Structural basis for action by diverse antidepressants on biogenic amine transporters

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The biogenic amine transporters (BATs) regulate endogenous neurotransmitter concentrations and are targets for a broad range of therapeutic agents including selective serotonin reuptake inhibitors (SSRIs), serotonin–noradrenaline reuptake inhibitors (SNRIs) and tricyclic antidepressants (TCAs)1–4. Because eukaryotic BATs are recalcitrant to crystallographic analysis, our understanding of the mechanism of these inhibitors and antidepressants is limited. LeuT is a bacterial homologue of BATs and has proven to be a valuable paradigm for understanding relationships between their structure and function5. However, because only approximately 25% of the amino acid sequence of LeuT is in common with that of BATs, and as LeuT is a promiscuous amino acid transporter6, it does not recapitulate the pharmacological properties of BATs. Indeed, SSRIs and TCAs bind in the extracellular vestibule of LeuT7,8 and act as non-competitive inhibitors of transport9. By contrast, multiple studies demonstrate that both TCAs and SSRIs are competitive inhibitors for eukaryotic BATs and bind to the primary binding pocket10,11. Here we engineered LeuT to harbour human BAT-like pharmacology by mutating key residues around the primary binding pocket. The final LeuBAT mutant binds the SSRI sertraline with a binding constant of 18 nM and displays high-affinity binding to a range of SSRIs, SNRIs and a TCA. We determined 12 crystal structures of LeuBAT in complex with four classes of antidepressants. The chemically diverse inhibitors have a remarkably similar mode of binding in which they straddle transmembrane helix (TM) 3, wedge between TM3/TM8 and TM1/TM6, and lock the transporter in a sodium- and chloride-bound outward-facing open conformation. Together, these studies define common and simple principles for the action of SSRIs, SNRIs and TCAs on BATs.

We used the structure of wild-type LeuT in complex with the competitive inhibitor tryptophan (PDB code 3F3A) as a template for mutant design (Fig. 1a). We analysed residues within a 10 Å radius of the primary binding pocket of the LeuT–Trp complex (Fig. 1a) together with a LeuT/human serotonin transporter (SERT) amino acid sequence alignment to identify about 20 residues which point towards the primary binding pocket and are divergent from SERT (Supplementary Fig. 1). These residues are located in both bundle and scaffold domains12,13, sodium binding sites14,15, the chloride binding site16,17 and the extracellular vestibule. Previous studies have demonstrated the importance of many of these residues in SERT pharmacology18–21. By tracking the binding constant (Ka) of [3H]paroxetine, we introduced these mutations into LeuT, focusing initially on ‘first shell’ residues predicted to interact directly with inhibitors and next on ‘second shell’ residues (Supplementary Table 1). The Ka values for paroxetine and mazindol binding to the final LeuBAT mutant, deemed Δ13 LeuBAT (Supplementary Table 1), are 431 ± 24 nM and 112 ± 18 nM, respectively (Supplementary Fig. 2). Notably, the Ka of Δ13 for mazindol is similar to that of SERT (103 ± 4.7 nM)22. Because uptake experiments using the Δ6 or Δ13 variants reconstituted into liposomes show that the constructs are not active in transporting either serotonin or dopamine (Supplementary Fig. 3), further experiments would be required to engineer a variant of LeuBAT that possesses both high-affinity inhibitor binding and transport activity.

For the Δ13 LeuBAT construct we performed competition experiments using [3H]paroxetine and multiple cold SSRIs, SNRIs and a TCA (Fig. 1b–d and Supplementary Table 2). Notably, sertraline possesses the highest affinity (Ka = 18 ± 2 nM; Ki = 14 ± 2 nM, Fig. 1c, d), thus approaching the reported value for sertraline binding to SERT (0.3 nM)23. To demonstrate that the Δ6 and Δ13 variants possess increased affinities for inhibitors relative to wild-type LeuT, we determined the Ka values for sertraline and mazindol binding to wild-type LeuT (Table 1). The substrate alanine, which binds to the primary pocket of wild-type LeuT, could not suppress the binding of sertraline to wild-type LeuT (Supplementary Fig. 2h), consistent with the conclusion that these drugs bind within the extracellular vestibule of wild-type LeuT.

We determined crystal structures of LeuBAT in complex with a panel of SSRIs, SNRIs and a TCA using the Δ5, Δ6 and Δ13 variants (Supplementary Table 3). For the Δ5 and Δ6 mutants, we determined structures for the Δ5–mazindol, Δ6–sertraline, Δ6–desvenlafaxine, Δ6–duloxetine and Δ6–mazindol complexes at resolutions of 2.3–2.7 Å. For the Δ13 variant, we determined seven structures with sertraline, paroxetine, fluoxetine, fluvoxamine, duloxetine, desvenlafaxine and clomipramine (CMI) at resolutions of 2.85–3.31 Å (Supplementary Fig. 4 and Supplementary Table 3). Because the binding positions of inhibitors is similar between the Δ6 and Δ13 constructs (Supplementary Fig. 5), we used the higher resolution structures of the Δ6 complexes for analysis and validation of the drug-binding sites in Δ13.

All LeuBAT structures adopt an outward-facing open conformation (Figs 2a and 3), similar to that of wild-type LeuT in complex with tryptophan (root mean squared deviation (r.m.s.d.) of 0.48 Å for Cα atoms). All drugs bind to the primary binding pocket, interact with both the bundle (TM1, TM6) and scaffold (TM3, TM8) domains, and are lodged between the extracellular gate residues Arg 30 and Asp 404 (ref. 3). These observations invalidate the notion that SSRIs and TCAs elicit their effects on SERT by binding in the extracellular vestibule6,7,24.

We find two sodium ions, Na1 and Na2, bound to sites similar to those in the LeuT–Trp complex with the following distinction. In the wild-type LeuT structures the α-carboxyl group of Leu or Trp participates in the coordination of Na1, whereas in the LeuBAT structures a water molecule is found at the equivalent position, bridging the carboxylate of Asp 24 and Na1 (Supplementary Fig. 6). We detected electron density for a chloride ion in the LeuBAT–paroxetine structure ~4.5 Å from Na1, coordinated by Tyr 47, Ser 254, Asn 286 and Ser 290, as predicted previously (Supplementary Fig. 6)6,19,24.

We solved structures of four LeuBAT–SSRI complexes: sertraline, paroxetine, fluoxetine and fluvoxamine. Sertraline occupies the primary binding pocket surrounded by TM1, TM3, TM6 and TM8 and buries 438.6 Å2, or 93%, of its surface area (Fig. 2). The amine group forms a salt bridge with the carboxyl group of Asp 24 whereas the tetrahydronaphthalene...
ring participates in hydrophobic interactions with Tyr 21 and is sandwiched between Val 104, Tyr 108 and Phe 259 (Fig. 2). The two chlorine atoms on the dichlorophenyl ring insert into a groove formed by Pro 101, Val 104, Ala 105, Ser 356 and Gly 359. We suggest that these extensive hydrophobic and van der Waals interactions contribute to the high affinity of sertraline for LeuBAT and human SERT.

The other SSRIs—paroxetine, fluoxetine and fluvoxamine—also bind to the primary binding pocket in an orientation similar to that of sertraline. Consistent with previous SERT–paroxetine and SERT–fluoxetine models, the amine groups are proximal to the carboxyl group of Asp 24 (Fig. 3a, b). The amine groups of the inhibitors also form direct hydrogen bonds with main-chain carbonyl groups of Tyr 21, Ala 22 and/or Phe 253. The benzodioxol group of paroxetine and trifluoromethylphenyl rings from fluoxetine and fluvoxamine insert to the same groove, as does the chlorophenyl ring of sertraline, forming hydrophobic interactions with Val 104, Tyr 108 and Phe 259, and/or van der Waals interactions with the main-chain carbonyl groups of Pro 101, Ala 105, Ser 356 and Gly 359. In addition, the fluorophenyl ring of paroxetine, the phenyl ring of fluoxetine and the ether chain of fluvoxamine extend into the extracellular vestibule, forming

Figure 1 | LeuBAT design and pharmacology. a, The representation of mutation positions around the primary binding pocket in the wild-type LeuT–Trp structure (PDB 3F3A). Bound tryptophan (yellow) and the mutated residues are in sticks. The transmembrane helices TM1, TM3, TM6, TM8 and TM10 around the pocket are highlighted as green, red, purple, orange and blue, respectively. Asterisks depict the glycine residue positions. b, Chemical structures of four SSRIs, two SNRIs, one TCA (CMI) and one stimulant (mazindol). c, Measurement of [3H]sertraline binding (filled circles) to LeuBAT by sertraline (filled diamonds), fluvoxamine (empty circles), fluoxetine (empty diamonds), duloxetine (empty inverted triangles), CMI (empty triangles) and desvenlafaxine (empty squares). Error bars, s.e.m., n = 3.

Figure 2 | LeuBAT Δ13–sertraline complex adopts an outward-facing open conformation. a, Cross-sections of the crystal structure of Δ13–sertraline showing the solvent-accessible surface area (blue). Bound sertraline is shown in yellow sticks. b, c, Zoom into the sertraline binding pocket viewed within the membrane plane (b) and from the extracellular side (c). Sertraline and key residues in the pocket are depicted in both sticks and spheres showing the van der Waals radii. Salt bridges and hydrogen bonds are in dashed lines. Phe 259 in b is omitted for clarity.
hydrophobic interactions, van der Waals contacts and hydrogen-bonding interactions with Tyr 107, Phe 253, Asp 404 and/or Thr 408.

In the SNRI complexes with duloxetine and desvenlafaxine, the inhibitors sit in the primary pocket with the amine groups interacting with the carboxyl group of Asp 24 and, in the case of duloxetine, also with the main-chain carbonyl of Tyr 21 (Fig. 3c, d). The naphthalene ring from duloxetine and the cyclohexanol ring from desvenlafaxine are sandwiched by hydrophobic groups Tyr 21, Val 104, Phe 259 and Tyr 108. The thiophene ring from duloxetine and the phenol ring from desvenlafaxine protrude into the extracellular vestibule and interact with Phe 253 by edge-to-face interactions. For desvenlafaxine, the hydroxyl group in the cyclohexanol moiety makes a hydrogen bond with the main-chain carbonyl group of Ser 355, whereas the phenol hydroxyl interacts with Asp 404 and the phenol group of Tyr 107.

The tricyclic antidepressant CMI binds to the primary binding pocket of Δ13 LeuBAT (Fig. 3e), in agreement with a human SERT–TCA model and in contrast to wild-type LeuT–TCA structures. The tricyclic ring is surrounded by hydrophobic residues including Tyr 21, Val 104, Tyr 108, Phe 253 and Phe 259. The chlorine atom in the tricyclic ring extends to the pocket formed by Ala 105, Ser 356 and Gly 359, similar to the chorine positions in sertraline or the trifluoromethyl moiety in fluoxetine. Not only are there previously proposed interactions observed in our LeuBAT–CMI structure, such as the salt bridge between Asp 24 (Asp 98 in SERT) and the tertiary aliphatic amine of the TCA, the structure is consistent with interactions between Ala 105 (Ala 173 in SERT) and the TCA 3-position, and with Phe 253 (Phe 335 in SERT) being near the TCA 7-position. Our structure is in harmony with the conclusion that Ser 438 in human SERT (Ser 355 in LeuBAT) is vicinal to the aminopropyl chain of TCA and that the S438T mutation affects the binding affinity because of steric clash.

The stimulant mazindol binds to the primary site and is surrounded by a hydrophobic pocket formed by Tyr 21, Val 104, Tyr 108 and Phe 259 (Fig. 3f). The amine nitrogen forms a salt bridge with the carboxyl group of Asp 24. The hydroxyl group hydrogen bonds with the carboxyl group of Asp 24 and the phenol group of Tyr 108. The chlorophenyl ring inserts into the pocket formed by Ala 105, Ser 356 and Gly 359, similar to sertraline. The importance of these interactions is supported by the fact that removal of the chlorophenyl ring or changes in the substitution on the phenyl ring decreases the affinity of mazindol to SERT.

By soaking A6 mutant crystals in 20 mM desvenlafaxine, we identified a second desvenlafaxine molecule in the extracellular vestibule (Supplementary Fig. 7). The second molecule occupies a similar position to the positions that TCAs, sertraline and fluoxetine occupy in wild-type LeuT structures reported previously. These results demonstrate that the n-octyl-β-D-glucoside molecule bound to the extracellular vestibule of wild-type LeuT is readily substituted by a drug molecule and that the extracellular vestibule is a site for the low-affinity, non-specific binding of small molecules.

Analysis of the sertraline (SSRI), duloxetine (SNRI) and CMI (TCA) complexes with LeuBAT allows us to identify three subsites within the primary binding site to which the pharmacophores of these chemically diverse inhibitors bind (Fig. 4a, b). Subsite A is defined by Asp 24, Tyr 21, Gly 256 and Ser 355 from TM1, TM6 and TM8, and accommodates the polar, amine moiety of the inhibitors. Asp 24 interacts with the amine groups of most drugs by salt bridge, which echoes the suggestion that Asp 98 in human SERT forms a similar interaction with the amine groups of SSRIs, TCAs and serotonin.

Subsite B includes residues from TM3, TM6 and TM8 and involves two types of interactions. First, nonpolar residues form hydrophobic interactions with the hydrophobic rings of the drugs. Phe 259 (Phe 341 in human SERT) together with Val 104 (Ile 172 in human SERT or Val 148 in the human noradrenaline transporter (NET)) define a non-polar ridge that accommodates the hydrophobic groups of the drugs. Previous studies showed that the F341Y mutation in human SERT reduces the potency of paroxetine and escitalopram. Here we suggest that the F341Y mutation leads to a clash with the fluoro phenyl ring of paroxetine. The second type of interaction in subsite B is the groove delineated by Pro 101, Ala 105, Gly 359 and Ser 356. This groove accommodates the polar groups in the drugs’ rings, such as the chloro, dichloro, trifluoromethyl and benzodioxol groups of CMI, sertraline, fluoxetine and paroxetine, respectively, and is capped by Tyr 108. A SERT–imipramine model suggests that the imipramine 3-position is vicinal to Ala 173 (Ala 105 in LeuBAT), and here we show that the chlorine atom in the 3-position of CMI forms a direct contact with this ridge.

Subsite C, distal to the primary, orthosteric binding site, is located in the extracellular vestibule, and is comprised of residues in TM6 and TM10, including Phe 253, Asp 404 and Thr 408. Subsite C interacts with bulky drugs such as paroxetine, desvenlafaxine and fluoxetine, and probably has a role in enhancing inhibitor affinity and specificity.

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Indeed, previous studies determined that mutation of E493Q in human SERT (Asp404 in LeuBAT) attenuates the potency of fluoxetine\(^7\).

To support the conclusion that the LeuBAT complexes represent reliable models for BAT–inhibitor complexes, we prepared and analyzed the individual Y21A, D24E, F259Y and S355T mutants in the context of the Δ13 LeuBAT construct. Y21A, D24E and S355T are in subsite A and F259Y is in subsite B. We next measured their \([^3]H\)sertraline \(K_d\) values and plotted log \(K_d\) of the LeuBAT mutants against log \(K_i\) of the homologous human SERT mutants\(^7\) (Fig. 4c). The resulting linear relationship suggests that the mutations in LeuBAT have similar effects to those in SERT, and suggests that LeuBAT represents a framework for understanding the pharmacology of human SERT. We further compared the pharmacological selectivity of LeuBAT with all human BATs by plotting log \(K_i\) (LeuBAT) versus log \(K_i\) (human dopamine transporter (DAT), NET or SERT) (Supplementary Fig. 8). Inspection of these plots suggests that the pharmacological properties of LeuBAT are a hybrid of human BATs. Moreover, we compared the TCA binding site of the LeuBAT–CMI complex with the recently determined structure of the Drosophila DAT–nortriptyline complex\(^8\) (Supplementary Fig. 9). We note that the overall outward-open conformation and the essential elements of inhibitor binding are shared between the two structures. Finally, we reverted residues Asp 24, Gly 256 and Gly 359 in LeuBAT to their LeuT identities and investigated their effects on the drug binding. These mutations profoundly diminish drug binding (Supplementary Fig. 10), thus supporting the conclusion that the LeuBAT–inhibitor crystal structures represent specifically bound ligand–transporter complexes.

Taken together, the LeuBAT complexes allow us to map crucial subsites within the primary, orthosteric binding site that are responsible for binding the pharmacophores of a chemically diverse group of SSRIs, SNRIs and TCAs (Supplementary Fig. 11); they show that these inhibitors act by binding to the outward-open conformation of the transporter; and, perhaps most importantly, they provide molecular guideposts for the development of new therapeutic agents.

**METHODS SUMMARY**

The LeuBAT mutants were expressed and purified as previously described\(^7\) except that lauryl maltose neopentyl glycol was used for solubilization and purification. After final purification by size-exclusion chromatography in n-octyl-β-D-glucoside, LeuBAT was concentrated to 2.5 mg ml\(^{-1}\) and supplemented with saturating serotonin or mazindol prior to crystalization. All LeuBAT–drug complexes except mazindol were formed by soaking LeuBAT crystals in crystallization solutions containing 3–20 mM of each drug. The structures were solved by molecular replacement using the LeuT–Trp structure as a search probe and then subjected to cryocrystallographic refinement. The functional activities of LeuBAT mutants were examined by[^1]^Hparoxetine, [^3]^Hsertraline and [^3]^Hmazindol saturation and competition binding assays.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions H.W. and E.G. designed the research; H.W., A.G. and R.R. performed protein expression and purification; H.W., A.G., K.H.W. and A.P. carried out ligand-binding and flux experiments; H.W. conducted crystallization and structure determination; H.W. and E.G. wrote the manuscript together with comments from all authors.

Author Information Coordinates and structure factors for the LeuBAT A13-paroxetine, Δ13-sertraline, Δ13-duloxetine, Δ13-desvenlafaxine, Δ13-fluoxetine, Δ13-fluvoxamine, Δ13-clopiomarline, Δ6-sertraline, Δ6-desvenlafaxine, Δ6-duloxetine, Δ6-mazindol and Δ5-mazindol crystal structures have been deposited in the Protein Data Bank with codes 4MM4, 4MM5, 4MM6, 4MM7, 4MM8, 4MM9, 4MMA, 4MMB, 4MMC, 4MMD, 4MME and 4MMF, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to E.G. (gouauxe@ohsu.edu).
METHODS

Protein expression and purification. The LeuBAT mutants were expressed and purified as described previously for wild-type LeuT\textsuperscript{13,24} with two exceptions. First, lauryl maltose neopentyl glycol (MNG-3) instead of n-dodecyl-β-D-maltoside (C\textsubscript{12}M) was used for solubilization and purification. Second, no drugs or substrate were added during purification. Cell membranes were solubilized using a buffer composed of 25 mM Tris-HCl (pH 8.0), 250 mM NaCl and 1% MNG-3 and the protein was then purified by immobilized-metal affinity chromatography (IMAC) in the presence of 0.02% MNG-3. For crystallization, LeuBAT was purified by size-exclusion chromatography in 25 mM Tris-HCl (pH 8.0), 150 mM NaCl and 40 mM n-octyl-β-D-glucoside.

Crystallization and crystal soaking. LeuBAT with saturated serotonin or mazindol was crystallized by vapour diffusion at 20 °C with two different precipitating solutions containing: 100 mM NaPi (pH 7.0), 100 mM NaCl, 32–34% PEG300; or 100 mM glycine (pH 9.4), 0.1 M Li\textsubscript{2}SO\textsubscript{4}, 29–31% PEG400. LeuBAT crystals grown in the presence of serotonin were soaked for 1 h in crystalization solution containing 4 mM paroxetine, 20 mM desvenlafaxine, 3.7 mM sertraline, 3.7 mM fluoxetine, 3 mM duloxetine, 3 mM fluvoxamine or 10 mM CMI. After soaking, crystals were directly flash frozen in liquid nitrogen before X-ray diffraction data collection.

Data collection and structure elucidation. Diffraction data sets were collected at the Advanced Light Source (beamlines 5.0.2 and 8.2.1) and Advanced Photon Source (Argonne National Laboratory, beamlines 24-ID-C and 24-ID-E). Data sets were processed using HKL2000 software\textsuperscript{30} and IMOSFLM\textsuperscript{31}. The LeuBAT-antidepressant structures were determined by molecular replacement using LeuT–Trp (PDB code 3F3A) as a search probe using Phaser\textsuperscript{32} in CCP4 suite\textsuperscript{33}. The drugs were generated with PyMOL (DeLano Scientific).

Binding and competition assays. For binding studies, protein from IMAC fractions (pH 7.5), 100 mM NaCl, 20% (v/v) glycerol, 0.02% MNG-3. Scintillation proximity assays (SPA) were performed as before\textsuperscript{37}. Sertraline binding was performed by incubating 4 nM protein with 1–2 mg ml\textsuperscript{-1} copper yttrium silicate (Cu-Ysi) SPA beads in buffer A in the presence of 0.3–300 nM [\textsuperscript{3}H]sertraline (4 Ci mmol\textsuperscript{-1}). For mazindol binding, 25 nM protein was incubated with 1–2 mg ml\textsuperscript{-1} Cu-Ysi SPA beads in buffer A in the presence of 1–3,000 nM [\textsuperscript{3}H]mazindol (2.5 Ci mmol\textsuperscript{-1}). For paroxetine binding, 50–100 nM protein was incubated with 1–2 mg ml\textsuperscript{-1} Cu-Ysi SPA beads in buffer A in the presence of 0.01–10 μM [\textsuperscript{3}H]paroxetine (1.04 Ci mmol\textsuperscript{-1}). Nonspecific binding was measured in the presence of 400 mM imidazole, 0.1 mM mazindol and/or 0.5 mM CMI. Plate readings were taken using a Wallac Microbeta plate counter. Data after overnight incubation was analysed by GraphPad Prism\textsuperscript{4} and fit into a single-site binding function. Experiments were performed three times.

Competition assays were performed by using 300 nM [\textsuperscript{3}H]paroxetine (4.16 Ci mmol\textsuperscript{-1}) in buffer A. For half-maximum inhibitory concentration measurements, the concentrations of inhibitors were varied from 3 nM to 6.7 mM. A protein concentration of 100 nM was used for the competition assays, except that 10 nM protein was used for the sertraline competition experiments. The K\textsubscript{i} values were calculated by using the Cheng–Prusoff equation.

To compare mazindol binding to the A13, A13(D24G), A13(G256S) and A13(G359I) mutants, proteins (50 nM) were incubated with 1 mg ml\textsuperscript{-1} Cu-Ysi SPA beads in buffer A in the presence of 100 nM [\textsuperscript{3}H]mazindol (27.8 Ci mmol\textsuperscript{-1}). For sertraline binding, proteins (27 nM) were incubated with 1 mg ml\textsuperscript{-1} Cu-Ysi SPA beads in buffer A in the presence of 15 nM [\textsuperscript{3}H]sertraline (80 Ci mmol\textsuperscript{-1}). Nonspecific binding was measured in the presence of 0.1 mM mazindol and sertraline, respectively.

Fluorescence-detection size-exclusion chromatography. Fluorescence-detection size-exclusion chromatography (FSEC) was performed as described previously\textsuperscript{35}. Before the FSEC experiment, 1.2–4.8 pmol of each variant protein was incubated with 45–90 pmol of the 2B12 monoclonal antibody\textsuperscript{39} for 1 h and then loaded onto a Superose 6 column pre-equilibrated with 20 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.02% MNG-3 detergent. The elution was monitored by an in-line fluorescence detector which was set with excitation at 280 nm and emission at 335 nm. The proteins in the absence of 2B12 were used as a control (Supplementary Fig. 10).

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