Electronic Supplementary Material (ESI) for Chemical Communications.

High-Throughput Label-Free Opioid Receptor Binding Assays using an Automated Desorption Electrospray Ionization Mass Spectrometry Platform

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Contents

Choice of opioid receptors for study

We utilized both μ and δ ORs for these reasons: (i) most opioid compounds interact with these receptors (ii) they are the most studied ORs and (iii) they are commercially available as membrane-bound preparations. The methodology could be expanded easily to κ or NOR receptors by utilizing the appropriate competitive ligand (for instance dynorphin or ketazocine, given that LeuEnk used in this study has little to no affinity for the κ OR) and a corresponding adequate internal standard.

Experimental section

Materials

Binding assays were developed and optimized using commercial membrane preparations of recombinant human opioid receptors (ChemiSCREEN, Sigma Aldrich). The competitive ligand, leucine enkephalin, (LeuEnk) and the internal standard, (D-Ala^2, D-Leu^5)-enkephalin (DADLE), were purchased from Bachem. Manual assay filtering through optimization was carried out using Whatman Puradisc GF/B 1 um syringe filters. Parallel plate filtering was performed using a MultiscreenHTS Vacuum Manifold with Multiscreen 96-well filter plates with a hydrophilic PVDF membrane (Millipore). Small molecules were purchased from Cayman Chemical, AstaTech, and Sigma Aldrich. All solvents used were Chromasolv LC-MS grade (Honeywell). Buffers were made in-house with components purchased from Sigma Aldrich.

Bioassay preparation

Both filter plates and syringe filters were utilized for the assays. Plates are compatible with the automated fluid-handling robotics whereas the syringe filters can be used for rapid testing of individual samples as well as experiment optimization. For conditioning, bovine serum albumin (BSA, 0.1% w/v) was slowly passed through the filters followed by a Tris buffer (pH 7.4, 10 mM) wash. This process was repeated four times, and the last buffer wash was removed just before use. Test compounds were added in appropriate amounts to achieve the desired testing concentrations, which in the case of dose-response curves varied from 5 to 1000 μM concentrations together with LeuEnk (final concentration: 500 nM) and an appropriate amount of Tris buffer (50 mM, pH 7.4). The samples were equilibrated at 37 °C prior to the addition of the opioid receptors. Receptor preparations were aliquoted as soon as received and individual aliquots were used for each experiment to avoid repeated freeze-thaw cycles. Before addition to the assay mixture, receptor aliquots were thawed at room temperature. A final concentration of 100 μg/mL membrane protein was used on each assay. The final assay volume was 200 μL. Once the receptors were added, samples were incubated for 1 hour at 37 °C. With every set of experiments, negative (without receptor) and positive (without competitive ligand) controls were prepared in triplicate. After incubation, samples were filtered and washed with 1.5 assay volumes of Tris buffer (pH 7.4, 50 mM). Filtrate and wash were collected together, and a volumetric aliquot (100 μL) was sampled. Finally, internal standard (DADLE, 1 μL, 20 μM) was added to this aliquot before automated transfer of the samples to 384-well plates. The utility of DADLE as an internal standard for LeuEnk quantitation was confirmed via a calibration curve (**Fig. S1**).

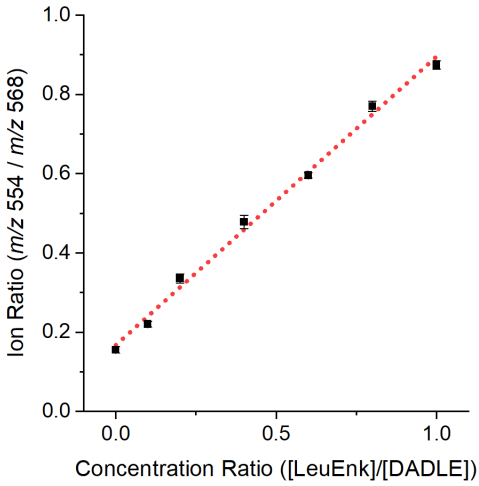


Fig. S1 Calibration curve of LeuEnk using DADLE as internal standard. A linear relationship (R^2 = 0.995) is observed up to equimolar concentrations of both compounds (maximum assay response). DADLE concentration was kept at 200 nM. Each data poin represents the average of three independent calibration solutions with 12 instrumental replicates each. Error bars indicate standard deviations. All coefficients of variation are below 5%.

High-throughput DESI-MS analysis

A Biomek i7 fluid handling workstation (Beckman Coulter) equipped with a 50-nL floating slotted 384-pin tool (V&P Scientific) was used to transfer samples from the final 384-well plates and spot them onto standard microplate size (127 x 85 mm) glass slides (Abrisa Technologies) coated with a porous PTFE membrane (Zitex G-115, Saint-Gobain) using a thin layer of low-VOC adhesive (Scotch Spray Mount, 3 M). Up to 6,144 samples can be spotted on one slide by offsetting the pinning position in-between transfers. At the highest density, spots (ca. 800 um) are separated by ca. 1 mm center-to-center. The spotted samples were analyzed using a Synapt G2-Si quadrupole time-of-flight mass spectrometer and a DESI stage equipped with a high-performance XS-generation sprayer and a prototype heated transfer capillary (Waters). The DESI spray was generated using methanol (2 uL/min) and nebulizing nitrogen gas (27 psi), with a spray voltage of 0.7 kV. The transfer capillary and the source temperature were set at 450 °C and 150 °C, respectively. MS analysis was performed in negative ion mode using the sensitivity setting, the target ion enhancement feature on (set to *m/z* 554), with a quadrupole MS profile ramp (100% scan time) as well as a mass range of *m/z* 530–580. Raw spectral data was processed and analyzed using custom Python and MATLAB (MathWorks) scripts, respectively.[1,2]

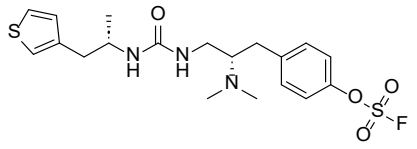
Late-stage functionalization of PZM21

All batch functionalization conditions were selected from a previous DESI-MS screening campaign.[3] All compounds were purified via preparative reverse-phase liquid chromatography. Synthesis conditions and compound characterization are provided below. All concentrations provided refer to the final concentrations in the reaction mixture. High-resolution (HR) MS experiments were performed using nanoelectrospray ionization (1 kV) with 5-µm pulled borosilicate capillaries (Sutter Instrument) and a Synapt G2-Si quadrupole time-of-flight mass spectrometer (Waters) using the high-resolution mode of the instrument in the positive ion mode. All HRMS spectra include LeuEnk (mainly observed as a $[M+Na]^+$ ion,

m/z 578) which was used as lock mass ion for accurate mass determination. Tandem MS (MS/MS) data was acquired under similar conditions using an LTQ ion trap mass spectrometer (Thermo Fisher).

*Fluorosulfurylation reaction (Compound **2**)*

1-(Fluorosulfonyl)-2,3-dimethyl-1H-imidazol-3-ium trifluoromethanesulfonate (FDIT) was used to install an SO_2F group on the phenol moiety of PZM21 (**1**). FDIT (30 mM) and PZM21 (20 mM, with 40 mM triethylamine to help with dissolution) were dissolved in acetonitrile, then mixed and incubated at room temperature for 3 hours to obtain product **2**. *Characterization:* Exact mass ($[M+H]^+$, from HRMS, **Fig. S2**): *m/z* 444.1424 (Mass error: 0.45 ppm); MS/MS: *m/z* 260 (20%), 277 (48%), 303 (100%), 399 (20%).



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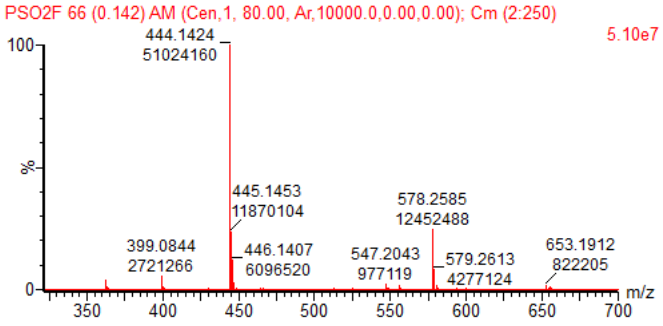
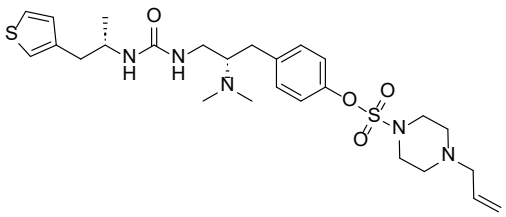


Fig. S2 HRMS of compound **2**, $[M+H]^+$ *m/z* 444.1.

*SuFEx Click reaction (Compound **3**)*

Fluorosulfurylated PZM21 (**2**) was obtained following the procedure above and then incubated for 6 hours with a 1-allylpiperazine solution (20 mM in acetonitrile, with 20 mM DABCO) to form product **3**. *Characterization:* Exact mass ($[M+H]^+$, from HRMS, **Fig. S3**): *m/z* 550.2520 (Mass error: 0.73 ppm); MS/MS: *m/z* 366 (20%), 383 (45%), 409 (100%), 505 (18%).



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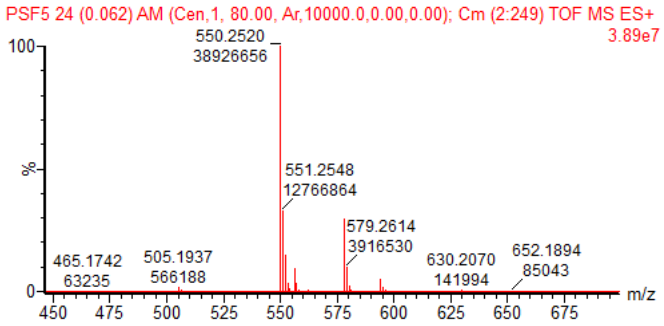
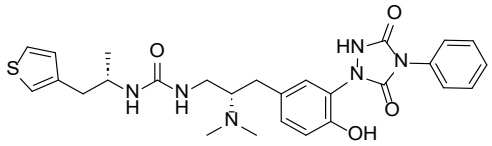


Fig. S3 HRMS of compound **3**, $[M+H]^+$ *m/z* 550.2.

*Ene-type click like reaction (Compound **4**)*

PZM21 (**1**, 20 mM with 40 mM triethylamine) and 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD, 30 mM) reagents were dissolved in acetonitrile. The solutions were then mixed with twice the volume of phosphate buffer (0.05 M, pH 7.4) and incubated for 6 hours to form product **4**. *Characterization:* Exact mass ($[M+H]^+$, from HRMS, **Fig. S4**): *m/z* 537.2281 (Mass error: 0.37 ppm); MS/MS: *m/z* 353 (5%), 370 (10%), 396 (100%), 492 (5%).



(**4**)

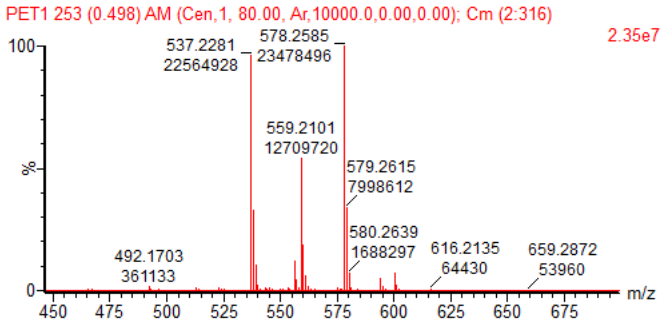


Fig. S4 HRMS of compound **4**, $[M+H]^+$ *m/z* 537.2. Note that the peak at *m/z* 559.2 corresponds to the sodium adduct, $[M+Na]^+$, of compound **4**.

Reported opioid K_d values

Table S1. Ranges of literature K_d values for different opioids and the δ and μ OR

OR	Opioid	K_d (nM)	References
δ	Naltrindole	0.07 - 0.3	4 - 6
	Naltriben	0.16 - 0.39	4-- 6
	PZM21	241 - 540	7 - 9
	Tramadol	5400 - 17000	10,11
	O-desmethyltramadol	690 - 3190	10,11
μ	Naltrexone	0.2-0.4	12,13
	Naloxone	1.2-1.5	12,13
	PZM21	1.1-31	7 - 9
	Tramadol	4400-12500	10,11
	O-desmethyltramadol	17-6660	10,11

Sensitivity calculation

The calculated sensitivity of the DESI-MS method was based on the amount of material deposited (150 nL), and the concentration of LeuEnk in a typical OR control where the maximum amount of LeuEnk is bound, and thus it has the lowest free concentration of LeuEnk to be quantified. In such a control, specifically for the δ OR which provides the largest response, the ratio of LeuEnk to DADLE is ca. 10%. LeuEnk and DADLE have almost identical ionization efficiencies as assessed through calibration experiments and as can be observed in the spectra of OR-free controls (*see* top **Fig. 1D**) where the concentration of both peptides is identical (200 nM). Thus, the lowest LeuEnk concentration being quantified can be estimated at 20 nM, equivalent to 1.6 pg of LeuEnk deposited per spot. Finally, as the spots are subsampled due to the 1-to-16 ratio between the areas of the DESI spray (200 um) and the sample spot (800 uM), and accounting for a small oscillation (assumed to complete the area of two DESI spray sizes) for a final subsampling factor is 1-to-8, and the amount of LeuEnk detected is estimated as on the order of 200 fg.

Droplet flight time estimation

The droplet flight time is estimated as ca. 100 ms based on the droplet velocities measured when the DESI process was originally characterized (Venter et al. *Anal. Chem.* **2006**, 78, 24, 8549-8555) and considering the flight distance (ca. 10 cm) in the instrument used.

Assay response calculation

The assay response (AR) was calculated using the ion ratio (LeuEnk, I_{554}, to DADLE, I_{568}) normalized to controls without test compound in the presence of ORs (OR+) and absence of ORs (OR-). For a test compound (TC) of a particular concentration (x), the assay response would be:

$$AR_{TC_x} = \frac{\left(\frac{I_{554}}{I_{568}}\right)_{TC_x} - \left(\frac{I_{554}}{I_{568}}\right)_{OR+}}{\left(\frac{I_{554}}{I_{568}}\right)_{OR-} - \left(\frac{I_{554}}{I_{568}}\right)_{OR+}}$$

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