Three Mutations Convert the Selectivity of a Protein Sensor from Nicotinic Agonists to S-Methadone for Use in Cells, Organelles, and Biofluids

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ABSTRACT: We report a reagentless, intensity-based S-methadone fluorescent sensor, iS-methadoneSnFR, consisting of a circularly permuted GFP inserted within the sequence of a mutated bacterial periplasmic binding protein (PBP). We evolved a previously reported nicotine-binding PBP to become a selective S-methadone-binding sensor, via three mutations in the PBP’s second shell and hinge regions. iS-methadoneSnFR displays the necessary sensitivity, kinetics, and selectivity—notably enantioselectivity against R-methadone—for biological applications. Robust iS-methadoneSnFR responses in human sweat and saliva and mouse serum enable diagnostic uses. Expression and imaging in mammalian cells demonstrate that S-methadone enters at least two organelles and undergoes acid trapping in the Golgi apparatus, where opioid receptors can signal. This work shows a straightforward strategy in adapting existing PBPs to serve real-time applications ranging from subcellular to personal pharmacokinetics.
used in Ca\textsuperscript{2+} sensors (the GCaMP series) and in neurotransmitter sensors.\textsuperscript{18,21} Our strategy consisted of (1) screening each methadone enantiomer against a previously reported nicotine biosensor, iNicSnFR3a, and its variants\textsuperscript{19} and (2) iterative site-saturation mutagenesis to select for S-methadone and against cholinergic ligands (Figure 2a). We performed chiral resolution on racemic methadone to isolate (+)-S-methadone and (−)-R-methadone (assigned by optical rotation\textsuperscript{22}) with analytical purity and 99% enantiomeric excess (Figure S1).

While there is no structural homology or pharmacological overlap between nicotinic and \(\mu\)-opioid receptors, several variants of nicotinic drug biosensors displayed weak fluorescence responses to S-methadone (Figure 2b). Although the PBP had no enantioslective pressure for binding its achiral ligand choline, all variants screened to date displayed enantioselectivity for S-methadone (Figure S2). Dose–response relations were fit to the Hill equation to determine an EC\textsubscript{50} and \(\Delta F\textsubscript{max}/F_0\). In the linear portion of the dose–response relation we define the increase in fluorescence per micromolar, “S-slope”, as a metric of biosensor sensitivity: \((\Delta(F/F_0)/\Delta[\text{ligand}])\) at [drug] \(\ll\) EC\textsubscript{50}.\textsuperscript{23} For a Hill coefficient of \(\sim 1.0\), the S-slope equals the ratio \((\Delta F\textsubscript{max}/F_0)/\text{EC}_{50}\). A variant of iNicSnFR3a, iNicSnFR3b, provided the largest dynamic range for both S-methadone and R-methadone (Figure S2) and served as the input to several rounds of directed evolution.

We selected for both an increase in sensitivity to S-methadone and a decrease in sensitivity to nicotinic ligands. We chose mutation sites based on a crystal structure of iNicSnFR1 (PDB:6EFR) and directed evolution of iNicSnFR3a.\textsuperscript{14} The resulting sensor displayed a \(\sim 16\)-fold improvement in sensitivity over iNicSnFR3a; \(\Delta F/F_0\) increased to 3.76 ± 0.16 at 1 μM, the representative plasma maintenance concentration.\textsuperscript{8} (Figure 2b). Notably, iS-methadoneSnFR displayed sensitivity to S-methadone that exceeded the sensitivity for any of the original cholinergic ligands and displayed a marked shift in ligand selectivity (Figure 2c). iS-methadoneSnFR displayed near-zero response for physiologically or pharmacologically relevant steady-state acetylcholine (ACh), choline, varenicline, and nicotine concentrations (\(\sim 1\) μM, 10 to 20 μM,\textsuperscript{24} 0 to 100 nM,\textsuperscript{25} and \(\sim 25\) to \(\sim 500\) nM, respectively\textsuperscript{26} (see Figure 4a)).

We characterized iS-methadoneSnFR’s binding using docking and biochemical studies. Although only three mutations were required to generate iS-methadoneSnFR from iNicSnFR3a/3b, advantageous mutations were rare: \(\sim 1\%\) of all mutations screened were accepted as improvements. Docking S-methadone into recently reported structures of liganded iNicSnFR3a\textsuperscript{27} showed that the N-methyl groups of S-methadone lie 4.6 and 5.5 Å from the aromatic groups of Y357 and Y65, respectively (slightly greater than the distance from the beta carbons of varenicline to these two groups). In the initial round of mutations, most sites yielded no improvement, except for a W436F mutation spatially near Y357 and Y65 (Figure S3). We previously reported nicotine and varenicline making cation–π interactions with Y65 and Y357 in iNicSnFR3a (PDB:7S7T and 7S7U, respectively).\textsuperscript{27} Each nicotinic ligand bears a protonated nitrogen lying midway on the axis of the aromatic centroids of Y65 and Y357 (Figure 3a). In the subsequent round, second-shell mutation N11V created additional volume next to F12, in the second shell. Finally, the third round yielded L490A, allowing for greater flexibility in the hinging of the PBP.

Leucine mutagenesis among individual binding pocket aromatic residues showed the primacy of Y65, Y357, F12, and Y460 (Figure 3b). An aromatic side-chain screen across these four positions revealed a necessity of Tyr in the first shell positions Y65, Y357, and Y460 (Figure 3c). Substituting a noncanonical side chain, O-methyltyrosine, yielded a near-null biosensor at residue 65 but not at 12 (Figure S4). These data suggest that S-methadone’s amine directly interacts with the first shell residues, as with nicotinic drugs, and the phenolic –OH is necessary for hydrogen bonding. The three accepted mutations represent a 94 Å\textsuperscript{3} reduction in van der Waals volume, comparable to the 132 Å\textsuperscript{3} increase in ligand volume from varenicline to methadone, as though the accepted mutations allowed S-methadone better access to aromatic residues critical to binding both classes of drugs. Therefore, the PBP has an aromatic binding pocket for protonated amines, and other regions of the binding site can be tuned to accommodate the remainder of the ligand’s steric bulk and functional groups.

iS-methadoneSnFR satisfied our sensitivity, selectivity, and biophysical criteria for a useful biosensor. Fluorescence dose–response relations showed an excellent dynamic range, \(\Delta F\textsubscript{max}/F_0\) of 15.3 ± 0.2, and an EC\textsubscript{50} of 3.2 ± 0.2 μM, near the relevant plasma concentrations for maintenance therapy.\textsuperscript{9} Isothermal titration calorimetry (ITC) determined a \(K_d\) of 1.9 ± 0.2 μM, in good agreement with the fluorescence EC\textsubscript{50} (Figure 4c). ITC also demonstrated a single binding site (stoichiometry \(\approx 0.92\)) with an entropically driven conformational change. iS-methadoneSnFR had little or no response (S-slope < 0.1 μM\textsuperscript{-1}) to other neurotransmitters (Figure 4a) and other opioids (Figure 4b). The S-slope for S-methadone was \(\sim 20\times\) that for R-methadone. When we added R-methadone to S-
methadone, fluorescence was modestly elevated at lower [S-methadone], but all responses converged at the $\Delta F_{\text{max}}/F_0$ for S-methadone alone (Figure S5). 1-s stopped-flow kinetics were obtained using racemic methadone (Figure S4) and determined an apparent $k_{\text{on}}$ of 0.13 $\mu$M$^{-1}$ s$^{-1}$ (Figure 4d). The final 10 ms of the 1 s stopped-flow traces were fitted by a Hill equation with EC$_{50}$ $\sim$ 8 $\mu$M (Figure S6) for the racemate, which was approximately double the EC$_{50}$ for S-methadone alone (as expected if the binding strongly favors the S-enantiomer).

Therapeutic use of opioids would be improved by quantitative, real-time, minimally invasive or noninvasive measurements in sweat, saliva, and interstitial fluid.$^{28,29}$ The selectivity and high aqueous solubility of iS-methadoneSnFR enable its use in such applications. We tested the biosensor in PBS:biofluid samples and found robust responses in the pharmacologically relevant concentration range (Figure 5). iS-methadoneSnFR, like all GFP-based biosensors, displays smaller responses at pH $<$ ~7 (Figure S7). Because biofluids, particularly sweat, have variable and/or acidic pH, 3× PBS pH

Figure 2. (a) Directed evolution strategy. (b) Fluorescence responses to S-methadone. iNicSnFR3a (black) has several variants (faded curves), of which one has markedly better sensitivity, owing to the N11E mutation (blue). This lead was evolved to iS-methadoneSnFR (red), which included reoptimization at position 11. Only the final biosensor had sufficient sensitivity at 1 $\mu$M (vertical black line; the relevant maintenance concentration). (c) Shift in selectivity from iNicSnFR3a (black) to iS-methadoneSnFR (red) measured by S-slope (see text). Note the scale change at the axis break.

Figure 3. (a) PDB:7S7T(iNicSnFR3a, varenicline bound) showing cation–π interactions with Y65 and Y357. S-methadone was docked into 7S7T. (b) Fluorescence dose–response relations of cation–π residue Leu mutants. (c) Aromatic side-chain screen through critical positions identified in (b) with resulting S-slope. Note the break in y-axis.
7.4 was used to partially buffer a mixture with the biofluid. Still, the response at 1 μM and below in the biofluids provide at least ∼200% dynamic range.

At the subcellular level, membrane-permeant weakly basic opioid drugs, but not impermeant derivatives or endogenous opioid peptides, enter the endoplasmic reticulum, and can act as pharmacological chaperones, altering the folding and trafficking of their receptors.10 Opioid drugs also activate their receptors in endosomes and the Golgi apparatus.11 We targeted iS-methadoneSnFR to the plasma membrane (Figure 6a), endoplasmic reticulum (Figure 6b), and Golgi apparatus (Figure 6c) of HeLa cells using targeting sequences. We

Figure 4. Selectivity and biophysical properties of iS-methadoneSnFR. (a) iS-methadoneSnFR vs endogenous neurotransmitters and choline. Responses to ACh and choline had S-slopes < 0.1 μM⁻¹. (b) iS-methadoneSnFR vs other clinically used opioids. The response to R-methadone was near zero at ∼1 μM. Weak or no responses were observed for other drugs tested. EDDP is 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, the major metabolite of methadone. (c) Isothermal titration calorimetry of purified iS-methadoneSnFR. Thirty μM of the biosensor was mixed with 2 μL injections of 300 μM S-methadone. (d) Stopped-flow kinetic measurements with racemic methadone.

Figure 5. iS-methadoneSnFR dose–response relation in biofluids. 1:1 mixture of drug:biosensor in 3× PBS pH 7.4 with either human sweat or human saliva and 1:3 mixture with mouse serum (no pH adjustment of any biofluid).

Figure 6. Spinning disk confocal imaging of HeLa cells transfected with (a) iS-methadoneSnFR_PM, (b) _ER, and (c) _Golgi (470 nm excitation, 535 nm emission, 100× 1.4 NA objective). Scale bar = 10 μm. (d) S-slope plotted for each organelle response to 0–250 nM S-methadone. Points are average responses to a 1 min pulse of [S-methadone]. PM n = 11 cells; ER n = 10; Golgi n = 11.
applied pulses of S-methadone (0 to 250 nM in 50 nM steps) to measure the linear portion of the dose–response relation (S-slope) in widefield imaging (Figure S8). The results indicate that ample S-methadone is available in the ER for potential chaperoning. The Golgi showed the largest S-slope among the three compartments (1.7× that of PM), despite having the lowest pH (Figure 6d). After correcting the S-slope for pH dependence, we find an accumulation factor of 2.9× to 4.4× across the Golgi pH range of 6.3 to 6.830 (Figure S8). Accumulation of opioids such as methadone in acidic compartments31 may lead to intensified G-protein coupled signaling. We also validated iS-methadoneSnFR for time-resolved measurements in primary hippocampal neurons, encouraging mechanistic studies in tissues and in vivo (Figure S9).

Along with other sensors of opioid signaling,11,32 this study establishes the first genetically encoded fluorescent protein biosensor for an opioid drug, enabling real-time quantification. Furthermore, the enantioselectivity encourages biosensor development to investigate “chiral switching” of other drugs where a single enantiomer substitutes a clinically used racemate.33 One enantiomer may serve previously unstudied indications. For example, S-methadone is used for opioid addiction treatment. S-Methylamphetamine can monitor drug concentration near receptors during administration by the experimenter or the subject, a common manipulation for studying mechanisms of reward, analgesia, and drug abuse. To meet immediate needs for diagnostics, iS-methadoneSnFR can also provide in situ readouts in the laboratory or home.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c02323.

Experimental details, reagents, supporting experimental figures, and amino acid and nucleotide sequences of iS-methadoneSnFR (PDF)

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Notes
The authors declare the following competing financial interest(s): Anand K. Muthusamy, Henry A. Lester, Loren L. Looger, and Jonathan S. Marvin have filed a patent application that includes iS-methadoneSnFR. Constructs reported in this manuscript will be deposited in Addgene. Sequences and dose response metrics will be made available in a GitHub repository.

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