Identification and Characterization of ML352: A Novel, Noncompetitive Inhibitor of the Presynaptic Choline Transporter

Elizabeth A. Ennis,† Jane Wright,† Cassandra L. Retzlaff,‡ Owen B. McManus,§ Zhinong Lin,† Xiaofang Huang,† Meng Wu,† Min Li,§ J. Scott Daniels,† Craig W. Lindsley,‡†∥⊥ Randy D. Blakely‡⊥§

†Departments of Pharmacology,‡Chemistry, and §Psychiatry, ‡Center for Neuroscience Drug Discovery, ⊥Specialized Center for Probe Development, Vanderbilt University Medical Center, Nashville, Tennessee 37232, United States
§Johns Hopkins University Ion Channel Center, Baltimore, Maryland 21205, United States

ABSTRACT: The high-affinity choline transporter (CHT) is the rate-limiting determinant of acetylcholine (ACh) synthesis, yet the transporter remains a largely undeveloped target for the detection and manipulation of synaptic cholinergic signaling. To expand CHT pharmacology, we pursued a high-throughput screen for novel CHT-targeted small molecules based on the electrogenic properties of transporter-mediated choline transport. In this effort, we identified five novel, structural classes of CHT-specific inhibitors. Chemical diversification and functional analysis of one of these classes identified ML352 as a high-affinity (Kᵢ = 92 nM) and selective CHT inhibitor. At concentrations that fully antagonized CHT in transfected cells and nerve terminal preparations, ML352 exhibited no inhibition of acetylcholinesterase (AChE) or cholineacetyltransferase (ChAT) and also lacked activity at dopamine, serotonin, and norepinephrine transporters, as well as many receptors and ion channels. ML352 exhibited noncompetitive choline uptake inhibition in intact cells and synaptosomes and reduced the apparent density of hemicholinium-3 (HC-3) binding sites in membrane assays, suggesting allosteric transporter interactions. Pharmacokinetic studies revealed limited penetrative properties, in keeping with a key role in autonomic function, motor control, attention, learning, and memory, and reward. Consequently, multiple, devastating disorders have been linked to perturbations of cholinergic signaling, including Alzheimer’s disease (AD), Parkinson’s disease (PD), dystonia, myasthenia, schizophrenia, and addiction, among others. Cholinergic agents, including acetylcholinesterase inhibitors (AChEIs) and muscarinic/nicotinic receptor agonists and antagonists, are used to treat symptoms in diseases with either diminished or excessive cholinergic signaling, as in AD, nicotine addiction, and dystonia. Although these agents have proven to be useful, their efficacy is reduced by dose-limiting side effects and, in some cases, a constitutive mode of cholinergic manipulation. Sustained expression of CHT is dependent on the efficient acquisition of choline by cholinergic terminals, an activity exclusively mediated by the high-affinity choline transporter (CHT, SLC5A7). Not surprisingly, full loss of CHT in transgenic mice produces a time-dependent elimination of cholinergic signaling that is incompatible with life. Less radical genetic manipulations of CHT also result in significant biochemical, physiological, and behavioral effects, in keeping with a key contribution of the transporter to ACh signaling. Thus, CHT heterozygous animals exhibit reduced ACh levels and ACh release, basal and exercise-induced tachycardia, and diminished neuromuscular signaling and motor endurance. Elevated CHT expression leads to increased ACh levels and reduced motor fatigue. With respect to CNS function, CHT heterozygous animals demonstrate reduced performance on tasks that require sustained attention in the presence of distraction and in tests of behavioral flexibility as well as diminished dopamine elevations following cocaine or nicotine challenge, whereas CHT overexpressing mice exhibit anxiety/depression behaviors. In keeping with these rodent studies, functional polymorphisms in the SLC5A7 gene have been linked to neuromuscular disorders, elevated distractibility and ADHD, and depression. CHT is conspicuous in being absent from current efforts to pharmacologically manipulate cholinergic function, but it may possess advantages in therapeutic targeting related to its activity-dependent support of cholinergic signaling, mediated by a

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steady-state enrichment on cholinergic synaptic vesicles, where it can move to the plasma membrane in response to cholinergic neuron activation.\textsuperscript{24,34,35} This feature also suggests that CHT-targeted antagonists may display use-dependence, thereby limiting drug effects to states of intense cholinergic signaling. CHT-mediated choline transport can be effectively attenuated by the competitive antagonist hemicholinium-3 (HC-3).\textsuperscript{36–38} Unfortunately, many of the properties of HC-3, including the presence of two choline-like quartenary nitrogens as well as its limited CNS penetrance and challenging chemical synthesis, restrict the use of the molecule as a tool for mechanistic studies or as a starting point for the development of imaging or therapeutic agents. However, in the more than 50 years since the first synthesis of HC-3,\textsuperscript{39,40} no other widely utilized CHT-targeted agents have been developed. To expand CHT pharmacology, we took advantage of the electrogenic nature of CHT-supported choline uptake to implement a membrane potential-based, high-throughput screen for CHT modulators.\textsuperscript{41,42}

Although CHT has been known to play a critical role in dictating cholinergic signaling capacity for many decades,\textsuperscript{39,43,44} the transporter is conspicuously absent from targets engaged for the therapeutic manipulation of cholinergic signaling. In part, this may be due to the understanding that full elimination of transporter function in vertebrates, as seen with CHT knockout mice,\textsuperscript{20} is incompatible with life. In CHT knockout mice, however, loss of CHT expression occurs throughout life, irrespective of demand, and thus the model may poorly represent the therapeutic limitations associated with CHT antagonism. Possibly, attenuated cholinergic signaling, rather than full inhibition, may offer an effective treatment for disorders where hypercholinergic function has been proposed as a major etiological component. For example, the uncontrolled movements associated with dystonia are commonly treated with anticholinergic agents to reduce both central and peripheral control of motor function.\textsuperscript{13,45} Hypercholinergic function has also been associated with depression and anxiety behaviors.\textsuperscript{46,47}

In the latter case, the nonspecific muscarinic ACh receptor antagonist scopolamine has received significant attention as a rapidly acting antidepressant.\textsuperscript{48,49} Finally, ACh receptor stimulation is intimately involved in the modulation of reward circuits, where anticholinergics have been shown to reduce aspects of reward signaling\textsuperscript{50,51} and CHT heterozygous mice have been found to demonstrate reduced dopamine release in response to cocaine and nicotine.\textsuperscript{26}

The importance of CHT in determining ACh signaling capacity, the therapeutic potential of CHT antagonism, and the limitations of HC-3 noted above encouraged us to pursue a high-throughput screen to identify novel CHT modulators. Here, we report the results of our screen for CHT inhibitors. We describe a novel, non-choline-based, CHT-targeted inhibitor (ML352) that demonstrates nanomolar CHT antagonism as well as selectivity for CHT in relation to multiple transporters, ion channels, and receptors. Our kinetic studies with ML352 are the first to demonstrate the possibility of allosteric modulation of the transporter and offer a novel path to the development of cholinergic therapeutics.

\section*{RESULTS AND DISCUSSION}

\textbf{High-Throughput Screen for CHT Antagonists.} To establish a screen for novel CHT inhibitors, we capitalized on the significantly elevated surface expression of the human transporter bearing alanine substitutions for two amino acids, L531 and V532, that constitute a strong dileucine-type endocytic sequence.\textsuperscript{41} In addition to the greatly enhanced choline-activated membrane depolarization achieved in hCHT LV-AA cells, the removal of strong endocytic sequences lessens the possibility that compounds that reduce choline-induced membrane depolarization do so by triggering transporter endocytosis. Using these cells, we instituted a triple-add protocol that involved addition of vehicle or inhibitor in the absence of choline, followed 1 min later by the addition of an EC_{20} concentration (500 nM) of choline, followed 2 min later by the addition of an EC_{50} concentration (60 \mu M) of choline. To identify noncompetitive and potentially allosteric inhibitors distinct from HC-3, we focused on compounds that reduced signal at the EC_{50} choline concentration, also capturing the superior signal/noise characteristics associated with signals generated at or near the CHT \textit{V}_{max}. Figure 1A demonstrates the fluorescence emission from the triple-add protocol following addition of choline alone or choline in the presence of an inhibitor, a structural predecessor to the focus of this article, ML352.

Figure 1. High-throughput screen for CHT inhibitors. (A) Example of screening assay. Triple-add protocol for screening library compounds for choline-induced membrane depolarization in HEK hCHT LV-AA cells. Time points for addition of vehicle, EC_{20} choline (500 nM), and EC_{50} (60 \mu M) choline are noted as arrows, left to right, respectively (solid line). Depolarization arising from three-add protocol in the presence of 500 nM VU0475863, one of the final 15 hit compounds and a progenitor of ML352, is shown in overlay (dotted line). (B) Screening workflow. Shown is our progression through the 9 major phases of the screen with the respective number of compounds that entered (small left-hand boxes) and exited (small right-hand boxes) each phase.
Using the triple-add format, we screened >300,000 compounds from the NIH Molecular Library Small Molecule Repository (MLSMR) compound collection (http://mli.nih.gov/mli/secondary-menu/mlscn/ml-small-molecule-repository/) at 60 μM, and, based on a cutoff criteria of 3 SD from the mean EC\textsubscript{50} choline-induced fluorescence, we identified 2635 preliminary hits, a 0.86% hit rate. We were able to validate CHT depolarization inhibition by 1714 of these compounds available for retesting, an ~65% replication rate. These compounds were then tested in a counterscreen against nontransfected HEK 293 cells, where we found that 90% of these compounds lacked CHT-independent depolarization-inducing activity, yielding 1544 molecules for further analysis. Next, 5-point concentration response curves (CRC) were performed using the hCHT LV-AA based membrane depolarization assay. In this effort, we found that 57%, or 877, of the compounds displayed dose-dependent inhibitory activity. From this group, we selected 393 molecules based on having IC\textsubscript{50} values lower than 10 μM to more carefully analyze in 10-point CRC assays, which yielded 228 molecules that retained high potency for inhibition of choline-induced depolarization. Of these compounds, 191 displayed no inherit fluorescence and retained their EC\textsubscript{50} inhibitory potency upon repeat testing. Inspection of the molecules in this group revealed 5 major structural classes, comprising 36 molecules, whose structures also indicated suitability for chemical modifications and in vivo use. As membrane depolarization that arises with CHT activity is nonstoichiometrically linked to choline flux,\textsuperscript{42} we next selected 15 molecules derived from 3 of these classes for 5-point CRC tests, now targeting inhibition of [3H]choline uptake, again using hCHT LV-AA cells. In the latter tests, 12 molecules demonstrated significant, dose-dependent inhibitory activity, 5 of which belong to the one class selected for chemical diversification (Figure 2A). Details of our chemical diversification efforts will be described elsewhere. In this effort, we produced VU0476328 N-((3-isopropylisoxazol-5-yl)methyl)-4-methoxy-3-((1-methylpiperidin-4-yl)oxy)benzamide (Figure 2B; hereafter designated ML352) as our most potent derivative and describe its characterization below.

**Potency and Specificity of ML352.** First, we sought to confirm the potency and specificity of ML352 for inhibition of hCHT LV-AA in transfected HEK 293 cells. In these studies (Figure 3A), we found that ML352 inhibited [3H]choline uptake with high affinity (K\textsubscript{i} = 92 ± 2.8 nM), with data well-fit to a single-site inhibition model (r = 0.935) In Figure 4B, we demonstrate that ML352 choline uptake inhibition was retained when tested in mouse forebrain synaptosomes (K\textsubscript{i} = 166 ± 12 nM). We also observed a lack of inhibitory activity at the dopamine transporter (DAT), the norepinephrine transporter (NET), and the serotonin transporter (SERT) in transfected HEK 293 cells as well as an insensitivity of [3H]choline accumulation in nontransfected HEK 293 cells to ML352 (<20% at 5 μM, data not shown). Regardless, these assays rule out a nonspecific effect on membrane integrity or alterations of ion gradients as being the basis for CHT inhibition. Additionally, little or no activity (>50% inhibition at 10 μM) was detected against 68 G-protein-coupled receptors (GPCRs), ion channels, and transporters in the EuroFin Lead Profiling Screen (Table 1), highlighting a clean ancillary pharmacology profile. The lack of choline and HC-3-mimicking quartenary nitrogens in the progenitor of ML352 suggested the possibility of limited interactions with other proteins that recognize choline or ACh. Indeed, in brain preparations, we also found no ability of ML352 to inhibit the biosynthetic enzyme ChAT and the metabolizing enzyme AChE at concentrations of ML352 well above the CHT K\textsubscript{i} (Figure 3C,D). Evaluation of ML352 and its future derivatives for interactions with other targets is, of course, incomplete, and more extensive evaluations, both in vitro and in vivo, will continue to be pursued, as should studies using these agents to manipulate cholinergic physiology and behavior.

**Kinetic Mode of ML352 Antagonism of CHT.** To establish a mechanism for CHT antagonism by ML352 and to classify the
molecule as similar to or distinct from HC-3 in action, we pursued studies of the kinetic basis of ML352 antagonism of choline transport activity (Figure 4A,B). In studies examining the activity of the inhibitor at varying concentrations of choline in transport assays with hCHT LV-AA transfected cells, we observed saturable choline transport with a $K_m$ for choline of $2.5 \pm 0.4 \mu M$, consistent with prior reports (Figure 4A). When combined with fixed concentrations of ML352, we observed no significant effect on the choline $K_m$ ($200 \text{ nM ML352}, K_m = 4.4 \pm 1.2 \mu M; 800 \text{ nM, } K_m = 4.4 \pm 2.0 \mu M$), whereas a concentration-dependent reduction in transport $V_{max}$ relative to vehicle-treated control was evident ($V_{max} 200 \text{ nM ML352} = 70.4 \pm 5.6\%$; 800 nM, $V_{max} = 30.3 \pm 4.2\%$). Analysis of ML352 inhibitory actions on choline uptake in mouse forebrain synaptosomes yielded similar effects ($V_{max} 300 \text{ nM ML352} = 57.2 \pm 3.4\%$) (Figure 4B). Together, these findings are consistent with the potent antagonism of CHT-dependent membrane potential of ML352 precursors when tested at the EC$_{50}$ choline concentration and point to a noncompetitive mode of CHT inhibition, distinct from the competitive mode of inhibition shown by HC-3. A noncompetitive mechanism is a potentially useful feature of ML352 inhibition in that extracellular choline has been reported at near saturating levels in brain extracellular fluid, and much higher concentrations of competitive antagonists, like HC-3, might be needed to effect CHT antagonism in vivo, leading to off-target actions.

HC-3 is the sole CHT antagonist in common use, and is limited essentially to mechanistic studies of cholinergic signaling. Developed in the late 1950s by Schueler and colleagues, HC-3 is a bicyclic compound with two quartenary nitrogens that mimics choline’s structure. The latter property affords orthosteric, high-affinity interactions with CHT. The choline features of HC-3, however, also provide for interactions with other choline targets such as CTL1-type choline transporters involved in choline lipid biosynthesis and choline kinase, with curvilinear binding kinetics reflecting multisite and/or cooperative binding interactions further limiting its use. With respect to in vivo utility, constitutive positive charge of HC-3 impedes CNS penetration and thus limits the molecule’s use in the potential treatment of brain disorders or in imaging CNS cholinergic innervation. Whereas HC-3 has proven to be useful in documenting CHT density in both membrane binding assays and autoradiographic studies, PET studies that document cholinergic neuron projections and/or terminals are currently limited, with a primary focus on derivatives of the vesicular choline transporter antagonist vesamicol. Depending on access of novel CHT ligands to intracellular versus extracellular conformations, targeting CHT may afford labeling
of activated cholinergic neurons where synaptic CHT density is elevated relative to less active states.\textsuperscript{24,34,35} To assess whether the binding site occupied by ML352 is kinetically independent of that occupied by HC-3, we pursued \[^{3}H\]HC-3 binding inhibition studies using membranes from HEK hCHT cells (Figure 4C,D). First, we observed that ML352 demonstrated dose-dependent inhibition of HC-3 binding, well-fit by a single-site inhibition model (\(r = 0.948\)) with a \(K_i\) of 128.6 ± 15.3 nM (\(n = 3\)). Next, we performed saturation binding studies using \[^{3}H\]HC-3, observing good (\(r = 0.93\)), single-site fit to binding data with a \(K_d\) of 8.5 ± 1.1 nM (Figure 4D), similar to previous observations.\textsuperscript{53} In support of uptake inhibition findings, we observed a significant reductions in \[^{3}H\]HC-3 binding \(B_{\text{max}}(B_{\text{max}} 200 \text{nM ML352} = 80.3 \pm 3.8\%); 800 \text{nM, 48.9 \pm 4.1\%). ML352 had no significant effect on \[^{3}H\]HC-3 \(K_d\) at 200 \text{nM (17.4 \pm 2.3\)), whereas an increased \(K_d\) was detected at 800 nM (23.4 ± 5.0)). \[^{3}H\]HC-3 binding with mouse and rat brain membranes exhibited multisite binding kinetics, as previously described,\textsuperscript{59} precluding accurate assessment of kinetic parameters in native tissues. Nonetheless, our studies provide strong support for the idea that choline uptake inhibition by ML352 is due to drug interactions at a site distinct from that occupied by either the substrate or HC-3.

\textbf{ML352 Modulation of CHT Surface Expression.} Recently, HC-3 was reported to induce elevated surface expression of CHT.\textsuperscript{64} To determine whether this property is shared by ML352, we performed cell-surface biotinylation studies, testing ML352 after 15 min incubations, in parallel with vehicle, HC-3, or choline treated cells. We used HEK 293 cells stably transfected with WT CHT for these studies, as the hCHT LV-AA mutant carries a compromised trafficking sequence that could obscure drug-induced changes in cell-surface expression. As shown in Figure 5, we replicated the findings that HC-3 elevates steady-state CHT surface expression (76 ± 16\% of control), although we obtained no evidence for the previously reported choline-dependent reductions.\textsuperscript{64} ML352 at saturating concentrations (5 \text{\mu}M) also induced a significant increase in hCHT surface expression (43.4 ± 4.7\% of control). No impact of drugs was seen on total CHT protein expression.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Inhibitory mechanisms involved with ML352 antagonism of CHT. (A) ML352 noncompetitively inhibits choline uptake in hCHT LV-AA cells. Inclusion of ML352 at 200 or 800 nM in saturation choline transport assays reveals a progressive decrease in the choline transport \(V_{\text{max}}\) without changing choline \(K_m\) (\(n = 3\)). (B) ML352 noncompetitively inhibits choline uptake in mouse forebrain synaptosomes. Inclusion of ML352 at 300 nM in saturation choline transport assays reveals a decrease in the choline transport \(V_{\text{max}}\) without a change in choline \(K_m\) (\(n = 3\)). (C) Inhibition of \[^{3}H\]HC-3 binding to transfected cell membranes by ML352. Binding assays revealed a ML352 \(K_i\) of 128.6 ± 15.3 nM (\(n = 3\)). (D) ML352 exhibits noncompetitive inhibition of \[^{3}H\]HC-3 binding to transfected cell membranes. Increasing concentrations of \[^{3}H\]HC-3 ± 200 nM ML352 yielded a significant reduction in binding \(B_{\text{max}}\) with no change in \[^{3}H\]HC-3 \(K_d\). At 800 nM ML352, a further reduction in \(B_{\text{max}}\) was detected along with an increase in \[^{3}H\]HC-3 \(K_d\) (\(n = 4\)).}
\end{figure}
Further studies are needed to determine the basis for these changes, for example, whether they arise from enhanced rates of surface delivery or recycling vs diminished protein conformations. Table 1. Off-Target Interactions of ML352

| Target               | % Inhibition | Target               | % Inhibition |
|----------------------|--------------|----------------------|--------------|
| Adenosine A₁         | −5           | Histamine, H₁       | 7            |
| Adenosine A₂A        | 0            | Imidazoline I₂, Central | 16          |
| Adenosine A₃         | 4            | Interleukin IL-1     | −2           |
| Adrenergic α₁A       | 23           | Leukotriene, Cysteinyl CysLT₁ | −3       |
| Adrenergic α₁B       | 3            | Melatonin MT₁       | 5            |
| Adrenergic α₁D       | 24           | Muscarinic M₁       | 32           |
| Adrenergic α₂A       | 43           | Muscarinic M₂       | 12           |
| Adrenergic β₁        | 2            | Muscarinic M₃       | 26           |
| Adrenergic β₂        | 43           | Neuropeptide YY₁     | −2           |
| Androgen (Testosterone) AR | 8  | Neuropeptide YY₂     | 7            |
| Bradykinin B₁       | 6            | Nicotinic Acetylcholine | 13         |
| Bradykinin B₂       | −3           | Nicotinic Acetylcholine α₁, Bungarotoxin | 7 |
| Calcium Channel L-Type, Benzothiazepine | 3 | Opiate δ₁ (OP1, DOP) | −2 |
| Calcium Channel L-Type, Dihydropyridine | 13 | Opiate κ (OP2, KOP) | 22 |
| Calcium Channel N-Type | −1 | Opiate μ (OP3, MOP) | 8          |
| Cannabinoid CB₁     | 4            | Phorbol Ester       | 5            |
| Dopamine D₁         | 9            | Platelet Activating Factor (PAF) | −4        |
| Dopamine D₂A        | 18           | Potassium Channel [KATP] | 4 |
| Dopamine D₃         | 15           | Potassium Channel hERG | 16 |
| Dopamine D₄A        | −3           | Prostanoid EP₂     | −7           |
| Endothelin ET₁a     | −3           | Purinergic P₂X      | 22           |
| Endothelin ET₁b     | −2           | Purinergic P₂Y      | 19           |
| Epidermal Growth Factor (EGF) | 5 | Rolipram | 1 |
| Estrogen Erα        | 4            | Sertonin (5-Hydroxytryptamine) 5-HT₁A | 40 |
| GABA₅, Flunitrazepam, Central | 6 | Sertonin (5-Hydroxytryptamine) 5-HT₂B | −7 |
| GABA₅, Muscimol, Central | 9 | Sertonin (5-Hydroxytryptamine) 5-HT₃ | 2 |
| GABA₅,α₁            | −9           | Sigma δ₁           | −1           |
| Glucocorticoid      | −1           | Sodium Channel, Site 2 | 20       |
| Glutamate, Kainate  | −2           | Tachykinin NK₁     | 22           |
| Glutamate, NMDA, Agonism | 1 | Thyroid Hormone | 6 |
| Glutamate, NMDA, Glycine | 4 | Transporter, Dopamine (DAT) | 4 |
| Glutamate, NMDA, Phencyclidine | −5 | Transporter, GABA | 11 |
| Histamine, H₁       | 37           | Transporter, Norepinephrine (NET) | 12 |
| Histamine, H₂       | 20           | Transporter, Serotonin (5-hydroxytryptamine) (SERT) | 2 |

valuers represent the percent inhibition of radioligand binding to designated targets at 10 μM ML352.

Figure 5. ML352 causes an increase in CHT surface expression in hCHT transfected cells. (A) Saturating concentrations, 5 μM, of ML352 or HC-3 were incubated for 15 min with wild-type (WT) hCHT transfected HEK 293 cells, followed by cell surface biotinylation and analysis of surface proteins as described in Methods. A representative western blot of surface protein levels is shown. (B) Quantitation of CHT surface expression studies. Both HC-3 and ML352 significantly elevated CHT surface protein levels (*P < 0.05, Dunnett’s posthoc comparison vs vehicle treated samples (n = 4) following a significant one-way ANOVA treatment effect (P < 0.001)).
In summary, we have successfully broadened the pharmacology of CHT antagonism, providing a novel tool for the study of CHT in both heterologous and native preparations and a platform for further drug development. Certainly, issues related to the potential broad physiological actions of CHT antagonism must be considered as one evaluates the potential clinical utility of CHT inhibitors, although relatively nonspecific reagents are commonly used in medical practice (e.g., amphetamines for the treatment of ADHD, clozapine for the treatment of schizophrenia). Importantly, CHT is highly regulated with respect to its contribution to cholinergic signaling due to a steady-state enrichment of the transporter on cholinergic synaptic vesicles.4,65,66 As a consequence of its vesicular localization, CHT exhibits activity-dependent shuttling to the plasma membrane in response to presynaptic excitation. The latter property may afford CHT inhibitors a unique profile of activity-dependent, cholinergic signaling inhibition, exerting their most prominent effects under conditions of excessive ACh signaling. The noncompetitive nature of CHT inhibition by ML-352 may also provide a spectrum of properties related to in vivo use distinct from the actions of HC-3.

### METHODS

**Reagents and Cells.** All biochemical reagents were of research grade and, unless stated otherwise, obtained from Sigma-Aldrich (St. Louis, MO, USA). Commercially available replicates and derivatives of molecules identified as hits in our HTS screen were obtained from Ambiber (Orleans, France) as dry powders. HEK 293 and HEK 293 cells stably transfected with either the human CHT (hCHT) or mutant hCHT LV- AA (L531A, V532A) were described previously41 and maintained according to American Type Culture Collection (ATCC, Manassas, VA, USA) guidelines using MEM Earles media containing 1X fetal bovine serum, 2 mM glutamine, 100 IU penicillin, and 100 μg/mL streptomycin (Fisher Scientific, Pittsburgh, PA, USA). All studies used cells cultured at 70–100% confluence.

**Choline-Induced Membrane Potential Assay (384-Well HTS Format).** HEK 293 cells stably expressing hCHT or hCHT LV-AA or control cells were assessed for basal and choline-induced changes in membrane depolarization as previously described41 by plating into 384-well black walled, clear bottom poly-n-lysine coated plates (Biotcoat, BD Biosciences, San Jose, CA, USA) at 20,000 cells in 20 μL/well, dispensing using a Thermo Electron Multidrop reagent dispenser. Plated cells were grown overnight at 37 °C. The following day, the culture medium was removed, plates were washed 3X with an ELX microplate washer (Biotek, Winooski, VT, USA), and 20 μL/well of 1.67 μg/mL of the membrane potential dye (Molecular Devices, Sunnyvale, CA, USA, R8042) was added in assay buffer (Hanks balanced salt solution (HBSS), Gibco) containing 20 mM HEPES, pH 7.3, by a dispenser (Thermo). Cells were incubated for 30 min at 37 °C in an atmosphere of 95% air/5% CO2 prior to addition of compounds at 2.5X their final concentration in HBSS. For control plates, a choline concentration that was later used in inhibitor screening assays. Cell plates and compound plates were loaded into a Hamamatsu FDSS 6000 kinetic imaging plate reader. Baseline fluorescence signals were collected for 9 s at 0.5 Hz prior to addition of media, 5 μM HC-3, or test compounds at various concentrations, diluted from DMSO stocks. After a subsequent 2 min 16 s of recording, choline chloride at EC90 was added (time point 2 min 25 s), followed 2.5 min later by the addition of EC90 choline (time point 6 min), with data collected for an additional 2.5 min. Fluorescence was captured by Hamamatsu FDSS 6000 imaging software, and data were exported to Microsoft Excel for graphical and statistical analysis.

**Choline-Induced Membrane Potential Assay (96-Well Flex-Station Format).** HEK 293T cells stably expressing hCHT LV-AA or
control cells were assayed for choline-induced membrane depolarization as previously described.41 Briefly, cells were plated into 96-well, black-walled, clear bottom poly-n-lysine coated plates (BioCoat BD Biosciences). Cells were plated in 50 μL/well at 45 000 cells/well and allowed to grow for 48 h. Cells were preincubated in HBSS/HEPES 1× dye (60 μL of blue membrane potential dye, R8042 at 1.67 μg/mL, Molecular Devices, Sunnyvale, CA, USA) for 30 min at 37 °C in an atmosphere of 95% air/5% CO2. Tested compounds were added at this preincubation step. Membrane potential-associated fluorescence was detected using a FlexStation (Molecular Devices) microplate fluorimeter, recording baseline (1 min) and following automated addition of choline (20 μL) to achieve 100 nM and 10 μM final concentrations in HBSS/HEPES 1× dye. Fluorescence was recorded for 2–4 min with sampling every 1.5 s. Data were analyzed by SoftMax Pro (Molecular Devices) and exported to Microsoft Excel for further evaluation.

**Neurotransmitter Transport Activity Assay (96-Well Format).** Cells were plated into poly-n-lysine coated, 96-well, white cell culture plates (Culturplate-96, PerkinElmer, Waltham, MA, USA) at 45 000 HEK293 hLTV-AA cells or 20 000 HEK293 hDAT, hNET, or hSERT cells in 100 μL/well and allowed to grow for 48 h at 37 °C in an atmosphere of 95% air/5% CO2. Each plate was washed 3× with 100 μL of KRH using a Biotek 405 Touch microplate washer (Winooski, VT, USA). Wells were filled with 40 μL of KRH buffer with or without drugs using a multichannel pipet. Plates were incubated at 37 °C in an atmosphere of 95% air/5% CO2 for 15 min for choline uptake assays and 10 min for DA, NE, and 5-HT uptake assays. Following the first incubation, an addition of 10 μL of either 100 nM or 10 μM (final concentrations) [3H]-choline chloride (PerkinElmer, Waltham, MA, USA, NET109001MC, 1 mCi/mL) or 50 nM (final concentrations) [3H]-DA, (dihydroxyphencylamine,3,4-Ring-2,5,6-3H) (PerkinElmer, NET673001MC, 1 μCi/mL), [3H]NE (norepinephrine hydrochloride, DL-[7-3H(N)]) (PerkinElmer, NET048001MC, 1 μCi/mL), [3H]5-HT (hydroxytryptamine creatinine sulfate) (S-H), or 5-[1,2-3H(N)] (PerkinElmer, NET498001MC, 1 μCi/mL) in KRH buffer. Final concentrations of substrates solutions were made using 1× radioligand with 99% unlabeled neurotransmitter stock solution. Following a 10–15 min incubation with radiolabeled substrates, each plate was washed 3× with 100 μL of KRH buffer using a Biotek (location) 405 Touch microplate washer. A volume of 100 μL of MicroScint-20 (PerkinElmer, Waltham, MA, USA) was added to wells, and uptake was quantified by scintillation spectrometry using a TopCount instrument (PerkinElmer, Waltham, MA, USA). Counts obtained from wells were recorded by MicroScint-20 (PerkinElmer, Waltham, MA, USA). Accurate counts were determined employing the following equations: where Ks represents the slope from linear regression analysis (% test article remaining). An additional recovery (Yrec) was determined using nonlinear curve fitting in Prism 5 (GraphPad Inc., San Diego, CA, USA).

**Hemicholinium-3 (HC-3) Radioligand Binding Assays.** HEK 293 cells stably expressing CHT were plated on 150 mm sterile tissue culture dishes and allowed to grow to confluency. Cells were harvested from the plates with 50 mM Tris-HCl buffer (pH 7.5) and homogenized using a Polytron (Wheaton Instruments, Millville, NJ, USA) at a speed setting of 4. Homogenates were centrifuged for 15 min at 15 000g. Pellets were resuspended in 10 mL of 50 mM Tris-HCl buffer (pH 7.5) and centrifuged again for 15 min at 15 000g. The resulting pellets were resuspended in 2 mL of Tris base buffer. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL, USA). Hemicholinium-3 diacetate salt, [methyl-3H]HC-3 (PerkinElmer, Waltham, MA, USA), 120 μCi/mmole) binding assays were conducted in 50 mM TRIS-HCl buffer, 200 mM NaCl, pH 7.5, at 37 °C for 45 min. For saturation assays, serial dilutions from a 1% mix of radiolabeled and unlabeled antagonist was used to maintain specific activity. For ML352 competition binding assays, 100 μg of membranes was incubated for designated times in 10 nM [3H]-HC-3 ± varying drug concentrations. Nonspecific binding in competition assays was defined in parallel incubations with 20 μM unlabeled HC-3 and subtracted from total counts to define specific HC-3 binding. Nonspecific binding in saturation binding assays was defined using membranes from non-transfected HEK 293 cells. Radioactively labeled membranes were harvested on glass fiber filters (Whatman GF/F, Brandel, Gaithersburg, MD, USA) that were pretreated with 0.3% polyethyleneimine and 2% BSA and rinsed 4× with assay buffer (50 mM Tris-HCl, 200 mM NaCl, pH 7.5). Filters were solubilized in EcoScint H (National Diagnostics, Atlanta, GA) and allowed to shake overnight, and bound radiolabel was quantified by liquid scintillation counting (TriCarb 2900TR, PerkinElmer). HC-3 binding capacity (Bmax) and affinity (Kd) and ML352 K values were determined using nonlinear curve fit to a single-site inhibition model using Prism 5.

**Off-Target Screen.** ML352 was tested in the Lead Profiling Screen (Eurofin, Luxembourg), a binding assay panel of 68 G-protein coupled receptors, ion channels, and transporters, at 10 μM. The Lead Profiling Screen consists of 68 primary molecular targets, including several CNS targets recommended by the European Medicines Agency (EMEA) to evaluate drug dependence potential. For full assay details, see www.eurofinspanlabs.com.

**Animal Care and Husbandry.** All procedures with mice (C57BL/6j mice, Jackson Laboratories, Bar Harbor, ME, USA) and rats (Sprague–Dawley, Harlan, Indianapolis, IN, USA) were performed with animals at 10–20 weeks of age under an approved protocol that is reviewed annually by the Vanderbilt Institutional Animal Care and Use Committee (IACUC). Animals were housed prior to use on a 12:12 light–dark cycle with food and water provided ad libitum.

**Synaptosomal Transport Assays.** Male mice (C57BL/6j, Jackson Laboratories) at 8–10 weeks of age were sacrificed by rapid decapitation under urethane anesthesia, brains were removed, and brain regions were dissected on an ice-cold aluminum plate on ice. Crude synaptosomes (P2) were prepared from isolated forebrain tissue as previously described,35 with forebrain defined as all brain tissue anterior to a coronal plane abutting the superior colliculus. Aliquots (100 μL) of crude synaptosomes (P2) were incubated with 100 μL of 100 nM radiolabeled choline, dopamine, or serotonin (materials noted above) in Kreb’s bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 25 mM NaHCO3, 1.7 mM CaCl2, 10 mM glucose, 100 mM ascorbic acid) in the presence and absence of 10 μM HC-3, or other inhibitors, also as noted above, for 15 min for choline uptake assays and 10 min for all other neurotransmitters at 37 °C. Transport assays were terminated by transferring the tubes to an ice bath followed by rapid filtration over a Brandel cell harvester (Brandel Inc., Gaithersburg, MD, USA). Accumulated radioactivity was determined using a TopCount instrument (PerkinElmer, Chicago, IL, USA). Specific uptake was defined with parallel incubations with specific transporter inhibitors as noted for transfected cells. Saturation kinetic parameters were determined using Prism 5 as described in cell studies.

**Metabolism and Disposition Methods.** The metabolism of ML352 was investigated in vitro in rat hepatic microsomes (BD Biosciences, Billerica, MA, USA) using substrate depletion methodology (% test article remaining). A potassium phosphate-buffered reaction mixture (0.1 M, pH 7.4) of test article (1 μM) and microsomes (0.5 mg/mL) was preincubated (5 min) at 37 °C prior to the addition of NADPH (1 mM). The incubations, performed in 96-well plates, were continued at 37 °C under ambient oxygenation, and aliquots (80 μL) were removed at selected time intervals (0, 3, 7, 15, 25, and 45 min). Protein was precipitated by the addition of chilled acetone (160 μL), containing carbamazepine as an internal standard (50 ng/mL), and centrifuged at 3000 rpm (4 °C) for 10 min. Resulting supernatants were transferred to new 96-well plates in preparation for LC/MS/MS analysis. The in vitro half-life (t1/2, min, eq 1), intrinsic clearance (CLint, mL/min/kg, eq 2), and subsequent predicted hepatic clearance (CLPapp, mL/min/kg, eq 3) were determined employing the following equations:

\[ t_{1/2} = \ln(2)/k \]

where k represents the slope from linear regression analysis (% test article remaining).
plate was sealed and incubated for 4 h at 37 °C.

The plate was centrifuged (3000 rpm, 10 min), and 15 min post second dose in order to obtain blood and CNS determinations.

Following treatment of hCHT transfected HEK 293 cells with or without choline (10 μM), HC-3 (5 μM), or ML352 for 15 min, cells were biotinylated on ice for 1 h, followed by a 30 min incubation with 1× PBS with calcium, magnesium, and 100 mM glycine. Cells were extracted with 500 μL of RIPA buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton-X 100, 10 g/L deoxycholate), and biotinylated proteins were isolated using Ultraspin Avidin (Pierce, Rockford, IL, USA), eluted in Laemmli buffer, resolved by 10% acrylamide SDS-PAGE, and blotted to PVDF membrane (Millipore, Darmstadt, Germany). PVDF membranes were blocked in PBST + 5% nonfat dry milk (PBST-M) for 1 h and then probed with 10 mL of polyclonal rabbit (1:500 to 1:1000) anti-CHT antibody for CHT overnight at 4 °C. Blots were rinsed 3× for 5 min in PBST and incubated in HRP-conjugated secondary antibody (goat anti-rabbit, 1:5000 in PBS-TM, Jackson Immunoresearch, West Grove, PA, USA) for 1 h at room temperature. Blots were rinsed 3× for 5 min in PBST and developed with chemiluminescence (Western Lightning Enhanced Chemiluminescence kit, PerkinElmer, Waltham, MA, USA). Blots developed using a LAS4000 Chemiluminescent Imaging System (GE Healthcare, Buckinghamshire, England). ImageQuant software (GE Healthcare, Buckinghamshire, England) was used to determine the band densities. In order to compensate for interexperiment variability, CHT bands were normalized to CHT protein detected in total protein extracts. Cumulative were analyzed by a one-way ANOVA with Dunnett’s planned comparisons of drug vs vehicle, with P < 0.05 taken as significant (Prism 5.0).

**AUTHOR INFORMATION**

Corresponding Author
*Tel: 615-936-1700. E-mail: randy.blakely@vanderbilt.edu.

Author Contributions
Corey R.Hopkins,Randy D. Blakely, Jane Wright, Elizabeth A. Ennis, Owen B. McManus, Zhihong Lin, Xiaofang Huang, Meng Wu, and Min Li participated in the establishment and execution of the high-throughput screen at the Johns Hopkins Ion Channel Center. Elizabeth Ennis completed the majority of the compound screening, cell, synaptosomal, specificity, and biotinylation assays. Jane Wright and Cassandra L. Retzlaff aided in the completion of the synaptosomal uptake assays, and the cell surface biotinylation assays, respectively. J. Scott Daniels and Craig W. Lindsley directed the drug metabolism and pharmacokinetic experiments. All of the aforementioned authors participated in the discussions that guided the work at various stages. Elizabeth A. Ennis and Randy D. Blakely wrote the text for this article with input on manuscript revisions provided by all of the authors.

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