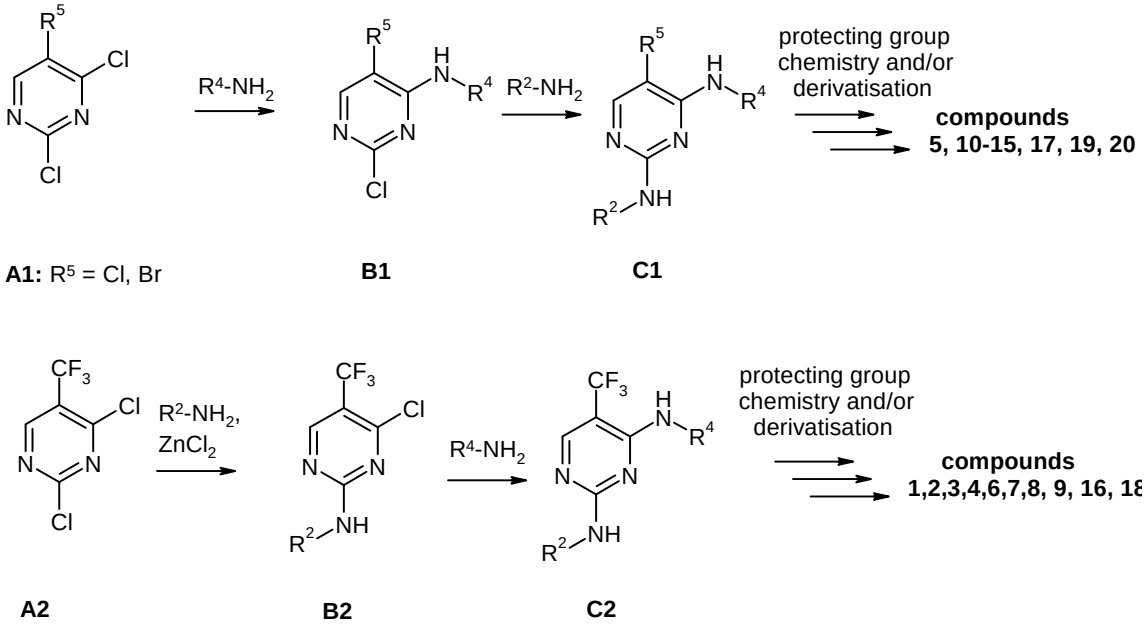
Electronic Supplementary Material (ESI) for MedChemComm.

Title

Discovery of novel amino-pyrimidine inhibitors of the insulin-like growth factor 1 (IGF1R) and insulin receptor (INSR) kinases; parallel optimization of cell potency and hERG inhibition

Supporting Information

General formula scheme and summary of the synthesis route



In principle, 2,4-diaminopyrimidine derivatives may be prepared from correspondingly substituted 2,4-dichloropyrimidines. The reactivity of those is controlled by the substituent in position 5, with the result that different synthesis strategies have to be adopted depending on the nature of this substituent.

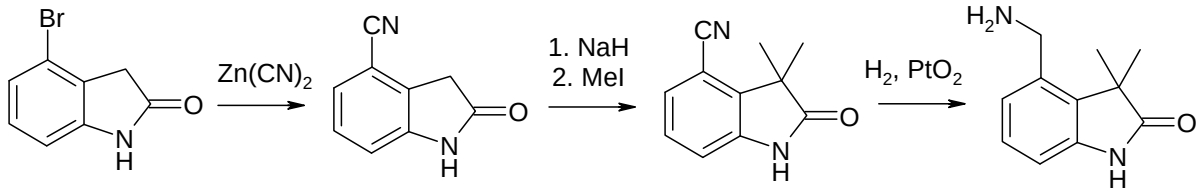
For all the substituents with the exception of the trifluoromethyl group (-CF_3) functionalization may be carried out in the 4 position as the first step. By reacting the 2,4-dichloro compound **A1** with the corresponding amine **R^4**-NH_2 the intermediate **B1** is obtained, which may in turn be converted into the 2,4-diaminopyrimidine **C1** by reaction with a (hetero)aromatic amine **R^2**-NH_2.

2,4-Dichloropyrimidine **A2** substituted in position 5 by a trifluoromethyl group may be reacted in the presence of zinc chloride with a (hetero)aromatic amine **R^2**-NH_2 and thus yields the 4-chloropyrimidine **F2**, which may in turn be reacted with amines **R^4**-NH_2 and converted into the 2,4-diaminopyrimidine **C2**.

The 2,4-diaminopyrimidines **C1** and **C2** may on the one hand be end compounds **1-20** or on the other hand may be prepared using correspondingly protected amines in positions 2 and 4, deprotected by conventional methods and then converted into the actual end compounds **1-20.**

Instead of protected amines it is also possible to use synthesis components which can be directly functionalized or derivatised without recourse to protective groups.

1. Preparation of the oxindole components (= amine R^4-NH_2)



Preparation of 4-cyanooxindole.

4-bromoxindole (2.0 g, 9.43 mmol), zinc cyanide (680 mg, 5.68 mmol), tris(bisbenzylideneacetone)dipalladium (250 mg, 0.27 mmol) and 1,1'-diphenylphosphineferrocene (300 mg, 0.54 mmol) are stirred in anhydrous DMF (10 mL) for 2 h at 140°C under argon. The reaction mixture is cooled and poured onto 30 % aqueous ethanol. The precipitate is filtered off, digested with water and EtOH and dried.

$[M+H]^+$ = not ionisable, t_{ret} = 0.44 min.

Preparation of 4-cyano-3,3-dimethyloxindole.

4-cyanooxindole (6.80 g, 43.0 mmol) in anhydrous THF (40 mL) is combined with sodium hydride (60 % dispersion in white oil, 7.0 g, 175 mmol) at - 72°C under argon and stirred for 20 min at this temperature. Methyl iodide (4.0 mL, 64.3 mmol) is added, the reaction mixture is slowly heated to RT and stirred for 48 h. The mixture is combined at - 72°C with saturated ammonium chloride solution (38 mL) and divided between water and EtOAc. The aqueous phase is exhaustively extracted with EtOAc, the combined organic phases are washed with water and saturated saline solution, dried on sodium sulphate, filtered and evaporated down. The crude product is crystallized or purified by column chromatography.

Additional alkylation at the nitrogen is obtained using 6 equiv. NaH and 7 equiv. methyl iodide. Moreover, an alkylating agent other than methyl iodide may be used.

$[M+H]^+$ = 187.2, t_{ret} = 0.72 min.

Preparation of 4-aminomethyl-3,3-dimethyloxindole .

4-cyano-3,3-dimethyloxindole (3.70 g, 19.9 mmol) in MeOH (35 mL) and 6 N HCl (15 mL) is mixed with platinum oxide (500 mg) and hydrogenated for 17 h at RT under a hydrogen pressure of 5 bar. The reaction mixture is filtered and evaporated down.

$[M+H]^+$ = 191.0, t_{ret} = 0.13 min.

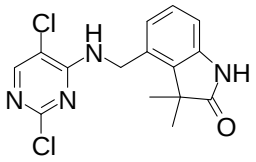
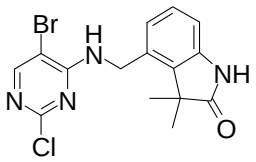
2. Preparation of the intermediates C1 / example compounds and 5, 10-15, 17, 19, 20

2.1.1 Synthesis of intermediate B-1

2,4,5-trichloropyrimidine **A1-1** (0.30 mL, 2.61 mmol) in anhydrous DCM is combined at 0°C with *N*-ethyldiisopropylamine (1.4 mL, 8.0 mmol) and 4-aminomethyl-3,3-dimethyloxindole (653 mg, 2.88 mmol) and stirred for 30 min at RT. The reaction mixture is washed with dil. ammonium chloride solution and water, the organic phase is dried on sodium sulphate, filtered, evaporated down and crystallized with water.

The bromine analogue **B1-1** is prepared analogously starting from 5-bromo-2,4-dichloropyrimidine.

Table S1

Intermediate	Structure	t_{ret} [min]	$[M+H]^+$
B-1		0.84	337
B1-1		1.01	381

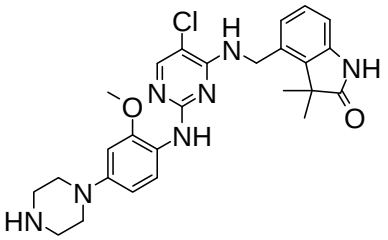
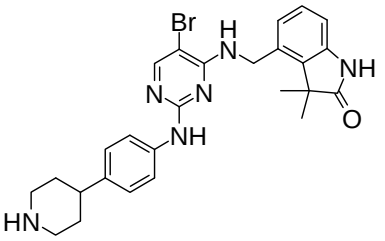
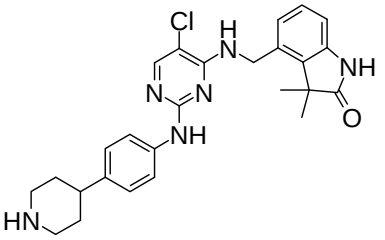
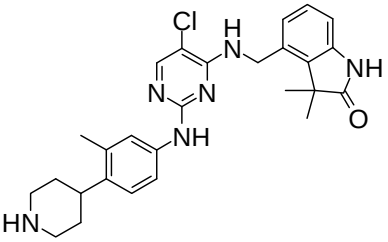
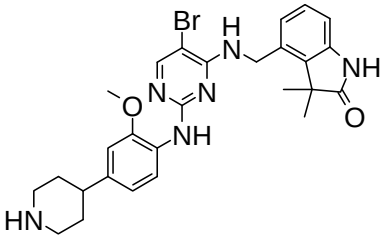
2.1.2 General synthesis of intermediates C1 and C1-1 starting from intermediates B1 and B1-1.

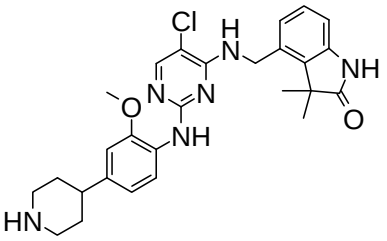
A mixture of the corresponding 2-chloropyrimidine **B** (0.22 mmol), the (hetero)aromatic amine R^2-NH_2 (0.29 mmol) and methanesulphonic acid (0.77 mmol) is stirred for 18 h in anhydrous 2-propanol (400 µL) at 90°C. The reaction mixture is purified by preparative HPLC-MS. The fractions containing the reaction product are freeze-dried.

Instead of methanesulphonic acid p-toluenesulphonic acid or trifluoroacetic acid may be used. Instead of 2-propanol, 4-methyl-2-pentanol may be used as solvent. If the amine used, R^2-NH_2, has a BOC protective group this is cleaved *in situ* under the acidic reaction conditions.

The following intermediates **C1** / example compounds are prepared by this method (Table S2):

Table S2

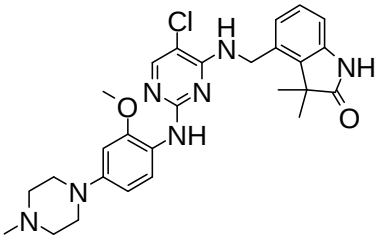
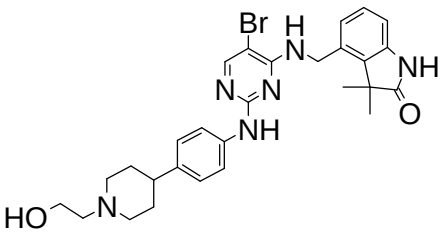
Intermediate / Example	Structure	t_{ret} [min]	$[M+H]^+$
C1-a		1.68	508/510
C1-1a		1.43	521/523
C1-b		1.39	477/479
C1-c		1.04	491/493
C1-1b		1.75	551/553

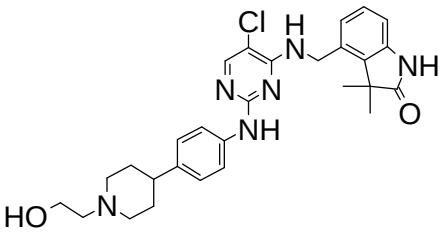
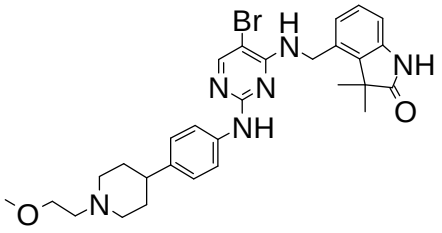
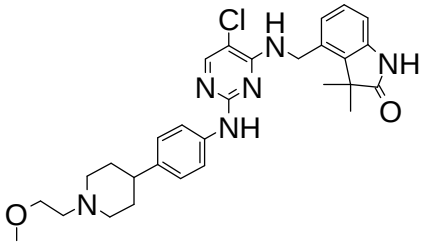
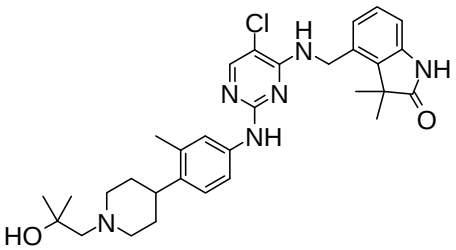
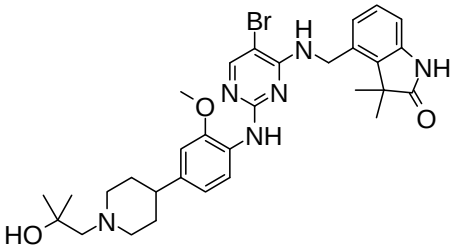
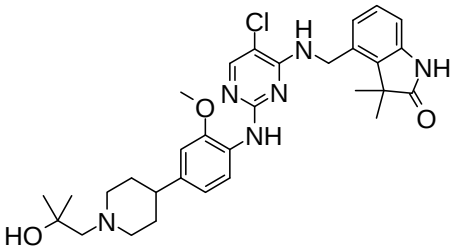
Intermediate / Example	Structure	t_{ret} [min]	$[M+H]^+$
C1-d		1.75	508/510

2.1.3 General synthesis of compounds 5, 12-15, 17, 19, 20 starting from intermediates C1 and C1-1 via reductive amination.

The corresponding compound **C1** or **C1-1** is taken up in anhydrous NMP, combined with the carbonyl compound (3 equivalents) and sodium triacetoxyborohydride (2.9 equivalents) and stirred for 1.5 h at rt. The reaction mixture is combined with formic acid and purified by preparative HPLC-MS. The fractions containing the reaction product are freeze-dried.

Table S3

Intermediate / Example	Structure	t_{ret} [min]	$[M+H]^+$
5		1.77	522/524
12		2.63	565/567

Intermediate / Example	Structure	t_{ret} [min]	$[M+H]^+$
13		2.57	522/524
14		2.73	579/581
15		4.91	535/537
17		1.31	563/565
19		2.14	623/625
20		1.4	580/582

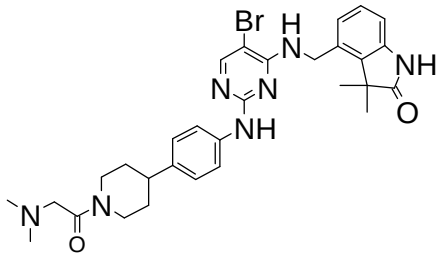
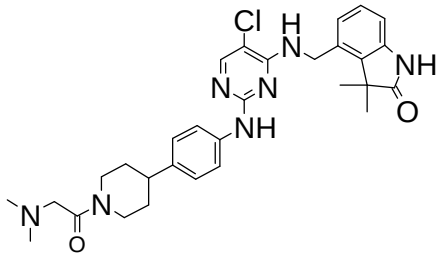
1H-NMR data set of a representative example (20).

^{1}H NMR (500 MHz, DMSO-d_6) δ 10.40 (s, 1H), 7.92 (s, 1H), 7.63 (t, J = 5.9 Hz, 1H), 7.43 (d, J = 9.0 Hz, 1H), 7.33 (s, 1H), 7.11 (t, J = 7.8 Hz, 1H), 6.79 (d, J = 7.9 Hz, 1H), 6.75 (d, J = 7.6 Hz, 1H), 6.52 (d, J = 2.4 Hz, 1H), 6.14 (d, J = 8.7 Hz, 1H), 4.67 (d, J = 6.1 Hz, 2H), 4.09 (s, 1H), 3.74 (s, 3H), 3.01 (t, J = 4.8 Hz, 4H), 2.63 (t, J = 4.8 Hz, 4H), 2.24 (s, 2H), 1.35 (s, 6H), 1.11 (s, 6H).

2.1.3 Synthesis of compounds 10 and 11 starting from intermediates C1 and C1-1 via acylation.

The corresponding arylpiperazine or -piperidine is taken up in anhydrous DCM, combined with the appropriate acid chloride (1.4 equivalents) and triethylamine (3 equivalents) and stirred for 3 h at rt. The reaction mixture is evaporated down, the residue is taken up in DMSO and purified by preparative HPLC-MS. The fractions containing the reaction product are freeze-dried.

Table S4

Intermediate / Example	Structure	t_{ret} [min]	$[M+H]^+$
10		2.75	608/610
11		2.7	562/564

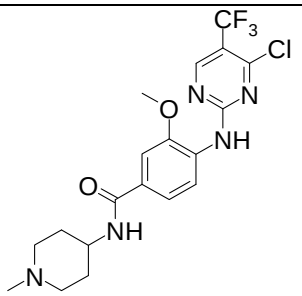
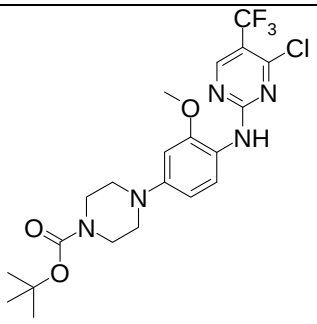
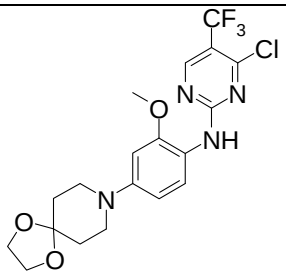
3. Preparation of the intermediates C2 / example compounds and 1-4, 6-9, 16, 18

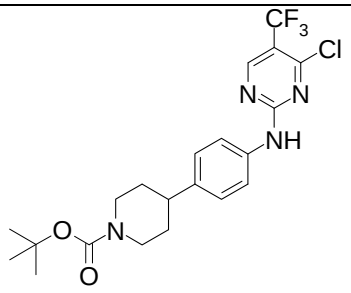
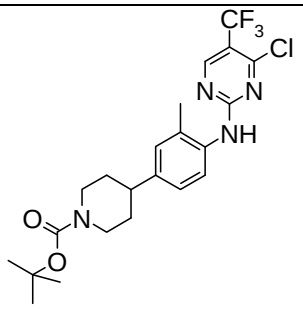
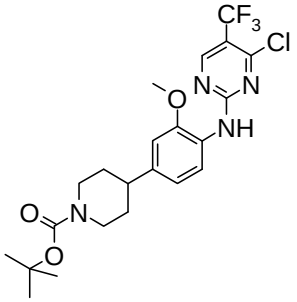
3.1 General synthesis of intermediates B2

The preparation of 2-(hetero)arylamino-4-chloro-5-trifluoromethylpyrimidine derivatives **B2** is carried out analogously to WO 2005/023780. The crude products obtained are optionally purified by chromatography.

The following intermediates **B2** are obtained (Table S5):

Table S5

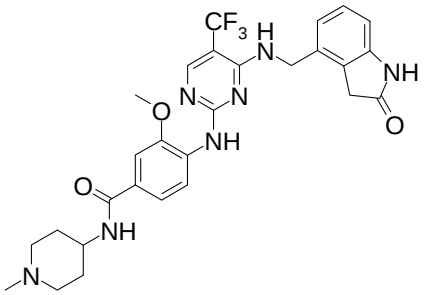
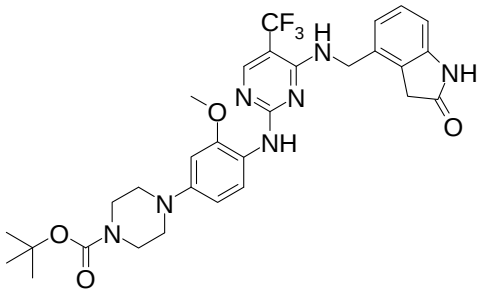
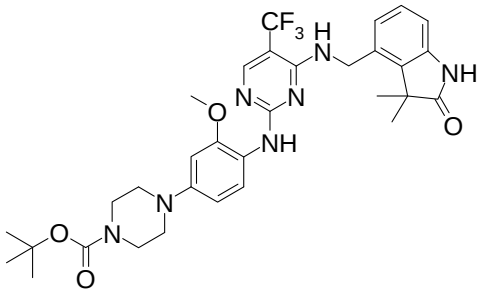
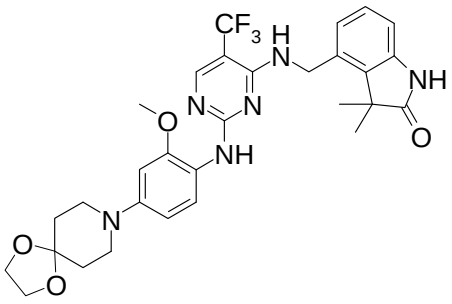
Intermediate	**Structure**	**t_{ret} [min]**	**$[M+H]^+$**
B2-a		0.83	444
B2-b		0.95	549
B2-d		1.24	445

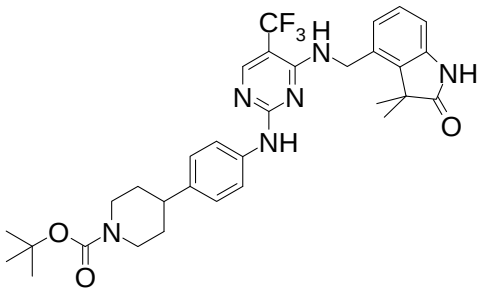
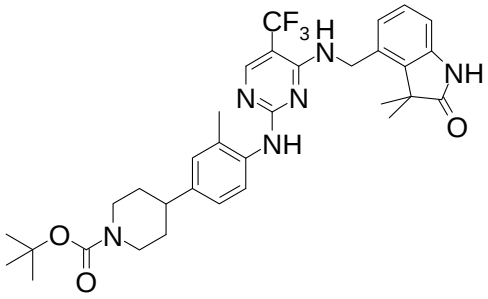
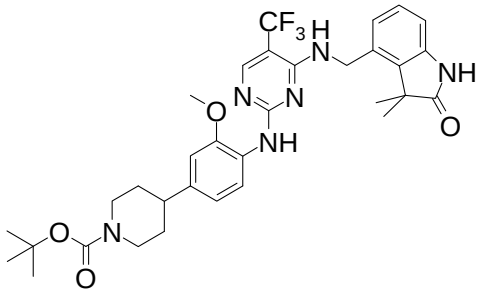
Intermediate	Structure	t_{ret} [min]	$[M+H]^+$
B2-e		2ю61	401
B2-f		1.61	469 (M-H)
B2-g		2.02	431 (M+H-tBu)

3.2 General synthesis of intermediates/example compounds C2 or starting from intermediates B2.

The reaction of 4-chloropyrimidine components **B2** with corresponding amines R4-NH2 is carried out according to the method used to synthesize intermediate **B1**.

Table S6

Intermediate (Example)	Structure	t_{ret} [min]	$[M+H]^+$
1		1.19	525
C2-b		1.11	614
C2-c		1.22	642.3
C2-d		n.a.	n.a.

Intermediate (Example)	Structure	t_{ret} [min]	$[M+H]^+$
C2-e		2.24	611
C2-f		1.99	625
C2-g		1.43	641.4

3.3 General synthesis of example compounds 2-4, 6-9, 16,18 starting from intermediates B2.

After deprotection with acid (TFA for BOC-deprotection, HCl for cleaving the dioxolan in **C2-d**) the respective arylpiperazine- or arylpipieridine-intermediates are liberated. They can be either reductively aminated to generate compounds **2, 3, 4, 8** or acylated to generate compounds **6, 9** or alkylated to synthesize compounds **7, 16** and **18.** .

3.3.1 General procedures for derivatisation

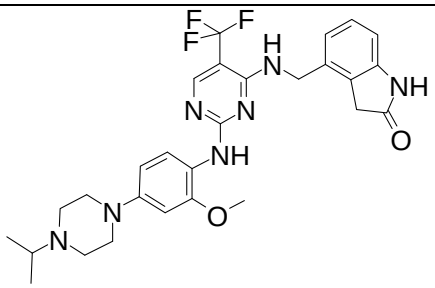
Reductive amination: The corresponding arylpiperazine or -piperidine is taken up in anhydrous NMP, combined with the carbonyl compound (3 equivalents) and sodium triacetoxyborohydride (2.9 equivalents) and stirred for 1.5 h at rt. The reaction mixture is combined with formic acid and purified by preparative HPLC-MS. The fractions containing the reaction product are freeze-dried.

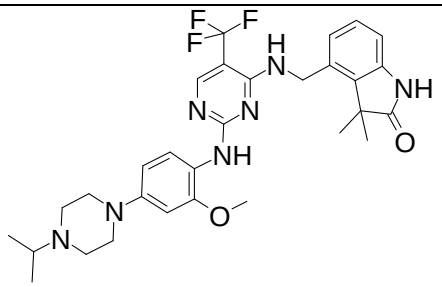
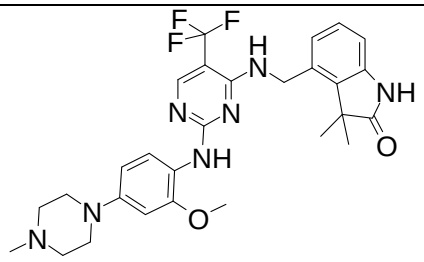
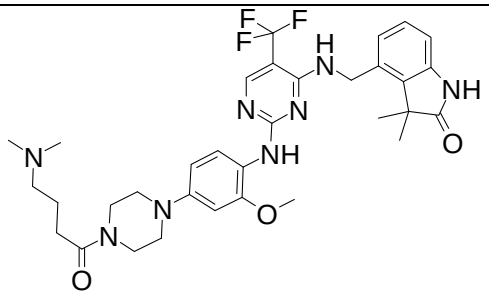
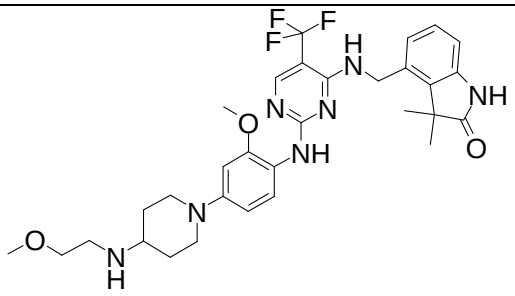
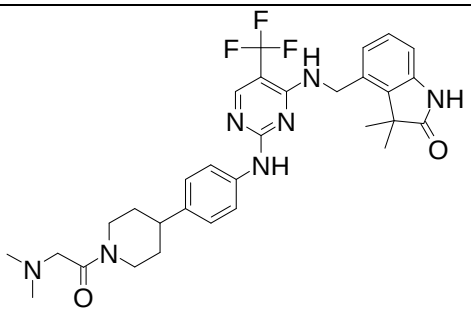
Acylation: The corresponding arylpiperazine or -piperidine is taken up in anhydrous DCM, combined with the appropriate acid chloride (1.4 equivalents) and triethylamine (3 equivalents) and stirred for 3 h at rt. The reaction mixture is evaporated down, the residue is taken up in DMSO and purified by preparative HPLC-MS. The fractions containing the reaction product are freeze-dried.

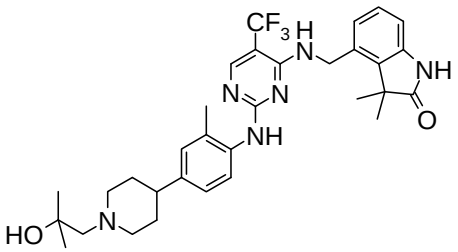
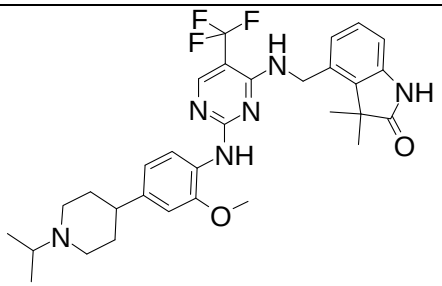
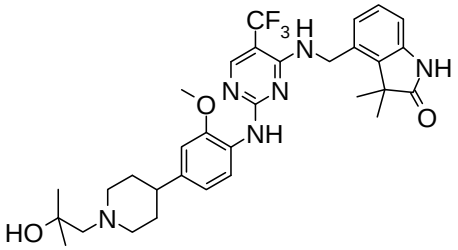
Alkylation: The corresponding arylpiperazine or -piperidine is taken up in anhydrous DMSO, combined with the alkylhalide (1.2 equivalents) and triethylamine (3 equivalents) and stirred for 12 h at rt. The reaction mixture is purified by preparative HPLC-MS. The fractions containing the reaction product are freeze-dried.

In case of compounds **16** and **18** 2,2-dimethyloxirane is used instead of a alkyl-halide. This reaction is carried out in methanol at 50 °C overnight without adding a base. Normal phase chromatography on silica gel with DCM/MeOH/NH3 95/5/0.5 generated the pure free base of **16** and **18**.

Table S7

Intermediate (Example)	Structure	t_{ret} [min]	$[M+H]^+$
2		1.89	556

Intermediate (Example)	Structure	t_{ret} [min]	$[M+H]^+$
3		2.03	584
4		1.86	556
6		1.93	655
7		1.90	614
9		3.05	596

Intermediate (Example)	Structure	t_{ret} [min]	$[M+H]^+$
16		1.36	597
8		2.29	583
18		1.38	613

1H-NMR data set of a representative example (18).

^{1}H NMR (500 MHz, DMSO-d_6) δ 10.39 (s, 1H), 8.21 (s, 1H), 7.89 (m, 1H), 7.57 (s, 1H), 7.45 (t, J=5.6 Hz, 1H), 7.11 (t, J = 7.8 Hz, 1H), 6.83 - 6.70 (m, 3H), 6.45 (s, 1H), 4.73 (d, J = 5.2 Hz, 2H), 3.99 (s, 1H), 3.75 (s, 3H), 3.05 (m, 2H), 2.12 – 2.24 (m, 5H), 1.58-1.66 (m, 4H), 1.33 (s, 6H), 1.10 (s, 6H).

Description of LCMS Method

HPLC Agilent 1100 Series

Column XBridge 2.5 µm, C18, 20 x 2.1 mm column

MS 6100 Series LC/MS (API-ES +/- 3000 V, Single Quadrupol, G6130B

Eluent A: 20 mM NH4HCO3 (pH = 9.2)

B: acetonitrile (HPLC grade)

Spectrum	range: 230 – 400 nm	
Injection	5 µL standard injection	
Run time	3 min.	
Flow	1.0 mL/min	
Column temperature	60 °C	
Gradient	0.0 – 1.5 min	10 % - 95 % B
	1.5 – 2.0 min	95 % B
	2.0 – 2.1 min	95 % - 10 % B
	2.1 – 3.0 min	10 % B

Biological / Pharmacological assays

Xenograft and Allograft Studies

Animals were kept under specific pathogen-free conditions (AAALAC accredited facility) and treated according to the institutional, governmental and European Union guidelines (GV-SOLAS, Felasa, Austrian Animal Protection Laws). The animals studies were reviewed and approved by the internal ethics committee and the local governmental committee (Amt der Wiener Landesregierung, Magistratsabteilung 58, Vienna, Austria).

Xenograft tumors were established from cultured GEO or RD-ES cells (5 x 10^6 cells in matrigel and PBS + 5% FCS (1:1)) by subcutaneous (s.c.) injection of 100 µL cell suspension into the right flanks of female BomTac:NMRI-Foxn1nu mice. Allograft tumors were established from murine NIH-3T3 fibroblasts stably transfected to express the human IGF1R (3T3-hIGF1R). 2.5 x 10^6 cells 3T3-hIGF1R cells were injected s.c. (in matrigel and PBS + 5% FCS (1:1).

Tumors were considered to be established once they had reached an average volume of approximately 100 mm^3. Animals were randomized (n=7/group) and treated with either vehicle (0.5% Natrosol) or the compound formulated with 0.5% Natrosol. All doses were calculated relative to the mouse body weight on the treatment day. Compounds and the vehicle control were administered orally (p.o.) daily.

Tumor volumes were determined three times a week using a digital caliper. Body weights of mice were measured daily as an indicator of tolerability of the compounds. TGI values were calculated as follows: TGI = 100 x {1-[(treated final day – treated day1) / (control final day – control day1)]}.

IGF1R in vitro kinase assay

A fusion protein of the cytoplasmic domain of the human IGF1R (amino acids 963 – 1367) with Glutathione-S-Transferase (IGF1R-GST) was used for IGF1R kinase assays. The assays were performed using a DELFIA® (dissociation-enhanced lanthanide fluorescence immunoassay) (Perkin Elmer) format. All steps were performed at room temperature (RT). Serial dilutions of compounds were mixed with 30 µl IGF1R-GST solution (diluted in 67 mM HEPES pH 7.4, 15 µg/ml pEY, 1.7 µg/ml bio-pEY, 13.3 mM $MgCL_2$, 3.3 mM Dithiothreitol, 0.0033% Brij 35, 2 ng IGF1R-GST) in 96-well plates. The reaction was initiated by the addition of 10 µl of an ATP solution and incubated for 40 minutes. The reaction was terminated by the addition of 50 µl of a stop solution (250 mM EDTA, 20 mM HEPES pH 7.4). 90 µl of each reaction was transferred to streptavidin-coated 96-well plates and incubated for 2 hours. Plates were washed three times with 200 µl PBS and then incubated for 1 hour with 100 µl of a europium-labelled antibody specific to phospho-tyrosinse (1/2000 diluted in Perkin Elmer DELFIA assay buffer). Following three further wash steps with 200 µl DELFIA wash buffer, 100 µl DELFIA Enhancement Solution was added to each well and plates incubated for a further 10. The fluorescence intensity was measured using a Wallac Victor TRF Reader. IC_{50} values for each compound were generated using the sigmoidal curve analysis program Graph Pad Prism.

In vitro cell proliferation assays

2000 TC-177 cells or 1000 HCT 116 cells were seeded per well in 180 µl IMDM + 10% fetal calf serum (FCS) + penicillin/streptomycin into 96-well microtitre plates. All incubation steps were performed in a cell culture incubator (37 °C in a humidified atmosphere of 95% O_2/5% CO_2). The following day, serial dilutions of compounds were transferred onto the cell layers. Cells were cultivated for a further 72 hours. 20 µl of Alamar Bluet™ (Serotec Ltd, Düsseldorf, Germany) was added to each well and the plates incubated for 7 hours. Fluorescence (extinction wavelength of 544 nm and emission at 590 nm) was then measured and EC_{50} values for each compound were generated using Graph Pad Prism.

herg-channel assay

Cells

HEK (human embryonic kidney) 293 cells were stably transfected with hERG cDNA. Cells determined for use in patch clamp experiments were cultivated without antibiotic.

Pipettes and solutions

Cells were superfused with a bath solution containing (mM): NaCl (137), KCl (4.0), $MgCl_2$ (1.0), $CaCl_2$ (1.8), Glucose (10), HEPES (10), pH 7.4 with NaOH. Patch pipettes were made from borosilicate glass tubing (Hilgenberg, Malsfeld, FRG) using a horizontal puller (DMZ-Universal Puller, Zeitz-Instrument, Martinsried, FRG) and filled with pipette solution containing (mM): K-aspartate (130), $MgCl_2$ (5.0), EGTA (5.0), K_2ATP (4.0), HEPES (10.0), pH 7.2 with KOH. Resistance of the pipettes was in the range of between 2 and 5 MΩ.

Stimulation and recording

Membrane currents were recorded using an EPC-10 patch clamp amplifier (HEKA Electronics, Lambrecht, FRG) and PatchMaster software (HEKA). The current signals were Bessel filtered at 2.5 kHz before being digitized at 5 kHz.

hERG-mediated membrane currents were recorded at 28 °C, using the whole-cell configuration of the patch-clamp technique. Transfected HEK293 cells were clamped at a holding potential of -60 mV and hERG-mediated inactivating tail currents were elicited using a pulse pattern with fixed amplitudes (activation/inactivation: 40 mV for 2000 ms; recovery: -120 mV for 2 ms; ramp to 40 mV in 2 ms; inactivating tail current: 40 mV for 50 ms) repeated at 15 s intervals. During each inter-pulse interval 4 pulses scaled down by a factor of 0.2 were recorded for a P/n leak subtraction procedure. R_s compensation was employed up to a level that safely allowed recording devoid of ringing. The remaining uncompensated R_s was recorded as well as temperature and holding current.

Compound preparation and application

The concentrations of the test items were applied sequentially on each of three different cells investigated. A steady state level of baseline current was measured for at least 90 s prior to the application of the first test article concentration.

The test items were dissolved in DMSO to yield a master stock solution of 1000-fold the intended final maximum concentration. This stock was diluted further in DMSO in a semi-logarithmic fashion to stock solutions fold 1000-fold the final concentrations intended to be used for a concentration-response curve. Final dilutions in extracellular buffer were prepared freshly from these stocks by a single 1:1000 dilution step each before starting the experiments.

Data analysis

Peak current amplitudes were measured 3 ms after the ramp to +40 mV. For baseline and each concentration the peak currents of the three last sweeps before application of the next concentration were averaged. Residual currents (I/I0) were calculated for each cell as the fraction of actual average peak current and average baseline peak current. Current inhibition was expressed as (1 - I/I0) * 100% and averaged for the three cells analyzed.

From the current inhibition versus concentration data the IC_{50} was estimated using the Hill equation and a least squares fit procedure.

Assessment of IGF1R phosphorylation in GEO tumors

Mice bearing GEO tumors with a size between 105 to 181 mm^3 were randomized and orally dosed with either vehicle control (0.5% Natrosol) (n=6) or compound 17 (n=4). Mice were sacrificed 6 hours post-application and GEO tumors explanted and immediately frozen at -80°C. Excised tumors were mechanically homogenized in liquid nitrogen using a mortar and pestle. After homogenization and prior to thawing of the tissue powder, cell lysis buffer supplemented with protease and phosphatase inhibitors (Biorad) was added at a ratio of 3:1 relative to the tumor weight. The mixtures were then transferred to tubes and incubated on ice for 10 minutes to enable lysis. The lysates were then centrifuged for 10 minutes (10000 rpm, 4°C) in a bench-top centrifuge. Supernatants were then transferred to new tubes and stored at -80°C prior to further use. A Bio-Plex™ kit (BioRad) was used according to the manufacturer's instructions to measure the levels of phosphorylated IGF1R

(Tyrosine 1131) in each lysate sample. Data are presented as the mean and standard deviation, normalized as percentage of control values. The statistical evaluation of the data was performed using unpaired two-tailed parametric t-test using the software package GraphPad Prism Bioanalytic Software version 5.01.