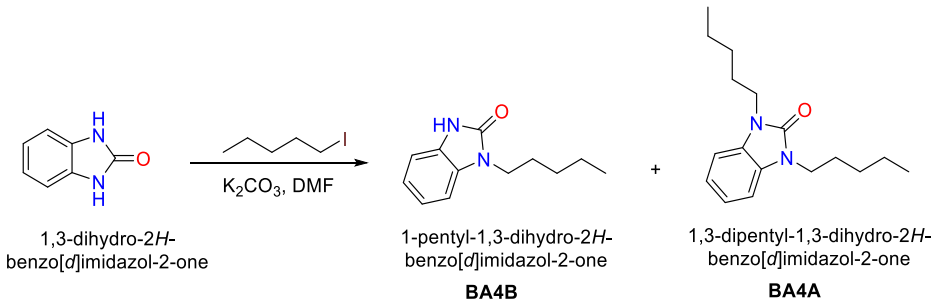
To a solution of commercial 1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.55 mmol, 60 mg) in DMF (3 ml), potassium carbonate (2 equiv., 1.11 mmol, 150 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodomethane (3 equiv., 1.66 mmol, 105 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated and the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure 1, yielding **BA2A** as a white solid (19% yield, >95% HPLC purity at 254 nm). ^{1}H NMR (400 MHz, DMSO) δ 7.16 – 7.10 (m, 2H), 7.10 – 7.04 (m, 2H), 3.35 (s, 6H). ^{13}C NMR (101 MHz, DMSO) δ 129.54, 120.75, 107.46, 26.79.

*1-propyl-1,3-dihydro-2H-benzo[d]imidazol-2-one and 1,3-dipropyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA3B**), 1,3-dipropyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA3A**)*



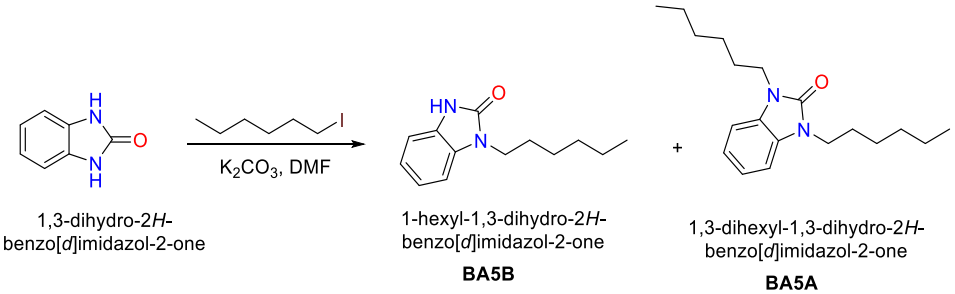
To a solution of commercial 1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.55 mmol, 60 mg) in DMF (3 ml), potassium carbonate (2 equiv., 1.11 mmol, 150 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodopropane (3 equiv., 1.66 mmol, 162 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated and the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure 1, yielding **BA3B** as a white solid (4% yield, >95% HPLC purity at diode array), and **BA3A** as a white solid (19% yield, >95% HPLC purity at 254 nm). **BA3B**: ^{1}H NMR (400 MHz, DMSO) δ 10.81 (s, 1H), 7.15 – 7.08 (m, 1H), 7.03 – 6.94 (m, 3H), 3.74 (t, *J* = 7.1 Hz, 2H), 1.65 (q, *J* = 7.2 Hz, 2H), 0.86 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 154.24, 130.50, 128.44, 120.53, 120.33, 108.54, 107.60, 41.23, 21.11, 10.98. **BA3A**: ^{1}H NMR (400 MHz, DMSO) δ 7.19 (dd, *J* = 5.7, 3.2 Hz, 1H), 7.04 (dd, *J* = 5.7, 3.2 Hz, 1H), 3.80 (t, *J* = 7.0 Hz, 2H), 1.66 (q, *J* = 7.2 Hz, 2H), 0.85 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 153.43, 128.91, 120.61, 107.75, 41.70, 21.12, 10.95.

*1-pentyl-1,3-dihydro-2H-benzo[d]imidazol-2-one and 1,3-dipentyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA4B**), 1,3-dipentyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA4A**)*



BA4B was prepared according to General Procedure 2. To a solution of 1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.55 mmol, 60 mg) in DMF (10 ml), potassium carbonate (2 equiv., 1.1 mmol, 150 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodopentane (3 equiv., 1.66 mmol, 217 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The resulting suspension was filtered and the collected solid washed with water (20 mL) and dried under vacuum. Purified by purification procedure 1, yielding **BA4B** as a white solid (7% yield, >95% HPLC purity at diode array), and **BA4A** as a white solid (5% yield, >95% HPLC purity at diode array). **BA4B**: ^{1}H NMR (400 MHz, DMSO) δ 10.79 (s, 1H), 7.15 – 7.02 (m, 1H), 7.02 – 6.91 (m, 3H), 3.75 (t, *J* = 7.1 Hz, 2H), 1.70 – 1.53 (m, 2H), 1.26 (ddt, *J* = 18.3, 8.7, 5.4 Hz, 5H), 0.83 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 154.11, 130.14, 128.12, 120.54, 120.36, 108.56, 107.56, 30.60, 28.25, 27.42, 21.68, 13.78. **BA4A**: ^{1}H NMR (400 MHz, DMSO) δ 7.17 (dd, *J* = 5.7, 3.1 Hz, 1H), 7.04 (dd, *J* = 5.7, 3.1 Hz, 1H), 3.83 (t, *J* = 7.0 Hz, 2H), 1.64 (t, *J* = 7.2 Hz, 2H), 1.33 – 1.21 (m, 5H), 0.83 (t, *J* = 7.1 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 153.36, 128.90, 120.64, 107.73, 28.24, 27.43, 21.74, 21.70, 13.79.

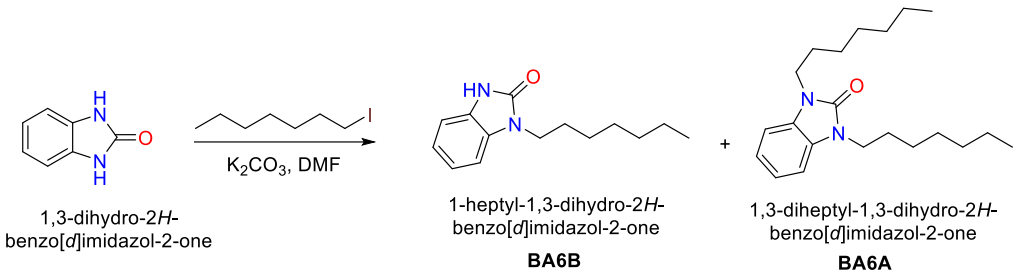
*1-hexyl-1,3-dihydro-2H-benzo[d]imidazol-2-one and 1,3-dihexyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA5B**), 1,3-dihexyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA5A**)*



To a solution of 1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 1.18 mmol, 128 mg) in DMF (10 ml), potassium carbonate (2 equiv., 2.3 mmol, 330 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodohexane (2 equiv., 2.37 mmol, 349 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated and

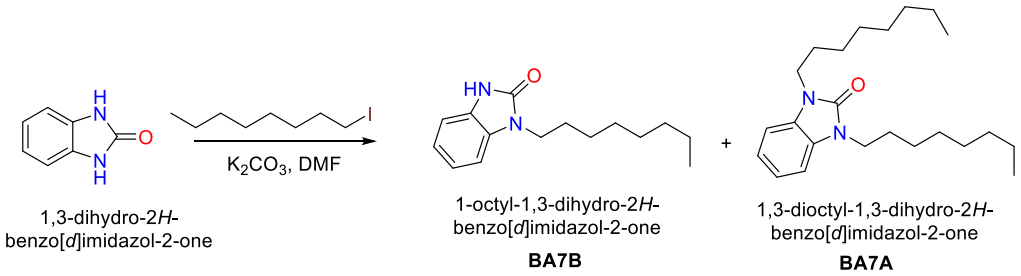
the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure 3, yielding **BA5B** as a white solid (1% yield, >95% HPLC purity at diode array), and **BA5A** as a white solid (2% yield, >95% HPLC purity at diode array).

*1-heptyl-1,3-dihydro-2H-benzo[d]imidazol-2-one and 1,3-diheptyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA6B**), 1,3-diheptyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA6A**)*



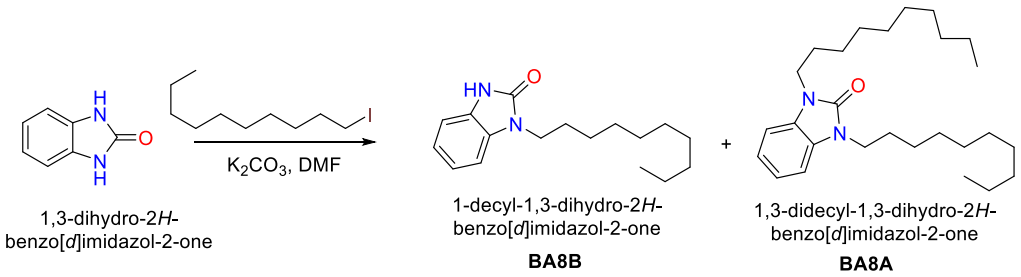
To a solution of commercial 1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 2. 8 mmol, 303 mg) in DMF (3 ml), potassium carbonate (1.2 equiv., 3.3 mmol, 464 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodoheptane (1.2 equiv., 2.3 mmol, 551 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated and the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure 3, yielding **BA6B** as a white solid (1% yield, >95% HPLC purity at diode array), and **BA6A** as a white solid (3% yield, >95% HPLC purity at diode array). **BA6B**: ^{1}H NMR (400 MHz, DMSO) δ 10.78 (s, 1H), 7.13 – 7.06 (m, 1H), 7.02 – 6.93 (m, 3H), 3.76 (t, *J* = 7.1 Hz, 2H), 1.68 – 1.55 (m, 2H), 1.33 – 1.15 (m, 9H), 0.84 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 154.62, 130.67, 128.64, 121.03, 120.85, 120.78, 109.06, 108.86, 108.06, 31.59, 28.70, 28.21, 26.53, 22.40, 14.31.

*1-octyl-1,3-dihydro-2H-benzo[d]imidazol-2-one and 1,3-dioctyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA7B**), 1,3-dioctyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA7BA**)*



To a solution of commercial 1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 1.18 mmol, 128 mg) in DMF (3 ml), potassium carbonate (2 equiv., 2.37 mmol, 464 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodododecane (2 equiv., 2.37 mmol, 430 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated and the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure 3, yielding **BA7B** as a white solid (1% yield, >95% HPLC purity at diode array), and **BA7A** as a white solid (3% yield, >95% HPLC purity at diode array).

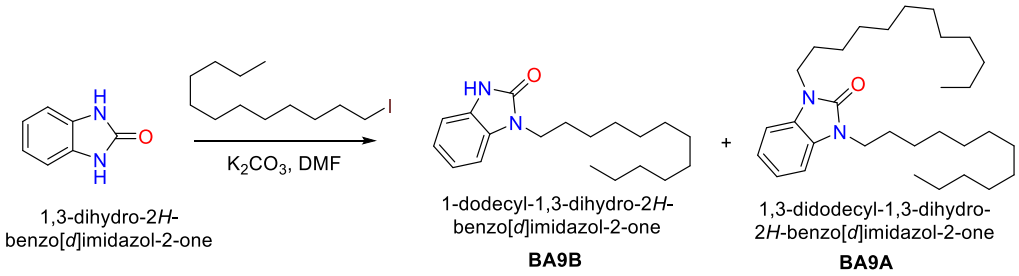
*1-decyl-1,3-dihydro-2H-benzo[d]imidazol-2-one and 1,3-didecyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA8B**), 1,3-didecyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA8A**)*



To a solution of commercial 1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 1.18 mmol, 128 mg) in DMF (3 ml), potassium carbonate (2 equiv., 2.37 mmol, 464 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodododecane (2 equiv., 2.37 mmol, 505 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated and the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure

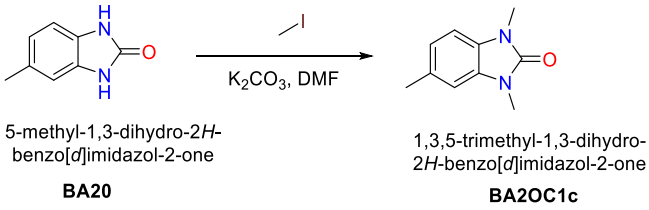
3, yielding **BA8B** as a white solid (0.5% yield, >95% HPLC purity at diode array), and **BA8A** as a white solid (4% yield, >95% HPLC purity at diode array).

*1-dodecyl-1,3-dihydro-2H-benzo[d]imidazol-2-one and 1,3-didodecyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA9B**), 1,3-didodecyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA9A**)*



To a solution of commercial 1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 1.18 mmol, 128 mg) in DMF (3 ml), potassium carbonate (2 equiv., 2.37 mmol, 464 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iododododecane (2 equiv., 2.37 mmol, 583 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated and the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure 3, yielding **BA9B** as a white solid (0.5% yield, >95% HPLC purity at diode array), and **BA9A** as a white solid (2.5% yield, >95% HPLC purity at diode array).

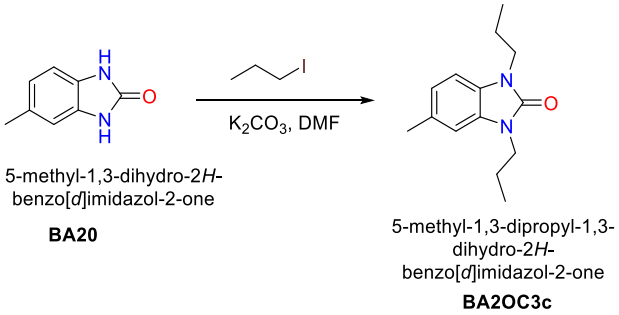
*1,3,5-trimethyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA2OC1c**)*



To a solution of 5-methyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.55 mmol, 60 mg) in DMF (3 ml), potassium carbonate (1.5 equiv., 0.6 mmol, 80 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodomethane (2 equiv., 0.8 mmol, 55 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated

and the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure 1, yielding **BA2OC1c** as a white solid (28% yield, >95% HPLC purity at Diode Array). ^{1}H NMR (500 MHz, DMSO) δ 6.99 (d, *J* = 7.9 Hz, 1H), 6.95 (dd, *J* = 1.6, 0.8 Hz, 1H), 6.87 (ddd, *J* = 7.9, 1.6, 0.8 Hz, 1H), 3.29 (s, 6H), 2.35 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 154.33, 130.43, 130.17, 127.96, 121.68, 108.53, 107.67, 27.30, 27.23, 21.52.

*5-methyl-1,3-dipropyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA2OC3c**)*



5-methyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

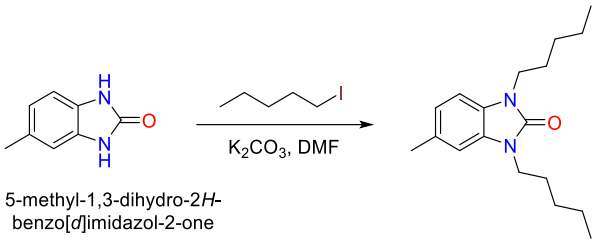
BA20

5-methyl-1,3-dipropyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

BA2OC3c

To a solution of 5-methyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.55 mmol, 60 mg) in DMF (3 ml), potassium carbonate (1.5 equiv., 0.6 mmol, 80 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodopropane (2 equiv., 0.8 mmol, 79 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated and the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure 1, yielding **BA2OC3c** as a white solid (15% yield, >95% HPLC purity at Diode Array). ^{1}H NMR (500 MHz, DMSO) δ 7.07 – 6.99 (m, 2H), 6.85 (d, *J* = 8.1 Hz, 1H), 3.77 (q, *J* = 8.7, 7.1 Hz, 4H), 2.34 (s, 3H), 1.69 – 1.62 (m, 4H), 0.90 – 0.82 (m, 6H). ^{13}C NMR (126 MHz, DMSO) δ 154.06, 130.28, 129.53, 127.32, 121.56, 108.74, 107.95, 42.20, 42.15, 21.61, 21.49, 11.43.

*5-methyl-1,3-dipentyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA23**)*



5-methyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

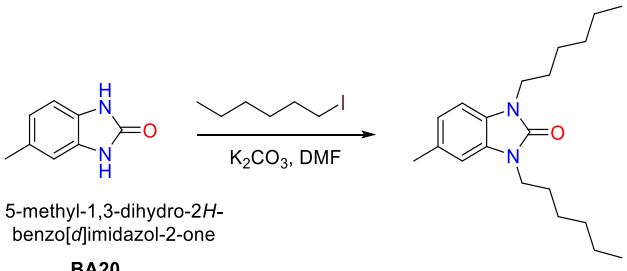
BA20

5-methyl-1,3-dipentyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

BA23

To a solution of 5-methyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.55 mmol, 60 mg) in DMF (3 ml), potassium carbonate (1.5 equiv., 0.6 mmol, 80 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodopentane (2 equiv., 0.8 mmol, 105 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated and the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure 1, yielding **BA23** as a white solid (31% yield, >95% HPLC purity at Diode Array). ^{1}H NMR (500 MHz, DMSO) δ 7.05 – 6.97 (m, 2H), 6.84 (dd, *J* = 7.7, 2.0 Hz, 1H), 3.79 (t, *J* = 6.8 Hz, 4H), 2.34 (s, 3H), 1.63 (q, *J* = 7.4 Hz, 4H), 1.32 – 1.19 (m, 9H), 0.83 (tdd, *J* = 7.0, 4.8, 1.7 Hz, 6H). ^{13}C NMR (126 MHz, DMSO) δ 153.94, 130.23, 129.48, 127.27, 121.53, 108.63, 107.84, 40.54, 40.50, 28.70, 27.90, 22.16, 21.46, 14.21.

*1,3-dihexyl-5-methyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA26**)*



5-methyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

BA20

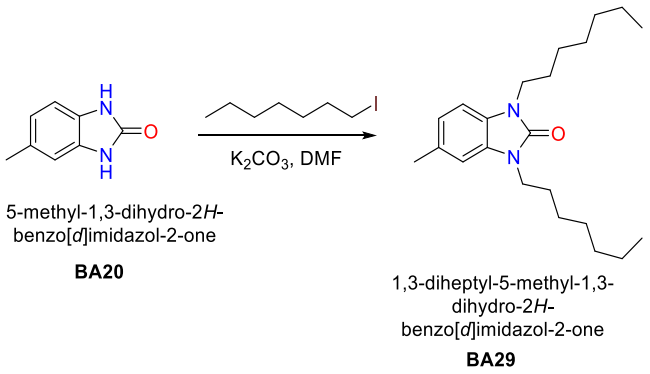
1,3-dihexyl-5-methyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

BA26

To a solution of 5-methyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.55 mmol, 60 mg) in DMF (3 ml), potassium carbonate (1.5 equiv., 0.6 mmol, 80 mg) was added. The reaction mixture was cooled

to 0 °C, then 1-iodohexane (2 equiv., 0.8 mmol, 120 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated and the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure 1, yielding **BA26** as a white solid (27% yield, >95% HPLC purity at Diode Array). ^{1}H NMR (500 MHz, DMSO) δ 7.03 – 6.96 (m, 2H), 6.84 (d, *J* = 7.9 Hz, 1H), 3.79 (t, *J* = 7.0 Hz, 4H), 2.34 (s, 3H), 1.67 – 1.61 (m, 4H), 1.60 (s, 1H), 1.24 (h, *J* = 6.6, 4.7 Hz, 13H), 0.82 (t, *J* = 6.9 Hz, 6H). ^{13}C NMR (126 MHz, DMSO) δ 153.92, 130.19, 129.48, 127.26, 121.49, 108.60, 107.79, 40.55, 40.50, 31.25, 28.16, 26.16, 22.42, 21.43, 14.15.

*1,3-diheptyl-5-methyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA29**)*



5-methyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

BA20

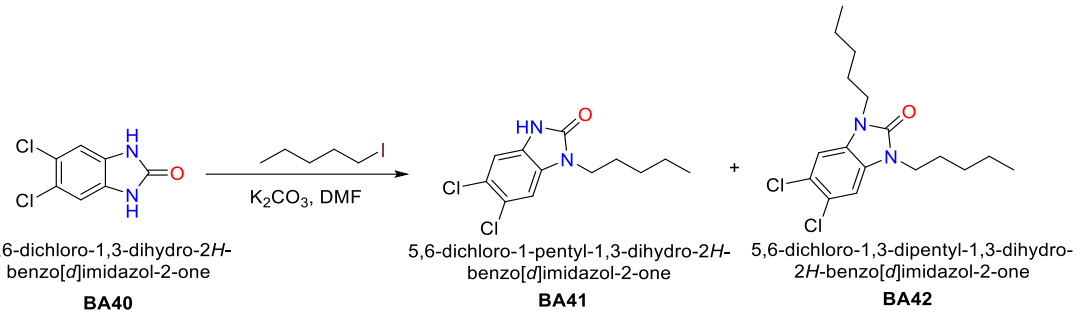
1,3-diheptyl-5-methyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

BA29

To a solution of 5-methyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.55 mmol, 60 mg) in DMF (3 ml), potassium carbonate (1.5 equiv., 0.6 mmol, 80 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodoheptane (2 equiv., 0.8 mmol, 133 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated and the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure 1, yielding **BA29** as a white solid (15% yield, >95% HPLC purity at Diode Array). ^{1}H NMR (500 MHz, DMSO) δ 7.04 – 6.96 (m, 2H), 6.84 (d, *J* = 7.9 Hz, 1H), 3.78 (t, *J* = 7.0 Hz, 4H), 2.34 (s, 3H), 1.66 – 1.58 (m, 4H),

1.31 – 1.15 (m, 20H), 0.82 (td, *J* = 7.0, 2.8 Hz, 6H). ^{13}C NMR (126 MHz, DMSO) δ 153.95, 130.22, 129.47, 127.25, 121.53, 108.66, 107.87, 40.56, 40.49, 31.64, 28.71, 28.18, 26.46, 22.41, 21.48, 14.27.

*5,6-dichloro-1-pentyl-1,3-dihydro-2H-benzo[d]imidazol-2-one and 5,6-dichloro-1,3-dipentyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA41**), 5,6-dichloro-1,3-dipentyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA42**)*



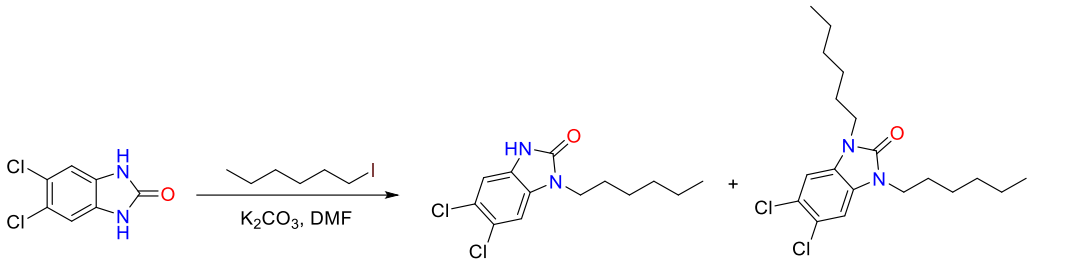
5,6-dichloro-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one **BA40**

5,6-dichloro-1-pentyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one **BA41**

5,6-dichloro-1,3-dipentyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one **BA42**

To a solution of 5,6-dichloro-1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.25 mmol, 50 mg) in DMF (3 ml), potassium carbonate (1.5 equiv., 0.6 mmol, 80 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodopentane (2 equiv., 0.5 mmol, 100 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated and the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure 3, yielding **BA41** as a white solid (9% yield, >95% HPLC purity at Diode Array), and **BA42** as a white solid (41% yield, >95% HPLC purity at Diode Array). **BA41**: ^{1}H NMR (500 MHz, DMSO) δ 11.16 (s, 0H), 7.46 (s, 1H), 7.16 (s, 1H), 3.77 (t, *J* = 7.5 Hz, 2H), 1.61 (p, *J* = 7.6 Hz, 2H), 1.42 – 1.14 (m, 4H), 0.84 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 154.14, 130.37, 128.26, 122.62, 122.51, 109.89, 109.23, 28.15, 27.28, 21.67, 13.78. **BA42**: ^{1}H NMR (500 MHz, DMSO) δ 7.52 (s, 2H), 3.84 (t, *J* = 7.3 Hz, 4H), 1.67 – 1.60 (m, 4H), 1.32 – 1.20 (m, 8H), 0.84 (t, *J* = 7.3 Hz, 6H). ^{13}C NMR (126 MHz, DMSO) δ 153.33, 128.90, 122.88, 109.28, 40.43, 28.10, 27.26, 21.65, 13.70.

*5,6-dichloro-1-hexyl-1,3-dihydro-2H-benzo[d]imidazol-2-one and 5,6-dichloro-1,3-dihexyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA43**), 5,6-dichloro-1,3-dihexyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA44**)*



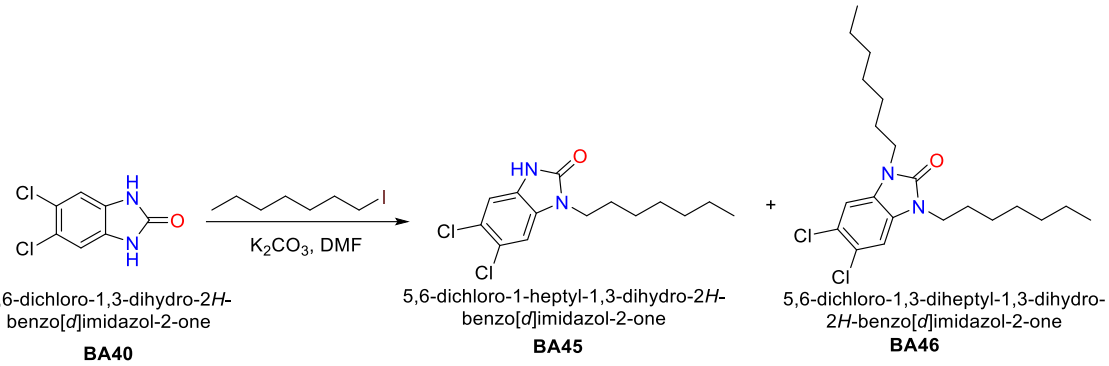
5,6-dichloro-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one **BA40**

5,6-dichloro-1-hexyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one **BA43**

5,6-dichloro-1,3-dihexyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one **BA44**

To a solution of 5,6-dichloro-1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.25 mmol, 50 mg) in DMF (3 ml), potassium carbonate (1.5 equiv., 0.6 mmol, 80 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodohexane (2 equiv., 0.5 mmol, 72 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated and the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure 3, yielding **BA43** as a white solid (15% yield, >95% HPLC purity at Diode Array), and **BA44** as a white solid (7% yield, >95% HPLC purity at Diode Array). **BA43**: ^{1}H NMR (500 MHz, DMSO) δ 11.14 (s, 1H), 7.48 (s, 1H), 7.16 (s, 1H), 3.77 (t, *J* = 7.2 Hz, 2H), 1.63 – 1.57 (m, 2H), 1.30 – 1.23 (m, 6H), 0.87 – 0.81 (m, 3H). ^{13}C NMR (126 MHz, DMSO) δ 154.59, 130.81, 128.69, 123.11, 122.99, 110.43, 110.26, 109.73, 31.24, 28.03, 26.11, 22.42, 14.31. **BA44**: ^{1}H NMR (500 MHz, DMSO) δ 7.55 (s, 2H), 3.82 (t, *J* = 7.0 Hz, 4H), 1.60 (t, *J* = 7.0 Hz, 4H), 1.26 – 1.21 (m, 12H), 0.86 – 0.79 (m, 6H). ^{13}C NMR (126 MHz, DMSO) δ 153.85, 129.41, 123.34, 109.95, 109.84, 40.94, 31.18, 27.98, 26.02, 22.41, 14.24.

*5,6-dichloro-1-heptyl-1,3-dihydro-2H-benzo[d]imidazol-2-one and 5,6-dichloro-1,3-diheptyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA45**), 5,6-dichloro-1,3-diheptyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA46**)*



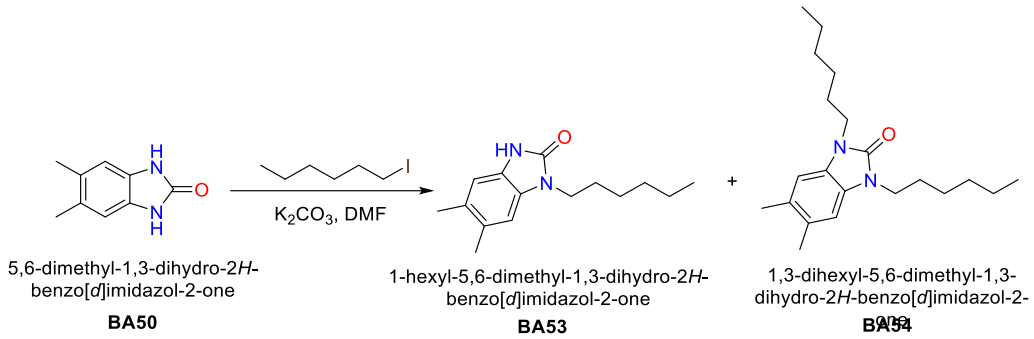
5,6-dichloro-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one
BA40

5,6-dichloro-1-heptyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one
BA45

5,6-dichloro-1,3-diheptyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one
BA46

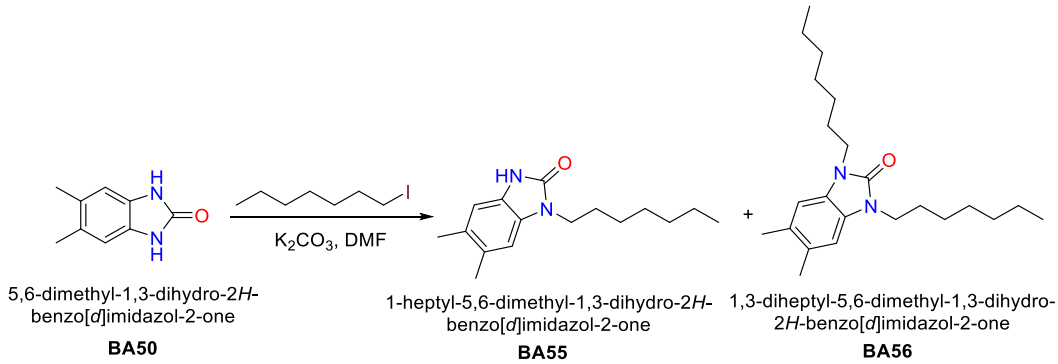
To a solution of 5,6-dichloro-1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.25 mmol, 50 mg) in DMF (3 ml), potassium carbonate (1.5 equiv., 0.6 mmol, 80 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodoheptane (2 equiv., 0.5 mmol, 80 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. The solvents evaporated and the crude dissolved in DMSO (1 ml). The precipitated solid was centrifuged and the solvent was decanted. The resulting solid was dried under vacuum, and purified by purification procedure 3, yielding **BA45** as a white solid (7% yield, >95% HPLC purity at Diode Array), and **BA46** as a white solid (12% yield, >95% HPLC purity at Diode Array). **BA45**: ^{1}H NMR (500 MHz, DMSO) δ 11.18 (s, 1H), 7.47 (s, 1H), 7.16 (s, 1H), 3.76 (t, *J* = 7.1 Hz, 2H), 1.63 – 1.57 (m, 2H), 1.29 (s, 2H), 1.28 – 1.17 (m, 8H), 0.84 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (126 MHz, DMSO) δ 154.62, 130.83, 128.74, 123.08, 122.97, 110.40, 110.33, 109.77, 109.69, 31.60, 28.68, 28.06, 26.41, 22.42, 14.34. **BA46**: ^{1}H NMR (500 MHz, DMSO) δ 7.54 (s, 2H), 3.82 (t, *J* = 6.9 Hz, 4H), 1.61 (p, *J* = 7.0 Hz, 4H), 1.28 – 1.18 (m, 16H), 0.86 – 0.80 (m, 6H). ^{13}C NMR (126 MHz, DMSO) δ 153.85, 129.40, 123.34, 109.86, 40.91, 31.61, 28.65, 28.00, 26.33, 22.40, 14.35, 14.27.

*1-hexyl-5,6-dimethyl-1,3-dihydro-2H-benzo[d]imidazol-2-one and 1,3-dihexyl-5,6-dimethyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA53**), 1,3-dihexyl-5,6-dimethyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA54**)*



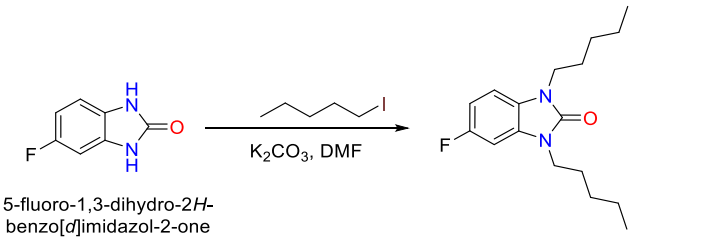
To a solution of 5,6-dimethyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.37 mmol, 60 mg) in DMF (2 ml), potassium carbonate (1.5 equiv., 0.5 mmol, 77 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodohexane (2 equiv., 0.74 mmol, 109 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. Additional DMF (3 ml) was added to the reaction mixture, by which the precipitated solid was centrifuged and the solvent was decanted. Purified by purification procedure 3, yielding **BA53** as a white solid (17% yield, >94% HPLC purity at diode array), and **BA54** as a white solid (5% yield, >95% HPLC purity at diode array). **BA53**: ^{1}H NMR (500 MHz, DMSO) δ 10.56 (s, 1H), 6.88 (s, 1H), 6.76 (s, 1H), 3.71 (t, *J* = 7.1 Hz, 2H), 2.22 (s, 3H), 2.19 (s, 3H), 1.60 (t, *J* = 7.3 Hz, 2H), 1.25 (q, *J* = 4.1 Hz, 6H), 0.85 (s, 2H). **BA54**: ^{1}H NMR (500 MHz, DMSO) δ 6.95 (d, *J* = 1.5 Hz, 2H), 3.76 (td, *J* = 7.0, 1.6 Hz, 4H), 2.23 (d, *J* = 1.6 Hz, 6H), 1.61 (t, *J* = 7.0 Hz, 4H), 1.29 – 1.18 (m, 13H), 0.82 (td, J = 6.2, 5.5, 2.0 Hz, 6H).

*1-heptyl-5,6-dimethyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA55**), 1,3-diheptyl-5,6-dimethyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA56**)*



5,6-dimethyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA50**) was prepared according to General Procedure 1. To a solution of 5,6-dimethyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.37 mmol, 60 mg) in DMF (2 ml), potassium carbonate (1.5 equiv., 0.5 mmol, 77 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodoheptane (2 equiv., 0.74 mmol, 110 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. Additional DMF (3 ml) was added to the reaction mixture, by which the precipitated solid was centrifuged and the solvent was decanted. Purified by purification procedure 3, yielding **BA55** as a white solid (8% yield, >95% HPLC purity at diode array), and **BA56** as a white solid (4% yield, >95% HPLC purity at diode array). **BA55**: ^{1}H NMR (500 MHz, DMSO) δ 10.56 (s, 1H), 6.88 (s, 1H), 6.75 (s, 1H), 3.71 (t, *J* = 7.1 Hz, 2H), 2.22 (s, 3H), 2.19 (s, 3H), 1.60 (p, *J* = 7.1 Hz, 2H), 1.31 – 1.16 (m, 9H), 0.84 (t, *J* = 7.0 Hz, 3H). **BA56**: ^{1}H NMR (500 MHz, DMSO) δ 6.94 (s, 2H), 3.76 (t, *J* = 7.0 Hz, 4H), 1.67 – 1.56 (m, 4H), 1.22 (ddp, *J* = 14.4, 11.3, 4.7 Hz, 17H), 0.83 (t, *J* = 6.9 Hz, 6H).

*5-fluoro-1,3-dipentyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA63**)*



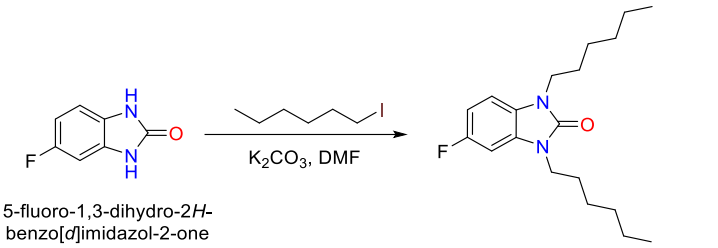
5-fluoro-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one
BA60

5-fluoro-1,3-dipentyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one
BA63

To a solution of 5-fluoro-1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.33 mmol, 50 mg) in DMF (2 ml), potassium carbonate (1.5 equiv., 0.5 mmol, 77 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodohexane (2 equiv., 0.66 mmol, 86 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. Additional DMF (3 ml) was added to the reaction mixture, by which the precipitated solid was centrifuged and the solvent was decanted. Purified by purification procedure 3, yielding **BA63** as a white solid (43% yield, >95% HPLC

purity at diode array). ^{1}H NMR (500 MHz, DMSO) δ 7.17 (ddd, *J* = 13.1, 8.9, 3.5 Hz, 2H), 6.87 (ddd, *J* = 10.8, 8.5, 2.5 Hz, 1H), 3.84 (td, *J* = 7.1, 1.7 Hz, 4H), 1.68 – 1.61 (m, 4H), 1.34 – 1.21 (m, 8H), 0.84 (td, *J* = 7.1 Hz, 6H). ^{13}C NMR (126 MHz, DMSO) δ 159.23, 157.36, 154.18, 130.20, 130.10, 125.68, 108.46, 107.15, 106.95, 96.78, 96.62, 96.55, 96.39, 41.04, 40.79, 40.67, 28.64, 28.22, 27.82, 27.77, 27.38, 22.14, 14.20, 14.10.

*5-fluoro-1,3-dihexyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA66**)*

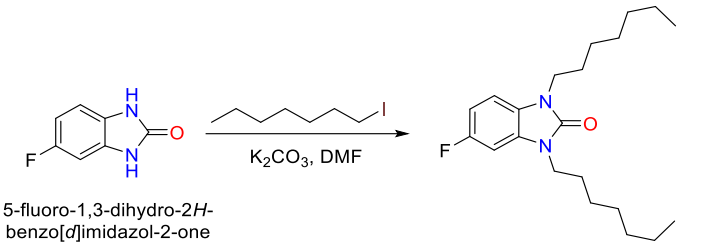


5-fluoro-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one
BA60

5-fluoro-1,3-dihexyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one
BA66

To a solution of 5-fluoro-1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.33 mmol, 50 mg) in DMF (2 ml), potassium carbonate (1.5 equiv., 0.5 mmol, 77 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodohexane (2 equiv., 0.66 mmol, 97 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. Additional DMF (3 ml) was added to the reaction mixture, by which the precipitated solid was centrifuged and the solvent was decanted. Purified by purification procedure 3, yielding **BA66** as a white solid (35% yield, >95% HPLC purity at diode array). ^{1}H NMR (500 MHz, DMSO) δ 7.20 – 7.12 (m, 2H), 6.86 (ddd, *J* = 10.6, 8.5, 2.4 Hz, 1H), 3.83 (t, *J* = 7.0 Hz, 4H), 1.63 (t, *J* = 6.9 Hz, 4H), 1.28 – 1.21 (m, 12H), 0.85 – 0.75 (m, 6H). ^{13}C NMR (126 MHz, DMSO) δ 159.22, 157.36, 154.18, 130.20, 130.10, 125.68, 108.59, 108.52, 107.14, 106.95, 96.75, 96.68, 96.53, 96.44, 40.81, 40.70, 40.55, 31.21, 28.47, 28.07, 28.02, 27.63, 26.09, 22.38, 14.13.

*5-fluoro-1,3-diheptyl-1,3-dihydro-2H-benzo[d]imidazol-2-one (**BA69**)*



5-fluoro-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

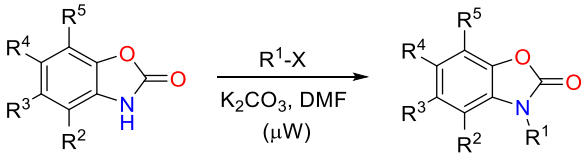
BA60

5-fluoro-1,3-diheptyl-1,3-dihydro-2*H*-benzo[*d*]imidazol-2-one

BA69

To a solution of 5-fluoro-1,3-dihydro-2H-benzo[d]imidazol-2-one (1 equiv., 0.33 mmol, 50 mg) in DMF (2 ml), potassium carbonate (1.5 equiv., 0.5 mmol, 77 mg) was added. The reaction mixture was cooled to 0 °C, then 1-iodoheptane (2 equiv., 0.66 mmol, 107 µl) was slowly added, and the solution was stirred at room temperature overnight. Upon reaction completion, water was added. Additional DMF (3 ml) was added to the reaction mixture, by which the precipitated solid was centrifuged and the solvent was decanted. Purified by purification procedure 3, yielding **BA69** as a white solid (36% yield, >95% HPLC purity at diode array). ^{1}H NMR (500 MHz, DMSO) δ 7.16 (ddd, *J* = 13.0, 8.8, 3.5 Hz, 2H), 6.86 (ddd, *J* = 10.7, 8.5, 2.6 Hz, 1H), 3.82 (t, *J* = 7.0 Hz, 4H), 1.63 (t, *J* = 7.1 Hz, 4H), 1.29 – 1.10 (m, 20H), 0.82 (t, *J* = 6.9 Hz, 6H). ^{13}C NMR (126 MHz, DMSO) δ 159.21, 157.35, 154.19, 130.19, 130.09, 125.67, 108.50, 108.43, 107.15, 106.95, 96.81, 96.65, 96.42, 40.79, 40.68, 31.59, 28.68, 28.06, 27.63, 26.39, 22.38, 14.27, 14.18.

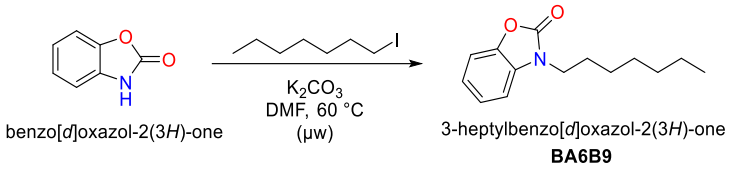
General Procedure 3



Benzooxazol, potassium carbonate, and alkyl derivative were placed in a **10 ml** process vial, equipped with a stirring bar. Upon the addition of DMF, the solution was stirred for 30 sec. at room temperature. Then, the vial was fitted with a Snap-On cap, and the mixture was heated under microwave irradiation (ramp = 2 min., P_{max} = 150 W) for 3 h at 60 °C. The resulting mixture was partitioned between DCM and water. The aqueous layer was extracted three times with DCM, and the combined organic layers

were washed with water and brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification of the crude by chromatography methods yielded the corresponding product.

*3-heptylbenzo[d]oxazol-2(3H)-one (**BA6B9**)*



benzo[*d*]oxazol-2(3*H*)-one

3-heptylbenzo[*d*]oxazol-2(3*H*)-one

BA6B9

Commercial benzo[d]oxazol-2(3H)-one (1 equiv., 0.74 mmol, 100 mg), potassium carbonate (3 equiv., 2.2 mmol, 307 mg), and 1-iodoheptane (5 equiv., 3.7 mmol, 607 µl) were placed in a **10 ml** process vial, equipped with a stirring bar. Upon the addition of DMF (2 ml), the solution was stirred for 30 sec. at room temperature. Then, the vial was fitted with a Snap-On cap, and the mixture was heated under microwave irradiation (ramp = 2 min., P_{max} =150 W) for 3 h at 60 °C. The resulting mixture was partitioned between DCM and water. The aqueous layer was extracted three times with DCM, and the combined organic layers were washed with water and brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. Purification of the crude by purification procedure 2 yielded **BA6B9** as a white solid (64.3% yield, >95% HPLC purity at diode array). ^{1}H NMR (400 MHz, DMSO) δ 7.32 (ddd, *J* = 12.0, 7.8, 1.2 Hz, 2H), 7.22 (td, *J* = 7.7, 1.2 Hz, 1H), 7.13 (td, *J* = 7.8, 1.4 Hz, 1H), 3.81 (t, *J* = 7.1 Hz, 2H), 1.68 (p, *J* = 7.1 Hz, 2H), 1.32 – 1.20 (m, 8H), 0.83 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 142.36, 131.46, 124.31, 122.55, 110.04, 109.60, 42.05, 31.52, 28.59, 27.50, 26.33, 22.38, 14.29.

***Ex-vivo* experiments**

Animals

Adult male Sprague-Dawley rats (n=56, 200-250g) were obtained from Envigo Laboratories (Jerusalem, Israel). Experiments were approved by the institutional ethics committee of Ben-Gurion University of the Negev, Israel (Protocols IL-25-04-2019B) and were carried out in strict accordance with the Guide

for the Care and Use of Laboratory Animals of the National Institutes of Health. The animals were kept under standardized conditions: 12:12 light: dark cycles at 20–24°C and 30–70% relative humidity. Animals were free-fed autoclaved rodent chow and had free access to reverse osmosis filtered water. Hearts were excised from the animals under deep pentobarbital anesthesia.

Perfused isolated hearts: Langendorff method

The isolated heart experiment setup was performed as previously described (7, 8). Briefly, each animal was anesthetized with 2% isoflurane and received an intraperitoneal (IP) injection of pentobarbital (60mg/kg), followed by IP injection of Heparin (1000U/kg). Following full anesthesia the heart was rapidly excised and placed into ice-cold Tyrode's buffer solution. Tyrode's Buffer consists of 140mM NaCl, 5.4mM KCl, 0.5mM MgCl2, 2.5mM CaCl2, 0.39mM NaH2PO4, 10mM HEPES and 11mM glucose, the solution was titrated to pH 7.4 with NaOH. The aorta was cannulated and connected to a pre-heated (37°c) and oxygenated perfusion system with Tyrode's solution while perfusion pressure was maintained at ~70mmHg throughout the experiment. Prior to the beginning of the experiment, the heart was perfused with Tyrode's solution to stabilize for 20 min. For hemodynamic measurements, the left atrial appendage was excised, and a latex balloon was inserted into the left ventricle (LV) through the mitral valve. Once positioned, the balloon was inflated with water to reach an end-diastolic pressure of 5~10mmHg. Coronary perfusion pressure and LV pressure were recorded by a pressure amplifier (ETH-256C amplifier and B-100 probes, iWorx, NH, USA). Electrophysiological signals were recorded from the high right atrium (HRA) via a miniature quadripolar hook electrode (for simultaneous pacing and recording) and from the LV via a bipolar hook electrode (9). Electrical signals were filtered (1-2kHz) and recorded by two voltage amplifiers (Model 3000, A-M Systems, Carlsberg, WA, USA). Signals were interfaced with a PC using an A/D converter (PCI-6024E, National Instruments, Austin TX, USA) and a custom-designed program developed with LabView programing language (National Instruments, Austin, TX, USA) to control signal acquisition, data saving and off-line analysis. Measured physiological

parameters during the experiment included perfusion flow (ml/min) under constant pressure as a marker for coronary resistance, LV developed pressure, maximal dP/dt during contraction (+dP/dt), minimal dP/dt during relaxation (-dP/dt), heart rate, and PR interval. Tram-34 and BA6b9 were applied for a 20-min incubation period and physiological parameters were recorded.

Effective refractory period measurements

Utilizing quadripolar electrode inserted on the HRA and two bipolar electrodes (recording and pacing) on the LV, allowed measurement of the effective refractory period (ERP) at the atrial level (atrial effective refractory period, AERP), atrioventricular level (AVERP) and ventricular level (VERP). For ERP measurement, a programmed S1S2 stimulation protocol was performed using double diastolic threshold intensity. The protocol consisted of ten S1-S2 intervals of 150ms followed by an S1-S2 interval that was reduced by 1ms each time until pacing capture failed 3 consecutive times. AERP, AVERP and VERP were measured and recorded prior and after exposure to TRAM-34 and BA6b9.

Carbachol-induced AF induction

For evaluation of TRAM-34 and BA6B9 AF induction atrial electrodes were inserted and following 20 min recovery and AERP measurement all preparations were exposed to 0.3μM carbachol, either alone or with 10μM TRAM-34 or 10μM BA6B9. Following exposure to the drugs burst pacing episodes (5 sec, 50Hz) were applied using increasing stimulus intensities (2X, 3X, 4X, 5X and 6X diastolic threshold). The burst pacing was applied 2 times for each stimulus intensity. To quantify sustained AF induction, we created a simple AF Induction Score (AFIS) ranking the induction based on the pacing level leading to sustained AF induction. The ranking was as follows: induction at 2X diastolic threshold received the highest score 5, at 3X the score was 4, at 4X the score was 3, at 5X the score was 2, at 6X the score was 1. If no induction occurred the obtained score was zero. Sustained AF was defined as AF lasting ≥5 minutes.

Supplementary References

1. G. Q. Chen, E. Gouaux, Overexpression of a glutamate receptor (GluR2) ligand binding domain in Escherichia coli: Application of a novel protein folding screen. *P Natl Acad Sci USA* **94**, 13431-13436 (1997).
2. A. Alaimo *et al.*, The use of dansyl-calmodulin to study interactions with channels and other proteins. *Methods Mol Biol* **998**, 217-231 (2013).
3. P. Bankhead *et al.*, QuPath: Open source software for digital pathology image analysis. *Sci Rep* **7**, 16878 (2017).
4. C. H. Lee, R. MacKinnon, Activation mechanism of a human SK-calmodulin channel complex elucidated by cryo-EM structures. *Science* **360**, 508-+ (2018).
5. M. Pekka, N. Lennart, Structure and Dynamics of the TIP3P, SPC, and SPC/E Water Models at 298 K. *J. Phys. Chem. A* **105**, 9954-9960 (2001).
6. J. Huang, A. D. MacKerell, Jr., CHARMM36 all-atom additive protein force field: validation based on comparison to NMR data. *J Comput Chem* **34**, 2135-2145 (2013).
7. Y. Etzion *et al.*, New insights into the atrial electrophysiology of rodents using a novel modality: the miniature-bipolar hook electrode. *Am J Physiol Heart Circ Physiol* **295**, H1460-1469 (2008).
8. M. Mor *et al.*, INO-8875, a highly selective A1 adenosine receptor agonist: evaluation of chronotropic, dromotropic, and hemodynamic effects in rats. *J Pharmacol Exp Ther* **344**, 59-67 (2013).
9. M. Murninkas *et al.*, A new implantable tool for repeated assessment of supraventricular electrophysiology and atrial fibrillation susceptibility in freely moving rats. *Am J Physiol-Heart C* **320**, H713-H724 (2021).

Supplementary Figure Legends

Figure S1: Synthesis scheme strategy. (*A*) Chemical structure of 1-EBIO. (*B*) The general procedure 1 is detailed in Supplementary Methods and yielded compounds BA10, BA20, BA30, BA40, BA50, BA60, BA70, BA80, BA90, BA100. (*C*) The general procedure 2 is detailed in Supplementary Methods and yielded compounds 2 BA2a, BA3a, BA3b, BA4a, BA4b, BA5a, BA5b, BA6a, BA6b, BA7a, BA7b, BA8a, BA8b, BA9a, BA9b, BA20C1c, BA20C3c, BA23, BA26, BA29, BA41, BA42, BA43, BA44, BA45, BA46, BA53, BA54, BA55, BA56, BA63, BA66, BA69. (*D*) The general procedure 3 is detailed in Supplementary Methods and yielded compound BA6b9.

Figure S2: Effect of BA6b9 on WT SK4 current with matched-control and washout. Representative raw trace of WT SK4 current in a transfected CHO cell. Whole-cell SK4 K^+ currents are activated using a voltage ramp protocol from -100mV to +60mV for 150 ms. Green traces show the control current with 3 overlapping traces reflecting the stability of the current. Then, BA6b9 is applied and deep purple traces show the current after 1 min, 2 min and 3 min application of the drug. After reaching steady block at 3 min (2 overlapping traces), a magenta trace shows the washout of the drug.

Figure S3: Molecular docking of BA6b9, 1-EBIO and BA40 to the SK4 channel. Docking was performed to the Ca2+-bound state I (6CNN) of the SK4 channel cryo-EM structure; the S1-S4 helices and the SK4 proximal C-terminus (helices A and B) are shown in deep purple and cyan, respectively. PIP2 is shown in deep teal stick and BA6b9, 1-EBIO and BA40 are displayed in green sticks.

Figure S4: BA6b9 effect on the SK channel family and cardiac voltage-gated K^+ channels. (*A*) Multiple protein sequence alignment of human SK1, SK2, SK3 and SK4 channels (T-coffee server: http://tcoffee.crg.cat/apps/tcoffee/do:tmcoffee) showing that at the S4-S5 linker region, residues R191 and H192 of the SK4 channel protein are not conserved in SK1-SK3 channel isoforms. (*B*) Statistical summary of the pharmacological effects of 20 μM BA6b9 on the SK channel family showing that it does

not affect significantly human SK1, rat SK2 and human SK3 with 118%, 134% and 95% of control respectively. The graph also shows that 20 µM BA6b9 does not affect significantly human Kv1.5, Kv2.1, KCNQ1/KCNE1 and Herg with 96%, 98%, 99% and 101% of control, respectively. The voltage-gated K+ channels were activated by a human ventricular action potential serving as a voltage command. (*C*) Representative trace of human SK1 currents in the absence and presence of 20 µM BA6b9, showing that the drug has no effect. (*D*) Representative trace of human Herg currents in the absence and presence of 20 µM BA6b9, showing that the drug has no effect.

Figure S5: Immunofluorescence staining of SK4 channels in transfected CHO cells. (*A-D*) Transfected CHO cells with SK1, SK2, SK3 and SK4 channels, respectively. Cells are also shown in bright field allowing to discriminate in the same field fluorescent cells and non-fluorescent cells. CHO cells are labeled with a primary mouse monoclonal anti-SK4 channel antibody (sc-365265, Santa Cruz) and a secondary antibody (Alexa Fluor 488, polyclonal donkey anti-mouse antibody, Jackson ImmunoResearch) and observed using confocal laser scanning microscopy with a x63 objective. Results show that the anti-SK4 channel antibody specifically labels only cells that overexpress SK4 channels and do not label those that overexpress SK1, SK2 and SK3 channels. (*E*) Non-transfected CHO cells are shown as a negative control.

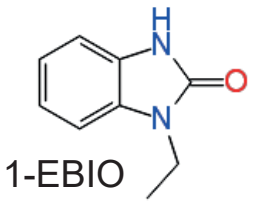
Figure S6: Immunohistochemistry of SK4 channels in human cardiac tissue. Representative left atrial (A) and left ventricular (B) paraffin embedded sections of a human heart. Sections are exclusively labeled with anti-SK4 antibody and stained with DAB as described in “Materials and Methods”. Note the positive staining of SK4 channel in a blood vessel both in the atrium (A) and in the ventricle (B).

Figure S7: Electrophysiological evaluation of Ba6b9 effects ex vivo. Example of recordings obtained from an isolated rat heart before (control) and after perfusion with BA6b9. A quadripolar electrode was inserted on the right atrium (RA) for simultaneous pacing and recording. Additional bipolar electrodes

was inserted on the right ventricle (RV). In all recordings the upper trace denotes RA recording, middle trace denotes RV recording and lower trace marks the atrial pacing (Stim). Left plots: Evaluation of AERP during S1-S2 programmed stimulation protocol with the lowest capture interval that successful conducted in the atria (capture). Left plots: Traces in which S1-S2 was reduced by 1ms leading loss of atrial capture. Not markedly prolonged atrial refractoriness (AERP) in the presence of BA6b9.

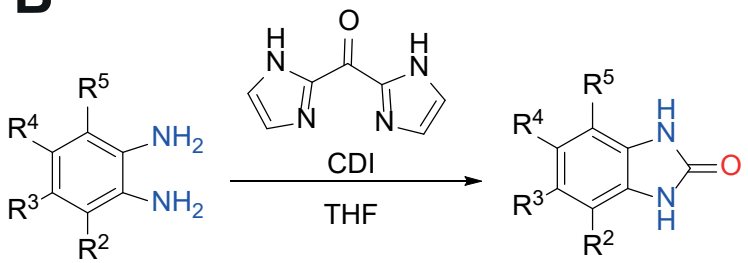
Figure S8: Effects of Tram-34 and BA6b9 on hemodynamic parameters in isolated rat hearts. Data are analyzed by two-tailed paired t-test. (*A,E*)While 10 µM Tram-34 has no effect (n=10), 10 µM BA6b9 significantly reduces the developed pressure, a measure of the left ventricular filling pressure (n=6, t=2.626, df=5, P=0.0468). (*B,F*)10 µM Tram-34 significantly decreases dP/dt Max, a measure of left ventricular contractility (n=10, t=2.613, df=9, P=0.0281), while 10 µM BA6b9 has no effect. (*C,G*) 10 µM Tram-34 significantly reduces dP/dt min, a measure of left ventricular relaxation (n=10, t=3.549, df=9, P=0.0062) and 10 µM BA6b9 is without effect. (*D,H*) While 10 µM BA6b9 has no effect on perfusion flow velocity, a marker for coronary perfusion pressure, 10 µM Tram-34 significantly reduces it (n=12, t=5.126, df=11, P=0.0003).

A



B

General procedure 1

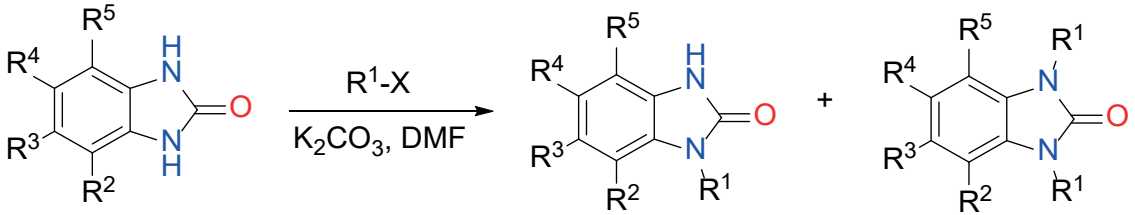


R^2=H
R^3=H, CH_3, Cl
R^4=H,CH_3, OMe, CN, NO_2, Cl, Br, F
R^5=H, Me, NO_2

BA10, BA20, BA30, BA40, BA50
BA60, BA70, BA80, BA90, BA100

C

General procedure 2

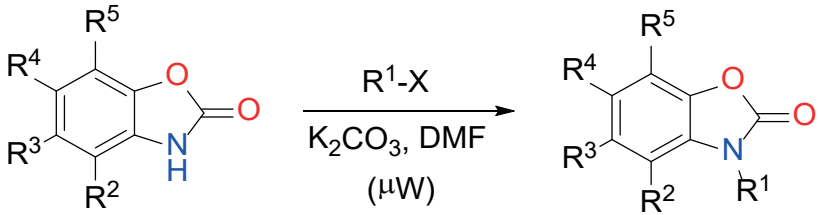


R^1=H,alkyl
R^2, R^5=H, NO_2
R^3=H, CH_3, Cl
R^4=H,CH_3, Cl, F

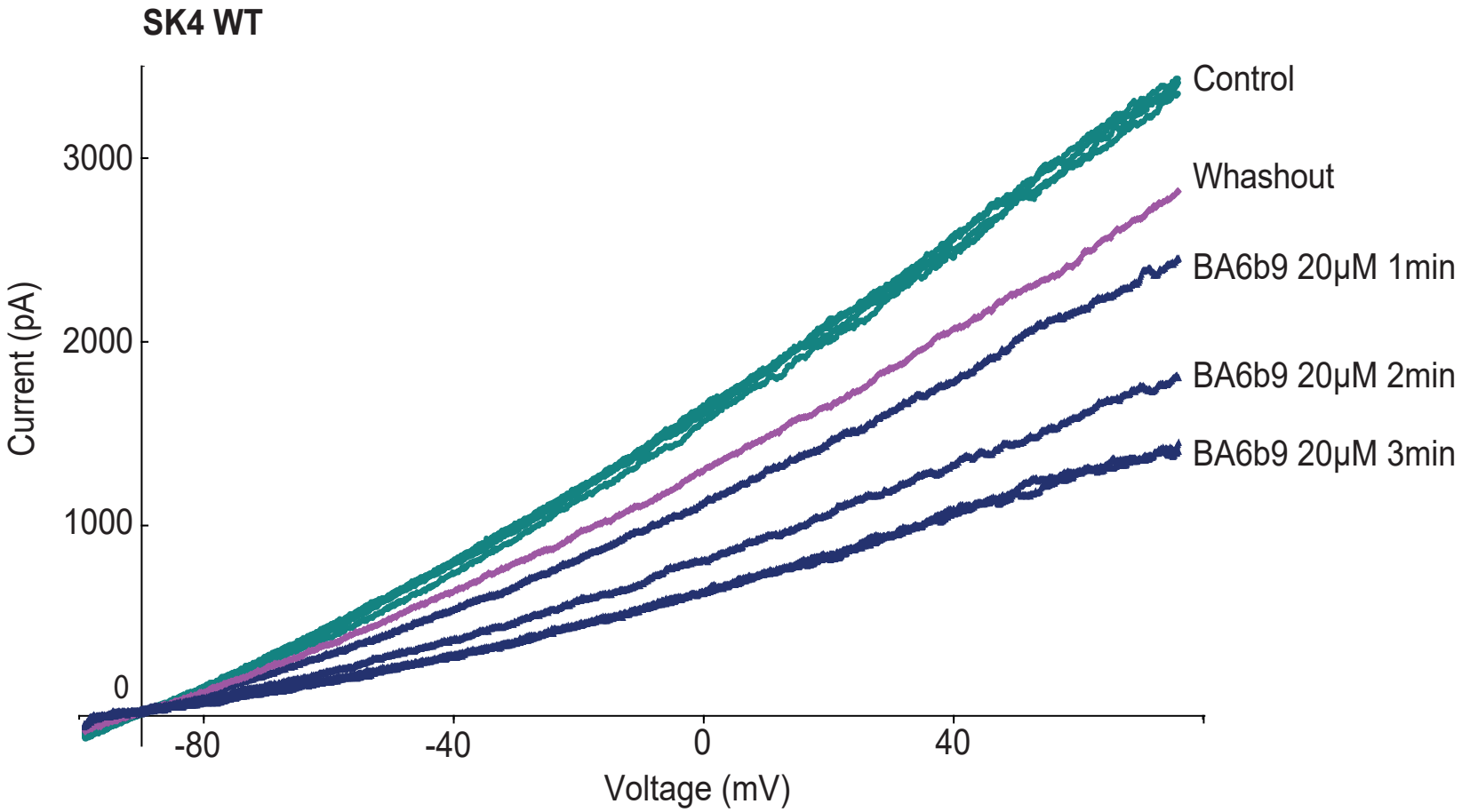
BA2a, BA3a, BA3b, BA4a, BA4b, BA5a, BA5b
BA6a, BA6b, BA7a, BA7b, BA8a, BA8b, BA9a
BA9b, BA20C1c, BA20C3c, BA23, BA26, BA29
BA41, BA42, BA43, BA44, BA45, BA46, BA53
BA54, BA55, BA56, BA63, BA66, BA69

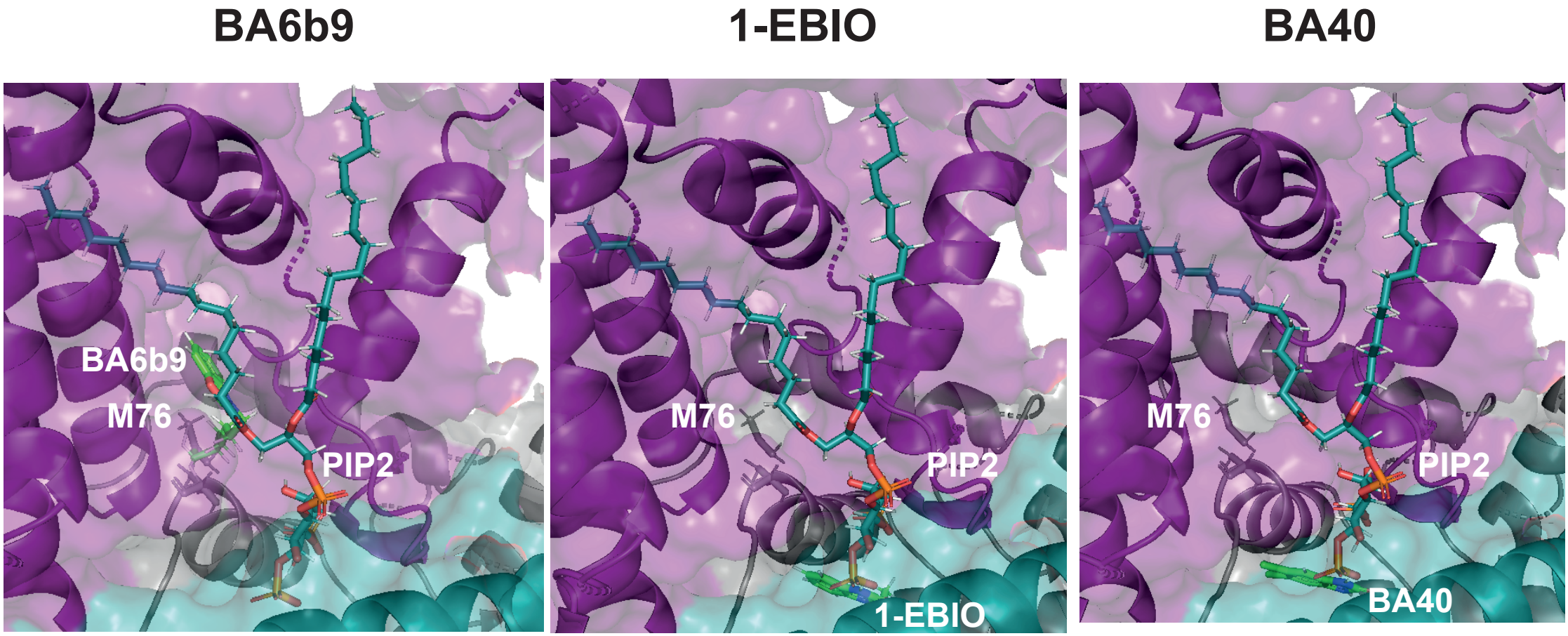
D

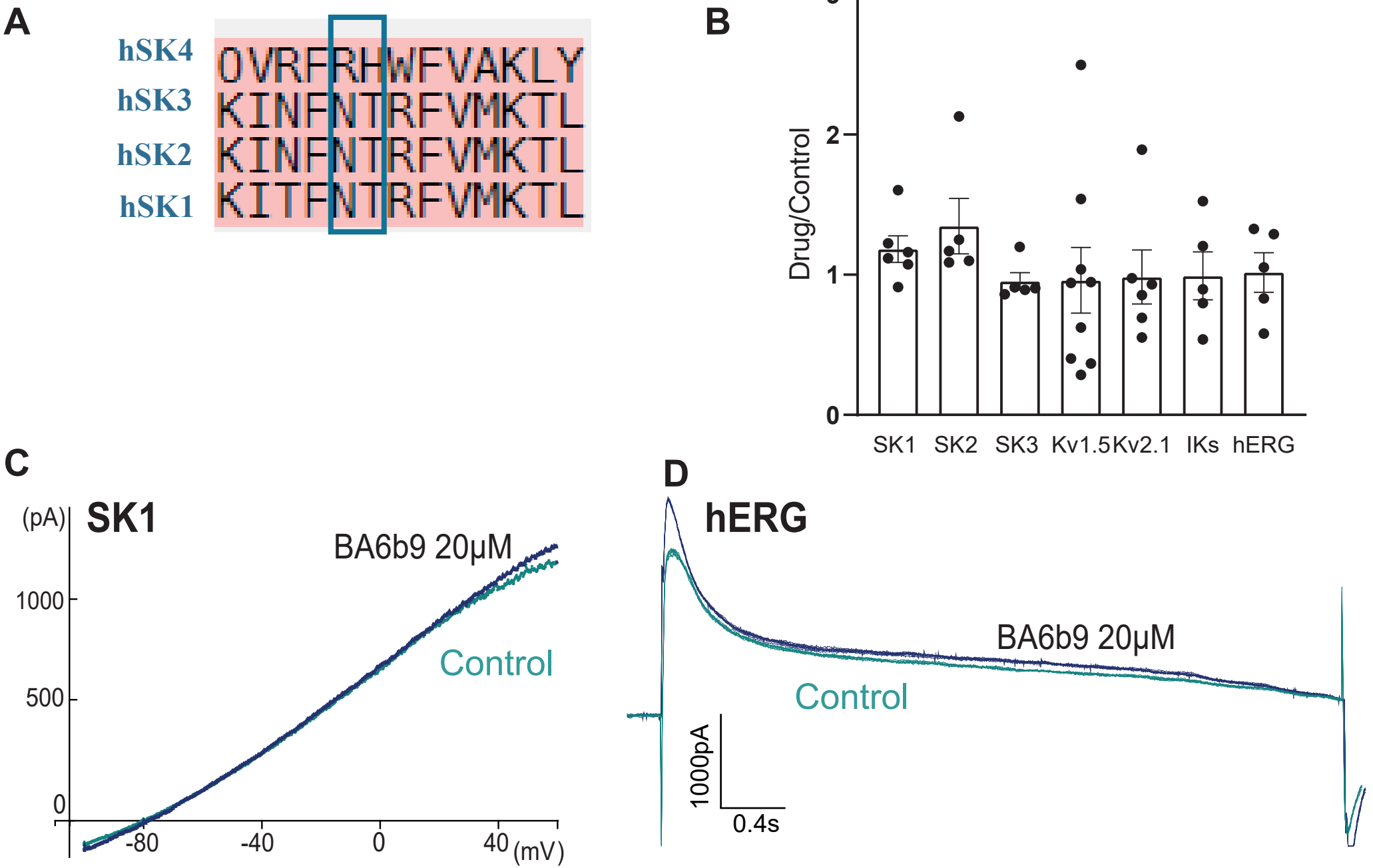
General procedure 3



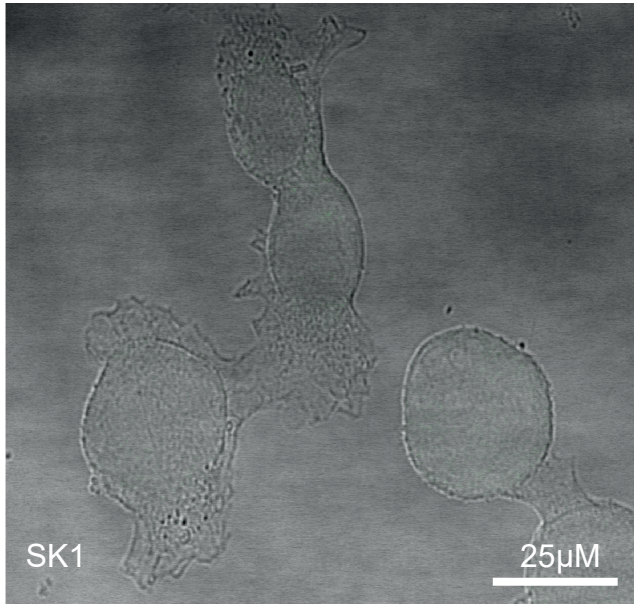
BA6b9


SK4 WT
Control
Whashout
BA6b9 20µM 1min
BA6b9 20µM 2min
BA6b9 20µM 3min
Current (pA)
3000
2000
1000
0
-80
-40
0
40
Voltage (mV)

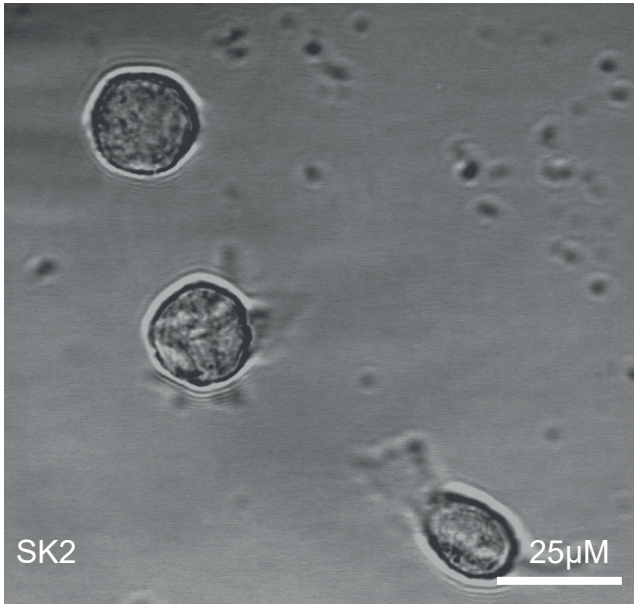

BA6b9
1-EBIO
BA40
BA6b9
M76
PIP2
M76
PIP2
1-EBIO
M76
PIP2
BA40


A
hSK4
hSK3
hSK2
hSK1
QVRFRHWFVAKLY
KINFNTRFVMKTL
KINFNTRFVMKTL
KITFNTRFVMKTL
B
Drug/Control
3
2
1
0
SK1
SK2
SK3
Kv1.5
Kv2.1
IKs
hERG
C
(pA)
SK1
BA6b9 20μM
Control
1000
500
0
-80
-40
0
40
(mV)
D
hERG
BA6b9 20μM
Control
1000pA
0.4s

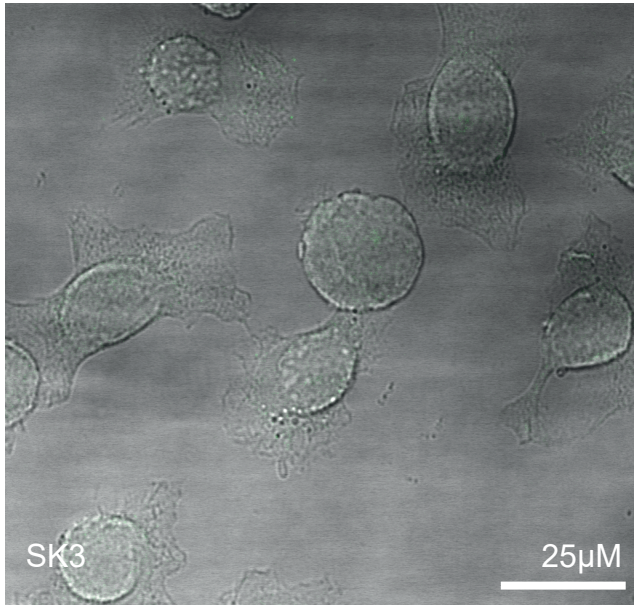
A



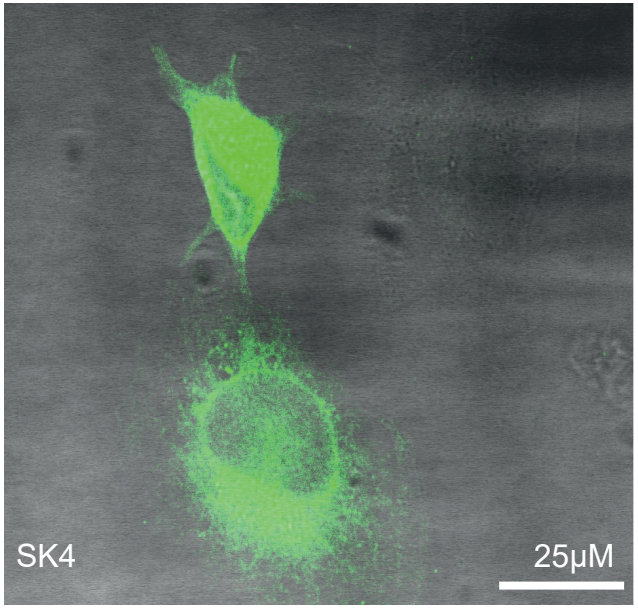
B



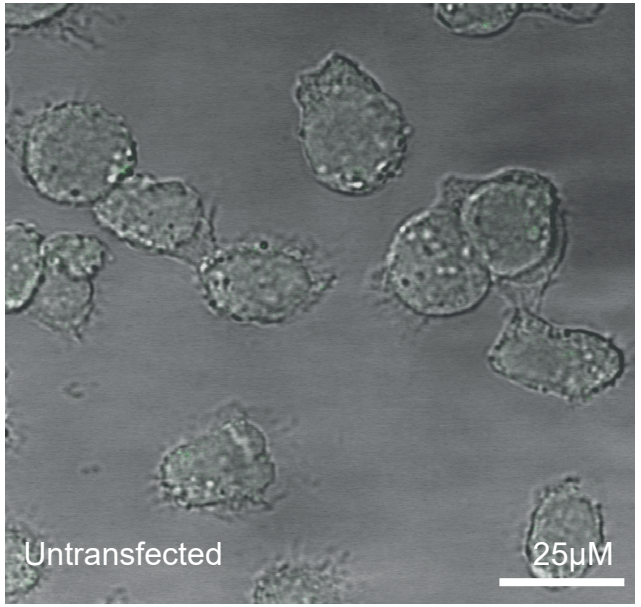
C



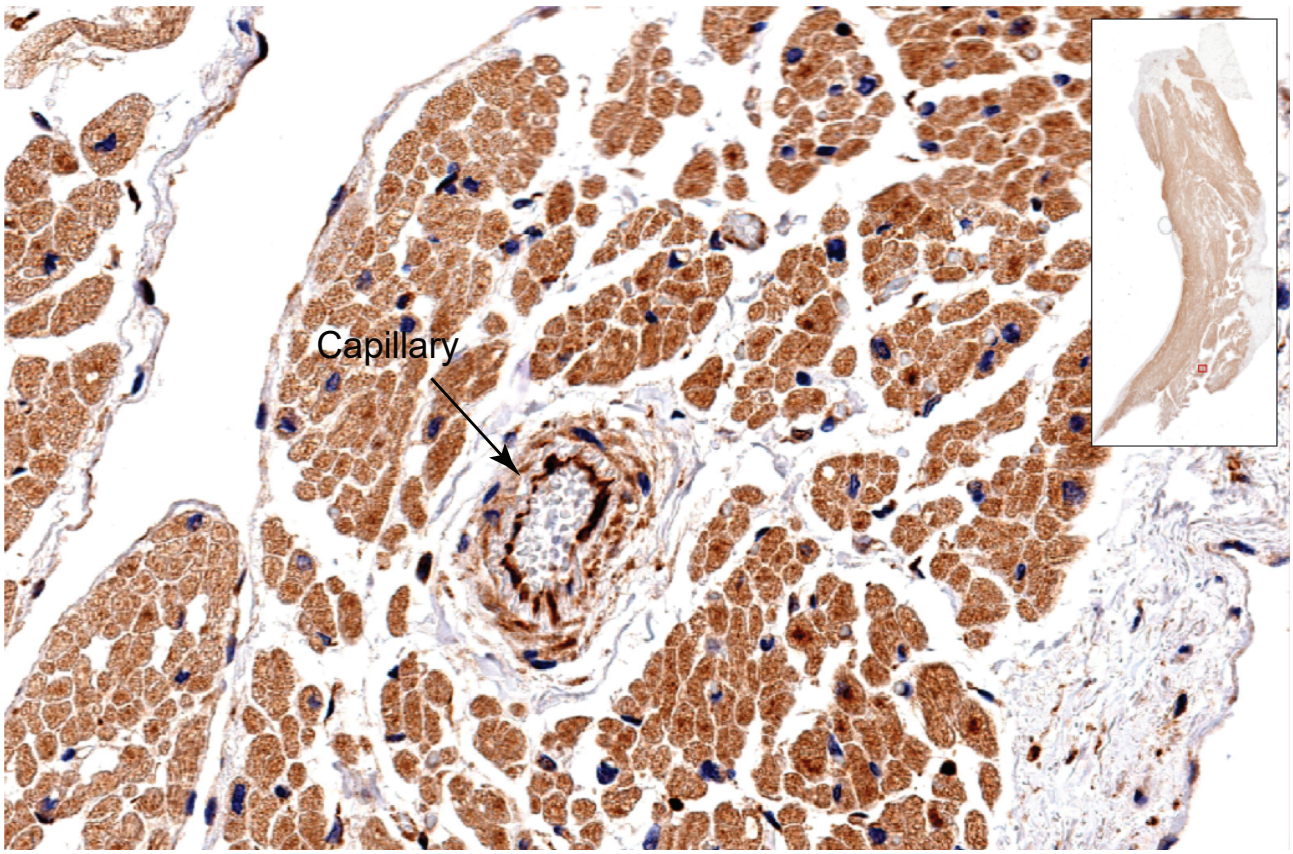
D



E



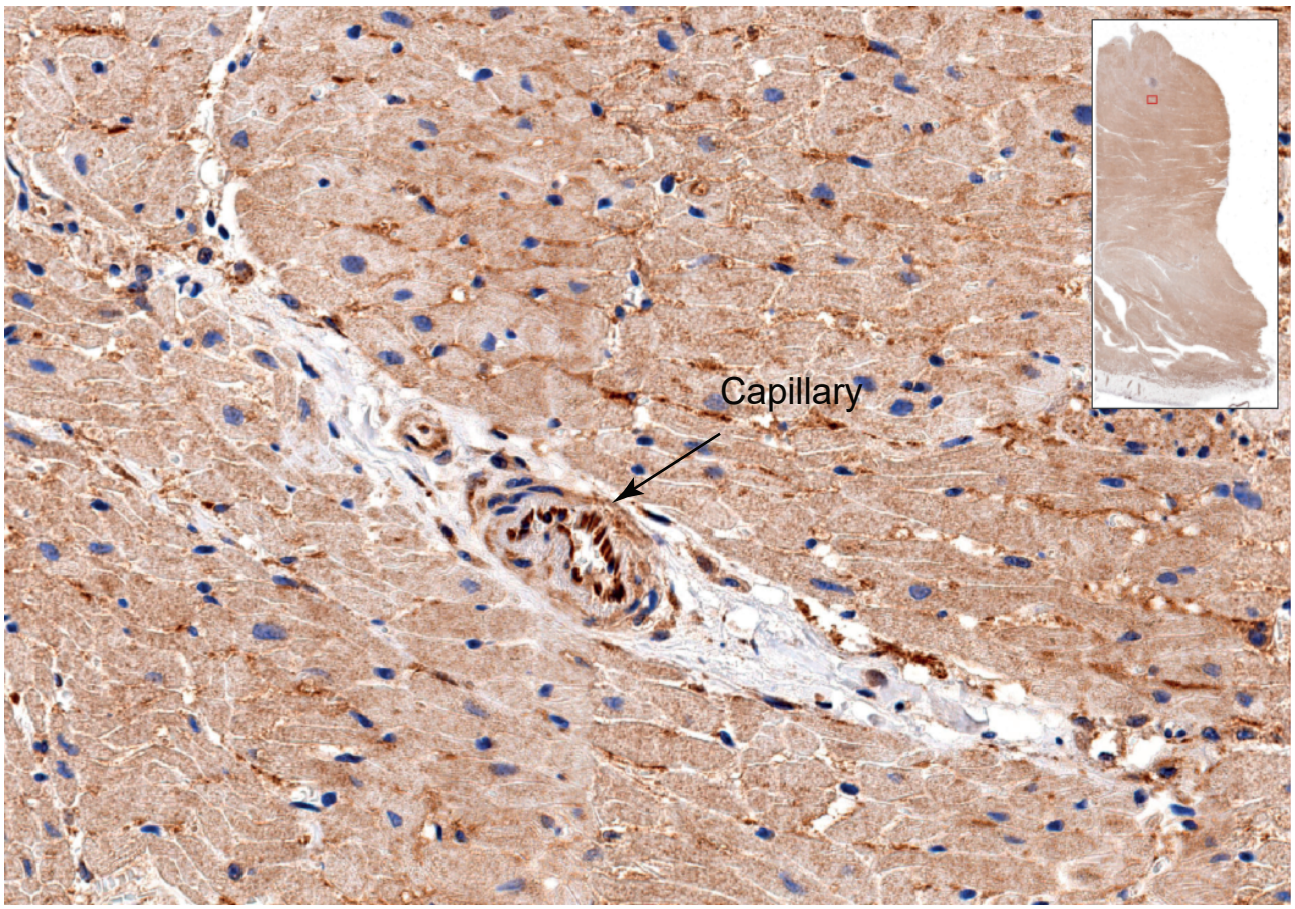
A



Human Left Atrium

50 µm

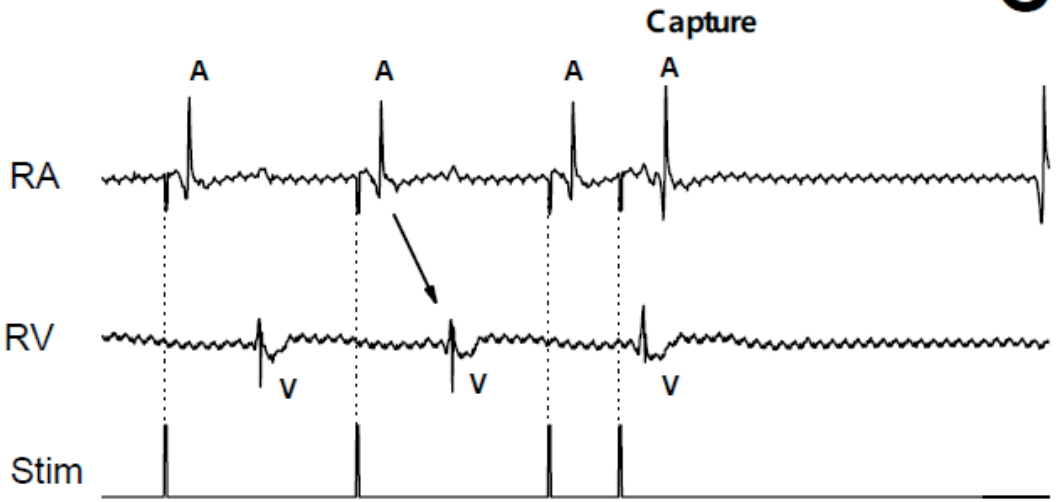
B



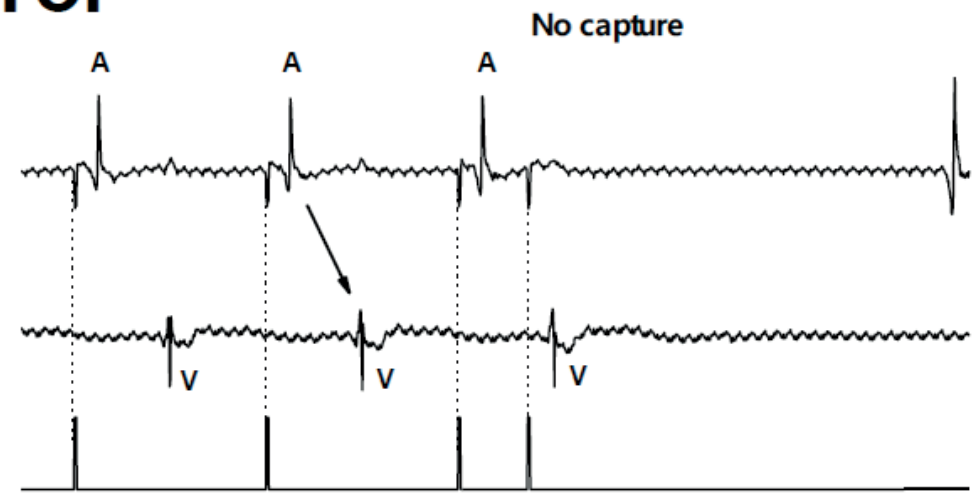
Human Left Ventricle

50 µm

Control

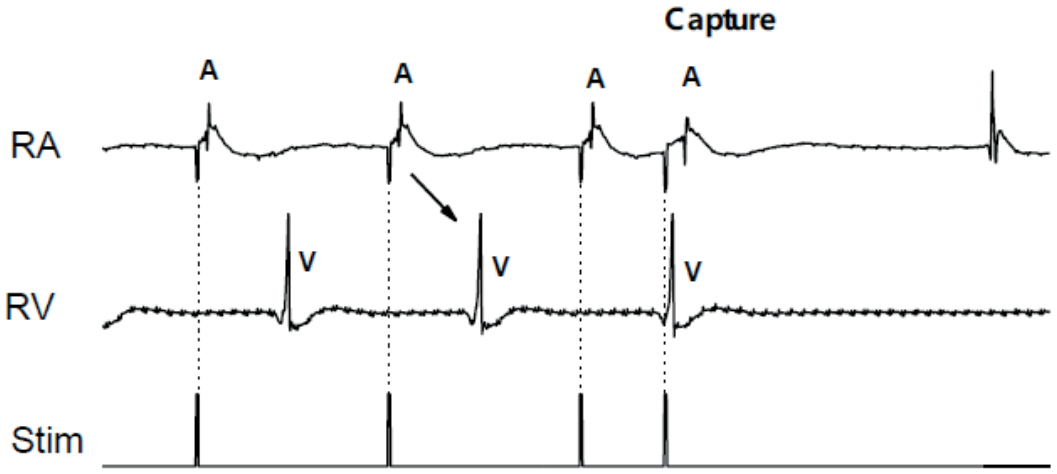


S1-S2=56

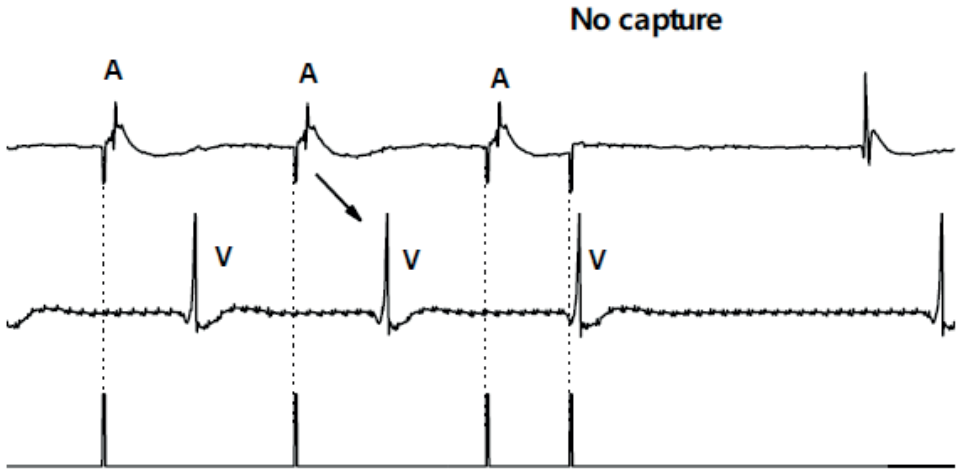


S1-S2=55

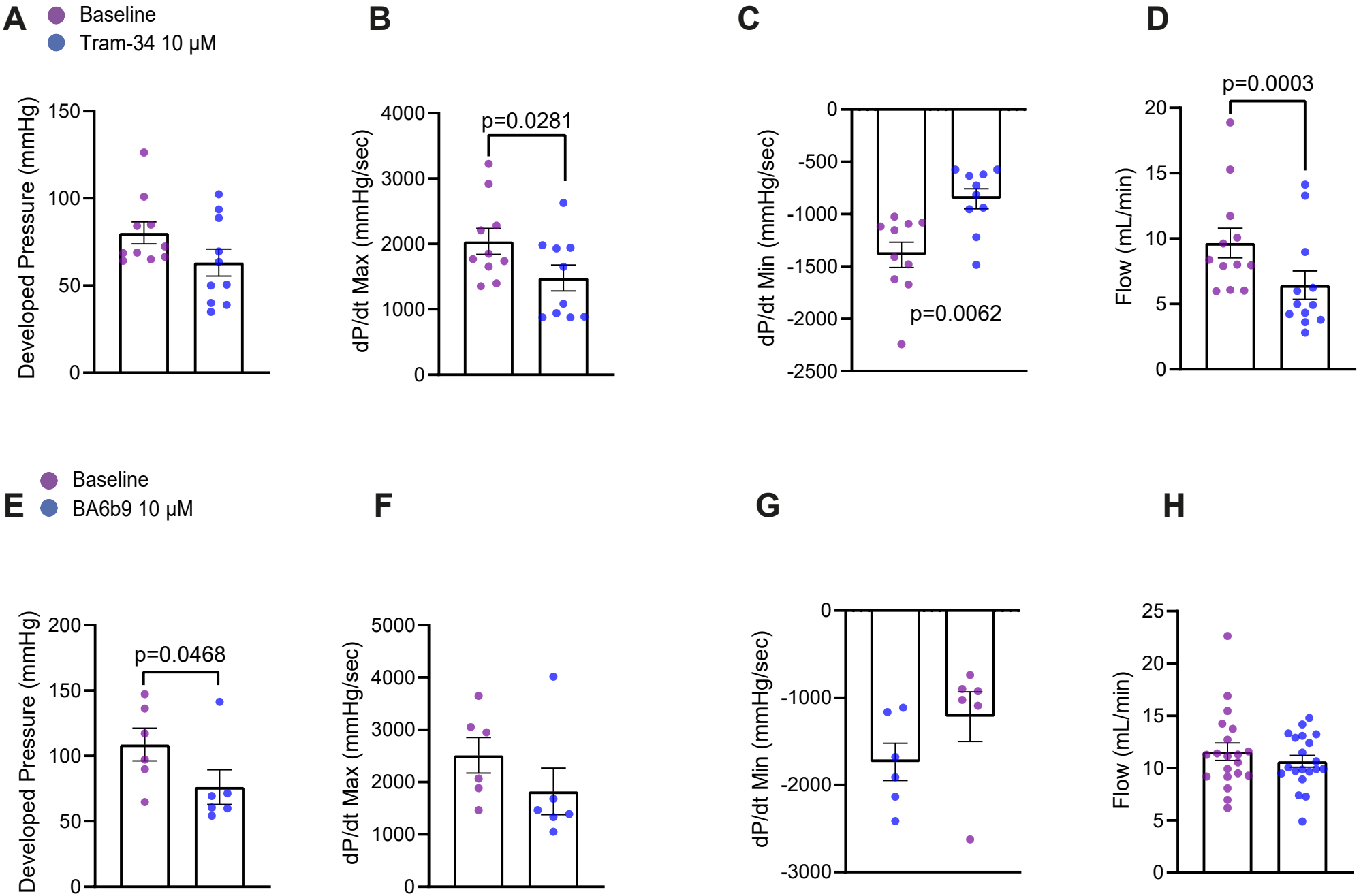
BA6b9



S1-S2=67



S1-S2=66


A
Baseline
Tram-34 10 μM
Developed Pressure (mmHg)
150
100
50
0
B
dP/dt Max (mmHg/sec)
4000
3000
2000
1000
0
p=0.0281
C
dP/dt Min (mmHg/sec)
0
-500
-1000
-1500
-2000
-2500
p=0.0062
D
Flow (mL/min)
20
15
10
5
0
p=0.0003
E
Baseline
BA6b9 10 μM
Developed Pressure (mmHg)
200
150
100
50
0
p=0.0468
F
dP/dt Max (mmHg/sec)
5000
4000
3000
2000
1000
0
G
dP/dt Min (mmHg/sec)
0
-1000
-2000
-3000
H
Flow (mL/min)
25
20
15
10
5
0