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Chemocentric Informatics Approach to Drug Discovery: Identification and Experimental Validation of Selective Estrogen Receptor Modulators as Ligands of 5-Hydroxytryptamine-6 Receptors and as Potential Cognition Enhancers

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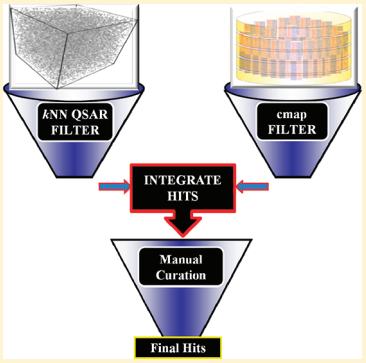
Supporting Information

ABSTRACT: We have devised a chemocentric informatics methodology for drug discovery integrating independent approaches to mining biomolecular databases. As a proof of concept, we have searched for novel putative cognition enhancers. First, we generated Quantitative Structure−Activity Relationship (QSAR) models of compounds binding to 5-hydroxytryptamine-6 receptor (5-HT_6R), a known target for cognition enhancers, and employed these models for virtual screening to identify putative 5-HT_6R actives. Second, we queried chemogenomics data from the Connectivity Map (http://www.broad.mit.edu/cmap/) with the gene expression profile signatures of Alzheimer's disease patients to identify compounds putatively linked to the disease. Thirteen common hits were tested in 5-HT_6R radioligand binding assays and ten were confirmed as actives. Four of them were known selective estrogen receptor modulators that were never reported as 5-HT_6R ligands. Furthermore, nine of the confirmed actives were reported elsewhere to have memory-enhancing effects. The approaches discussed herein can be used broadly to identify novel drug−target−disease associations.



INTRODUCTION

Target-oriented drug discovery is one of the most popular modern drug discovery approaches.[1−5] Target-oriented approaches rely on established functional associations between activation or inhibition of a molecular target and a disease. Modern genomics approaches including gene expression profiling, genotyping, genome-wide association, and mutagenesis studies continue to serve as useful sources of novel hypotheses linking genes (proteins) and diseases and providing novel putative targets for drug discovery.

In recent years, functional genomics approaches have been increasingly complemented by chemical genomics,[6−11] i.e., large scale screening of chemical compound libraries in multiple biological assays.[12−16] The resulting data (either generated within chemical genomics centers or collected and curated from published literature) have been deposited in many public and private databases such as the NIMH Psychoactive Drug Screening Program K_i Database (K_i-DB),[17] PubChem,[18] ChEMBL,[19] WOMBAT,[20] and others (reviewed in ref 21).

Various *in silico* techniques have been exploited for analyzing target-specific biological assay data. A recent publication by Kortagere and Ekins[22] could serve as a good summary of most common target-oriented computational drug discovery approaches including (1) structure based virtual screening (docking and scoring) using either experimentally characterized (with X-ray or NMR) or predicted by homology modeling structure of the target protein, (2) chemical similarity searching using known active compounds as queries, (3) pharmacophore based modeling and virtual screening, (4) quantitative structure−activity relationship (QSAR) modeling, and (5) network or pathway analysis.

Data resulting from large-scale gene or protein expression or metabolite profiling (often collectively referred to as 'omics' approaches[23−26]) can be explored not only for specific target identification but also in the context of systems pharmacology to identify networks of genes (or proteins) that may collectively define a disease phenotype. For example, 'omics' data can be used to query genes or proteins, or post-translationally modified states of proteins that are over (or under-)-expressed in patients suffering from a particular disease. These types of data can be found in a number of public repositories such as the Gene Expression Omnibus (GEO),[27,28] GEOmetadb,[29] the Human Metabolome Database (HMDB),[30,31] Kinase SARfari,[32] the Connectivity Map (cmap),[33,34] the Comparative Toxicogenomics Database (CTD),[35] STITCH,[36,37] GenBank,[38,39] and others. Importantly, many of these databases integrate, in some way, chemical effects on biological systems providing an

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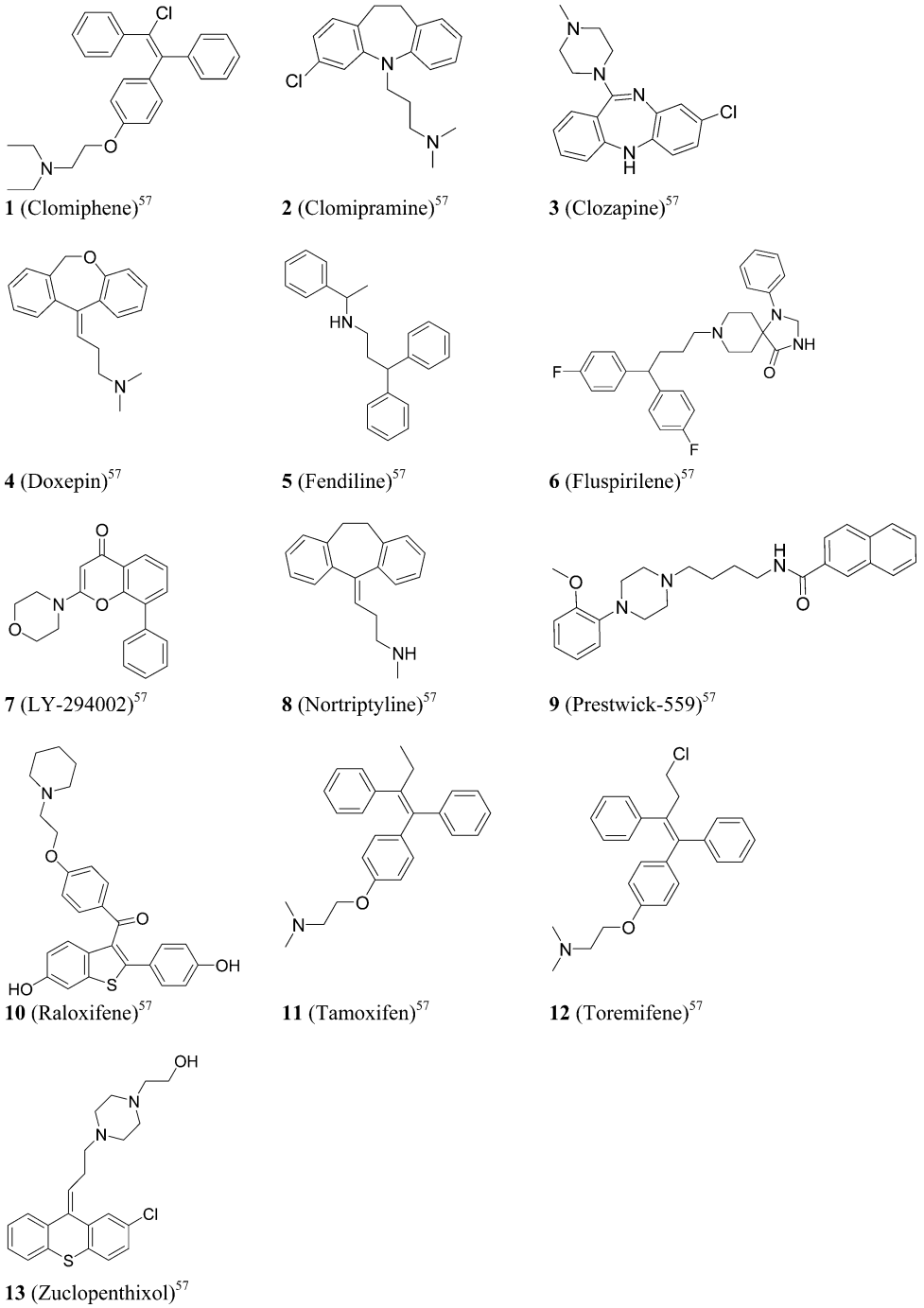

Figure 1. Computational hits.

opportunity to explore diverse computational approaches, individually or in parallel, to modeling and predicting the relationships between drug structure, its bioactivity profile in short-term biological assays, and its effects *in vivo*.

Recently, a group of scientists at the Broad Institute established the Connectivity Map (cmap) database to catalog the biological responses of a large number of diverse chemicals in terms of their gene expression profiles.[33] Indeed, insights into disease pathology and underlying mechanisms can be revealed by the disease 'gene signature', i.e., those genes whose expression varies consistently between patients and healthy individuals (controls).[40] Gene-expression profiling has been often applied to elucidate the mechanisms underlying the roles of biological pathways in a disease,[41,42] reveal arcane subtypes of a disease,[43,44] and estimate cancer prognosis.[45,46] At the same time, the treatment of cultured human cells with chemical compounds that target a disease can produce a drug related 'gene signature', i.e., differential expression profile of genes in response to the chemical.[40,47−49] It has been shown that examining the correlations between gene expression profiles characteristic of a disease and those modulated by drugs may lead to novel hypotheses linking chemicals to either etiology or treatments for a disease.[33,43,50−56]

The cmap database provides an unusual but intriguing example of what we shall call a *chemocentric* 'omics' database and methodology for generating independent and novel drug

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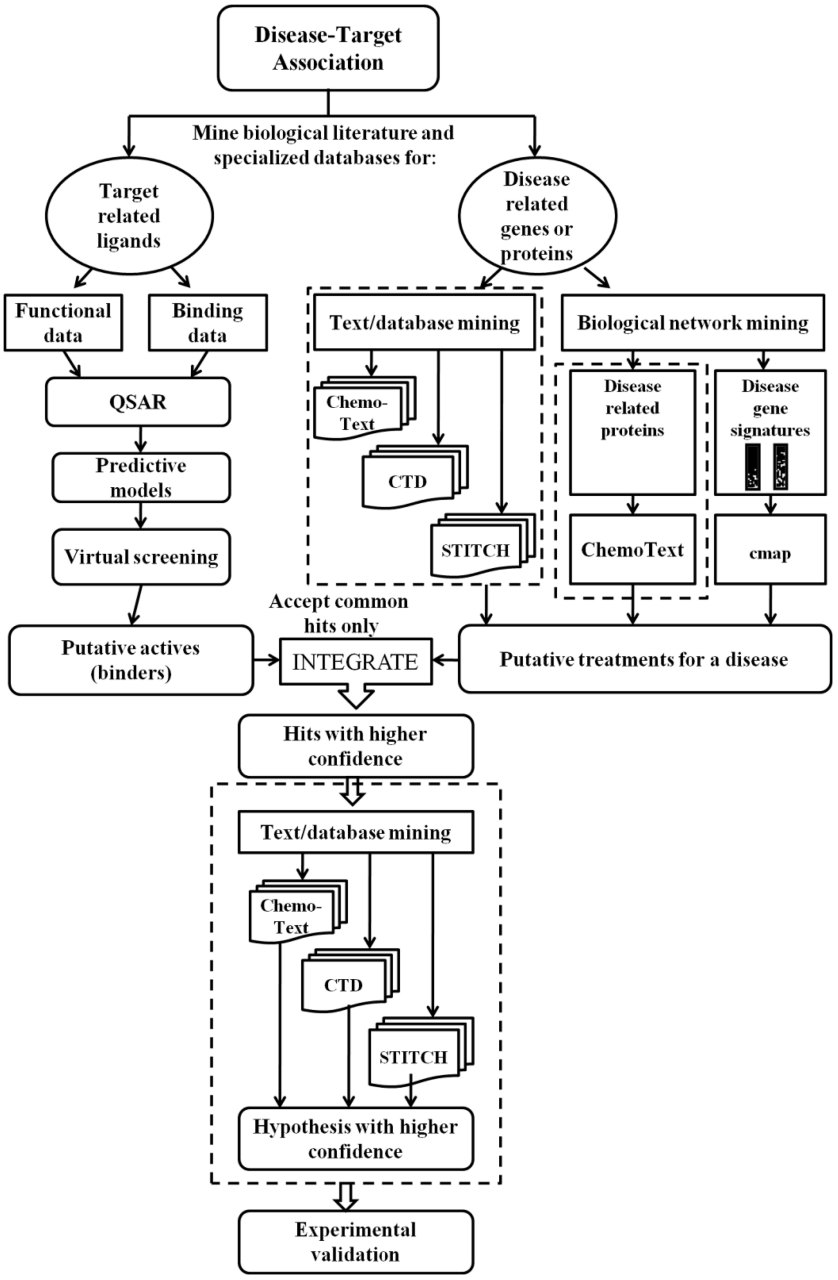

Figure 2. Study design for the integrated informatics approach for drug discovery integrating network mining, text mining of biological literature, the analysis of disease gene signatures, and efficient cheminformatics techniques to discover novel drugs with desired polypharmacology.

discovery hypotheses. Indeed, there exists a wealth of information buried in the biological literature and numerous specialized chemical databases[17−20,57] linking chemical compounds and biological data (such as targets, genes, experimental biological screening results; cf. ref 58). The chemocentric exploration of these sources, either individually or in parallel, opens up vast possibilities for formulating novel drug discovery hypotheses concerning the predicted biological or pharmacological activity of investigational chemical compounds or known drugs. The integration and cross-validation of such independent structural hypotheses can increase the quality of the final hit list of predicted actives.

Herein, we describe a novel integrative *chemocentric informatics* approach to drug discovery that integrates computational hits generated from independent analysis of both traditional target-specific assay data and those resulting from large scale genomics and chemical genomics studies. As a proof of concept, we have focused on the Alzheimer's disease as one of the most debilitating neurodegenerative diseases with complex etiology and polypharmacology. We have considered and cross-examined two independent but complementary approaches to the discovery of novel putative anti-Alzheimer's drugs. First, we have employed a traditional target-oriented cheminformatics approach to discovering anti-Alzheimer's agents. We have built QSAR models of ligands binding to the 5-hydroxytryptamine-6 receptor (5-HT_6R). It has been shown that 5-HT_6R antagonists can produce cognitive enhancement in animal models,[59] and it has been suggested that this receptor may be a potential target for treating cognitive deficits in Alzheimer's disease.[60] We have then used models developed with the rigorous predictive QSAR modeling workflow established and implemented in our laboratory[61] for virtual

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screening (VS) of the World Drug Index database (WDI)[57] and DrugBank[62] to identify putative cognition enhancing agents as compounds predicted to interact with 5-HT$_6$R. Second, we have explored (chemo)genomic data available from the cmap project[33,34] to link chemical compounds and Alzheimer's disease without making explicit hypotheses about target-specific mechanisms of action, i.e., treating Alzheimer's disease as a complex polypharmacological disease.

We then cross-examined and combined common hits regarded as structural hypotheses resulting from both approaches toward common integrated hits supported by two independent lines of computationally based evidence. Thirteen common hits (Figure 1) were tested in 5-HT$_6$R binding assays using the resources of the NIMH Psychoactive Drug Screening Program (PDSP),[17] and ten were confirmed experimentally as actives. Unexpectedly, we found that the confirmed actives included several selective estrogen receptor modulators (SERMs) that were never reported earlier as 5-HT$_6$R ligands suggesting that they may be potential cognitive enhancers. Indeed, we have identified clinical evidence in biomedical literature in support of this hypothesis. We believe that approaches discussed in this study can be applied to a large variety of systems to identify novel drug−target−disease associations.

MATERIALS AND METHODS

Integrative Chemocentric Informatics Approach. We have devised an integrative workflow focused on the discovery of new drug candidates and finding new uses for existing drugs by integrating predictions generated from different data types and methods. Currently, the workflow (Figure 2) incorporates three major components: (1) a module for QSAR-based VS of chemical libraries to identify new ligands for target proteins, (2) a network-mining module to identify small molecule therapeutics for specific diseases without necessarily knowing the underlying target-specific mechanism; this module explicitly relies on cmap,[33,34] an external online database (www.broadinstitute.org/cmap/) that links the effects of different drugs and diseases using gene expression profiles, and (3) ChemoText,[58] an in-house repository of relationships between chemicals, diseases, proteins, and biological processes. The first two modules have been employed extensively for studies reported herein.

We start our study with identifying established disease−target associations (e.g., 5-HT$_6$R is implicated in treating Alzheimer's disease). Then we mine the biological literature and specialized databases to extract ligands known to interact with the biological target of interest. Activity data could be either binding affinities (K_i values) or functional data (IC_{50} values for agonists and antagonists). Binding and functional data could be either continuous (e.g., K_i and IC_{50} values) or categorical (e.g., active vs nonactive or agonist vs antagonist) in nature. At this stage, we use our QSAR-based VS module (see Figure 3; predictive QSAR workflow) to generate robust predictive QSAR models that can be employed for VS of chemical libraries to derive new hypotheses about putative actives (agonists or antagonists).

In parallel, we mine the biological literature for gene signatures associated with the disease and/or for all related protein targets implicated in the disease state. We use these disease related genes and proteins to query specialized databases to extract information about disease−protein (gene)−chemical connections. For example, we use disease

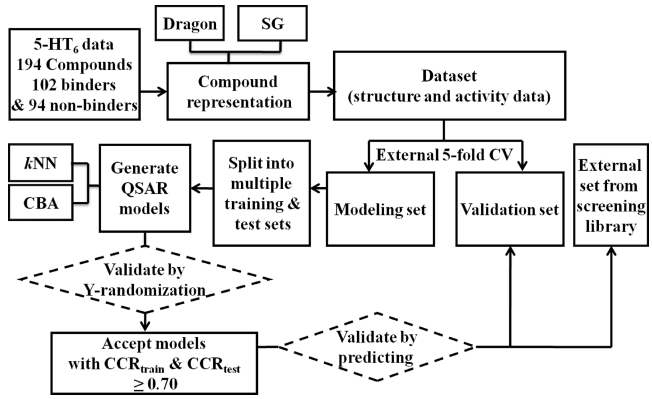

Figure 3. Workflow for QSAR model building, validation, and virtual screening as applied to the 5-HT$_6$R data set.

gene signatures to query the cmap for putative treatments, and we use related proteins to query ChemoText for related chemicals to establish new disease−protein (gene)−chemical connections. After a thorough analysis of all data, we select hit compounds that are expected to be novel treatments for the disease (cf. Figure 2).

Finally, we integrate hypotheses derived from the QSAR-based VS approach with those derived from text/network mining. The common structural hits identified by both approaches are considered for further experimental validation. We assume that the quality of the final structural hypotheses resulting from independent approaches to knowledge mining in chemocentric databases is intrinsically better than that in any computational hit generated in respective independent studies.

Databases and Data Sets. *PDSP K_i-DB.* PDSP K_i-DB[17] (http://pdsp.med.unc.edu/pdsp.php) includes published binding affinities (K_i) of drugs and chemical compounds for receptors, neurotransmitter transporters, ion channels, and enzymes. It currently lists more than 47000 K_i values for more than 700 molecular targets. K_i-DB represents a curated, fully searchable database of both published data and data internally derived from the NIMH-PDSP. The experimental data for Alzheimer's disease related target 5-HT$_6$R were extracted from the PDSP K_i-DB available in the public domain. The complete 5-HT$_6$R data set included binding affinity data for 250 compounds.

World Drug Index (WDI). WDI[57] is an authoritative database for marketed and developmental drugs providing information about internationally recognized drug names, synonyms, trade names, trivial names, trial preparation codes, compound structures, and activity data. Herein, we used WDI for QSAR-based VS to identify putative 5-HT$_6$R ligands.

DrugBank. DrugBank[62] (http://www.drugbank.ca) is a unique bioinformatics and cheminformatics resource that combines detailed drug data (i.e., chemical, pharmacological, and pharmaceutical) with comprehensive drug target information (i.e., sequence, structure, and pathway). Currently, the database contains nearly 4800 drug entries. Herein, we used DrugBank for virtual screening using QSAR models to identify putative 5-HT$_6$R ligands among known drugs.

PubChem. PubChem[18] is a public repository of chemical structures and their activities obtained from a variety of biological assays. The PubChem compound repository presently contains more than 30 million unique structures with biological property information provided for many of the

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compounds. Herein, we used PubChem to obtain all chemical structures for our data sets in SDF file format.

Connectivity Map (cmap). The cmap[33,34] (http://www.broadinstitute.org/cmap/) is a unique database for using chemical genomics in drug discovery framework. It provides researchers with a systematic solution for the discovery of the functional connections among drugs, genes, and diseases. The database used in this study (cmap build 02) included 7056 genome-wide expression profiles representing 6100 individual treatment instances with 1309 bioactive small molecules (i.e., drugs and other biologically active compounds). All gene expression profiles included in the cmap were derived from treating cultured human cells (MCF7, PC3, HL60, SKMEL5, HepG2, and SHSY5Y) with chemical compounds.

NetAffx. NetAffx[63,64] (http://www.affymetrix.com) gene ontology mining tool is a web-based, interactive tool that permits traversal of the gene ontology graph in the context of microarray data. It accepts a list of Affymetrix probe sets and renders a gene ontology graph as a heat map colored according to significance measurements. It also details and annotates probe sets on Affymetrix GeneChip microarrays. In this study, we used NetAffx to populate our disease gene signatures with Affymetrix U133A probe sets.

ChemoText. ChemoText[58] is an in-house repository of chemical entities, and activity terms (indicating biological effects) extracted from annotations provided in Medline records. This resource has different applications in drug discovery projects. First, we can use ChemoText in a discovery-mode to formulate independent hypotheses about chemical−disease associations according to Swanson's ABC rule.[65] Second, we can use it as an information retrieval tool to gather relevant data about chemical−protein (or gene)−disease connections derived from biomedical literature. In this study, we used ChemoText to retrieve all available biological information about the final computational hits predicted by our integrative approach. This analysis helped us in assessing the novelty of the produced hypotheses and in validating some of them.

COMPUTATIONAL METHODS

QSAR Modeling and QSAR-Based Virtual Screening. *Preprocessing of the Data Set.* We used a workflow for chemical data curation that was developed in our lab and published recently.[66] First, all molecules were "washed" using the "Wash Molecules" application in MOE (v.2007.09).[67] Using this tool, we processed chemical structures by carrying out several standard operations including 2D depiction layout, hydrogen correction, salt and solvent removal, chirality, and bond type normalization (all details can be found in the MOE manual[67]). Second, we used ChemAxon's Standardizer (v. 5.2.6)[68] to harmonize the representation of aromatic rings. Third, we checked all normalized molecular structures in the data set for duplicated compounds using the "Sort Unique Entries" application in MOE. Our analysis resulted in the detection and removal of 56 duplicate chemical entries leaving 194 unique normalized molecular structures. These 194 unique, organic compounds, including 102 actives and 92 nonactives (see Table S1 of Supporting Information) were used for binary QSAR studies. We assigned the activity class for each compound based on its K_i value(s) obtained from the PDSP and according to PDSP specifications as reported at the PDSP Web site (http://pdsp.med.unc.edu/). Compounds with K_i values ≥10 μM were considered nonactives and assigned to class 0, whereas compounds with K_i values <10 μM were considered actives and assigned to class 1.

Data Set Division for Model Building and Validation. Following our predictive QSAR modeling workflow (summarized in a recent review[61]), all QSAR models generated to classify 5-HT$_6$R actives vs nonactives were validated by predicting both test and external validation sets. The original data set of 194 compounds (102 actives and 92 nonactives) was randomly split into 5 different subsets of nearly equal size to allow for external 5-fold cross-validation (CV)[69,70] where the data set compounds were ranked from 1 to n (n = total number of compounds in the data set), then the first compound went to the external set, and the following 4 compounds were included in the modeling set. Then the sixth compound went to the external set, and the following 4 compounds went to the modeling set. This process was continued until all compounds were divided between the external and the modeling sets. In this protocol, each subset including 20% of the original data set was systematically employed as the external validations set, while the remaining 80% of the compounds constituted the modeling set.

Another level of internal validation was achieved by comparing model performance for training and test sets. This approach is always employed as part of our predictive QSAR modeling workflow[61,71] to emphasize the fact that training-set-only modeling is not sufficient to obtain reliable models that are externally predictive.[72] Thus, for each collection of descriptors, the modeling sets (each including 80% of the original data set) were further partitioned into multiple chemically diverse training and test sets of different sizes using the Sphere Exclusion method implemented in our laboratory.[73] Only models with external predictive accuracy for test sets above certain threshold (see Selection and Validation of QSAR Models in Supporting Information) were retained for the consensus prediction of the external validation sets. Finally, models that demonstrated the highest predictive power for both evaluation sets were used in consensus fashion for virtual screening of external compound libraries. The model building and validation approach is illustrated schematically in Figure 3.

Combinatorial QSAR Modeling. Two QSAR modeling approaches of different nature were used concurrently to generate classification models for 5-HT$_6$R actives vs nonactives (Figure 4). The first approach relied on *k*-nearest neighbor (*k*NN) model optimization method combined with Dragon descriptors, and the second employed classification based on association (CBA) and subgraphs (SG) descriptors. All details about QSAR methods, evaluation of generated models, and consensus prediction are available in the Supporting Information.

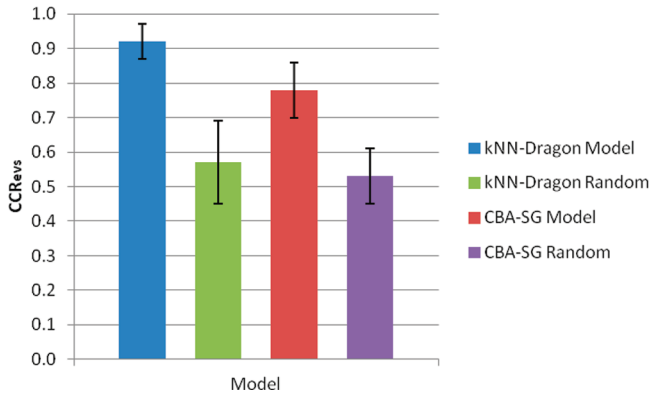

Figure 4. Comparison of the QSAR approaches to classify 5-HT$_6$R actives vs nonactives based on CCR$_{evs}$.

Virtual Screening. To identify putative ligands, validated consensus *k*NN-Dragon models generated for 5-HT$_6$R ligands were used for virtual screening of both the 59000 molecules within the WDI[57] chemical library and 1300 DrugBank[62] compounds included in the cmap database. The identified hits (by consensus agreement between all accepted *k*NN-Dragon models) were then evaluated additionally using CBA-SG classifier when there was a need to reduce the size of the VS library generated with *k*NN-Dragon models.

Biological Network Mining. *Querying the cmap with Alzheimer's Disease Gene Signatures.* The cmap[33,34] was used to discover unexpected connections between chemicals, genes, and Alzheimer's disease by generating a detailed map that links gene patterns associated with Alzheimer's to corresponding patterns produced by drug candidates and a variety of genetic perturbations included in the cmap database. The effects of different drugs and diseases are described using genomic signatures, the full complement of genes that are turned on and off by a particular drug or disease. We start by querying the online database (cmap: http://www.broadinstitute.org/cmap/) with gene signatures characteristic of Alzheimer's disease. Then, a computer program, that uses sophisticated pattern-matching methods, matches the barcodes based on the patterns shared among Alzheimer's gene signature and drugs included in the cmap.

Alzheimer's Disease Gene Signatures. In order to query the cmap, a disease gene signature should exist. Two lists of genes are required to perform the query: a list of up-regulated genes and a list of down-regulated genes characteristic of a disease. Query signatures can be obtained from two major sources: (1) biological literature; gene signatures of diseases can be extracted through the National Library of Medicine's PubMed system (http://www.ncbi.nlm.nih.gov/pubmed); (2) GEO[27,28] database, a gene expression/molecular abundance repository supporting MIAME[74] (Minimum Information About a Microarray Experiment) compliant data submissions, and a curated, online resource for gene expression data browsing, query, and retrieval. For the purposes of this study, two independent reports of gene-expression changes in brain tissues from Alzheimer's patients were used to derive gene signatures (i.e., lists of genes up- and down-regulated in Alzheimer's disease) to query the cmap. Signature 1 (from the hippocampus) consisted of 40 genes reported by Hata, R. et al.,[75] and signature 2 (from cerebral cortex) consisted of 25 genes reported by Ricciarelli, R. et al.[76] NetAffx was then used to map gene symbols and Unigene identifiers to populate gene signature lists with Affymetrix U133A probe sets to query the cmap.

Hypothesis Integration. We cross-examined and integrated structural hypotheses generated independently from both QSAR-based VS and biological network mining efforts to identify and accept common hits only. This step of hypotheses integration was based on structural identity comparisons. All chemical structures of cmap compounds were retrieved from DrugBank[62] using their DrugBank identifiers. Identical structures only were then accepted for further analysis. All chemical structures labeled as identical were also subjected to a manual curation step where structures and names of the chemical compounds were compared in different databases to make sure they both refer to the same chemical entity. Common hits were then considered for further experimental validation.

Experimental Validation in Radioligand Binding Assays. Final common hit compounds from QSAR-based VS and cmap negative connections with Alzheimer's were purchased and submitted to PDSP for experimental target validation. The experimental details of radioligand binding assays are available at the PDSP Web site.[17]

RESULTS AND DISCUSSION

QSAR Modeling of 5-HT$_6$R Actives vs Nonactives. *k*NN with Dragon descriptors was employed to classify modeling set compounds into 5-HT$_6$R actives vs nonactives. As part of 5-fold cross-validation process, the data set was divided, into five subsets of nearly equal size. Four parts (selected systematically) formed modeling sets with 155 compounds each, and the fifth part, containing 39 compounds, was considered as a validation set. Each of the five modeling sets derived to collect 5-fold CV statistics was additionally subdivided into multiple training and test sets (28−40 divisions) using the Sphere Exclusion algorithm as described in Computational Methods. Multiple QSAR models were generated independently for all training sets and applied to the test sets. Generally, we accept models with CCR values above or equal to 0.70 for both the training and test sets.

However, because we were able to generate thousands of acceptable models, we used more conservative criteria (i.e., CCR_{train} and CCR_{test} above or equal to 0.90) for model selection to predict external compounds. Basically, for each train/test split, *k*NN will generate at least one model. Increasing the "Number of Runs" option will multiply the number of generated models. The "descriptors per model" parameter can again multiply the number of generated models; if *k*NN tries to make a model with 5 descriptors, a model with 6 descriptors, and a model with 7 descriptors in each case, the total number of models will be tripled. So, to calculate the number of models that will be generated, (number of train-test splits)·(number of runs)·(number of different values "descriptors per model" can take). This is an important thing to consider before starting a modeling run: Too few models will be unlikely to create a good predictor, while too many models can take a very long time to generate. If the data set is a high quality data set (i.e., there are very good distinctive features between actives and inactives), then we may be able to generate thousands of acceptable models. However, these models differ in their statistical parameters. See Selection and validation of QSAR Models in Supporting Information.

Results of Y-randomization tests confirmed that *k*NN-Dragon classification models with CCR_{train} and CCR_{test} values above or equal to 0.90 were robust. None of the models with randomized class labels of the training set compounds had CCR_{train} and CCR_{test} above 0.65 or CCR_{evs} above 0.55 for any split.

The CBA method was used to classify the data set using SG descriptors. The modeling sets (described above) were used to build the classifier in CBA[77] using an initial pool of about 400 SG descriptors. The classifier gave an average CCR_{train} of 0.92 (i.e., the average resulted from five different tests). Then, the external validation sets consisting of 39 compounds were used to assess the robustness of the classifier. The average CCR_{test} was 0.78, which is not as high the CCR value for the training set but is still statistically acceptable.

Clearly, *k*NN (mean CCR_{evs} = 0.92) performed better than CBA (mean CCR_{evs} = 0.78) on the external validation sets (cf. Figure 4). Therefore, we chose to use *k*NN-Dragon models for VS of external drug libraries. Nevertheless, we maintained CBA-SG models as an additional filter to suggest smaller sets of compounds as 5-HT$_6$R putative actives selected from the list of virtual hits obtained with *k*NN-Dragon models and therefore predicted by both models as putative actives.

QSAR-Based Virtual Screening. Since our models proved reasonably accurate based on external validation sets, we used the best models to mine two external databases of approved and potential drugs for putative 5-HT$_6$R ligands. The use of applicability domain assures reliable predictions by the models. Therefore, we used two types of applicability domains in the virtual screening of compound databases. First, we used a global applicability domain that acted as a filter and ensured some level of global similarity between the predicted compounds and the compounds in the modeling set. Second, we defined a local applicability domain for each of the individual classification models.

We first screened the WDI database[57] of about 59000 compounds (approved or investigational drugs) (Figure 5). This original collection had many duplicates (i.e., many salt forms for the same chemical entity), and these duplicates were

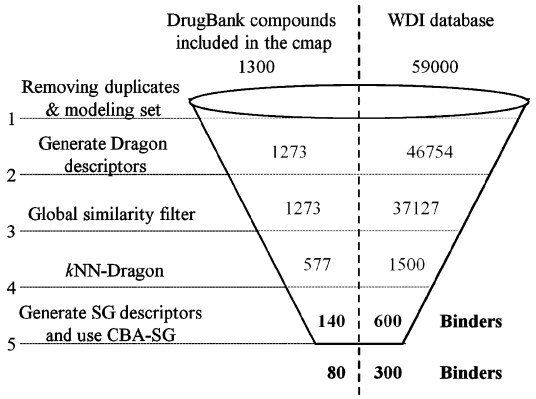

Figure 5. QSAR-based virtual screening of two chemical databases: the WDI and DrugBank compounds included in the cmap.

removed using MOE. We also removed all compounds that were duplicates of those molecules that were included in our modeling and external validation sets. Dragon descriptors were generated for the remaining 46859 unique compounds in the database; of these, 9732 compounds were excluded because Dragon was unable to calculate at least one of the descriptors generated for the modeling set. The remaining 37127 compounds were then subjected to a global applicability domain filter for the modeling set using a strict Z cutoff of 0.5 (which formally places the allowed pairwise distance threshold at the mean of all pairwise distance distribution for the training set plus one-half of the standard deviation). Then, all *k*NN-Dragon models with CCR_{train} and CCR_{test} above or equal to 0.90 were employed in consensus fashion to predict 1500 compounds remaining after several filtering steps, which resulted in the identification of the 600 predicted actives. In an effort to reduce the number of hits, we have generated SG descriptors for these 600 molecules and applied the CBA-SG classifier which filtered out half of these compounds, leaving 300 compounds as putative actives for 5-HT_6R. However, in this study we explicitly focused on compounds from DrugBank[62] that were employed in the cmap project. These VS hits from WDI should be viewed as hypothetically active compounds awaiting the experimental confirmation; the list of the top scoring hits is included in the Supporting Information (Table S2).

Additionally, we screened 1300 DrugBank compounds included in the cmap database. Dragon descriptors were computed for 1273 unique compounds. These compounds were then subjected to a global applicability domain filter for the modeling set using a strict Z cutoff of 0.5. Consequently, we placed the allowed pairwise distance threshold at the mean of all pairwise distance distribution for the training set plus one-half of the standard deviation, which resulted in 577 predictions within the applicability domain. Next, validated consensus *k*NN-Dragon models (i.e., all models with CCR_{train} and CCR_{test} above or equal to 0.90) were used to predict these 577 compounds, resulting in the identification of 140 unique compounds predicted to be 5-HT_6R actives.

Searching the Connectivity Map for Potential Anti-Alzheimer's Agents. We used two gene signatures for the Alzheimer's disease (designated as S1 and S2) to query the cmap database in an attempt to link genes associated with the disease to potential therapeutic agents. These two signatures were based on two independent rank-ordered gene lists provided by two different Gene Set Enrichment Analysis (GSEA) studies.[75,76] The two disease signatures were compared with predefined signatures of therapeutic compounds included in the cmap and ranked according to a connectivity score (ranging from +1 to −1), representing relative similarity to the disease gene lists. The connectivity score itself is derived using a nonparametric, rank-based, pattern-matching strategy based on the Kolmogorov−Smirnov statistic.[78] Connectivity scores are calculated using the online tools available at the cmap (http://www.broadinstitute.org/cmap/). All instances in the database are then ranked according to their connectivity scores; those at the top (+) are most strongly correlated to the query signature and looked at as disease causes, and those at the bottom (−) are most strongly anticorrelated and considered as possible therapeutics.

The majority of chemicals included in the cmap database are represented by multiple independent replicates. Most compounds are profiled in three different cell lines, some at different concentrations. These are called instances for the same chemical and defined as "a treatment and control pair and the list of probe sets ordered by their extent of differential expression between this treatment and control pair".[79] The instance is the basic unit of data and metadata in cmap. Instances of the same compound might have similar or dissimilar connectivity scores with the query signature. We have higher confidence in the derived connections when gene signatures are conserved across diverse cell types and experimental settings. However, Lamb and colleagues[33,34] indicated that the nonconsistent scoring of different instances of the same chemical may represent (1) a cellular-context-dependent difference in activity, (2) a concentration-discriminated effect, or (3) poor reproducibility between replicates. Therefore, the best connections are those where multiple, autonomous instances of the same chemical have consistently high (or low) scores. However, inconsistently scoring compounds should not necessarily be dismissed since their significance as potential treatments for a disease can be boosted by additional evidence, such as predictions from QSAR models.

In this study, we were interested in compounds whose chemogenomics profiles were negatively correlated with the Alzheimer's disease gene signatures. Hits with statistically significant, negative connectivity scores could be potential treatments for the Alzheimer's disease; however, the list of negatively correlated molecules might be long and must be analyzed carefully before suggesting hypotheses of possible mechanisms for controlling or mediating the disease. Examples of top negative connections with both signatures S1 and S2 are shown in Tables 1 and 2, respectively (see Tables S2 and S3 in Supporting Information for full listings of connections and their connectivity scores). Although the two gene signatures (i.e., for the Alzheimer's disease) used to query the cmap shared no common genes, both queries resulted in a common list of negative connections that were given a higher confidence in our studies. All chemical structures for each chemical compound included in the cmap were obtained from the DrugBank[62] and mapped based on the DrugBank identifiers provided by the cmap database.

Hypothesis Generation: Integrating Independent Hypotheses from QSAR-Based VS and cmap Analysis. We combined hypotheses produced from two different data sets and using two different computational methods (Figure 2): (1) QSAR-based datamining of chemical databases in an effort to identify novel ligands for 5-HT_6R and (2) network-mining using two signatures for Alzheimer's disease to query the cmap

Table 1. Top 20 Negative Connections from the cmap with S1

compd	rank[a]	cell	score	instance_ID
naproxen	6100	PC3	−1	7146
sulfacetamide	6099	MCF7	−0.990	1695
amprolium	6098	HL60	−0.930	1979
aminoglutethimide	6097	MCF7	−0.913	7463
ioxaglic acid	6096	HL60	−0.897	2966
dexpanthenol	6095	MCF7	−0.871	7455
suxibuzone	6094	MCF7	−0.870	7163
chlorphenesin	6093	HL60	−0.862	1432
metixene	6092	HL60	−0.853	2451
fulvestrant	6091	MCF7	−0.843	5565
seneciphylline	6090	MCF7	−0.841	2797
troglitazone	6089	MCF7	−0.839	6991
dicloxacillin	6088	HL60	−0.834	2445
phentolamine	6087	HL60	−0.831	2362
monocrotaline	6086	MCF7	−0.828	6771
lymecycline	6085	HL60	−0.823	2953
bezafibrate	6084	PC3	−0.815	6653
6-benzylaminopurine	6083	HL60	−0.812	2351
terbutaline	6082	MCF7	−0.811	3202
clorgiline	6081	MCF7	−0.805	3219

[a]The rank order is generated from estimating the connectivity scores of 6100 individual treatment instances with S1. A rank order of 6100 corresponds to the compound with the strongest negative connectivity S1, while a rank order of 1 corresponds to the compound with the strongest positive connectivity with S1.

and identify possible anti-Alzheimer's therapeutics. Our procedure for integrating hypotheses was based on structural identity for chemical compounds derived from both approaches mentioned above. Compounds with negative connectivity scores, representing genes expressed in an opposite fashion to the imported Alzheimer's disease query, which implies their potential benefits to be candidate treatments, were compared with 5-HT$_6$R hits predicted from QSAR-based VS, and identical compounds were regarded as common hits.

Table 2. Top 20 Negative Connections from the cmap with S2

compd	rank[a]	cell	score	instance_ID
trifluoperazine	6100	HL60	−1	2389
1	6099	MCF7	−0.982	4994
ethotoin	6098	HL60	−0.977	2196
sulfafurazole	6097	HL60	−0.973	1603
quercetin	6096	MCF7	−0.964	4846
triflusal	6095	HL60	−0.925	1717
alfuzosin	6094	PC3	−0.903	4644
metitepine	6093	HL60	−0.890	1616
trioxysalen	6092	MCF7	−0.885	6216
7	6091	MCF7	−0.883	258
tanespimycin	6090	HL60	−0.873	6184
spironolactone	6089	MCF7	−0.871	6255
nifurtimox	6088	MCF7	−0.859	4953
iobenguane	6087	HL60	−0.847	1729
undecanoic acid (u0125)	6086	PC3	−0.845	663
monorden	6085	MCF7	−0.841	5947
primidone	6084	PC3	−0.833	6723
calcium pantothenate	6083	MCF7	−0.828	4775
phthalylsulfathiazole	6082	HL60	−0.826	3033
ceforanide	6081	PC3	−0.824	6751

[a]The rank order is generated from estimating the connectivity scores of 6100 individual treatment instances with S2. A rank order of 6100 corresponds to the compound with the strongest negative connectivity S2, while a rank order of 1 corresponds to the compound with the strongest positive connectivity with S2.

The primary goal for integrating hit lists produced by two independent approaches in this study was initially to overcome some of the inherent hit scoring problems in classification QSAR and achieve higher success rates in experimental testing of the VS hits. In other words, we often select for further experimental validation those QSAR hits with consensus scores above or equal to 0.90 (referred to as consensus scores in Materials and Methods). However, many novel scaffolds that are significantly different (i.e., structurally and possibly therapeutically) from the training set compounds might have lower consensus scores ranging from 0.50 to 0.90 despite the fact that they might be actives too. Thus, this process of integrating hypotheses derived independently from different types of data and using multiple prediction methods allowed us to fish out these low-confidence QSAR hits (that yet could be highly important ligands) for further analysis. As a result, we posit that integrating independent hypotheses is likely to improve the overall experimental success rates of hit compounds identified *in silico*.

Scoring and Integrating Structural Hypotheses to Identify Putative Anti-Alzheimer's Agents. Our method for integrating hit lists was derived from a combination of voting and statistical metrics. In the first step, we used two different scoring functions to rank the computational hits generated independently from both QSAR and cmap. In the QSAR study, we used the *k*NN consensus score which takes into account the total number of models used to predict the compound's activity and the number of models that predicted the compound to belong to a specific class correctly. We considered all computational hits that had an average predicted value (i.e., consensus score) above or equal to 0.50 for further inspection. Our analysis resulted in 140 putative 5-HT$_6$R actives among cmap compounds and with *k*NN consensus scores ranging from 0.50 to 1.00 (see Figure 7)

However, we used the connectivity scores[33] to rank the hits resulting from querying the cmap with Alzheimer's disease gene signatures. Because we were interested in identifying novel treatments for Alzheimer's disease, we ranked hits with larger negative connectivity scores at the top and gave them higher confidence. Such compounds were hypothesized to have higher chances to reverse the Alzheimer's gene signatures and therefore might have immense therapeutic value in Alzheimer's disease. We considered for further analysis all compounds that had at least one instance of negative connection with any of the two gene signatures used to query the cmap (S1 and S2) so as not to miss any important connections. Our analysis resulted in identifying 881 negative connectivity instances with S1 and 861 instances with S2 (Figure 6).

Finally, we combined the hypotheses generated from both QSAR and cmap analyses and accepted common hits only. We identified 97 compounds that were both predicted to be active at 5-HT$_6$R and had at least one instance of negative connectivity with S1 and 106 compounds that had at least one instance of negative connectivity with S2. Accepting only common hits among S1 and S2 resulted in 73 putative hits (see Figure 7). At this stage, we applied a manual curation where we inspected all available data for these 73 hits. Each of the 73

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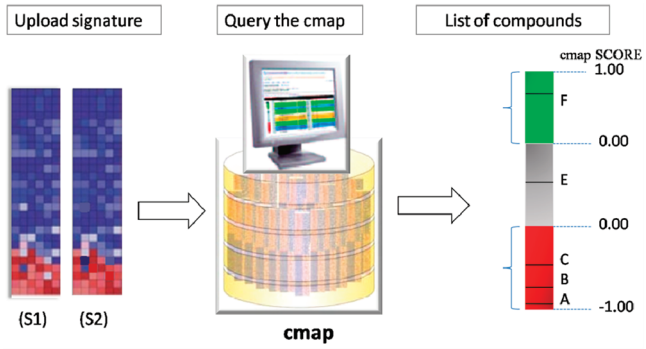

Figure 6. Querying the connectivity map with Alzheimer's disease gene signatures (S1 and S2).

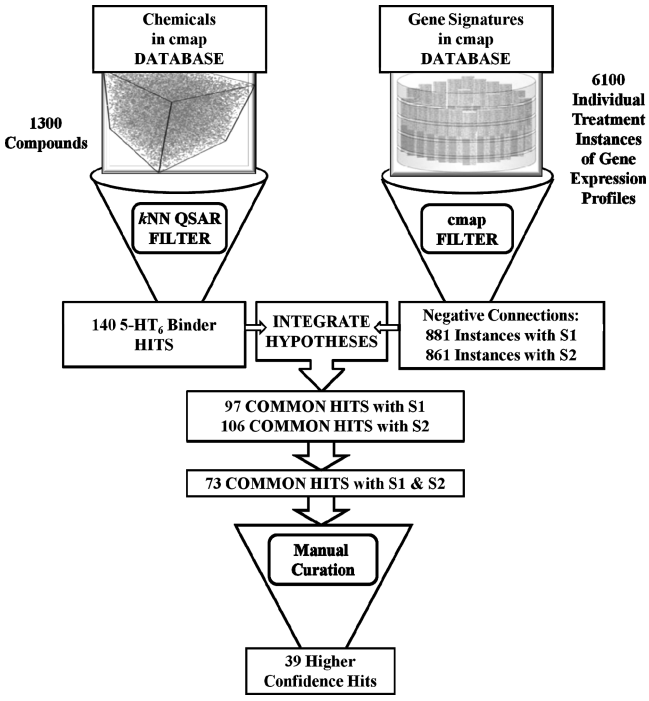

Figure 7. Integrating hypotheses from QSAR modeling and cmap negative connections.

common hits had three scores (*k*NN consensus score, cmap connectivity score with S1, and cmap connectivity score with S2) to be considered in the final decision to prioritize hits for further testing. Therefore, we estimated the average connectivity scores for all predicted hits across all treatment instances for each of the S1 and S2 hits. Then, we excluded those compounds that had high positive connectivity scores in some treatment instances of the same compound. Finally, we retained 39 compounds that had acceptable negative average connectivity scores at least with one signature (see Figure 7). We hypothesized that these compounds could be tested as putative 5-HT$_6$R hits and potential cognition enhancing agents in the Alzheimer's disease. One of the final 39 hits, vinpocetine, is worthy of special attention as there is new evidence that this compound may play a role in the treatment of Parkinson's disease and Alzheimer's disease.[80,81] Details on all these 39 VS hits are provided in Tables 3 and 4.

Each of the 39 common hits had three scores (*k*NN consensus score, cmap connectivity score with S1, and cmap connectivity score with S2) to be considered in the final decision to prioritize hits for further experimental testing. We plotted the mean connectivity scores vs *k*NN QSAR consensus scores generating separate plots for S1 and S2 (see Figure 8) to analyze these hits in further details.

Additionally, another level of confidence was achieved (besides considering both *k*NN CS and cmap scores) by giving more emphasis to molecules that belonged to the same pharmacological or therapeutic group or had very high structural similarity to hits of higher confidence. This step permitted the retrieval of some compounds that had less significant negative connectivity scores with the disease (e.g., null connectivity or even low positive connectivity scores in few instances). We noticed that the 39 putative actives belonged to several major therapeutic groups (see Table 4): antipsychotics, antidepressants, antihistamines, selective estrogen receptor modulators (SERMs), and calcium channel blockers. Among

Table 3. Final 39 Computational Hits from QSAR-Based VS and cmap

cmap name	cmap score1	cmap score2	num. *k*NN models	*k*NN CS	*k*NN pred.	CBA pred.
acepromazine	−0.528	−0.496	441	0.93	B	B
alimemazine	−0.121	−0.117	438	1.00	B	B
astemizole	−0.349	−0.237	328	0.91	B	B
bepridil	−0.134	−0.409	393	0.89	B	B
bromperidol	−0.239	−0.213	428	0.83	B	B
cetirizine	−0.495	−0.327	421	0.92	B	B
chlorprothixene	−0.277	−0.298	442	0.90	B	B
cinchocaine	−0.004	−0.335	423	0.58	B	B
cinnarizine	−0.349	−0.149	414	0.98	B	B
citalopram	−0.003	−0.260	429	0.71	B	NB
1	−0.378	−0.265	409	0.91	B	B
2	−0.192	−0.310	437	0.96	B	B
cloperastine	−0.273	−0.353	443	0.88	B	B
3	0.093	−0.058	422	0.97	B	B
diltiazem	−0.128	−0.336	433	0.72	B	NB
4	0.027	−0.259	444	0.95	B	B
5	−0.303	−0.228	393	0.84	B	NB
flavoxate	−0.127	−0.112	403	0.71	B	NB
6	0.055	−0.138	351	0.98	B	B
imipramine	−0.400	−0.214	427	0.96	B	B
laudanosine	−0.226	−0.174	411	0.78	B	NB
7	−0.028	−0.078	428	0.71	B	B
meclozine	−0.365	−0.171	439	0.95	B	B
mepacrine	−0.236	−0.301	418	0.53	B	B
methylergometrine	−0.400	−0.509	441	0.98	B	B
naftifine	−0.198	−0.148	359	0.85	B	B
8	0.011	−0.354	433	0.93	B	B
phenoxybenzamine	−0.461	−0.309	444	0.79	B	NB
piperidolate	−0.168	−0.119	431	0.69	B	NB
9	−0.247	−0.206	435	0.96	B	B
nitrarine dihydrochloride	−0.086	−0.213	381	0.72	B	B
promazine	−0.210	−0.307	424	0.97	B	B
10	0.047	−0.058	356	0.56	B	NB
11	0.300	−0.220	435	0.93	B	B
telenzepine	−0.419	−0.114	387	0.68	B	B
terfenadine	−0.183	−0.512	416	0.51	B	NB
vanoxerine	−0.450	−0.233	374	1.00	B	NB
vinpocetine	−0.177	−0.132	376	0.76	B	B
13	−0.152	0.144	434	0.98	B	B

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Table 4. Therapeutic Classes of the 39 Final Computational Hits from QSAR-Based VS and cmap

cmap name	theraputic class/use
acepromazine	antipsychotic
alimemazine	antipruritic, sedative, hypnotic and antiemetic
astemizole	anti-histamine
bepridil	calcium channel blocker once used to treat angina
bromperidol	neuroleptic, used as an antipsychotic in the treatment of schizophrenia
cetirizine	second-generation antihistamine
chlorprothixene	typical antipsychotic drug of the thioxanthene class
cinchocaine	local anesthetic
cinnarizine	antihistamine, which is mainly used for the control of nausea and vomiting due to motion sickness
citalopram	antidepressant drug of the selective serotonin reuptake inhibitor (SSRI) class
1	SERM
2	tricyclic antidepressant
cloperastine	cough suppressant
3	atypical antipsychotics
diltiazem	calcium channel blocker
4	psychotropic agent with tricyclic antidepressant and anxiolytic properties
5	calcium channel blocker
flavoxate	anticholinergic with antimuscarinic effects
6	antipsychotic
imipramine	tricyclic antidepressant
laudanosine	Benzyltetrahydroisoquinoline alkaloid; interacts with GABA, opioid, and nicotinic acetylcholine receptors.
7	Morpholino derivative of quercetin; it is a potent inhibitor of phosphoinositide 3-kinase s (PI3Ks).
meclozine	antihistamine considered to be an antiemetic
mepacrine	Antiprotozoal, antirheumatic, and an intrapleural sclerosing agent; it is known to act as a histamine *N*-methyltransferase inhibitor
methylergometrine	psychedelic alkaloid
naftifine	allylamine antifungal drug
8	second-generation tricyclic antidepressant
phenoxybenzamine	nonspecific, irreversible alpha antagonist
piperidolate	antimuscarinic
9	drug used in scientific research, which acts as a moderately selective dopamine D_3 receptor partial agonist
nitrarine dihydrochloride	hyhpotensive, spasmolytic, coronary dilator and sedative
promazine	antipsychotic
10	SERM
11	SERM
telenzepine	anticholinergic or sympatholytic
terfenadine	antihistamine formerly used for the treatment of allergic conditions
vanoxerine	piperazine derivative, which is a potent and selective dopamine reuptake inhibitor (DRI)
vinpocetine	Vinpocetine has been identified as a potent anti-inflammatory agent that might have a potential role in the treatment of Parkinson's disease and Alzheimer's disease.[80,81]
13	typical antipsychotic drug

these groups, predicting SERMs to have activity at 5-HT_6R was the most surprising.

Hypothesis Testing: Evaluation of Computational Hits at Human Cloned 5-HT_6 Receptors. Common hits from QSAR-VS studies and cmap were taken forward for biological validation, in binding assays, for the 5-HT_6 receptor. As discussed above, we identified 39 chemicals, out of 59000 molecules included in the WDI,[57] and 1300 compounds included in the cmap, as consensus hits and putative actives for 5-HT_6R with higher chances of having potential cognition enhancement effects in Alzheimer's disease; none of these hits was included in the training set used to develop QSAR models. Then, we prioritized 12 compounds from a list of 39 molecules plus an additional compound; a SERM that was among the top QSAR-VS hits that was not included in the cmap (**1**−**13**,[57] see Tables 3 and 5) for further experimental validation in 5-HT_6R radioligand binding assays. It should be noted that compound **12** was tested because it was a SERM predicted with a high CS of 0.93, and we wanted to test it because three other SERMS were among our common hit list of 39 compounds. Our final selection was based on different criteria: (1) we tested some compounds with high consensus scores and stronger negative connectivity with Alzheimer's disease, (2) some compounds were selected because they belonged to the same therapeutic class as several other predicted hits and were not known before to bind to 5-HT_6R such as selective estrogen receptor modulators (SERMs) (e.g., **1**, **11**, and **12**), (3) we tested some compounds with low *k*NN CS (e.g., **10** having a *k*NN CS of 0.56) if other hits that belonged to the same therapeutic class had high consensus scores (e.g., **11** and **12** having *k*NN CS equal to 0.93 and **1** having a *k*NN CS of 0.91), and (4) we also tried to test predictions that had strong negative connectivity

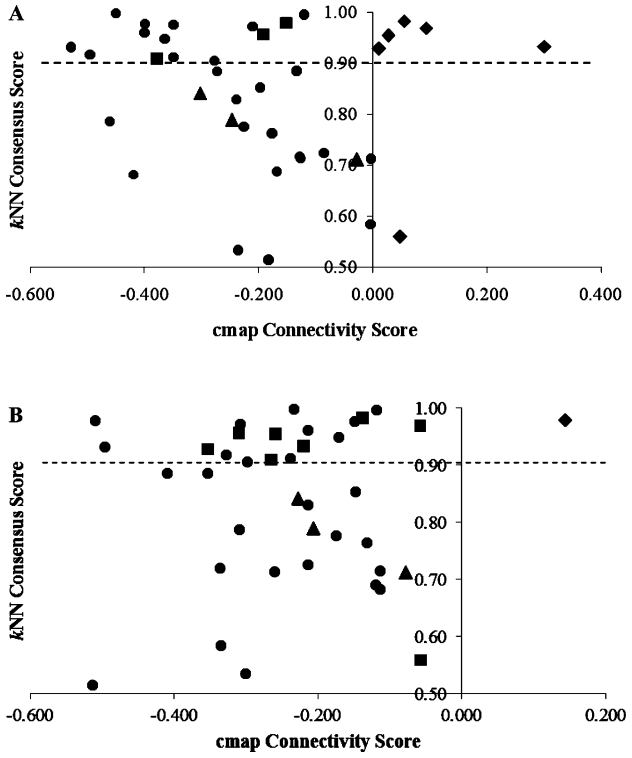

Figure 8. Plots for *k*NN scores vs cmap connectivity scores for 39 final common hits from QSAR-based VS and cmap for (A) Alzheimer's disease signature S1 and (B) Alzheimer's disease signature S2. Squares, compounds predicted and validated as 5-HT_6R actives having negative connectivity scores with Alzheimer's disease gene signatures; diamonds, compounds predicted and experimentally validated as 5-HT_6R actives but having positive connectivity scores with one of the Alzheimer's disease gene signatures; triangles, compounds predicted as 5-HT_6R actives having negative connectivity scores with Alzheimer's disease gene signatures but found nonactives in radioligand binding assays against 5-HT_6R; circles, compounds predicted as 5-HT_6R actives which have negative connectivity scores with Alzheimer's disease gene signatures but were not experimentally tested.

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Table 5. Experimental Validation Results for the 13 Computational Hits Predicted as 5-HT$_6$R Ligands and Had Negative Connections with Alzheimer's Disease Gene Signatures

compd	PDSP ID CID[a]	score1[b]/cell score2[c]/cell	*k*NN CS[d]	CBA pred.[e]	Ki (nM)
1	13499	−0.602/PC3	0.91	B[f]	1,956.0
	1548953	−0.982/MCF7			
2	13494	−0.768/PC3	0.96	B	112.0
	2801	−0.814/MCF7			
3	24842	−0.590/PC3	0.97	B	17.0[g]
	2818	−0.652/MCF7			
4	13495	−0.463/MCF7	0.95	B	105.0
	667477	−0.777/HL60			
5	14821	−0.520/MCF7	0.84	NB[h]	NB
	3336	−0.683/HL60			
6	14815	−0.493/MCF7	0.98	B	1,188.0
	3396	−0.551/HL60			
7	13502	−0.790/MCF7	0.69	B	NB
	3973	−0.883/MCF7			
8	13503	−0.555/PC3	0.96	B	214.0
	4543	−0.586/MCF7			
9	13498	−0.741/MCF7	0.79	B	NB
	3038495	−0.619/HL60			
10	13505	−0.626/HL60	0.56	NB	750.0
	5035	−0.619/HL60			
11	13506	0/MCF7	0.93	B	1,041.0
	2733526	−0.531/MCF7			
12[i]	16514	N/A	0.93	B	4,125.0
	3005573	N/A			
13	13510	−0.609/PC3	0.98	B	169.0
	5311507	−0.746/HL60			
success rate	77% for predictions with *k*NN CS ≥ 0.5		100% for predictions with *k*NN CS ≥ 0.9		

[a]CID, PubChem compound ID. [b]cmap score1, the highest negative connectivity score for this compound with S1 (or the smallet positive in case all other scores are positive). [c]cmap score2, the highest negative connectivity score for this compound with S2 (or the smallest positive in case all other scores are positive). [d]CS, consensus score. [e]CBA pred., predicted binding to 5-HT$_6$ receptors by CBA. [f]B, active. [g]PDSP certified data. [h]NB, nonactive. [i]Toremifene was not included in the cmap database but was prioritized because 3 other related SERMs were hits from both cmap and QSAR-based VS.

scores with one query signature but had much weaker negative connectivity with the second signature to see if there is one specific signature that was generating better results.

We found that 10 of these 13 predicted actives were confirmed experimentally to inhibit 5-HT$_6$R radioligand binding thereby achieving a success hit rate of 77% in this proof-of-concept study (see Table 5). One of these 10 confirmed hits was clozapine, which is known to bind 5-HT$_6$R but was not included in our training set. Binding affinity (K_i) values for the nine predicted hits were in the range 17−4125 nM, with six compounds having K_i values <1 μM. These six highest affinity compounds were **3** (K_i = 17 nM),[17] **4** (K_i = 105 nM, Figure 9A), **2** (K_i = 112 nM, Figure 9B), **13** (K_i = 169 nM, Figure 9C), **8** (K_i = 214 nM, Figure 9D), and **10** (K_i = 750 nM, Figure 9E).

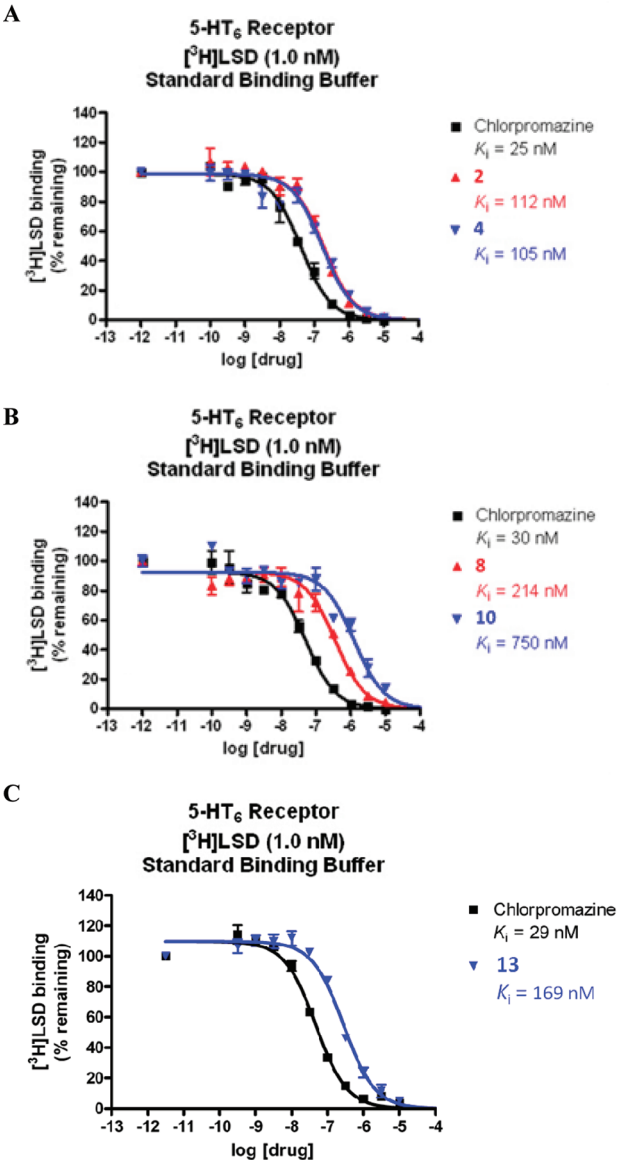

Figure 9. Competition binding isotherms at 5-HT$_6$R for several predicted actives: (A) **2** (red triangle) and chlorpromazine (square), and **4** (blue triangle) and chlorpromazine (square); (B) **8** (red triangle) and chlorpromazine (square), and **10** (blue triangle) and chlorpromazine (square); (C) **13** (triangle) and chlorpromazine (square), versus [3H]LSD.

Among the tested compounds, we found that compounds having negative connectivity scores and *k*NN CS above 0.90 were all true actives at 5-HT$_6$R achieving a success rate of 100%. We also found that lowering the threshold to 0.50 resulted in 3 false positives which decreased the success rate down to 77%. It was strikingly important that we were able to prioritize a VS hit (i.e., **10**) with very low *k*NN CS of 0.56 and insignificant negative connectivity scores with Alzheimer's (see Table 5) and validate that this compound was a true active of 5-HT$_6$R and a potential cognition enhancer. This is a clear

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example on the importance of integrating independent hypotheses to prioritize for testing the otherwise less significant computational hits.

Mining of the biomedical literature using ChemoText identified possible neuroprotective, in addition to cognitive- and memory-enhancing, effects for most of the computational hits (see Table 6), although there is no evidence that 5-HT$_6$R-active compounds are neuroprotective. The list of all 39 compounds predicted by our integrative approach as putative 5-HT$_6$R actives with possible anti-Alzheimer's effects is shown in Table 5.

Table 6. Significance of the Tested Hits in Relation to Cognition, Neuroprotection, and Anti-Alzheimer's Effects

compd	predicted	K_i	significance to Alzheimer's disease prevention/treatment
1	active	1956.0	unknown
2	active	112.0	neuroprotective[101]
3	active	17.0	used in combination therapy for Alzheimer's[102]
4	active	105.0	unknown
5	active	NB	GABA receptor modulator[103,104] and may inhibit amyloid-β protein oligomerization as other related antihypertensives[105]
6	active	1188.0	possible anti-Alzheimer's effects[106]
7	active	NB	can inhibit central sensitization and neuroinflammation[107,108]
8	active	214.0	possible anti-Alzheimer's effects[109]
9	active	NB	unknown
10	active	750.0	possible anti-Alzheimer's effects[96]
11	active	1041.0	neuroprotective[110]
12	active	4125.0	unknown
13	active	169.0	facilitates memory in rats[111]

SERMs Identified as 5-HT$_6$R Ligands. Several selective estrogen receptor modulators (SERMs) were predicted as 5-HT$_6$R ligands and also had negative connections with the Alzheimer's disease gene signatures. Compounds **1**, **10**, and **11** had negative connections with Alzheimer's disease gene signatures in the cmap database.[33,34] Compound **12** was not included in the cmap but was predicted as 5-HT$_6$R active by QSAR-based VS. Although anti-Alzheimer's effects of these drugs were observed previously and attributed to their modulation of estrogen receptors (ERs), the evidence about ER modulators or hormone replacement therapy in postmenopausal women to prevent or treat the Alzheimer's disease has been inconclusive and sometimes even contradictory.[82−84] Although postmenopausal estrogen depletion is a known risk factor for Alzheimer's disease, estrogen-containing hormone therapy initiated during late postmenopausal period does not improve episodic memory (an important early symptom of Alzheimer's disease), leads to no improvement or adverse effects on overall cognitive performance and Alzheimer's disease in postmenopausal women,[84−86] and it increases the risk of dementia.[83,84] Be that as it may, there is still substantial evidence from both preclinical and human studies that ovarian steroids have significant effects on neuroregulatory pathways.[87−94] However, critical gaps exist in our knowledge of both the effects on brain function of declining ovarian steroid secretion during reproductive aging and the role of ovarian steroid hormone therapy in the prevention or treatment of brain diseases.[82]

Raloxifene Identified as a 5-HT$_6$R Antagonist with Potential Utility in Alzheimer's Disease. Raloxifene is a selective estrogen receptor modulator used to prevent or treat osteoporosis; recently, it was also approved by the FDA as an anticancer drug for reducing the risk of invasive breast cancer in postmenopausal women.[95] It was one of the low confidence QSAR-based VS hits because of the low structural similarity with modeling set compounds. Therefore, we would have avoided testing this compound if it had not been predicted from the cmap to have a negative connection with Alzheimer's disease. Another level of confidence was obtained from having other compounds that belonged to the same pharmacological group (SERMs) that were predicted as 5-HT$_6$R actives with high confidence (i.e., consensus scores above 0.90) and had negative connections with Alzheimer's disease. This example highlights the value of the integrated informatics approach in increasing the hit rates of QSAR-based VS. Experimental testing had indeed confirmed that raloxifene binds to 5-HT$_6$R with a K_i of 750 nM (Table 5 and Figure 9B).

Yaffe and co-workers examined the data from the Multiple Outcomes of Raloxifene Evaluation (MORE) trial and indicated that raloxifene given at a dose of 120 mg/day, but not 60 mg/day, led to reduced risk of cognitive impairment in postmenopausal women.[96] Additionally, recent studies pointed out that raloxifene enters the brain in relevant quantities and exerts a measurable effect in humans.[97] It is possible that raloxifene's anticipated anti-Alzheimer's effects could be due to complex polypharmacological profile effecting several protein targets and signaling pathways involved in memory, cognition, inflammation, oxidative control, and other important biological processes underlying the Alzheimer's disease etiology, and not limited to its canonical targets (i.e., estrogen receptors). Currently, raloxifene is in phase II clinical trials for Alzheimer's disease in postmenopausal women.[98] This example can thus be considered as a proof of concept for the ability of our approach to increase the confidence in the identified hits that are structurally and biologically dissimilar to training set compounds.

CONCLUSIONS

We have developed a novel integrative chemocentric informatics approach that could be used as a tool for generating and cross-validating drug discovery hypotheses. Our approach integrates different *in silico* strategies and different data types and sources to increase the confidence in the final hypotheses. The study design was composed of three major parts: (1) QSAR-based datamining of chemical libraries to identify new ligands for target proteins, (2) network-mining to identify chemicals that could treat specific diseases; and (3) integrating hits derived from 1 and 2.

This approach has been applied to study the 5-HT$_6$R system in relation to cognition enhancement strategies which may be useful for Alzheimer's and, perhaps, similar diseases with impaired cognition. Disease gene signatures for Alzheimer's disease have been used to query the cmap database to formulate testable hypotheses about potential treatments. Common compound hits from QSAR/VS studies against 5-HT$_6$R and the cmap were tested at 5-HT$_6$R. Our approach identified 39 drugs, as potential 5-HT$_6$R antagonists, out of 1300 molecules included in DrugBank.[62] Thirteen hits with the highest confidence level were tested in binding assays, and 10 compounds were confirmed as 5-HT$_6$R ligands achieving a success rate of 77%. We noticed that this study design can be

applied to many other protein targets involved in the etiology of Alzheimer's disease.

We shall emphasize that our approach could be used to aid in the process of prioritizing computational hits that would not be picked by an individual model contributing to the integrative approach presented in this study. For instance, QSAR/VS hits could emphasize connections from the cmap that one would not focus on otherwise, especially as the size of the database continues to grow. In diseases like Alzheimer's, with little knowledge about specific etiology and the lack of drug gene signatures generated from neuronal cell lines, it is hard to decide *a priori* which negative connections are more important to be viewed as potential therapeutics. Reciprocally, one can use strong connections revealed by cmap to focus on weak hits resulting from QSAR/VS studies as was demonstrated here for raloxifene. Thus, herein we have hypothesized and proved that integrating results generated from the cmap with predictions generated from QSAR-based VS increased the confidence in the final hit list of predicted actives.

EXPERIMENTAL SECTION

Radioligand Binding Assays. This screen was performed by the National Institute of Mental Health Psychoactive Drug Screening Program (PDSP).[17] Radioligands were purchased by PDSP from Perkin-Elmer or GE Healthcare. Competition binding assays were performed using transfected or stably expressing cell membrane preparations as previously described (Shapiro et al.;[99] Roth et al.[100]) and are available online (http://pdsp.med.unc.edu). All experimental details are available online (http://pdsp.med.unc.edu/UNC−CH%20Protocol%20Book.pdf).

Chemistry. Chemical compounds predicted as hits from the virtual screening were obtained from commercial suppliers according to their availability. All compounds were ordered to have above or equal to 95% purity. Additionally, all compounds were subjected to purity assessment using LC/MS by the Center for Integrative Chemical Biology and Drug Discovery at UNC-Chapel Hill (see Supporting Information). LC/MS spectra of all compounds were acquired from an Agilent 6110 Series system with UV detector set to 220 nm. Samples were injected (5 μL) onto an Agilent Eclipse Plus 4.6 × 50 mm, 1.8 μM, C18 column at room temperature. A linear gradient from 10% to 100% B (MeOH + 0.1% Acetic Acid) in 5.0 min was followed by pumping 100% B for another 2 min with A being H_2O + 0.1% acetic acid. The flow rate was 1.0 mL/min.

ASSOCIATED CONTENT

Supporting Information

5-HT_6R data sets, virtual screening hits, cmap negative connections with Alzheimer's disease gene signatures, connectivity scores, further computational details, and LC/MS purity spectra for all tested compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

5-HT_6R, 5-hydroxytryptamine-6 receptors; CARs, Class Association Rules; CBA, classification based on association; CCR, correct classification rate; CCR_{train}, correct classification rate for training set; CCR_{test}, correct classification rate for test set; CCR_{evs}, correct classification rate for external validation set; CCR_{rand}, correct classification rate of the random models using the external validation set; cmap, the connectivity map; Cp, compound; CS, consensus score; CV, cross-validation; E, enrichment; En, normalized enrichment; FN, false negative; FP, false positive; HTS, high throughput screen; *k*NN, *k* nearest neighbor; LOO−CV, leave-one-out cross-validation; MFD, most frequent descriptors; MOE, molecular operating environment; MZ, MolConnZ descriptors; PDSP, NIMH Psychoactive Drug Screening Program; QSAR, quantitative structure−activity relationships; S1, Alzheimer's disease gene signature 1; S2, Alzheimer's disease gene signature 1; SA, simulated annealing; SE, sensitivity; SG, subgraph; SP, specificity; TP, true positive; TN, true negative; VS, virtual screening; WDI, World Drug Index

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