Supporting Information

Development of a Cell-Selective and Intrinsically Active Multikinase Inhibitor Bioconjugate

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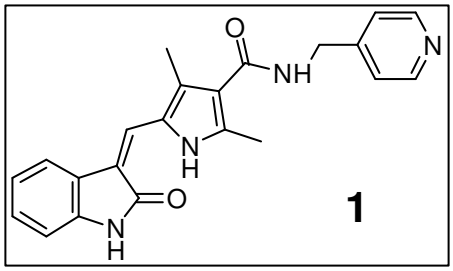
1. Materials and Methods

All chemicals used in this study were of analytical grade or equivalent and obtained from Sigma Aldrich (Zwijndrecht, the Netherlands) and used without further purification unless otherwise stated. The multikinase inhibitor precursor (*Z*)-2,4-dimethyl-5-((2-oxoindolin-3-ylidene)methyl-1*H*-pyrrole-3-carboxylic acid was synthesized by Vichem Chemie (Budapest, Hungary). Universal Linkage System™ (monochloro-mononitrato(ethylenediamine)platinum(II)) was provided by Kreatech (Amsterdam, the Netherlands). HPLC grade acetonitrile (ACN), methanol (MeOH), isopropanol (IPA), formic acid (FA) and *N,N'*-dimethylformamide (DMF) which was dried over molecular sieve before use were purchased from Biosolve (Valkenswaard, the Netherlands). Products were characterized by $^{1}H/^{195}Pt$ NMR and LC-MS/MS. $^{1}H/^{195}Pt$ NMR spectra were recorded on a Varian Gemini (300 MHz; 298 K; Varian Associates Inc., NMR Instruments, Palo Alto, CA) NMR spectrometer. The LC-MS/MS equipment consisted of an Accela pump and autosampler and a TSQ Quantum Ultra quadrupole mass spectrometer with heated electrospray ionization (Thermo Fisher Scientific, San Jose, CA, USA). Data were recorded on and the system was controlled by a Dell Precision T3400 personal computer, equipped with the Thermo Fisher Xcalibur software (version 2.07). 5-µl injections were made on an Aquity UPLC® BEH C18 column (30 × 2.1mm × 1.7 µm; Waters, Milford, USA) with the corresponding VanGuard pre-column (5 × 2.1mm × 1.7 µm; Waters, Milford, USA). Column temperature was maintained at 40˚C and the autosampler was maintained at 4˚C. A 20-90% gradient of solvent B over 2.5 min with a 0.5 ml/min flow rate was used. Solvent A consisted of 0.1% (v/v) formic acid in water and solvent B of methanol. The eluate was totally led into the electrospray probe, oriented at "+1" in the X-, at "1" in the Y- and at "C" in the Z-direction, from 0.7 min after injection. Electrospray (ESI+) settings were a 4000 V spray voltage, 350°C capillary and vaporizer temperatures and a 1.0 mTorr argon collision pressure. The mass resolution was set at 0.7 full with at half height (unit resolution) for both separating quadrupoles.

2. Molecular Modeling of an oxindole-based inhibitor in the ATP-binding pocket of c-KIT

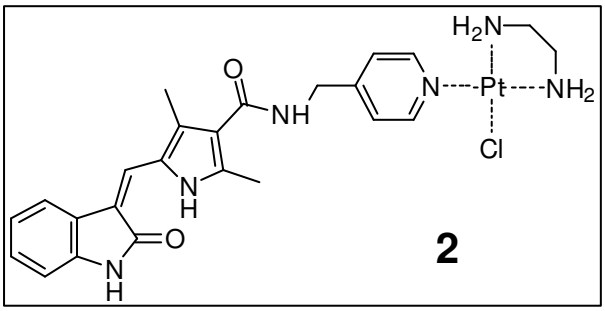
YASARA (www.yasara.org; version 10.6.1) was used to evaluate how oxindole-based multikinase inhibitors generally bind to their targets. Therefore, protein structures co-crystallized with either sunitinib (3G0E) or intedanib (3C7Q) were downloaded from www.pdb.org and their orientation in the binding pocket of their target kinases was studied. YASARA was additionally used to generate a graphic representation of how compound **2** would bind to c-KIT by modification of the structure used in 3G0E.

3. Synthesis of the multikinase inhibitor (1), multikinase inhibitor platinum complex (2) and drug-lysozyme conjugate (3)



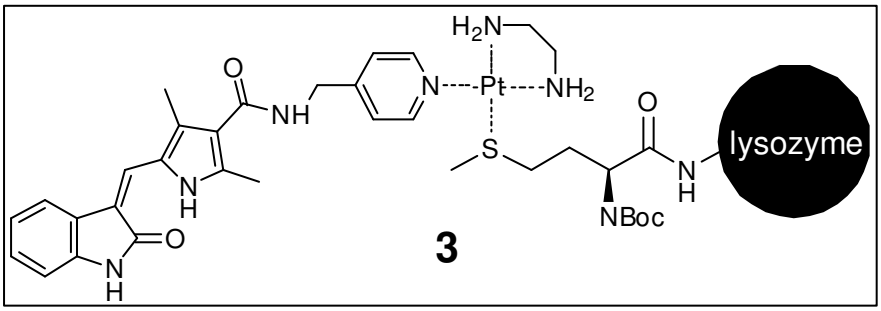
Synthesis of multikinase inhibitor (1) ((*Z*)-2,4-dimethyl-5-((2-oxoindolin-3-ylidene)methyl)-*N*-(pyridin-4-ylmethyl)-1*H*-pyrrole-3-carboxamide)

Compound **1** was synthesized according to methods described by Sun *et al*[1]. In brief, (*Z*)-2,4-dimethyl-5-((2-oxoindolin-3-ylidene)methyl-1*H*-pyrrole-3-carboxylic acid (1.0 g, 3.5 mmol) was suspended in 40 ml dry DMF and 4-(aminomethyl)pyridine (0.38 g, 3.5 mmol), EDC (0.81 g, 4.2 mmol), HOBt (0.57 g, 6.0 mmol) and triethylamine (0.74 ml, 5.3 mmol) were added to the suspension and stirred at 50°C for 72 hours. The solvent was removed under reduced pressure and the residue was taken up with water and the product was filtered off yielding compound **1** (0.72 g, 54%). ^{1}H NMR (300 MHZ, DMF-d_7): δ (ppm): 10.87 (s, 1H, NH); 8.55 (d, 2H, 2CH, ^{3}J=4.2 Hz); 8.11 (t, 1H, NH); 7.85 (d, 1H, CH, ^{3}J=7.6 Hz); 7.79 (s, 1H, CH); 7.41 (d, 2H, 2CH, ^{3}J=5.0 Hz); 7.18 (t, 1H, CH, ^{3}J=7.6 Hz); 7.02 (t, 1H, CH, ^{3}J=7.6 Hz); 6.97 (d, 1H, CH); 4.60 (d, 2H, CH_2, ^{3}J=6.1); 2.56 (s, 3H, CH_3); 2.55 (s, 3H, CH_3). MS (ESI^+) calcd. for $C_{22}H_{20}N_4O_2H^+$ $[M+H]^+$ 373, detected 373.



Synthesis of multikinase inhibitor platinum(II) complex (2)

Compound **1** (15.0 mg, 40.3 μmol) was reacted with monochloro-mononitrato (ethylenediamine)platinum(II) (Universal Linkage System (ULS)^{TM}- nitrate; 14.2 mg , 40.3 μmol) in DMF for 2 hours at 37^{o}C to yield compound **2** (23.1 mg, 83.4%). ^{195}Pt NMR (300 MHz, DMF-d_7) -2492 ppm (N3PtCl). ^{1}H NMR (300 MHz, DMF- d_7): δ (ppm): 10.91 (s, 1H, NH); 8.80 (d, 2H, 2CH, ^{3}J=5.6 Hz); 8.23 (t, 1H, NH); 7.86 (d, 1H, CH); 7.81 (s, 1H, CH); 7.62 (d, 2H, 2CH, ^{3}J=6.2 Hz); 7.19 (t, 1H, CH, ^{3}J=7.7 Hz); 7.04 (t, 1H, CH, ^{3}J=7.4 Hz); 7.00 (d, 1H, CH); 6.31 (s, 2H, NH_2), 5.89 (s, 2H, NH_2); 4.69 (d, 2H, CH_2,^{3}J=5.7 Hz); 2.58 (s, 3H, CH_3); 2.57 (s, 3H, CH_3); 1.56 (t, 2H, CH_2); 1.29 (s, 2H, CH_2).MS (ESI^{+}) calcd. for $C_{24}H_{28}N_6O_2HPtCl$ $[M+H]^{+}$ 663, detected 663 $[M+H]^{+}$.



Conjugation of a multikinase inhibitor platinum(II) complex to methionylated lysozyme (3)

Methionylated lysozyme was synthesized as previously described by Fretz *et al.*[2]. Compound **2** (15.5 mg, 23.3 μmol) was reacted with methionylated lysozyme (112.1 mg, 7.8 μmol overnight in 0.02 M tricine/sodium nitrate buffer (pH 8.5). The product was purified by dialysis (molecular weight cut-off 10,000 Da) against ultrapure water (MilliQ, Millipore, Bedford, MA, USA) and lyophilized. MS (ESI^{+}) calcd. for

$C_{24}H_{28}N_6O_2HPt$-LZM $[M+H]^+$ 14930, detected 1493.0 $[M+H]^{10+}$ and 1357.2 $[M+H]^{11+}$ corresponding with 14930 $[M+H]^+$. Conjugation efficiency was determined as described previously[2]. Liberated **1** was quantified in samples containing **3** incubated overnight at 80°C in the presence of the strong platinum ligand thiocyanate (SCN; 0.5 M) thereby competitively displacing compound **1** from ULS by HPLC-photodiodearray (PDA) analysis at 431 nm. The HPLC system consisted of a Waters 717 autosampler, Waters 600 pumps and a Waters 2996 Photodiode Array Detector (Waters, Milford, USA). Data were recorded with Empower software (version2.0; Waters). 50-µl injections were made on SunFire™ C18 column (150 × 4.6mm × 5 µm; Waters). Gradient elution according to table S1 at 1 ml/min was used with solvent A consisting of 5 % ACN in water + 0.1 % TFA (w/w) and solvent B of 0.1 % TFA (w/w) in ACN. The molar coupling ratio was determined to be 0.9:1 (drug-ULS:LZM). ChemDraw Ultra 12.0 was used to prepare draw the above molecular structures and those in scheme 1.

Table S1

t [min]	%B
0	0
25	80
26	100
33	100
34.5	0

4. Determination of intrinsic activity

Cell lysate was prepared by lyzing serum-starved HK-2 cells using mammalian protein extraction reagent (M-PER) containing protease and phosphatase inhibitors (Thermo Fischer Scientific, Etten-Leur, the Netherlands). Protein concentrations were determined using the BCA protein determination assay (Thermo Fischer Scientific). HK-2 cell lysate (0.1 mg/ml) was pre-incubated with equimolar amounts of **1**, **2** complex, or the **3** drug conjugate at room temperature for 30 minutes. Phosphorylation was initiated by adding 1 µM ATP in Abl kinase assay buffer (50 mM Tris–HCl [pH

7.5], 10 mM $MgCl_2$, 1 mM ethyleneglycoltetraacetic acid (EGTA), 2 mM dithiothreitol (DTT), and 0.01% Brij-35). The reaction mixture was incubated at 37°C for 60 minutes. Phosphorylation was terminated by adding reducing sample buffer (Invitrogen, Breda, the Netherlands) containing a final concentration of 40 mM DTT and 0.1% (w/v) SDS. Total protein (10 μg) was separated by SDS-polyacrylamide gel electrophoresis using NuPage novex 4-12% bis-tris gradient gels (Invitrogen, Breda, the Netherlands). Proteins were electroblotted onto Immobilon P polyvinylidenefluoride (PVDF) membranes (Millipore). After blocking (1 hour at RT) with 3% (w/v) BSA in tris-buffered saline containing 0.1% (v/v) Tween-20, the membranes were incubated for 1 hour at RT with a murine monoclonal anti-phosphotyrosine (pY) primary antibody (pY-20; 1:500; Abcam, Cambridge, UK) coupled to horse radish peroxidase (HRP). β-actin (1:10000; AC-15; Abcam, Cambridge, UK) was used as a loading control. The proteins were visualized by a chemiluminescence-based detection reagent (SuperSignal West Femto; Thermo Fischer Scientific, Etten-Leur, the Netherlands) and band density was determined on a Gel Doc XRS Imaging system with Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

5. Cellular uptake and retention

Cell culturing

The human kidney proximal tubular cell line HK-2 (ATCC, LGC Standards, Middlesex, UK) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 3.7 g/L sodium bicarbonate, 1.0 g/L glucose, supplemented with 10% (v/v) fetal bovine serum, penicillin (100 U/L), streptomycin (100 μg/mL) and amphotericin B (0.25 μg/mL) at 37°C with 5% CO_2 in humidified air. All cell culture related media were obtained from PAA Laboratories GmbH (Pasching, Austria).

Cellular uptake

HK-2 cells (1.5×10^4 cells/well) were seeded onto 96-well plates. Cells were serum-starved overnight before the uptake experiments were initiated. Cellular uptake was studied by incubating the HK-2 cells with **1** (10 μM) or **3** (10 μM) for different time

intervals (t=0, 1, 2, 3, 24, 48, 72 h) at 4°C (only 1, 2, 3 h) or 37°C. Uptake was determined by measuring the cellular optical density (OD) at 431 nm of **1** and 700 nm (reference wavelength) with a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Values were normalized to the number of living cells (as determined with an Alamar Blue assay). The obtained data were analyzed and processed with Microsoft Excel (figure 3a).

Cellular retention

Cellular retention was studied by incubating serum-starved HK-2 cells (1.5×10^4 cells/well) with either 10 μM **1** or 10 μM **3**. After 24 h (indicated as t = 0 h), cells were washed thrice with phosphate buffered saline (PBS) and medium was replaced with blank serum-free DMEM medium every 24 hours. Cellular retention was determined by measuring the cellular $OD_{431/700}$ (normalized to the number of living cells (Alamar Blue). The obtained data were analyzed and processed with Microsoft Excel (figure 4a).

6. Cellular activity

PamChip® Peptide microarray

A microarray-based multiplex method with real-time detection was used to determine the inhibitory activity of **3** on the phosphorylation of peptide substrates that correspond with human proteins. In brief, HK-2 cell lysate (prepared from lysis with MPER) spiked with **1** (2 μM) or **3** (2 μM) were incubated on a tyrosine kinase PamChip® microarray (PamGene International BV, 's-Hertogenbosch, the Netherlands) with 10 μM ATP at 30°C in a total volume of 25 μL. Just prior to the incubations, the PamChip was blocked with 2% (w/v) BSA in water and washed three times with Abl kinase assay buffer. After application onto the array, the incubation mixture was pumped up and down through the array for 152 cycles at a rate of 2 cycles per minute. Peptide phosphorylation was detected with a monoclonal murine anti-phosphotyrosine primary antibody (pY-20) coupled to FITC. Images of each array were taken every fifth cycle.

Data generated on PamChip peptide microarrays were analyzed with Bionavigator software (PamGene). The software quantifies signal intensity in each spot and its background, calculates signal minus background[3]. The obtained data was ranked based on strongest pY inhibition compared to control, and graphics were prepared with Microsoft Excel (figure 3b).

IC_{50} determination

IC_{50} determinations of the compounds **1**, **2** and **3** against platelet derived growth factor receptor (PDGFR)-β and c-KIT were performed by SignalChem (Richmond, Canada). In brief, protein kinase assays were performed in duplicate at room temperature for 30 min in a final volume of 25 µL containing ~10-20 nM recombinant kinase, peptide substrate (0.2 µg/µL), 5 µM ^{33}P-ATP (0.04 µCi; PerkinElmer, Waltham, MA, USA), and the indicated compounds at concentrations ranging from 0.03 nM-1000 nM in kinase buffer. After the incubation period, the assay was terminated by spotting 10 µL of the reaction mixture onto Multiscreen phosphocellulose P81 plate. The Multiscreen phosphocellulose P81 plate was washed 3 times 15 minutes in a 1% phosphoric acid solution. The radioactivity on the P81 plate was counted in the presence of scintillation fluid in a Trilux scintillation counter. The data was analyzed with Prism 5.0 (Graphpad Software Inc, La Jolla, CA, USA).

In-cell Western blotting

After incubation, cells were washed thrice with ice-cold PBS and were fixed with 3% (v/v) formaldehyde (Sigma Aldrich) for 15 minutes. Cells were permeabilized by washing five times with 0.1% (v/v) triton X-100 (Sigma Aldrich) in PBS for 5 minutes and blocked for 1 h with 3% (w/v) BSA. After blocking, cells were incubated with a horseradish peroxidase coupled primary antibody raised against phosphorylated tyrosine residues (pY-20; Abcam, Cambridge, UK). After 1 h, cells were washed thrice with PBS and phosphorylation was visualized with a chemiluminescence-based detection reagent (SuperSignal West Femto; Pierce, Rockford, IL, USA). The signal intensities were determined on a Gel Doc XRS Imaging system with Quantity One

analysis software (Bio-Rad, Hercules, CA, USA). The obtained data were analyzed with Microsoft Excel (figure 4b).

7. References

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(2) Fretz, M. M., Dolman, M. E., Lacombe, M., Prakash, J., Nguyen, T. Q., Goldschmeding, R., Pato, J., Storm, G., Hennink, W. E., and Kok, R. J. (2008) Intervention in growth factor activated signaling pathways by renally targeted kinase inhibitors. *J Control Release 132*, 200-207.

(3) Hilhorst, R., Houkes, L., van den Berg, A., and Ruijtenbeek, R. (2009) Peptide microarrays for detailed, high-throughput substrate identification, kinetic characterization, and inhibition studies on protein kinase A. *Anal Biochem 387*, 150-161.