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Scaffold Ranking and Positional Scanning Utilized in the Discovery of nAChR-Selective Compounds Suitable for Optimization Studies

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Abstract

Nicotine binds to nicotinic acetylcholine receptors (nAChR), which can exist as many different subtypes. The $\alpha 4\beta 2$ nAChR is the most prevalent subtype in the brain and possesses the most evidence linking it to nicotine seeking behavior. Herein we report the use of mixture based combinatorial libraries for the rapid discovery of a series of $\alpha 4\beta 2$ nAChR selective compounds. Further chemistry optimization provided compound **301**, which was characterized as a selective $\alpha 4\beta 2$ nAChR antagonist. This compound displayed no agonist activity but blocked nicotine-induced depolarization of HEK cells with an IC_{50} of approximately 430 nM. **301** demonstrated nearly 500-fold selectivity for binding and 40-fold functional selectivity for $\alpha 4\beta 2$ over $\alpha 3\beta 4$ nAChR. In total over 5 million compounds were assessed through the use of just 170 samples in order to identify a series of structural analogues suitable for future optimization toward the goal of developing clinically relevant smoking cessation medications.

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ASSOCIATED CONTENT

Supporting Information

Detailed description of the scaffold ranking library samples and their corresponding dose response data against $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChR as well as 1H and ^{13}C NMR spectra for key compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions

All authors have given approval to the final version of the manuscript.

The authors declare no competing financial interest.

INTRODUCTION

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that mediate cation flux and are activated by nicotine, the major constituent of tobacco leading to addiction.^{1,2} In mammalian neural tissue, the nAChR is a pentamer usually made up of a combination of α and β subunits,³ although there are also fully functional homomeric receptor proteins, particularly the prominent $\alpha 7$ nAChR. The $\alpha 4\beta 2^*$ nAChR (where * denotes the possible presence of an additional subunit) is by far the most prevalent in the CNS,^{4,5} being expressed in large numbers throughout the brain. Addiction to cigarettes is primarily mediated by nicotine-induced activation of nAChR and, as with other abused drugs, nicotine addiction is thought to be primarily due to the activation of the dopaminergic mesocorticolimbic pathway.^{6,7} The $\alpha 4\beta 2^*$ nAChR is the most prevalent nicotinic receptor subtype in this region.^{4,8}

Despite the prevalence of the $\alpha 4\beta 2^*$ nAChR in the drug reward pathway, a considerable amount of research has been conducted on many different nAChR receptor types and individual subunits, with many different receptor types and subunits having been demonstrated to be involved in acquiring or maintaining addiction to nicotine or other abused drugs. Pharmacological and genetic evidence has suggested that the $\alpha 3\beta 4^*$ nAChR, found primarily in the medial habenula (MHb) and interpeduncular nucleus, is involved in addiction to cigarettes and other abused drugs. The relatively nonselective $\alpha 3\beta 4$ antagonist 18-methoxycoronaridine (18-MC) blocks the self-administration and conditioned place preference of many abused drugs.⁹ The more selective and higher affinity $\alpha 3\beta 4$ nAChR ligand AT-1001 potently blocks nicotine self-administration and reinstatement.¹⁰ These data are consistent with Genome Wide Association Studies (GWAS) that have implicated variants in the $\alpha 3$ - $\alpha 5$ - $\beta 4$ gene cluster on chromosome 15 to be associated with an increased risk of whether a smoker becomes nicotine dependent and to smoking a greater number of cigarettes per day.^{11,12} The GWAS, therefore, also suggest a possible involvement of the $\alpha 5$ subunit in tobacco dependence. This is consistent with behavioral experiments. In $\alpha 5$ null mice, there is an increase in nicotine conditioned place preference and nicotine self-administration at higher nicotine levels.^{13,14} Furthermore, viral vector-mediated reintroduction of $\alpha 5$ subunits into the MHb recovers wild-type nicotine self-administration behavior.¹⁴ The studies indicate that nAChRs containing the $\alpha 5$ subunit may be involved in limiting the reward mediated by higher nicotine doses. There is also considerable evidence that the $\alpha 6$ subunit, which is also found in high concentrations in the mesolimbic dopamine pathway, is involved in nicotine reward. $\alpha 6$ knockout (KO) mice do not self-administer nicotine, and inhibition of $\alpha 6$ -containing nAChR using conotoxin also blocks nicotine self-administration.¹⁵

Nevertheless, the greatest body of evidence accumulated for the involvement of a particular nAChR in smoking pertains to the $\alpha 4\beta 2^*$ nAChR. Neither $\beta 2$ - nor $\alpha 4$ -knockout mice self-administer nicotine.^{16,17} In addition, transgenic mice with a sensitized $\alpha 4$ subunit self-administer nicotine at lower doses,¹⁸ indicating that changes in the $\alpha 4$ alone is sufficient to modify nicotine self-administration. Pharmacological evidence for the involvement of the $\alpha 4\beta 2^*$ nAChR also exists. The selective $\alpha 4\beta 2$ antagonist dihydro- β -erythroidine

hydrobromide (DHβE) blocks nicotine self-administration in rats,¹⁹ and most important of all, the $\alpha 4\beta 2$ partial agonist varenicline is clinically used as a smoking cessation medication.

Unfortunately, although varenicline exhibits binding selectivity, this selectivity is not observed in functional assays. In functional assays, it is a full agonist at both $\alpha 3\beta 4$ and $\alpha 7$ nAChR with comparable potency to that observed at $\alpha 4\beta 2$.^{20,21} Varenicline users have also experienced significant adverse psychiatric effects, including depression and suicidal thoughts, limiting its clinical usefulness.²² Although other high affinity $\alpha 4\beta 2$ agonists and antagonists have been reported, generally, the selectivity compared to $\alpha 3\beta 4$ nAChR and functional activity at $\alpha 3\beta 4$ nAChR were generally not characterized.^{23–25} Here we report novel selective $\alpha 4\beta 2$ nAChR antagonists, derived from compounds originally identified from combinatorial libraries containing mixtures of small molecules.

Starting from a small molecule mixture-based combinatorial scaffold ranking library with more than 5 million individual compounds arranged into 34 samples, we selected a mixture-based library comprised of 45864 compounds systematically arranged into 110 mixtures for further screening. Upon screening of this positional scanning library, a number of mixtures showed affinity and selectivity for $\alpha 4\beta 2$ nAChR. From this single library, we identified 18 individual compounds with modest affinity but some with excellent selectivity for $\alpha 4\beta 2$ nAChR over $\alpha 3\beta 4$ nAChR. These lead compounds were modified to produce antagonists with high affinity and impressive selectivity in both receptor binding studies and in functional activity in vitro. The success of this approach shown herein demonstrates the ability of mixture-based combinatorial libraries to identify individual novel structures in the context of any low- to medium-throughput assay (Figure 1).

RESULTS AND DISCUSSION

Scaffold Ranking

To reduce the number of samples to test, we began by using our scaffold ranking library.^{26,27} As reported elsewhere, we have successfully utilized the scaffold ranking approach in efforts directed toward the identification of metalloprotease ADAM 17 ligands,²⁸ antimicrobials that inhibit tyrosine recombinases and Holliday junction-resolving enzymes,^{29,30} and novel antinociceptives through the use of a direct in vivo assay.³¹ We have described in some detail the method for constructing a scaffold ranking library,²⁶ as well as the advantages and limitations of the approach, elsewhere.²⁷ The scaffold ranking library utilized in this work contained 34 mixture samples that in total contained more than 5 million individual compounds (Supporting Information Table S1). As previously described,²⁶ each mixture sample contains only compounds with the same specific core scaffold, so only close structural analogues are contained in each mixture sample. The mixture samples tested in this work contained anywhere from as few as 10800 individual compounds (Library 1409 Supporting Information Table S1) to as many as 738192 (Libraries 1343–1347 Supporting Information Table S1). The median complexity was 45864 compounds per sample. Each of these individual compounds is represented at an approximately equal molar concentration within a given sample. Because the samples are organized to contain only structural analogues, if a particular individual compound is active then the mixture sample containing that compound may possess many more structural

analogues that are also active. Therefore the effective concentration of active compounds in a given mixture is, in general, significantly higher than it would be if only one compound in the mixture was active.³² In this manner, we are able to utilize these 34 mixture samples to prioritize which chemical scaffolds are likely to contain active individual compounds.

These 34 mixtures were screened at the four different concentrations 10, 5, 1, and 0.5 $\mu\text{g/mL}$ for their ability to block [^3H]epibatidine binding to $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChR, as described in Materials and Methods. Shown in Figure 2 are the results from the 10 $\mu\text{g/mL}$ screening (data from all doses is shown in Supporting Information Table S2).

1169 Library Screening

We identified positional scanning library 1169 for screening based on the results of the scaffold ranking library. Library 1169 contains 45864 individual bis-cyclic guanidines (see Scheme 1) systematically formatted into a positional scanning library containing 110 mixture samples (Table 1). For example, sample 1 contains in approximately equal molar amounts the 1092 individual compounds that have (1-phenyl-cyclopropyl)-methyl fixed at the R_1 position (Table 1). In this manner, the first 42 samples of library 1169 contain all 45864 compounds in the library sorted by R_1 functionality. Samples 43–68 contain the same 45864 compounds sorted by R_2 functionality, and samples 69–110 are sorted by R_3 functionality.

The 110 samples were screened for binding affinity exactly as conducted on the original scaffold ranking library. To use Harmonic Meaning to confirm the integrity of the screening,³² percent inhibition values were converted to xIC_{50} values, assuming a Hill coefficient of one (see Materials and Methods). The Harmonic Mean was then taken in each position for each receptor. The calculated μM harmonic mean values at the R_1 , R_2 , and R_3 positions were (respectively) 26.6, 28.0, and 34.1 for the $\alpha 4\beta 2$ receptor, and 78.6, 82.4, and 73.2 for the $\alpha 3\beta 4$ receptor; the deviation of at most 27% at each receptor represents excellent evidence of the integrity of the synthesis and screening. The xIC_{50} values for each sample are shown in Table 1 as well as the fold selectivity. In general, the samples produced a stronger response against $\alpha 4\beta 2$ than against $\alpha 3\beta 4$. The data shows significant differentiation in activity levels among the samples tested at each position, a key feature for deconvoluting a positional scanning library.²⁷ Moreover, some preliminary SAR can be seen from the library screening. For example when an aromatic group with a halogen substitution is fixed in the R_1 position (Table 1: **5**, **6**, **9**, **11**, and **15**), the sample produced strong inhibition for $\alpha 4\beta 2$. However, while these same fixed functionalities provided activity at the R_3 position (Table 1: **73**, **74**, **77**, **79**, and **83**) against $\alpha 4\beta 2$, a number of other samples with different functionalities fixed at this position also produced strong inhibition. At the R_2 position, the profile indicates that for activity an *R* configuration could be preferred over *S* for $\alpha 4\beta 2$ (Table 1: **53** over **44**, **59** over **51**, **62** over **61**, **66** over **65**, and **68** over **67**). The screening data also identifies several active samples that are highly selective for $\alpha 4\beta 2$ over $\alpha 3\beta 4$ (>4-fold selectivity, such as Table 1: **5**, **21**, **26**, **53**, **68**, and **106**). There were some samples that showed activity against both targets providing low levels of selectivity (<1.5-fold selectivity, Table 1: **15**, **65**, **66**, **74**, **79**, and **83**).

Selection of Individual Compounds for Synthesis

On the basis of the 1169 library screening data, 18 individual compounds were selected for synthesis (Scheme 1). These 18 compounds were selected based on three different criteria. The first set was chosen by selecting functionalities that were active at both $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChR, showing little selectivity for one receptor over the other. For the R_1 position, 3,4-dichlorophenethyl (**15**) was selected. While two other samples that also have aromatic functionalities with halogen groups 2-(3-fluoro-phenyl)-ethyl (**5**) and 2-(3-bromo-phenyl)-ethyl (**6**) were actually more active at $\alpha 4\beta 2$ than **15**, they were also more selective for $\alpha 4\beta 2$ in the library screening (Table 1). From the R_2 position, *R*-2-naphthylmethyl (**66**) and *S*-2-naphthylmethyl (**65**) were selected due to the fact they showed activity at both receptors. Additionally, by using these chiral isomers, the preference for the *R* configuration for activity at $\alpha 4\beta 2$ could be confirmed with individual compounds. From the R_3 position, three different halogen substituted aromatic functionalities that showed activity and low selectivity in the library screening were selected: 2-(3-bromo-phenyl)-ethyl (**74**), 2-(4-bromo-phenyl)-ethyl (**79**), and 3,4-dichlorophenethyl (**83**). There are therefore only six compounds in this set ($1 \times 2 \times 3$), identified as compounds **201** through **206** in Table 2. While this set of individual compounds was chosen with the purpose of reducing receptor selectivity, it should also be noted that this is the set of compounds that would have been made if the sole purpose was to choose the compounds most likely to be active at $\alpha 3\beta 4$. In contrast to the first set of compounds, the second set was chosen in order to maximize affinity at $\alpha 4\beta 2$ as well as selectivity for $\alpha 4\beta 2$ over $\alpha 3\beta 4$. Additionally, consideration was made to explore chemical diversity where appropriate. For the R_1 position in this set, three different functionalities were chosen: 2-(3-fluoro-phenyl)-ethyl (**5**), heptyl (**21**), and 4-methylpentyl (**26**). While sample **5** (Table 1) was not the only sample in the library with a substituted aromatic functionality at R_1 that showed activity and selectivity at $\alpha 4\beta 2$, it was the most active/selective sample and therefore was selected. The two aliphatic functionalities, heptyl and 4-methylpentyl, were chosen because they showed strong selectivity for $\alpha 4\beta 2$ (7.20- and 12.32-fold, respectively) and provided a different chemical characteristic. Likewise for the R_2 position in this set, two chemically diverse functionalities were selected: *R*-benzyl and *R*-cyclohexyl. Both mixture samples with these defined functionalities, **53** and **68**, respectively, showed affinity for $\alpha 4\beta 2$ and strong selectivity. For the R_3 position, in general, mixture samples with aliphatic groups defined showed slightly less potency but more selectivity for $\alpha 4\beta 2$ than samples defined with aromatic groups. Therefore, for this set, 4-*tert*-butyl-cyclohexyl-methyl was utilized, as it was among the most active mixtures with an aliphatic defined R_3 group and the most selective R_3 sample (R_3 samples are samples **69–110** in Table 1). This second set of individuals is shown in Table 2 as compounds **207–212**. A final set of six compounds was selected for synthesis from the library screening, compounds **213–218** shown in Table 2. This set contains the same functionalities at R_1 and R_2 as the previous set but replaces the R_3 group with 2-(3-bromo-phenyl)-ethyl (Table 1: **74**). This substitution changes the functionality used in the R_3 group from the most selective R_3 functionality in the library at this position to the most active at $\alpha 4\beta 2$ regardless of selectivity. In so doing, this set contains among it, the compound that combines the most active functionalities at each position (Table 2: **213**) against $\alpha 4\beta 2$.

Screening of Individual Compounds

The 18 individual compounds were synthesized and screened against $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChR (Table 2). The results further confirmed the SAR identified from the library screening. The first set of six compounds proved to be the least selective of the three sets, the second set the most selective, and the third set of six showed moderate selectivity. The predicted selectivity versus experimental results is further shown in Figure 3. The experimental selectivity exhibited a high correlation of $R = 0.91$ to predicted values ($R^2 = 0.83$), demonstrating the validity of choosing compounds to synthesize based upon the affinities of the individual functionalities in positions 1, 2, and 3 derived from mixture screening as well as the validity of our methodology of predicting binding affinities. Of the 18 individual compounds tested in these sets, the highest affinity compounds against $\alpha 3\beta 4$ came from the first set (**201**, **202**, and **205**); this was to be expected as this set contained the compounds that matched the most active functionalities against $\alpha 3\beta 4$ in the library screening. From the first set of six compounds, **201–206**, the apparent preference for R over S absolute configuration at the R_2 position that was seen at the library level for $\alpha 4\beta 2$ is also observed. When comparing the affinities of the second set of six compounds (**207–212**) against the last set of six (**213–218**), we note that the respective affinities against $\alpha 4\beta 2$ remain similar but the selectivity in general decreases significantly. The only difference in these two sets is the functionality used in the R_3 position. For compounds **207–212**, the aliphatic functionality 4-*tert*-butyl-cyclohexyl-methyl was utilized whereas the halogen substituted aromatic 2-(3-bromo-phenyl)-ethyl group was used for compounds **213–218**. The results indicate that the R_3 group change has little effect on the affinity of the compounds against $\alpha 4\beta 2$ but has a dramatic effect on their affinity for $\alpha 3\beta 4$. We sought to further exploit these observations in order to produce a set of compounds with increased affinity for $\alpha 4\beta 2$ as well as selectivity for $\alpha 4\beta 2$ over $\alpha 3\beta 4$.

Optimization of Individuals

Utilizing the acquired data, compounds **207** and **208** were selected for further optimization. Compound **207** was selected because it exhibits the highest affinity for $\alpha 4\beta 2$, is among the most selective for $\alpha 4\beta 2$, and has an aromatic functionality at the R_1 position that we identified for modification. Compound **208** was selected, as it is the $\alpha 4\beta 2$ selective analogue of **207** containing the same aromatic group in the R_1 position. The focus of the optimization was the modification of the R_1 functionality. While the 1169 library contains a diverse set of aromatic functionalities in the R_1 position, it does not contain any pyridyl groups. A number of reported nAChR ligands contain pyridyl groups, and some of the most potent, including nicotine, contain a 3-pyridyl group or substituted 3-pyridyl group such as epibatidine.³³ Using the same two-carbon spacer off the core cyclic guanidine scaffold, the 3-fluorophenyl group of **207** was replaced with a 3-pyridyl group in **301** (see Figure 4, Table 3). This substitution drastically increased its affinity at $\alpha 4\beta 2$, from 1587 to 118 nM while remaining relatively inactive at $\alpha 3\beta 4$, 61750 nM for **207** and 56480 nM for **301**, producing a high affinity and highly selective compound. The same R_1 modification to compound **208** also dramatically increased its $\alpha 4\beta 2$ affinity and selectivity, **306** (see Figure 4, Table 3). Modifying the R_2 chirality of **301** and **306** from R to S , compounds **302** and **307**, respectively, decreases the affinity at $\alpha 4\beta 2$ in line with what is observed in both the 1169

library samples (Table 1) as well as the individual compounds (Table 2) with regards to this change in absolute configuration at the R₂ position. Eliminating the R₂ position entirely from **301** and **306** provides the same analogue, **303**, which has a lower affinity to $\alpha 4\beta 2$ and selectivity than either parent compound. A change in absolute configuration in the core scaffold of **301** from *S* to *R*, compound **304**, also decreased its affinity at $\alpha 4\beta 2$, however, the same change to **306** provided a slightly more potent $\alpha 4\beta 2$ ligand, **308**. Combining the change in absolute configuration in the core scaffold from *S* to *R* with the elimination of the R₂ functionality provides the same analogue, **305**, which has the lowest affinity at $\alpha 4\beta 2$ of all the analogues tested in this optimization phase. Therefore, from this set of optimized hits, compound **301** was selected for further evaluation.

Biological Profiling of **301**

Compound **301** was further evaluated for binding affinity against $\alpha 3\beta 4\alpha 5$ nAChR and found to be weakly active with a K_d/K_i of 9062 nM. Compound **301** was tested for functional activity by measuring the ligand-induced change in membrane potential in intact HEK cells transfected with $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChR. Membrane potential changes were determined using a FlexStation, as described previously¹⁰ and in Materials and Methods. Compound **301** displayed no agonist activity in either cell line. As seen in Figure 5 and Table 3, **301** blocks nicotine stimulation at $\alpha 4\beta 2$ nAChR with an IC₅₀ of approximately 500 nM, which is slightly more potent than the selective $\alpha 4\beta 2$ nAChR antagonist DH β E. It is also very selective for $\alpha 4\beta 2$ nAChR, as it did not inhibit $\alpha 3\beta 4$ nAChR activation by 50% up to 10 μ M, the highest concentration tested (data not shown). **301** has no apparent agonist activity, so there is no reason to believe that this compound induces desensitization rather than direct inhibition of nicotine activity. To determine if antagonist activity is competitive, nicotine dose response curves were constructed in the presence of various concentrations of **301**. As seen in Figure 6, **301** causes a small rightward shift in the nicotine dose response curve but to a greater extent a decrease in maximal activity. These results appear to suggest a noncompetitive inhibition of nicotine's activity.

DISCUSSION AND CONCLUSION

Screening to identify novel structures with high affinity to nAChR started with a “scaffold ranking library,” which is a collection of 34 samples. Each of these 34 samples contains a mixture of individual compounds arranged so that compounds with the same structural core “scaffold” are contained in the same sample, overall more than 5 million individual compounds are contained in the 34 mixture samples. From the initial 34 mixtures, we identified a sample that demonstrated moderate affinity for $\alpha 4\beta 2$ nAChR. Therefore the complete positional scanning library associated with this sample was chosen for further screening. This positional scanning library, 1169, contains 45864 individual bis-cyclic guanidines systematically formatted into 110 samples allowing for the identification of individual compounds of high affinity, by methods that were discussed above and described previously.^{26,34–36} The series of individual compounds identified by the library provided useful SAR that was utilized to produce a series of further optimized compounds with high affinity and selectivity for nAChR subtypes. The highest affinity and most selective compounds appear to be pure antagonists when measuring membrane potential changes in

HEK cells transfected with $\alpha 4\beta 2$ nAChR. These compounds have low affinity ($IC_{50} > 10 \mu M$) for $\alpha 3\beta 4$ nAChR.

As we have demonstrated previously, mixture-based libraries provide a method for the efficient and rapid identification of novel structures that can be used directly from the libraries or for further chemical modification.^{26,37} Of the compounds described here, **301** is a novel compound, never previously demonstrated to bind to nAChR. In FlexStation assays, **301** appears to have no agonist activity at $\alpha 4\beta 2$ nAChR up to $10 \mu M$. When cells are pretreated with **301**, the increase in membrane potential induced by nicotine is blocked at concentrations of **301** similar to binding affinity. **301** has an IC_{50} for inhibition of nicotine activity of approximately 430 nM. This is at least 20-fold selective over its ability to block nicotine activity in cells transfected with $\alpha 3\beta 4$ nAChR. This 20-fold functional selectivity is encouraging because some very selective compounds, such as varenicline, have excellent binding selectivity but very poor functional selectivity.²¹

Interestingly, inhibition of nicotine stimulation appears to be noncompetitive, as increasing concentrations of **301** induce a progressive decrease in the maximal activity rather than a parallel shift to the right of a nicotine dose response curve. It seems unlikely that this would be due to desensitization of the receptor because **301** does not activate the receptor in these experiments, although it is possible that desensitization of the $\alpha 4\beta 2$ nAChR might occur. The $\alpha 4\beta 2$ partial agonist sazetidine A was originally described as a nonactivating desensitizer, but it was later determined that it was in fact a partial or full agonist in other cellular assays.^{38,39} Additional studies of **301** will be required to determine if it has agonist activity under some circumstances.

In conclusion, through the use of mixture-based libraries a total over 5 million compounds were assessed through the use of only 170 samples in order to provide a series of nAChR-selective compounds. One compound, **301**, has moderately high affinity and binding and functional selectivity for $\alpha 4\beta 2$ over $\alpha 3\beta 4$ nAChR; additional studies are underway on congeners and fragments of **301**. The success of this study demonstrates conclusively that mixture-based combinatorial libraries utilizing the scaffold ranking and positional scanning approaches can successfully be used in assays of low to medium throughput to develop novel lead compounds for further research.

MATERIALS AND METHODS

Chemistry General

The 1H and ^{13}C NMR spectra were obtained utilizing the Bruker 400'54 Ascend (400 and 100 MHz, respectively). NMR chemical shifts were reported in δ (ppm) using the δ 7.26 signal of $CHCl_3$ (1H NMR), the δ 4.79 signal of D_2O (1H NMR), and the δ 77.16 signal of $CDCl_3$ (^{13}C NMR) as internal standards. ^{13}C NMR spectra in D_2O were not adjusted (representative NMR is available in the Supporting Information). Mass spectra data was acquired using Shimadzu LCMS-2010. The reported final purity of the compounds were verified by Shimadzu HPLC and mass spectra under the following conditions: column, Phenomenex Luna 150 mm $\times$ 21.20 mm, 5 μm , C18; mobile phase, (A) H_2O (+0.1% formic acid)/(B) MeCN (+0.1% formic acid). Three gradient methods were used based on hydro-

phobicity (2% B to 20% B, 11 min) (25% B to 45% B, 31 min) (45% B to 65% B, 21 min); flow rate, 12 mL/min; detection, UV 214 nm. The purity of all final compounds were >95%. All chirality was generated from the corresponding amino acids. Under the reaction conditions described, no epimerization was observed and, for those compounds with multiple chiral centers, a single diastereomer was obtained.

Synthesis of Library 1169 and Individual Compounds and Construction of Scaffold Ranking Plate. General Synthesis of Bis-cyclic Guanidines F—Library 1169 and all individual compounds reported (**201–218** and **301–308**) were synthesized following the same general scheme (Scheme 1).^{40,41} The solid phase synthesis was performed using the “tea-bag” methodology.⁴² Initially, 100 mg of p-methylbenzylamine (MBHA) resin (1.1 mmol/g, 100–200 mesh) was sealed in a mesh “tea-bag,” neutralized with 5% diisopropylethylamine (DIEA) in dichloromethane (DCM) and subsequently swelled with additional DCM washes. Fmoc-Lys(Boc)-OH (Fmoc-L-Lys(Boc) for all compounds reported within except **304**, **305**, and **308** where Fmoc-D-Lys(Boc) was used) (6 equiv) was coupled in dimethylformamide (DMF) for 120 min in the presence of diisopropylcarbodiimide (DIC, 6 equiv) and 1-hydroxybenzotriazole hydrate (HOBt, 6 equiv) (**A**, Scheme 1). The Boc protecting group was removed with 55% TFA/DCM for 30 min and subsequently neutralized with 5% DIEA/DCM (3×). Boc-amino acids were coupled utilizing standard coupling procedures (6 equiv) with DIC (6 equiv) and HOBt (6 equiv) in DMF (0.1M) for 120 min (**B**, Scheme 1). The Boc protecting group was removed with 55% trifluoroacetic acid (TFA) in DCM for 30 min and subsequently neutralized with 5% DIEA/DCM (3×). Carboxylic acids are coupled using (6 equiv) in the presence of DIC (10 equiv) and HOBt (10 equiv) in DMF (0.1M) for 120 min (**C**, Scheme 1). The Fmoc protecting group was removed with 20% piperidine in DMF for 20 min, and the R₁ carboxylic acids were coupled in 10 equiv under the same solvent and activating reagent conditions. All coupling reactions were monitored for completion by the ninhydrin test. The reduction was performed in a 4000 mL Wilmad LabGlass vessel under nitrogen. A borane in 1.0 M tetrahydrofuran complex solution was used in 40-fold excess for each amide bond. The vessel was heated to 65 °C and maintained at temperature for 72 h. The solution was then discarded, and the bags were washed with THF and methanol. Once completely dry, the bags were treated overnight with piperidine at 65 °C and washed several times with methanol, DMF, and DCM (**D**, Scheme 1). Before proceeding, completion of reduction was monitored by a control cleavage and analyzed by LCMS. Urea cyclization (**E**, Scheme 1) was performed with a 5-fold excess of cyanogen bromide (CNBr) in a 0.1 M anhydrous DCM solution for each of the cyclization sites overnight. Following the cyclization, the bags were rinsed with DMF and DCM. The resin was cleaved with HF in the presence of anisole in an ice bath at 0 °C for 90 min (**F**, Scheme 1).

(R)-3-(3-Bromophenethyl)-1-(4-((S)-3-(3,4-dichlorophenethyl)-2-iminoimidazolidin-4-yl)butyl)-4-(naphthalen-1-ylmethyl)-imidazolidin-2-imine (201)—Using general scheme for the synthesis of **F**, compound **201** was synthesized using the following reagents: 3,4-dichlorophenylacetic acid (R₁), Boc-D-2-naphthylalanine (R₂), 3-bromophenylacetic acid (R₃). Final crude product was purified by HPLC as described above. ¹H NMR (400 MHz, CDCl₃): δ 8.51 (s, 2H), 7.86 (d, *J* = 7.6 Hz, 1H), 7.76 (d, *J* = 8.4

Hz, 1H), 7.67 (d, $J = 8.0$ Hz, 1H), 7.55 (t, $J = 7.2$ Hz, 1H), 7.50 (t, $J = 7.6$ Hz, 1H), 7.38 (t, $J = 8.0$ Hz, 2H), 7.32–7.29 (m, 3H), 7.20 (d, $J = 6.8$ Hz, 2H), 7.15–7.10 (m, 2H), 4.15–4.08 (m, 1H), 3.85–3.80 (m, 2H), 3.65–3.58 (m, 2H), 3.49 (dd, $J = 13.6, 4.4$ Hz, 1H), 3.40–3.28 (m, 4H), 3.24–3.14 (m, 3H), 2.94–2.88 (m, 3H), 2.82–2.72 (m, 2H), 1.65–1.54 (m, 1H), 1.50–1.35 (m, 3H), 1.25–1.10 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 158.97, 156.94, 140.78, 138.74, 134.10, 132.31, 132.01, 131.67, 131.27, 131.09, 130.71, 130.63, 130.61, 130.10, 129.36, 129.04, 128.44, 127.87, 127.81, 126.92, 126.23, 125.74, 122.74, 58.99, 57.37, 50.83, 46.14, 44.97, 44.65, 43.00, 35.58, 33.39, 32.72, 31.23, 26.52, 20.64. MS (ESI) m/z $[\text{M} + \text{H}]^+$: 719.0.

(R)-3-(4-Bromophenethyl)-1-(4-((S)-3-(3,4-dichlorophenethyl)-2-iminoimidazolidin-4-yl)butyl)-4-(naphthalen-1-ylmethyl)-imidazolidin-2-imine (202)—Using general scheme for the synthesis of **F**, compound **202** was synthesized using the following reagents: 3,4-dichlorophenylacetic acid (R_1), Boc-D-2-naphthylalanine (R_2), 4-bromophenylacetic acid (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.52 (s, 1H), 8.34 (s, 1H), 7.86 (d, $J = 7.2$ Hz, 1H), 7.76 (d, $J = 8.4$ Hz, 1H), 7.65 (d, $J = 8.0$ Hz, 1H), 7.55–7.49 (m, 2H), 7.41 (s, 1H), 7.38 (d, $J = 7.6$ Hz, 1H), 7.35 (d, $J = 7.6$ Hz, 2H), 7.30 (d, $J = 8.0$ Hz, 1H), 7.20 (d, $J = 7.2$ Hz, 2H), 7.08 (d, $J = 7.6$ Hz, 2H), 4.15–4.12 (m, 1H), 3.92–3.84 (m, 2H), 3.65–3.57 (m, 2H), 3.51 (dd, $J = 13.6, 4.0$ Hz, 1H), 3.42–3.25 (m, 5H), 3.22–3.15 (m, 2H), 2.94–2.90 (m, 1H), 2.88–2.75 (m, 4H), 1.65–1.56 (m, 1H), 1.50–1.37 (m, 3H), 1.27–1.13 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 158.89, 157.01, 138.81, 137.38, 134.12, 132.28, 131.95, 131.67, 131.33, 131.11, 130.91, 130.71, 130.60, 129.35, 129.10, 128.42, 127.78, 126.90, 126.24, 125.75, 122.73, 120.83, 59.05, 57.14, 50.87, 46.09, 44.92, 44.45, 43.01, 35.55, 33.25, 32.72, 31.15, 26.51, 20.57. MS (ESI) m/z $[\text{M} + \text{H}]^+$: 718.9.

(R)-3-(3,4-Dichlorophenethyl)-1-(4-((S)-3-(3,4-dichlorophenethyl)-2-iminoimidazolidin-4-yl)butyl)-4-(naphthalen-1-ylmethyl)-imidazolidin-2-imine (203)—Using general scheme for the synthesis of **F**, compound **203** was synthesized using the following reagents: 3,4-dichlorophenylacetic acid (R_1), Boc-D-2-naphthylalanine (R_2), 3,4-dichlorophenylacetic acid (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.61 (s, 2H), 7.83 (d, $J = 8.0$ Hz, 1H), 7.73 (d, $J = 8.4$ Hz, 1H), 7.67 (d, $J = 8.0$ Hz, 1H), 7.54–7.46 (m, 2H), 7.40–7.38 (m, 1H), 7.35 (d, $J = 7.6$ Hz, 1H), 7.27–7.25 (m, 2H), 7.21 (d, $J = 5.6$ Hz, 2H), 7.18–7.13 (m, 1H), 7.03 (d, $J = 6.4$ Hz, 1H), 4.15–4.02 (m, 1H), 3.94–3.85 (m, 2H), 3.56–3.47 (m, 3H), 3.35–3.25 (m, 5H), 3.20–3.13 (m, 2H), 2.97–2.92 (m, 1H), 2.88–2.70 (m, 4H), 1.58–1.50 (m, 1H), 1.42–1.25 (m, 3H), 1.17–1.05 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 159.17, 157.03, 138.83, 138.57, 134.06, 132.42, 132.21, 131.60, 131.31, 131.05, 130.91, 130.82, 130.64, 130.50, 129.35, 129.02, 128.77, 128.42, 127.77, 126.90, 126.24, 125.72, 122.65, 58.79, 57.20, 50.99, 46.26, 45.11, 44.48, 43.01, 35.66, 32.75, 31.29, 26.52, 24.80, 20.72. MS (ESI) m/z $[\text{M} + \text{H}]^+$: 709.0.

(S)-3-(3-Bromophenethyl)-1-(4-((S)-3-(3,4-dichlorophenethyl)-2-iminoimidazolidin-4-yl)butyl)-4-(naphthalen-1-ylmethyl)-imidazolidin-2-imine (204)—Using general scheme for the synthesis of **F**, compound **204** was synthesized using the following reagents: 3,4-dichlorophenylacetic acid (R_1), Boc-L-2-naphthylalanine (R_2), 3-

bromophenylacetic acid (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.64 (s, 2H), 7.84 (d, $J = 8.0$ Hz, 1H), 7.74 (d, $J = 8.4$ Hz, 1H), 7.65 (d, $J = 8.0$ Hz, 1H), 7.55–7.47 (m, 2H), 7.39 (s, 1H), 7.38 (d, $J = 8.0$ Hz, 1H), 7.38 (d, $J = 3.2$ Hz, 1H), 7.30–7.26 (m, 2H), 7.19 (d, $J = 7.2$ Hz, 1H), 7.16 (d, $J = 8.4$ Hz, 1H), 7.12–7.08 (m, 2H), 4.13–4.10 (m, 1H), 3.92–3.84 (m, 1H), 3.82–3.75 (m, 1H), 3.65–3.53 (m, 2H), 3.46 (dd, $J = 13.6, 4.0$ Hz, 1H), 3.38–3.25 (m, 4H), 3.19–3.12 (m, 3H), 2.91 (dd, $J = 13.6, 9.2$ Hz, 1H), 2.84–2.78 (m, 4H), 1.57–1.48 (m, 1H), 1.46–1.35 (m, 3H), 1.20–1.10 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3): δ 159.25, 157.01, 140.73, 138.88, 134.06, 132.28, 131.96, 131.65, 131.35, 131.08, 130.68, 130.61, 130.55, 130.07, 129.33, 129.00, 128.40, 127.86, 127.81, 126.90, 126.21, 125.73, 122.71, 58.78, 57.35, 50.86, 46.21, 45.07, 44.71, 42.98, 35.58, 33.32, 32.73, 31.30, 26.54, 20.66. MS (ESI) m/z $[\text{M} + \text{H}]^+$: 719.0.

(S)-3-(4-Bromophenethyl)-1-(4-((S)-3-(3,4-dichlorophenethyl)-2-iminoimidazolidin-4-yl)butyl)-4-(naphthalen-1-ylmethyl)-imidazolidin-2-imine (205)

—Using general scheme for the synthesis of **F**, compound **205** was synthesized using the following reagents: 3,4-dichlorophenylacetic acid (R_1), Boc-L-2-naphthylalanine (R_2), 4-bromophenylacetic acid (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.62 (s, 2H), 7.88–7.85 (m, 1H), 7.77 (d, $J = 8.0$ Hz, 1H), 7.65 (d, $J = 8.0$ Hz, 1H), 7.56–7.50 (m, 2H), 7.41–7.39 (m, 2H), 7.36 (d, $J = 8.4$ Hz, 2H), 7.31 (d, $J = 8.0$ Hz, 1H), 7.21 (d, $J = 6.8$ Hz, 1H), 7.18 (d, $J = 8.4$ Hz, 1H), 7.06 (d, $J = 8.4$ Hz, 2H), 4.20–4.16 (m, 1H), 3.91–3.89 (m, 2H), 3.58–3.55 (m, 2H), 3.51 (dd, $J = 14.0, 4.8$ Hz, 1H), 3.38–3.31 (m, 4H), 3.21–3.18 (m, 3H), 2.95 (dd, $J = 13.6, 8.8$ Hz, 1H), 2.85–2.80 (m, 4H), 1.61–1.50 (m, 1H), 1.50–1.38 (m, 3H), 1.25–1.13 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3): δ 159.28, 157.01, 138.76, 137.18, 134.13, 132.38, 132.00, 131.66, 131.26, 131.10, 130.86, 130.72, 130.69, 129.38, 128.98, 128.49, 127.81, 126.94, 126.29, 125.76, 122.69, 120.92, 58.76, 57.20, 50.96, 46.19, 45.17, 44.66, 43.07, 35.64, 33.22, 32.81, 31.05, 26.53, 20.56. MS (ESI) m/z $[\text{M} + \text{H}]^+$: 718.9.

(S)-3-(3,4-Dichlorophenethyl)-1-(4-((S)-3-(3,4-dichlorophenethyl)-2-iminoimidazolidin-4-yl)butyl)-4-(naphthalen-1-ylmethyl)-imidazolidin-2-imine (206)

—Using general scheme for the synthesis of **F**, compound **206** was synthesized using the following reagents: 3,4-dichlorophenylacetic acid (R_1), Boc-L-2-naphthylalanine (R_2), 3,4-dichlorophenylacetic acid (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.62 (s, 2H), 7.83 (d, $J = 8.0$ Hz, 1H), 7.74 (d, $J = 8.0$ Hz, 1H), 7.68 (d, $J = 8.0$ Hz, 1H), 7.55–7.47 (m, 2H), 7.38–7.35 (m, 2H), 7.28–7.25 (m, 2H), 7.22 (d, $J = 7.2$ Hz, 1H), 7.20 (s, 1H), 7.15 (d, $J = 7.6$ Hz, 1H), 7.01 (d, $J = 8.0$ Hz, 1H), 4.13–4.02 (m, 1H), 4.00–3.90 (m, 1H), 3.87–3.77 (m, 1H), 3.56–3.47 (m, 3H), 3.29–3.26 (m, 5H), 3.20–3.17 (m, 2H), 2.97 (q, $J = 8.8$ Hz, 1H), 2.85–2.70 (m, 4H), 1.58–1.50 (m, 1H), 1.47–1.28 (m, 3H), 1.17–1.05 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3): δ 159.19, 157.03, 138.84, 138.54, 134.09, 132.48, 132.26, 131.63, 131.36, 131.07, 130.91, 130.88, 130.82, 130.67, 130.55, 129.38, 129.00, 128.76, 128.45, 127.80, 126.93, 126.27, 125.75, 122.66, 58.79, 57.21, 51.03, 46.20, 45.11, 44.50, 43.00, 35.73, 32.74, 31.31, 26.52, 20.68. MS (ESI) m/z $[\text{M} + \text{H}]^+$: 709.0.

(R)-4-Benzyl-3-((4-tert-butylcyclohexyl)methyl)-1-(4-((S)-3-(3-fluorophenethyl)-2-iminoimidazolidin-4-yl)butyl)imidazolidin-2-imine (207)—Using general scheme for the synthesis of **F**, compound **207** was synthesized using the following reagents: 3-fluorophenylacetic acid (R_1), Boc-D-phenylalanine (R_2), 4-tert-butyl-cyclohexanecarboxylic acid, predominantly *trans*, 98+% (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.53 (s, 1H), 8.25 (s, 2H), 7.30 (t, $J = 7.2$ Hz, 2H), 7.24 (d, $J = 7.6$ Hz, 1H), 7.20 (t, $J = 6.8$ Hz, 1H), 7.12 (d, $J = 7.2$ Hz, 2H), 7.07 (d, $J = 7.6$ Hz, 1H), 7.03 (d, $J = 10.0$ Hz, 1H), 6.84 (t, $J = 8.4$ Hz, 1H), 4.04–3.99 (m, 1H), 3.92–3.84 (m, 1H), 3.81–3.61 (m, 1H), 3.53–3.42 (m, 3H), 3.37–3.26 (m, 2H), 3.24–3.17 (m, 3H), 3.11–2.95 (m, 2H), 2.90–2.80 (m, 2H), 2.73–2.65 (m, 1H), 1.83–1.75 (m, 1H), 1.73–1.68 (m, 2H), 1.65–1.45 (m, 4H), 1.43–1.30 (m, 3H), 1.20–1.11 (m, 2H), 1.08 (d, $J = 12.8$ Hz, 1H), 1.03–0.98 (m, 1H), 0.95–0.90 (m, 2H), 0.81 (s, 9H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 589.2.

(R)-3-((4-tert-Butylcyclohexyl)methyl)-4-(cyclohexylmethyl)-1-(4-((S)-3-(3-fluorophenethyl)-2-iminoimidazolidin-4-yl)butyl)imidazolidin-2-imine (208)—Using general scheme for the synthesis of **F**, compound **208** was synthesized using the following reagents: 3-fluorophenylacetic acid (R_1), Boc-D-cyclohexylalanine (R_2), 4-tert-butyl-cyclohexanecarboxylic acid, predominantly *trans*, 98+% (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, D_2O): δ 7.44 (q, $J = 7.6$ Hz, 1H), 7.19 (t, $J = 7.6$ Hz, 1H), 7.12 (t, $J = 8.0$ Hz, 2H), 4.06–4.00 (m, 1H), 3.80 (q, $J = 9.6$ Hz, 2H), 3.72–3.66 (m, 2H), 3.62–3.55 (m, 1H), 3.48–3.38 (m, 1H), 3.36–3.31 (m, 2H), 3.29–3.21 (m, 1H), 3.16 (d, $J = 7.6$ Hz, 1H), 2.99 (t, $J = 6.4$ Hz, 2H), 2.75–2.73 (m, 1H), 1.86–1.71 (m, 8H), 1.66–1.54 (m, 8H), 1.43–1.37 (m, 2H), 1.31–1.25 (m, 5H), 1.10–1.08 (m, 2H), 1.04–1.03 (m, 2H), 0.99–0.93 (m, 2H), 0.89 (s, 9H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 595.2.

(R)-4-Benzyl-3-((4-tert-butylcyclohexyl)methyl)-1-(4-((S)-3-heptyl-2-iminoimidazolidin-4-yl)butyl)imidazolidin-2-imine (209)—Using general scheme for the synthesis of **F**, compound **209** was synthesized using the following reagents: heptanoic acid (R_1), Boc-D-phenylalanine (R_2), 4-tert-butyl-cyclohexanecarboxylic acid, predominantly *trans*, 98+% (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.54 (s, 1H), 8.00 (s, 2H), 7.32 (t, $J = 7.2$ Hz, 2H), 7.27–7.25 (m, 1H), 7.14 (d, $J = 7.2$ Hz, 2H), 4.06–4.02 (m, 1H), 3.85–3.80 (m, 1H), 3.73–3.65 (m, 2H), 3.55–3.47 (m, 2H), 3.40–3.32 (m, 2H), 3.29–3.22 (m, 2H), 3.12–3.05 (m, 2H), 3.03–2.97 (m, 1H), 2.76–2.68 (m, 1H), 1.83–1.75 (m, 1H), 1.71–1.65 (m, 3H), 1.57–1.41 (m, 7H), 1.27–1.25 (m, 11H), 1.13–1.04 (m, 2H), 1.00–0.95 (m, 2H), 0.86 (d, $J = 6.4$ Hz, 3H), 0.81 (s, 9H). MS (ESI) m/z $[\text{M} + \text{H}]^+$: 565.2.

(R)-3-((4-tert-Butylcyclohexyl)methyl)-4-(cyclohexylmethyl)-1-(4-((S)-3-heptyl-2-iminoimidazolidin-4-yl)butyl)imidazolidin-2-imine (210)—Using general scheme for the synthesis of **F**, compound **210** was synthesized using the following reagents: heptanoic acid (R_1), Boc-D-cyclohexylalanine (R_2), 4-tert-butyl-cyclohexanecarboxylic acid, predominantly *trans*, 98+% (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.58 (s, 1H), 3.83–3.79 (m, 2H), 3.66 (t, $J = 8.8$ Hz,

2H), 3.50–3.44 (m, 4H), 3.37–3.30 (m, 1H), 3.19–3.13 (m, 1H), 3.09–3.03 (m, 1H), 2.96 (dd, $J = 15.2, 6.0$ Hz, 1H), 1.83–1.77 (m, 2H), 1.75–1.72 (m, 2H), 1.71–1.67 (m, 4H), 1.66–1.64 (m, 2H), 1.62–1.59 (m, 3H), 1.57–1.48 (m, 4H), 1.37–1.32 (m, 2H), 1.30–1.22 (m, 1H), 1.20–1.16 (m, 2H), 1.14–1.11 (m, 1H), 1.06 (d, $J = 8.8$ Hz, 1H), 1.03–1.00 (m, 2H), 0.97–0.93 (m, 3H), 0.88–0.86 (m, 3H), 0.82 (s, 9H). MS (ESI) m/z $[M + H]^+$: 571.3.

(R)-4-Benzyl-3-((4-tert-butylcyclohexyl)methyl)-1-(4-((S)-2-imino-3-(4-methylpentyl)imidazolidin-4-yl)butyl)imidazolidin-2-imine (211)—Using general scheme for the synthesis of **F**, compound **211** was synthesized using the following reagents: 4-methylvaleric acid (R_1), Boc-D-phenylalanine (R_2), 4-tert-butyl-cyclohexanecarboxylic acid, predominantly *trans*, 98+% (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.55 (s, 1H), 7.33 (t, $J = 7.2$ Hz, 2H), 7.28–7.25 (m, 1H), 7.13 (d, $J = 7.2$ Hz, 2H), 4.07–4.02 (m, 1H), 3.83–3.80 (m, 1H), 3.71–3.64 (m, 1H), 3.51–3.45 (m, 2H), 3.30–3.21 (m, 4H), 3.10–3.06 (m, 2H), 3.04–2.98 (m, 2H), 2.76–2.68 (m, 1H), 1.83–1.78 (m, 1H), 1.71 (d, $J = 10.4$ Hz, 1H), 1.66–1.58 (m, 3H), 1.56–1.50 (m, 5H), 1.44–1.38 (m, 2H), 1.27–1.23 (m, 2H), 1.16 (q, $J = 7.2$ Hz, 3H), 1.07–1.04 (m, 1H), 1.00–0.97 (m, 3H), 0.86 (d, $J = 6.8$ Hz, 6H), 0.83 (s, 9H). MS (ESI) m/z $[M + H]^+$: 551.2.

(R)-3-((4-tert-Butylcyclohexyl)methyl)-4-(cyclohexylmethyl)-1-(4-((S)-2-imino-3-(4-methylpentyl)imidazolidin-4-yl)butyl)-imidazolidin-2-imine (212)—Using general scheme for the synthesis of **F**, compound **212** was synthesized using the following reagents: 4-methylvaleric acid (R_1), Boc-D-cyclohexylalanine (R_2), 4-tert-butyl-cyclohexanecarboxylic acid, predominantly *trans*, 98+% (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.54 (s, 1H), 8.28 (s, 2H), 3.84–3.78 (m, 2H), 3.66 (t, $J = 9.2$ Hz, 2H), 3.48–3.42 (m, 4H), 3.34–3.30 (m, 1H), 3.19–3.13 (m, 1H), 3.05 (dd, $J = 14.4, 7.6$ Hz, 1H), 2.95 (dd, $J = 14.8, 5.6$ Hz, 1H), 1.80–1.77 (m, 1H), 1.71 (d, $J = 10.4$ Hz, 6H), 1.64–1.63 (m, 2H), 1.60 (d, $J = 8.0$ Hz, 4H), 1.53 (q, $J = 6.0$ Hz, 4H), 1.49–1.44 (m, 2H), 1.33–1.29 (m, 3H), 1.27–1.24 (m, 1H), 1.23–1.21 (m, 3H), 1.20–1.18 (m, 1H), 1.17–1.15 (m, 2H), 1.13–1.10 (m, 1H), 1.07–1.05 (m, 1H), 1.02–0.99 (m, 2H), 0.96–0.92 (m, 3H), 0.86 (d, $J = 6.8$ Hz, 6H), 0.82 (s, 9H). MS (ESI) m/z $[M + H]^+$: 557.2.

(R)-4-Benzyl-3-(3-bromophenethyl)-1-(4-((S)-3-(3-fluorophenethyl)-2-iminoimidazolidin-4-yl)butyl)imidazolidin-2-imine (213)—Using general scheme for the synthesis of **F**, compound **213** was synthesized using the following reagents: 3-fluorophenylacetic acid (R_1), Boc-D-phenylalanine (R_2), 3-bromophenylacetic acid (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.54 (s, 1H), 7.34 (d, $J = 8.0$ Hz, 1H), 7.31 (s, 1H), 7.28 (d, $J = 7.2$ Hz, 2H), 7.24 (d, $J = 7.2$ Hz, 1H), 7.22–7.19 (m, 2H), 7.15 (t, $J = 7.6$ Hz, 2H), 7.03 (d, $J = 6.8$ Hz, 3H), 6.85 (t, $J = 8.0$ Hz, 1H), 4.03–3.98 (m, 1H), 3.87–3.80 (m, 1H), 3.71–3.64 (m, 1H), 3.62–3.50 (m, 2H), 3.36 (t, $J = 9.6$ Hz, 2H), 3.32–3.20 (m, 4H), 3.12 (dd, $J = 9.6, 6.4$ Hz, 1H), 2.91 (d, $J = 4.0$ Hz, 1H), 2.87–2.80 (m, 4H), 2.60 (d, $J = 13.2, 7.6$ Hz, 1H), 1.62–1.50 (m, 1H), 1.45–1.30 (m, 3H), 1.21–1.14 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3) δ 162.98 ($J = 244.2$ Hz), 159.00, 156.85, 140.99 ($J = 7.6$ Hz), 140.77, 135.27, 132.02, 130.61, 130.35 ($J =$

7.1 Hz), 130.07, 129.19 ($J = 4.0$ Hz), 128.02, 127.50, 125.04, 122.67, 121.63, 115.95 ($J = 20.9$ Hz), 113.68 ($J = 21.7$ Hz), 58.94, 58.22, 50.64, 46.10, 45.02, 44.43, 43.12, 38.30, 33.20, 31.05, 26.42, 20.55. MS (ESI) m/z $[M + H]^+$: 619.0.

(R)-3-(3-Bromophenethyl)-4-(cyclohexylmethyl)-1-(4-((S)-3-(3-fluorophenethyl)-2-iminoimidazolidin-4-yl)butyl)imidazolidin-2-imine (214)—Using general scheme for the synthesis of **F**, compound **214** was synthesized using the following reagents: 3-fluorophenylacetic acid (R_1), Boc-D-cyclohexylalanine (R_2), 3-bromophenylacetic acid (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.56 (s, 2H), 7.35 (s, 1H), 7.32 (d, $J = 8.0$ Hz, 1H), 7.22–7.19 (m, 2H), 7.14 (t, $J = 7.6$ Hz, 1H), 7.02 (t, $J = 8.0$ Hz, 2H), 6.84 (t, $J = 8.0$ Hz, 1H), 3.95–3.85 (m, 2H), 3.58–3.50 (m, 2H), 3.50 (d, $J = 8.8$ Hz, 1H), 3.46–3.42 (m, 2H), 3.37–3.30 (m, 3H), 3.25–3.17 (m, 1H), 3.04–3.00 (m, 1H), 2.87–2.78 (m, 4H), 1.67–1.62 (m, 4H), 1.50–1.45 (m, 6H), 1.21 (d, $J = 6.4$ Hz, 2H), 1.16–1.11 (m, 5H), 0.91 (t, $J = 11.6$ Hz, 1H), 0.76 (t, $J = 6.8$ Hz, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ 162.96 ($J = 244.2$ Hz), 159.10, 156.94, 141.01 ($J = 7.3$ Hz), 140.80, 131.98, 130.53, 130.30 ($J = 8.1$ Hz), 130.01, 127.93, 124.98 (d, $J = 2.0$ Hz), 122.69, 115.90 ($J = 20.7$ Hz), 113.64 ($J = 20.7$ Hz), 58.83, 55.56, 51.89, 46.13, 45.14, 43.94, 43.07, 39.87, 34.28, 34.11, 33.20, 32.32, 31.30, 26.54, 26.27, 26.16, 20.66. MS (ESI) m/z $[M + H]^+$: 625.0.

(R)-4-Benzyl-3-(3-bromophenethyl)-1-(4-((S)-3-heptyl-2-imino-imidazolidin-4-yl)butyl)imidazolidin-2-imine (215)—Using general scheme for the synthesis of **F**, compound **215** was synthesized using the following reagents: heptanoic acid (R_1), Boc-D-phenylalanine (R_2), 3-bromophenylacetic acid (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.56 (s, 1H), 8.06 (s, 2H), 7.33 (d, $J = 8.8$ Hz, 2H), 7.27 (d, $J = 7.6$ Hz, 2H), 7.23 (t, $J = 7.2$ Hz, 2H), 7.15 (t, $J = 7.6$ Hz, 1H), 7.02 (d, $J = 6.8$ Hz, 2H), 4.17–4.05 (m, 1H), 3.85–3.83 (m, 1H), 3.65–3.63 (m, 2H), 3.52–3.48 (m, 1H), 3.37–3.30 (m, 5H), 3.11 (dd, $J = 9.6, 6.4$ Hz, 1H), 3.07–3.03 (m, 1H), 2.90 (d, $J = 4.4$ Hz, 1H), 2.87 (d, $J = 4.0$ Hz, 2H), 2.59 (dd, $J = 13.6, 8.0$ Hz, 1H), 1.67–1.65 (m, 1H), 1.49–1.41 (m, 5H), 1.26–1.23 (m, 10H), 0.83 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3): δ 158.93, 156.87, 140.78, 135.19, 132.00, 130.56, 130.02, 129.18, 129.13, 127.99, 127.48, 122.61, 58.22, 58.15, 50.57, 46.03, 45.01, 44.32, 41.97, 38.26, 33.17, 31.81, 31.22, 29.16, 27.19, 26.61, 22.68, 20.60, 14.17. MS (ESI) m/z $[M + H]^+$: 595.0.

(R)-3-(3-Bromophenethyl)-4-(cyclohexylmethyl)-1-(4-((S)-3-heptyl-2-iminoimidazolidin-4-yl)butyl)imidazolidin-2-imine (216)—Using general scheme for the synthesis of **F**, compound **216** was synthesized using the following reagents: heptanoic acid (R_1), Boc-D-cyclohexylalanine (R_2), 3-bromophenylacetic acid (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.56 (s, 1H), 8.31 (s, 1H), 7.35 (s, 1H), 7.33 (d, $J = 8.0$ Hz, 1H), 7.21 (d, $J = 7.2$ Hz, 1H), 7.15 (t, $J = 7.6$ Hz, 1H), 3.96–3.94 (m, 1H), 3.85–3.83 (m, 1H), 3.71–3.64 (m, 1H), 3.53 (t, $J = 8.8$ Hz, 2H), 3.44–3.42 (m, 2H), 3.35–3.31 (m, 3H), 3.04 (t, $J = 8.0$ Hz, 2H), 2.87–2.82 (m, 2H), 1.70–1.65 (m, 4H), 1.55–1.44 (m, 8H), 1.26–1.23 (m, 11H), 1.18–1.10 (m, 5H), 0.95–0.89 (m, 1H), 0.84 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3): δ 159.19, 156.98, 140.80, 131.98, 130.53, 129.99, 127.94, 122.67, 58.09, 55.56, 51.90, 46.05, 45.21, 43.93, 41.91,

39.89, 34.28, 34.11, 33.19, 31.82, 31.32, 29.15, 27.21, 26.65, 26.26, 26.16, 25.96, 22.69, 20.66, 14.18. MS (ESI) m/z $[M + H]^+$: 601.1.

(R)-4-Benzyl-3-(3-bromophenethyl)-1-(4-((S)-2-imino-3-(4-methylpentyl)imidazolidin-4-yl)butyl)imidazolidin-2-imine (217)—Using general scheme for the synthesis of **F**, compound **217** was synthesized using the following reagents: 4-methylvaleric acid (R_1), Boc-D-phenylalanine (R_2), 3-bromophenylacetic acid (R_3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, $CDCl_3$): δ 8.57 (s, 1H), 8.26 (s, 1H), 7.34 (s, 1H), 7.32–7.31 (m, 1H), 7.28 (d, $J = 7.2$ Hz, 2H), 7.24 (d, $J = 7.2$ Hz, 1H), 7.22–7.20 (m, 1H), 7.16 (t, $J = 7.6$ Hz, 1H), 7.02 (d, $J = 7.2$ Hz, 2H), 4.13–4.07 (m, 1H), 3.85–3.82 (m, 1H), 3.65–3.60 (m, 2H), 3.51–3.48 (m, 1H), 3.37–3.33 (m, 5H), 3.12 (dd, $J = 9.6, 6.4$ Hz, 1H), 3.06–3.03 (m, 1H), 2.90 (d, $J = 4.4$ Hz, 1H), 2.87 (d, $J = 4.0$ Hz, 2H), 2.59 (dd, $J = 13.6, 8.0$ Hz, 1H), 1.67–1.60 (m, 1H), 1.52–1.46 (m, 4H), 1.42–1.36 (m, 2H), 1.26–1.23 (m, 2H), 1.18–1.12 (m, 2H), 0.84 (d, $J = 6.8$ Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.11, 156.90, 140.76, 135.21, 132.01, 130.58, 130.03, 129.19, 129.14, 127.99, 127.49, 122.62, 58.16, 50.61, 46.04, 45.05, 44.35, 42.11, 38.28, 35.58, 33.17, 31.25, 27.93, 26.60, 25.02, 22.67, 22.59, 20.58. MS (ESI) m/z $[M + H]^+$: 581.0.

(R)-3-(3-Bromophenethyl)-4-(cyclohexylmethyl)-1-(4-((S)-2-imino-3-(4-methylpentyl)imidazolidin-4-yl)butyl)imidazolidin-2-imine (218)—Using general scheme for the synthesis of **F**, compound **218** was synthesized using the following reagents: 4-methylvaleric acid (R_1), Boc-D-cyclohexylalanine (R_2), 3-bromophenylacetic acid (R_3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, $CDCl_3$): δ 8.57 (s, 1H), 8.25 (s, 1H), 7.35 (d, $J = 8.0$ Hz, 1H), 7.32 (s, 1H), 7.22 (d, $J = 7.2$ Hz, 1H), 7.15 (t, $J = 8.0$ Hz, 1H), 3.96–3.92 (m, 1H), 3.85–3.83 (m, 1H), 3.75–3.65 (m, 1H), 3.53 (t, $J = 8.8$ Hz, 2H), 3.45–3.41 (m, 2H), 3.38–3.31 (m, 3H), 3.03 (t, $J = 8.0$ Hz, 2H), 2.86–2.84 (m, 2H), 1.70–1.63 (m, 4H), 1.54–1.43 (m, 10H), 1.38–1.25 (m, 2H), 1.20–1.09 (m, 8H), 0.84 (d, $J = 6.4$ Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.13, 156.95, 140.86, 131.99, 130.54, 129.98, 127.97, 122.66, 58.09, 55.57, 51.89, 46.05, 45.23, 43.96, 42.11, 39.88, 35.59, 34.28, 33.21, 32.31, 31.30, 27.93, 26.71, 26.16, 25.96, 25.02, 22.67, 20.63. MS (ESI) m/z $[M + H]^+$: 587.1.

(R)-4-Benzyl-3-((4-tert-butylcyclohexyl)methyl)-1-(4-((S)-2-imino-3-(2-pyridin-3-yl)ethyl)imidazolidin-4-yl)butyl)imidazolidin-2-imine (301)—Using general scheme for the synthesis of **F**, compound **301** was synthesized using the following reagents: 3-pyridinylacetic acid (R_1), Boc-D-phenylalanine (R_2), 4-tert-butylcyclohexanecarboxylic acid, predominantly *trans*, 98+% (R_3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, D_2O): δ 8.55–8.45 (m, 2H), 7.87 (d, $J = 8.0$ Hz, 1H), 7.51 (dd, $J = 7.2, 5.2$ Hz, 1H), 7.42 (t, $J = 7.2$ Hz, 2H), 7.36 (t, $J = 6.8$ Hz, 1H), 7.29 (d, $J = 6.8$ Hz, 2H), 4.36–4.30 (m, 1H), 3.85–3.79 (m, 1H), 3.73–3.65 (m, 3H), 3.61–3.54 (m, 1H), 3.45 (dd, $J = 10.4, 4.8$ Hz, 1H), 3.30–3.25 (m, 3H), 3.06–3.02 (m, 5H), 2.94 (dd, $J = 14.4, 4.0$ Hz, 1H), 1.86 (d, $J = 2.4$ Hz, 1H), 1.80 (d, $J = 7.6$ Hz, 2H), 1.75 (d, $J = 12.8$ Hz, 1H), 1.69–1.65 (m, 1H), 1.60–1.57 (m, 1H), 1.47–1.39 (m, 1H), 1.37–1.23 (m, 2H), 1.10–0.96 (m, 7H), 0.87 (s, 9H). ^{13}C NMR (100 MHz, D_2O): δ 158.23, 156.14, 148.37, 146.78, 138.85, 135.69, 134.87, 129.71, 128.84, 127.23, 124.73, 58.87, 56.84,

49.54, 47.88, 45.68, 44.10, 43.60, 42.76, 36.63, 31.72, 30.37, 29.92, 29.90, 27.56, 26.84, 25.71, 20.34. MS (ESI) m/z $C_{35}H_{54}N_7$ $[M + H]^+$: 572.2.

(S)-4-Benzyl-3-((4-tert-butylcyclohexyl)methyl)-1-(4-((S)-2-imino-3-(2-(pyridin-3-yl)ethyl)imidazolidin-4-yl)butyl)imidazolidin-2-imine (302)—Using general scheme for the synthesis of **F**, compound **302** was synthesized using the following reagents: 3-pyridinylacetic acid (R_1), Boc-L-phenylalanine (R_2), 4-tert-butylcyclohexanecarboxylic acid, predominantly *trans*, 98+% (R_3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, $CDCl_3$): δ 8.62 (s, 1H), 8.53 (s, 1H), 8.40 (d, $J = 3.6$ Hz, 1H), 7.78 (d, $J = 8.0$ Hz, 1H), 7.33 (d, $J = 7.2$ Hz, 2H), 7.28–7.21 (m, 2H), 7.14 (d, $J = 6.8$ Hz, 2H), 4.10–4.03 (m, 1H), 4.01–3.96 (m, 1H), 3.76 (dd, $J = 15.2$, 8.4 Hz, 1H), 3.65–3.55 (m, 2H), 3.48 (t, $J = 9.6$ Hz, 2H), 3.43–3.39 (m, 1H), 3.32–3.19 (m, 3H), 3.09–3.06 (m, 1H), 3.05–3.03 (m, 1H), 2.92–2.86 (m, 2H), 2.72 (dd, $J = 13.6$, 8.4 Hz, 1H), 1.79–1.77 (m, 2H), 1.72 (d, $J = 11.6$ Hz, 2H), 1.60 (t, $J = 7.6$ Hz, 2H), 1.51–1.47 (m, 1H), 1.42 (t, $J = 7.2$ Hz, 2H), 1.21–1.18 (m, 2H), 1.10–1.02 (m, 2H), 0.97–0.96 (m, 3H), 0.82 (s, 9H). ^{13}C NMR (100 MHz, D_2O): δ 158.22, 156.49, 148.49, 146.86, 138.60, 135.65, 134.72, 129.60, 128.80, 127.19, 124.60, 58.93, 57.44, 49.69, 48.59, 47.58, 45.77, 44.17, 42.80, 36.54, 35.80, 31.67, 30.59, 30.52, 29.97, 27.05, 26.47, 25.74, 20.48. MS (ESI) m/z calcd for $[M + H]^+$: 572.2.

(S)-1-((4-tert-Butylcyclohexyl)methyl)-3-(4-(2-imino-3-(2-(pyridin-3-yl)ethyl)imidazolidin-4-yl)butyl)imidazolidin-2-imine (303)—Using general scheme for the synthesis of **F**, compound **303** was synthesized using the following reagents: 3-pyridinylacetic acid (R_1), Boc-glycine (R_2), 4-tert-butylcyclohexanecarboxylic acid, predominantly *trans*, 98+% (R_3). Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, D_2O): δ 8.54–8.50 (m, 2H), 7.93 (d, $J = 7.6$ Hz, 1H), 7.55 (dd, $J = 7.6$, 5.2 Hz, 1H), 3.96–3.89 (m, 1H), 3.73 (d, $J = 9.2$ Hz, 1H), 3.71–3.67 (m, 4H), 3.64–3.62 (m, 1H), 3.37–3.31 (m, 4H), 3.13 (d, $J = 7.2$ Hz, 2H), 3.07–3.03 (m, 2H), 1.85–1.82 (m, 2H), 1.76 (d, $J = 10.0$ Hz, 2H), 1.69–1.51 (m, 6H), 1.31–1.25 (m, 2H), 1.01 (d, $J = 4.4$ Hz, 3H), 0.97–0.94 (m, 1H), 0.86 (s, 9H). ^{13}C NMR (100 MHz, D_2O): δ 158.24, 157.19, 148.17, 146.57, 138.99, 134.90, 124.73, 58.89, 50.95, 47.93, 47.62, 46.14, 45.73, 45.38, 42.65, 35.52, 31.68, 31.53, 30.43, 30.40, 26.89, 26.80, 26.31, 20.45. MS (ESI) m/z $[M + H]^+$: 482.1.

(R)-4-Benzyl-3-((4-tert-butylcyclohexyl)methyl)-1-(4-((R)-2-imino-3-(2-(pyridin-3-yl)ethyl)imidazolidin-4-yl)butyl)imidazolidin-2-imine (304)—Using general scheme for the synthesis of **F**, compound **304** was synthesized using the following reagents: 3-pyridinylacetic acid (R_1), Boc-D-phenylalanine (R_2), 4-tert-butylcyclohexanecarboxylic acid, predominantly *trans*, 98+% (R_3). The only variation of the general compound synthesis for compound 304 was utilizing Fmoc-D-Lys(Boc)-OH instead of Boc-L-Lys(Fmoc)-OH. Final crude product was purified by HPLC as described above. 1H NMR (400 MHz, $CDCl_3$): δ 8.93 (s, 2H), 8.61 (s, 1H), 8.52 (s, 1H), 8.41 (s, 1H), 7.76 (d, $J = 6.8$ Hz, 1H), 7.33 (t, $J = 7.2$ Hz, 2H), 7.27 (d, $J = 6.4$ Hz, 1H), 7.24–7.20 (m, 1H), 7.14 (d, $J = 7.2$ Hz, 2H), 4.10–4.04 (m, 1H), 4.01–3.96 (m, 1H), 3.76 (dd, $J = 14.4$, 8.4 Hz, 1H), 3.65–3.57 (m, 2H), 3.47 (t, $J = 9.6$ Hz, 2H), 3.43–3.39 (m, 1H), 3.29–3.23 (m, 3H),

3.09–3.06 (m, 1H), 3.05–3.02 (m, 1H), 2.94–2.84 (m, 2H), 2.72 (dd, $J = 13.2, 8.4$ Hz, 1H), 1.79–1.77 (m, 2H), 1.72 (d, $J = 11.2$ Hz, 2H), 1.65–1.55 (m, 2H), 1.51–1.37 (m, 3H), 1.24–1.16 (m, 2H), 1.08–1.04 (m, 2H), 0.99–0.94 (m, 3H), 0.82 (s, 9H). ^{13}C NMR (100 MHz, CDCl_3) δ 159.35, 157.28, 150.20, 147.99, 137.26, 135.16, 133.97, 129.30, 129.21, 127.58, 123.90, 58.63, 57.78, 50.34, 48.82, 47.87, 46.27, 45.19, 43.03, 37.76, 36.18, 32.54, 31.20, 30.71, 27.66, 26.85, 26.50, 20.61. MS (ESI) m/z $[\text{M} + \text{H}]^+$: 572.2.

(R)-1-((4-tert-Butylcyclohexyl)methyl)-3-(4-(2-imino-3-(2-(pyridin-3-yl)ethyl)imidazolidin-4-yl)butyl)imidazolidin-2-imine (305)—Using general scheme for the synthesis of **F**, compound **305** was synthesized using the following reagents: 3-pyridinylacetic acid (R_1), Boc-glycine (R_2), 4-tert-butyl-cyclohexanecarboxylic acid, predominantly trans, 98+% (R_3). The only variation of the general compound synthesis for compound **305** was utilizing Fmoc-D-Lys(Boc)-OH instead of Boc-L-Lys(Fmoc)-OH. Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, D_2O): δ 8.54–8.46 (m, 2H), 7.98 (d, $J = 8.0$ Hz, 1H), 7.59 (t, $J = 6.4$ Hz, 1H), 3.95–3.91 (m, 1H), 3.74 (d, $J = 9.2$ Hz, 1H), 3.73–3.67 (m, 5H), 3.65–3.59 (m, 1H), 3.37–3.31 (m, 3H), 3.13 (d, $J = 7.2$ Hz, 2H), 3.08–3.05 (m, 2H), 1.87–1.80 (m, 2H), 1.76 (d, $J = 10.8$ Hz, 3H), 1.69–1.62 (m, 4H), 1.59–1.51 (m, 1H), 1.34–1.25 (m, 2H), 1.01 (d, $J = 4.4$ Hz, 3H), 0.96 (d, $J = 12.4$ Hz, 1H), 0.87 (s, 9H). ^{13}C NMR (100 MHz, CCl_3D): δ 159.41, 158.06, 150.16, 147.98, 137.22, 134.05, 123.91, 58.64, 51.72, 47.91, 46.51, 46.20, 45.70, 45.41, 42.91, 36.26, 32.54, 31.36, 30.89, 30.78, 27.67, 26.78, 26.70, 20.73. MS (ESI) m/z $[\text{M} + \text{H}]^+$: 482.1.

(R)-3-((4-tert-Butylcyclohexyl)methyl)-4-(cyclohexylmethyl)-1-(4-((S)-2-imino-3-(2-(pyridin-3-yl)ethyl)imidazolidin-4-yl)butyl)imidazolidin-2-imine (306)—Using general scheme for the synthesis of **F**, compound **306** was synthesized using the following reagents: 3-pyridinylacetic acid (R_1), Boc-D-cyclohexylalanine (R_2), 4-tert-butyl-cyclohexanecarboxylic acid (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, D_2O): δ 8.55–8.45 (m, 2H), 7.88 (d, $J = 7.6$ Hz, 1H), 7.51–7.49 (m, 1H), 4.00–3.90 (m, 2H), 3.82 (t, $J = 9.2$ Hz, 1H), 3.77–3.72 (m, 1H), 3.68 (t, $J = 7.2$ Hz, 1H), 3.62–3.55 (m, 1H), 3.37–3.33 (m, 4H), 3.24–3.11 (m, 2H), 3.06–2.97 (m, 2H), 1.92–1.75 (m, 5H), 1.74–1.60 (m, 8H), 1.58–1.50 (m, 2H), 1.43–1.36 (m, 2H), 1.32–1.25 (m, 4H), 1.17 (d, $J = 10.8$ Hz, 1H), 1.02–0.92 (m, 7H), 0.87 (s, 9H). ^{13}C NMR (100 MHz, D_2O): δ 158.27, 156.96, 148.71, 146.99, 138.36, 124.56, 59.01, 55.68, 51.42, 48.32, 47.54, 45.81, 44.50, 42.87, 39.08, 36.01, 34.02, 33.63, 32.17, 31.89, 30.71, 30.12, 27.28, 26.68, 26.09, 25.85, 20.59. MS (ESI) m/z $[\text{M} + \text{H}]^+$: 578.2.

(S)-3-((4-tert-Butylcyclohexyl)methyl)-4-(cyclohexylmethyl)-1-(4-((S)-2-imino-3-(2-(pyridin-3-yl)ethyl)imidazolidin-4-yl)butyl)imidazolidin-2-imine (307)—Using general scheme for the synthesis of **F**, compound **307** was synthesized using the following reagents: 3-pyridinylacetic acid (R_1), Boc-L-cyclohexylalanine (R_2), 4-tert-butyl-cyclohexanecarboxylic acid, predominantly trans, 98+% (R_3). Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CCl_3D): δ 8.97 (s, 1H), 8.61 (s, 1H), 8.52 (m, 1H), 8.42 (s, 1H), 7.75 (d, $J = 6.8$ Hz, 1H), 7.25–7.22 (m, 1H), 4.01–3.93 (m, 1H), 3.82 (d, $J = 7.2$ Hz, 2H), 3.65 (t, $J = 9.2$ Hz, 2H), 3.58–3.52 (m, 3H), 3.44–3.37 (m, 2H), 3.25–3.21 (m, 1H), 3.16 (t, $J = 8.0$ Hz, 1H), 2.97 (dd, $J = 14.8, 5.6$ Hz, 1H), 2.90–2.86

(m, 1H), 1.81–1.78 (m, 2H), 1.72 (d, $J = 10.8$ Hz, 5H), 1.65–1.63 (m, 4H), 1.56–1.48 (m, 3H), 1.33–1.14 (m, 8H), 1.03–0.93 (m, 7H), 0.81 (s, 9H). ^{13}C NMR (100 MHz, CDCl_3): δ 159.41, 157.33, 150.19, 147.99, 137.24, 134.03, 58.63, 55.21, 51.81, 48.40, 47.87, 46.27, 45.31, 43.00, 39.55, 36.09, 34.43, 34.38, 32.53, 31.38, 31.21, 30.72, 27.66, 26.87, 26.59, 26.26, 26.06, 20.69. MS (ESI) m/z [$M + H$] $^+$: 578.2.

(R)-3-((4-*tert*-Butylcyclohexyl)methyl)-4-(cyclohexylmethyl)-1-(4-((R)-2-imino-3-(2-(pyridin-3-yl)ethyl)imidazolidin-4-yl)butyl)-imidazolidin-2-imine (308)—Using general scheme for the synthesis of **F**, compound **308** was synthesized using the following reagents: 3-pyridinylacetic acid (R_1), Boc- D -cyclohexylalanine (R_2), 4-*tert*-butylcyclohexanecarboxylic acid, predominantly *trans*, 98+% (R_3). The only variation of the general compound synthesis for compound **308** was utilizing Fmoc- D -Lys(Boc)-OH instead of Boc-L-Lys(Fmoc)-OH. Final crude product was purified by HPLC as described above. ^1H NMR (400 MHz, CDCl_3): δ 8.59 (s, 1H), 8.54–8.48 (m, 1H), 8.40 (s, 1H), 7.78 (d, $J = 7.2$ Hz, 1H), 7.23–7.21 (m, 1H), 4.03–3.96 (m, 1H), 3.82 (d, $J = 8.4$ Hz, 1H), 3.66 (t, $J = 9.2$ Hz, 3H), 3.61–3.58 (m, 2H), 3.54–3.52 (m, 1H), 3.46–3.40 (m, 2H), 3.26–3.23 (m, 1H), 3.17 (t, $J = 8.0$ Hz, 1H), 2.97 (dd, $J = 15.2, 5.6$ Hz, 1H), 2.91–2.85 (m, 1H), 1.78–1.61 (m, 12H), 1.56–1.51 (m, 4H), 1.29–1.24 (m, 4H), 1.23–1.13 (m, 4H), 1.05–0.99 (m, 3H), 0.96–0.93 (m, 2H), 0.80 (s, 9H). ^{13}C NMR (100 MHz, CDCl_3): δ 159.34, 157.26, 150.23, 147.98, 137.33, 133.96, 123.87, 58.66, 55.21, 51.84, 48.43, 47.84, 46.33, 45.43, 43.10, 39.55, 36.09, 34.37, 32.53, 31.39, 31.18, 30.83, 30.69, 27.66, 26.87, 26.73, 26.25, 26.05, 20.73. MS (ESI) m/z calcd for $\text{C}_{35}\text{H}_{60}\text{N}_7$ [$M + H$] $^+$, 578.49; found, 578.15.

Positional Scanning Library 1169—Utilizing Scheme 1, positional scanning library 1169 was synthesized. The positional scanning library incorporates both individual and mixtures of amino acids (R_2) and carboxylic acid (R_1 and R_3). The synthetic technique facilitates the generation of information regarding the likely activity of individual compounds from the screening of the library.^{26,35,36} Equimolar isokinetic ratios have been previously determined and calculated for each of the amino and carboxylic acids utilized for the respective mixtures.^{43,44} The bis-cyclic guanidine library 1169 has a total diversity of 45864 compounds ($42 \times 26 \times 42 = 45864$). The R_1 and R_3 positions as shown in Scheme 1 (**F**) each consist of 42 carboxylic acids and the R_2 contains 26 amino acids.

Scaffold Ranking Library—The scaffold ranking library contains one sample representing each of the positional scanning libraries. This sample contains an approximate equal molar amount of each compound in that library. So, for example, the sample 1169 in the scaffold ranking library contains 45864 compounds in approximately equal molar amounts. These samples can be prepared by mixing the cleaved products of the complete positional scanning library, as was the case for sample 1169, or they can be synthesized directly as a single mixture.^{26,27}

Calculation of xIC_{50}s

It has been shown³² that the harmonic mean of the IC_{50}s of constituents within a mixture accurately models the resulting IC_{50} of the mixture. Because percent responses are generally

used as the initial screening metric, it is often necessary (as it was in this case) to extrapolate IC_{50} s for samples using the equation

$$xIC_{50} = \min \left([x] \left(\frac{100}{\%_{Resp}} - 1 \right) \times 1000 \right)$$

Here $[x]$ is the concentration tested and $\%_{resp}$ is the percent inhibition value obtained at that concentration. Obviously, these values are extrapolations and not a substitute for actual experimentally determined IC_{50} s, but they are sufficient for the order-of-magnitude estimation used in the Harmonic Mean Analysis of position scanning libraries.

Prediction of Selectivity

For a sample with $IC_{50} x_1$ against $\alpha 3\beta 4$ and x_2 against $\alpha 4\beta 2$, we define the selectivity over $\alpha 3\beta 4$ as

$$s = \frac{x_1 - x_2}{x_2}$$

To predict the selectivity of an individual compound based on the values of the positional scanning mixture, we used the standard biometrical analysis approach⁴⁵⁻⁴⁷ of using the product of the mixture activity values. In particular, if a compound has functionality A_1 at position R_1 , A_2 at position R_2 , and A_3 at position R_3 , then its Biometrical Analysis Score would be

$$BA = \left(\frac{(\text{Activity of } A_1 \text{ Mixture at the } R_1 \text{ Position}) \times (\text{Activity of } A_2 \text{ Mixture at the } R_2 \text{ Position}) \times (\text{Activity of } A_3 \text{ Mixture at the } R_3 \text{ Position})}{(\text{Activity of } A_1 \text{ Mixture at the } R_1 \text{ Position}) \times (\text{Activity of } A_2 \text{ Mixture at the } R_2 \text{ Position}) \times (\text{Activity of } A_3 \text{ Mixture at the } R_3 \text{ Position})} \right)^{1/3}$$

Given BA values for $\alpha 3\beta 4$ and $\alpha 4\beta 2$, the predicted selectivity is then found using the same formula as above:

$$s_p = \frac{BA_1 - BA_2}{BA_2}$$

Cell Culture

KX $\alpha 3\beta 4R2$ and KX $\alpha 4\beta 2R2$ cells, containing rat $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChR respectively (obtained from Drs. Kenneth Kellar and Yingxian Xiao, Georgetown University), were cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 0.5% penicillin/streptomycin, and 0.4 mg/mL of geneticin and were maintained in an atmosphere of 7.5% CO_2 in a humidified incubator at 37 °C. For binding assays, cells were passaged on 150 mm dishes and harvested when confluent.

Binding Assays

Cells were harvested by scraping the plates with a rubber policeman, suspended in 50 mM Tris buffer pH 7.4, homogenized using a Polytron homogenizer, and the centrifugation was repeated twice at 20000g (13500 rpm) for 20 min. For binding, the cell membranes were incubated with the test compounds or mixtures in the presence of 0.3 nM of [³H]epibatidine. After 2 h of incubation, at room temperature, samples were filtered, using a Tomtec cell harvester, through glass fiber filters that had been presoaked in 0.05% polyethyleneimine. Filters were counted on a betaplate reader (Wallac). Nonspecific binding was determined by using 1 μM of AT-1001 or 0.1 μM of unlabeled epibatidine. IC₅₀ values were determined by using the program Graphpad/PRISM. K_i values were calculated using the Cheng-Prusoff transformation: $K_i = IC_{50}/(1 + L/K_d)^{48}$ where, L is radioligand concentration and K_d is the binding affinity of the radioligand, as determined previously by saturation analysis.

nAChR Functional Assays

nAChR functional activity was determined by measuring nAChR-induced membrane potential change, which can be directly read by Molecular Devices Membrane Potential Assay Kit (Blue Dye) (Molecular Devices), using the FlexStation 3 microplate reader (Molecular Devices). The HEK cells transfected with α3β4 or α4β2 nAChR were seeded in a 96-well plate (4000 cells per well) one day prior to the experiments. For agonist assays, after brief washing, the cells were loaded with 225 μL of HBSS assay buffer (Hank's Balanced Salt Solution with 20 mM of HEPES, pH 7.4), containing the blue dye, and incubated at 37°C. After 30 min, 25 μL of the appropriate compounds were dispensed into the wells by the FlexStation and nAChR stimulation-mediated membrane potential change was recorded every 3 s for 120 s by reading 550–580 nm fluorescence excited at 530 nm wavelength. For the antagonist assay, the cells were loaded with 200 μL of HBSS buffer containing the blue dye and incubated at 37 °C. After 20 min, 25 μL of test compounds were added, and after another 10 min, 25 μL of epibatidine (or nicotine) was added by the FlexStation to a final concentration of 100 nM, with fluorescence measured as described above. The change in fluorescence represents the maximum response minus the minimum response for each well. Graphpad PRISM was used to determine the EC₅₀ and IC₅₀ values.

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ABBREVIATIONS USED

nAChR	nicotinic acetylcholine receptors
DHβE	dihydro-β-erythroidine hydrobromide
18-MC	18-methoxycoronaridine
GWAS	Genome Wide Association Studies
MHb	medial habenula

KO	knockout
NAc	nucleus accumbens
ADAM 17	ADAM metalloproteinase domain 17
MBHA	<i>p</i> -methylbenzhydryl-amine resin
DIEA	diisopropylethylamine
DCM	dichloromethane
Fmoc	9-fluorenylmethoxycarbonyl
Boc	<i>tert</i> -butoxycarbonyl
DMF	dimethylformamide
DIC	diisopropylcarbodiimide
HOBt	hydroxybenzotriazole hydrate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
LCMS	liquid chromatograph/mass spectrometer
BA	biometric analysis
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

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Figure 1.

Approach used to identify potent and selective (over $\alpha 3\beta 4$) $\alpha 4\beta 2$ ligands. Starting with just 34 samples comprised of >5 million compounds in the Scaffold Ranking Library, a specific Positional Scanning Library (1169) was identified for screening. This library contained 110 samples, and the results from screening the library were used in the selection of 18 compounds that were synthesized and tested (Deconvolution). On the basis of these results, eight new optimized compounds were synthesized and tested and found to be potent and selective (over $\alpha 3\beta 4$) $\alpha 4\beta 2$ ligands. In total, only 170 samples were tested; this shows the broad utility of this approach for many assay formats, especially those limited for whatever reason by throughput.

Figure 2.

Scaffold Ranking Library data for the 10 $\mu\text{g/mL}$ dose (approximately 20 μM assuming an average molecular weight of 500 Da for each compound in a given mixture). Values are shown as percent inhibition of [^3H]epibatidine binding to $\alpha 4\beta 2$ (red) and $\alpha 3\beta 4$ (blue) nAChR. The numbers along the x-axis represent the specific Scaffold Ranking Library sample, sc = solvent control (0.1%DMF), and the EPI (no.) are different concentrations of epibatidine (EPI(1) = 1 nM, (10) = 10 nM, (100) = 100 nM, (1000) = 1000 nM).

Figure 3.

The values for “Projected Selectivity” and “Experimental Selectivity” shown in Table 2 for the 18 compounds are graphed in the scatter plot. Each dot corresponds to one of the 18 compounds. The Experimental Selectivity value is calculated using the following equation $(K_i \alpha 3\beta 4 - K_i \alpha 4\beta 2)/(K_i \alpha 4\beta 2)$. The Projected Selectivity value is a value “projected” from using the 1169 library data. The methodology used to determine the Projected Selectivity is detailed in Materials and Methods. In brief, first a projected activity value for each compound is determined for both receptors. This is done by taking the product of the activity value of the 1169 positional scanning sample that corresponds with the functionality used in the Table 2 individual compounds. Once this value is obtained for each of the compounds and for both receptors, the Projected Selectivity value can be determined using the same selectivity formula used for the Experimental Selectivity values.

Figure 4. Hit optimization of compounds **207** and **208**. The colored stars and arrows correspond to site specific analogue modifications.

Figure 5.

Antagonist activity of compound **301**. **301** has no agonist activity but potently blocks nicotine-induced increase in membrane potential in $\alpha 4\beta 2$ -containing cells. **301** has potential inverse agonist activity at very high concentrations.

Figure 6.

Compound **301** inhibits nicotine-induced $\alpha 4\beta 2$ nAChR stimulation in a dose-dependent manner. Increasing concentrations of **301** induce a progressive decrease in maximal activity with a slight change in the EC_{50} . EC_{50} and maximum stimulation were: 779 nM and 100%, 999 nM and 83%, 1809 nM and 38%, EC_{50} not measurable, maximum 0, for 0, 0.3, 1.0, and 3.0 μM **301**, respectively.

Scheme 1.
Synthesis of Bis-cyclic Guanidines

Table 1

The 110 Samples That Make up Library 1169, Their Associated xIC_{50} Values for $\alpha 4\beta 2$ and $\alpha 3\beta 4$ nAChR, and Fold Selectivity Are Reported^a

#	R1	R2	R3	$\alpha 3\beta 4$ xIC_{50} (μM)	$\alpha 4\beta 2$ xIC_{50} (μM)	Fold Selectivity $\alpha 3\beta 4 > 0$
1	(1-phenyl-cyclopropyl)-methyl	X	X	203	135	0.51
2	2-phenylbutyl	X	X	77	28	1.78
3	3-phenylbutyl	X	X	63	45	0.40
4	<i>m</i> -tolylethyl	X	X	65	31	1.07
5	2-(3-fluoro-phenyl)-ethyl	X	X	60	7	7.12
6	2-(3-bromo-phenyl)-ethyl	X	X	23	7	2.45
7	2-(3-trifluoromethyl-phenyl)-ethyl	X	X	30	33	-0.10
8	<i>p</i> -tolylethyl	X	X	121	17	6.22
9	2-(4-fluoro-phenyl)-ethyl	X	X	63	10	5.06
10	2-(3-methoxy-phenyl)-ethyl	X	X	110	41	1.71
11	2-(4-bromo-phenyl)-ethyl	X	X	33	14	1.33
12	2-(4-methoxy-phenyl)-ethyl	X	X	256	45	4.74
13	2-(4-ethoxy-phenyl)-ethyl	X	X	469	57	7.19
14	2-(4-isobutyl-phenyl)-propyl	X	X	157	81	0.95
15	3,4-dichlorophenethyl	X	X	12	10	0.21
16	2-(3,5-bis-trifluoromethyl-phenyl)-ethyl	X	X	100	50	0.99
17	3-(3,4-dimethoxy-phenyl)-propyl	X	X	121	41	1.97
18	phenethyl	X	X	75	18	3.11
19	3,4,5-trimethoxy-benzyl	X	X	1398	251	4.57
20	butyl	X	X	188	30	5.35
21	heptyl	X	X	129	16	7.20
22	isobutyl	X	X	213	122	0.74
23	2-methylbutyl	X	X	121	84	0.44
24	3-methylbutyl	X	X	191	45	3.26
25	3-methylpentyl	X	X	180	37	3.93
26	4-methylpentyl	X	X	241	18	12.32
27	4-methyl-benzyl	X	X	391	382	0.02
28	cyclopentyl-methyl	X	X	242	74	2.29
29	cyclohexyl-methyl	X	X	251	60	3.21
30	cyclohexyl-ethyl	X	X	220	33	5.77
31	cyclohexyl-butyl	X	X	98	17	4.62
32	cycloheptyl-methyl	X	X	193	41	3.65
33	(2-methyl-cyclopropyl)-methyl	X	X	134	91	0.47
34	cyclobutyl-methyl	X	X	106	55	0.93
35	3-cyclopentyl-propyl	X	X	63	15	3.24
36	cyclohexyl-propyl	X	X	56	21	1.69
37	4-methyl-1-cyclohexyl-methyl	X	X	76	36	1.14
38	4- <i>tert</i> -butyl-cyclohexyl-methyl	X	X	120	70	0.71
39	2-Biphenyl-4-yl-ethyl	X	X	36	36	0.02
40	adamantan-1-yl-methyl	X	X	75	78	-0.04
41	2-adamantan-1-yl-ethyl	X	X	236	65	2.65
42	2-Bicyclo[2.2.1]hept-2-yl-ethyl	X	X	110	24	3.60
43	X	S-methyl	X	62	47	0.32
44	X	S-benzyl	X	46	21	1.16
45	X	hydrogen	X	96	39	1.49
46	X	S-2-butyl	X	137	35	2.93
47	X	S-isobutyl	X	172	42	3.07
48	X	R-hydroxymethyl	X	911	69	12.30
49	X	(R,R)-1-hydroxyethyl	X	889	94	8.41

#	R1	R2	R3	$\alpha 3\beta 4$ xIC_{50} (μM)	$\alpha 4\beta 2$ xIC_{50} (μM)	Fold Selectivity $\alpha 3\beta 4 > 0$
50	X	S-isopropyl	X	505	61	7.35
51	X	S-4-hydroxybenzyl	X	58	25	1.37
52	X	R-methyl	X	67	40	0.68
53	X	R-benzyl	X	66	6	9.38
54	X	R-2-butyl	X	119	40	1.99
55	X	R-isobutyl	X	350	37	8.53
56	X	S-hydroxymethyl	X	1036	75	12.80
57	X	(S,S)-1-hydroxyethyl	X	329	45	6.38
58	X	R-isopropyl	X	127	49	1.60
59	X	R-4-hydroxybenzyl	X	62	15	3.21
60	X	S-phenyl	X	45	53	-0.16
61	X	S-propyl	X	170	64	1.67
62	X	R-propyl	X	90	28	2.18
63	X	S-butyl	X	65	45	0.46
64	X	R-butyl	X	53	17	2.14
65	X	S-2-naphthylmethyl	X	27	30	-0.08

^aLibrary 1169 is made up of 45864 bis-cyclic guanidines all containing the same central scaffold shown at the top of Table 1. The compounds are systematically synthesized into a positional scanning library containing the 110 samples shown in the table. By way of example, sample 1 is an approximate equal molar mixture of 1092 compounds. The 1092 compounds contain (1-phenyl-cyclopropyl)-methyl fixed in the R₁ position, and all 1092 combinations of the 26 R₂ (shown in Table 1 43–68) and 42 functionalities at R₃ (Table 1 69–110). Similarly, sample 83 contains the 1092 compounds generated from fixing R₃ with 3,4-dichlorophenethyl and utilizing all 1092 combinations of the 42 R₁ functionalities and 26 R₂ functionalities. The xIC₅₀ and fold selectivity values were calculated as described in the Materials and Methods section. The xIC₅₀ values are further color coded using a three-color scale: red (most active), white, blue (least active). The fold selectivity values are also color coded using a three-color scale: green (more selective for $\alpha 4\beta 2$), yellow, red (less selective for $\alpha 4\beta 2$). A value of 0 for fold selectivity means a sample produced the same activity in both receptors.

Table 2

The Functionalities, K_i Values, and Selectivity (Both Experimentally Determined and Projected) for the 18 Individual Compounds Synthesized Based on the Data Generated from the Screening of 1169 are Shown^a

Compound #	R1	R2	R3	$\alpha 3\beta 4$ K_i (μ M)	$\alpha 4\beta 2$ K_i (μ M)	Experimental Selectivity $\alpha 3\beta 4 > 0$	Projected Relative Selectivity $\alpha 3\beta 4 > 0$
201	3,4-dichlorophenylethyl	R-2-naphthylmethyl	2-(3-bromo-phenyl)-ethyl	3.0	1.9	0.6	0.8
202	3,4-dichlorophenylethyl	R-2-naphthylmethyl	2-(4-bromo-phenyl)-ethyl	4.0	2.5	0.6	0.5
203	3,4-dichlorophenylethyl	R-2-naphthylmethyl	3,4-dichlorophenethyl	347.4	47.5	6.3	0.2
204	3,4-dichlorophenylethyl	S-2-naphthylmethyl	2-(3-bromo-phenyl)-ethyl	76.0	43.4	0.8	0.4
205	3,4-dichlorophenylethyl	S-2-naphthylmethyl	2-(4-bromo-phenyl)-ethyl	2.7	3.1	-0.1	0.2
206	3,4-dichlorophenylethyl	S-2-naphthylmethyl	3,4-dichlorophenethyl	100.0	183.2	-0.5	-0.1
207	2-(3-fluoro-phenyl)-ethyl	R-benzyl	4-tert-butyl-cyclohexyl-methyl	25.7	1.3	19.0	6.6
208	2-(3-fluoro-phenyl)-ethyl	R-cyclohexyl	4-tert-butyl-cyclohexyl-methyl	106.9	4.0	25.9	5.3
209	heptyl	R-benzyl	4-tert-butyl-cyclohexyl-methyl	54.4	2.7	19.3	6.6
210	heptyl	R-cyclohexyl	4-tert-butyl-cyclohexyl-methyl	55.1	3.9	13.2	5.4
211	4-methylpentyl	R-benzyl	4-tert-butyl-cyclohexyl-methyl	68.4	2.3	28.5	7.9
212	4-methylpentyl	R-cyclohexyl	4-tert-butyl-cyclohexyl-methyl	78.4	3.5	21.2	6.5
213	2-(3-fluoro-phenyl)-ethyl	R-benzyl	2-(3-bromo-phenyl)-ethyl	20.4	1.8	10.1	3.9
214	2-(3-fluoro-phenyl)-ethyl	R-cyclohexyl	2-(3-bromo-phenyl)-ethyl	20.1	3.5	4.8	3.1
215	heptyl	R-benzyl	2-(3-bromo-phenyl)-ethyl	14.4	2.5	4.8	4.0
216	heptyl	R-cyclohexyl	2-(3-bromo-phenyl)-ethyl	23.4	2.8	7.2	3.2
217	4-methylpentyl	R-benzyl	2-(3-bromo-phenyl)-ethyl	47.8	2.6	17.5	4.8
218	4-methylpentyl	R-cyclohexyl	2-(3-bromo-phenyl)-ethyl	37.3	3.3	10.2	3.9

^aThe functionalities correspond to the core scaffold shown in Table 1. The activity data is presented as a K_i (μ M) for each individual compound against each of the two targets. The experimental selectivity is the value obtained from $(K_i \alpha 3\beta 4 - K_i \alpha 4\beta 2)/(K_i \alpha 4\beta 2)$. The projected relative selectivity value is generated from using only library 1169 data as described in the Materials and Methods section. The K_i values are further color coded using a three-color scale: red (most active), white, blue (least active). The fold selectivity values are also color coded using a three-color scale: green (more selective for $\alpha 4\beta 2$), yellow, red (less selective for $\alpha 4\beta 2$). A value of 0 for fold selectivity means a sample produced the same activity in both receptors. Note the strong correlation between the experimental and projected selectivity values ($R^2 = 0.831$), also shown in Figure 3.

Table 3Binding Affinity, Selectivity, and Antagonist Activity of Selected Individual Compounds^a

compd	<u>[³H]epibatidine binding</u>		selectivity (fold) (α 3 β 4 K_i / α 4 β 2 K_i)	α 4 β 2 antagonist potency IC ₅₀ (nM)
	α 3 β 4 K_i (nM)	α 4 β 2 K_i (nM)		
207	61750 ± 36030	1587 ± 301	39	26320 ± 980
208	88895 ± 18005	3731 ± 237	24	5319 ± 346
301	56480 ± 8920	118 ± 2	478	514 ± 84
302	23765 ± 5345	323 ± 152	74	1465 ± 43
303	27550 ± 7150	433 ± 79	64	2820 ± 82
304	63320 ± 3560	307 ± 78	206	3024 ± 125
305	51893 ± 27353	2217 ± 1194	23	2953 ± 594
306	59678 ± 15253	340 ± 148	176	2830 ± 75
307	78085 ± 8355	611 ± 220	128	3405 ± 125
308	33353 ± 4373	257 ± 113	130	1686 ± 373
DH β E	>10000	485 ± 33	>20	624 ± 24

^aExperiments were conducted a minimum of two times by methods described in Materials and Methods. Values shown are mean ± SEM for experiments conducted in triplicate at least twice.