Structure-based shape pharmacophore modeling for the discovery of novel anesthetic compounds

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ABSTRACT

Current anesthetics, especially the inhaled ones, have troublesome side effects and may be associated with durable changes in cognition. It is therefore highly desirable to develop novel chemical entities that reduce these effects while preserving or enhancing anesthetic potency. In spite of progress toward identifying protein targets involved in anesthesia, we still do not have the necessary atomic level structural information to delineate their interactions with anesthetic molecules. Recently, we have described a protein target, apoferritin, to which several anesthetics bind specifically and in a pharmacodynamically relevant manner. Further, we have reported the high resolution X-ray structure of two anesthetic/apoferritin complexes (Liu, R.; Loll, P. J.; Eckenhoff, R. G. *FASEB J.* **2005**, *19*, 567). Thus, we describe in this paper a structure-based approach to establish validated shape pharmacophore models for future application to virtual and high throughput screening of anesthetic compounds. We use the 3D structure of apoferritin as the basis for the development of several shape pharmacophore models. To validate these models, we demonstrate that (1) they can be used to effectively recover known anesthetic agents from a diverse database of compounds; (2) the shape pharmacophore scores afford a significant linear correlation with the measured binding energetics of several known anesthetic compounds to the apoferritin site; and (3) the computed scores based on the shape pharmacophore models also predict the trend of the EC_{50} values of a set of anesthetics. Therefore, we have now obtained a set of structure-based shape pharmacophore models, using ferritin as the surrogate target, which may afford a new way to rationally discover novel anesthetic agents in the future.

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1. Introduction

General anesthesia, introduced formally in the mid nineteenth century, is now delivered to 40 million patients per year in the United States. Despite its central role in healthcare, a molecular understanding of anesthesia or anesthetics is still very poor. Its definitions are at best operational and convey little understanding of the underlying neurobiology or the molecular mechanisms as to how these molecules interact with the body's receptors.[1] Contemporary anesthetics, especially the inhaled ones, are well recognized to have troublesome physiologic side effects, and may even have durable cognitive effects, especially at the extremes of age.[2,3] Thus, it is highly desirable to discover novel chemical entities that can be used as either probes to help understand the molecular mechanisms of anesthesia, or as lead compounds for further development into novel and safer anesthetic agents.

Most investigators now believe that anesthetics produce their effects by interacting directly with specific proteins via unique binding sites.[4,5] Some of these targets may be more important than others in producing the desired endpoint, and more mechanistically linked to some endpoints than others. For example, it is widely held that members of the Cys-loop ligand-gated ion channel family are uniquely sensitive and plausible targets underlying hypnosis.[6] The $GABA_A$ receptor, known through years of mechanisms research to be anesthetic sensitive, produces anxiolysis and amnesia, two essential and desirable components of general anesthesia.[7] In spite of knowing the probable involvement of $GABA_A$ in anesthesia, we do not have the necessary atomic level structural information to delineate its interactions with anesthetic molecules. High resolution structures of plausible targets, like $GABA_A$ receptors, are exceedingly difficult to obtain, owing to the low-abundance of these receptors, their complex heterooligomeric nature, and the fact that they are poorly soluble membrane proteins. Thus, structure-based rational drug discovery techniques cannot be directly applied to analyze these protein targets; structures of

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anesthetic binding sites from other, surrogate proteins, may be needed for this purpose. The protein data bank has the structures of only three protein complexes with clinically-used general anesthetics: human serum albumin,[8] horse apoferritin[9] and a de novo designed 4-helix bundle.[10,11]

In the pursuit of proteins to serve as an anesthetic template, one of our authors has discovered that apoferritin mimics features thought to exist for the GABA$_A$ receptor's anesthetic site.[5,9,12] In addition, the binding energetics of a wide range of anesthetic compounds to this apoferritin site correlates extremely well to the potency for producing anesthesia in a mammal.[13] This has laid a sound foundation for employing structure-based drug design (CADD) tools to discover improved GABAergic general anesthetics.

Although commercially available structure-based tools, such as Gold,[14] FRED,[15] DOCK,[16] AutoDock[17] and FlexX,[18] can be used for our purpose, we have employed a new shape pharmacophore modeling method developed in our laboratories due to its effectiveness and speed for virtual screening.[19] It uses the architecture and physicochemical texture of the binding pocket to perform virtual screening experiments. The binding pocket is modeled as an inverse shape with complementary functionalities to the binding pocket, and then used to screen in silico against large chemical libraries. More specifically, we first derive the shape from the X-ray structure of ligand-bound ferritin, then extract the complementary pharmacophore information with a procedure similar to that adopted by LigandScout.[20] We then combine the shape and pharmacophore information and represent it using the shape functions encoded in the OEShape ToolKit.[21] To validate the effectiveness of the shape pharmacophore models, we used a database of diverse chemical structures selected from Asinex database, and mixed them with known anesthetic agents. The results show that the known anesthetic agents are always ranked at the top, together with a few other new molecules. We further show that the scores of the known anesthetic agents correlate in a linear fashion to the binding data (K_D), even though the scoring functions were never calibrated using these data. The shape pharmacophore scores are also correlated, in a less linear fashion, with the EC$_{50}$ data of 14 known anesthetic compounds.

2. Materials and methods

2.1. X-ray structures of ferritin

Two X-ray crystal structures have been used in this work for creating the shape pharmacophore models. They are the structure of apoferritin complexed with isoflurane (1XZ3), and that of halothane complexed with apoferritin (1XZ1). Both structures were solved at 1.75 A resolution.[9] The dimer structures were generated based on the crystal symmetry information before being used by the SHAPE4 program[19] for virtual screening.

2.2. Binding pocket shape pharmacophore extraction

The SHAPE4 program employs a computational geometry algorithm (i.e., alpha-shape analysis) to detect the binding site atoms and generate a negative image of the binding pocket. This negative image is then represented by a set of spheres, and converted into the shape representation functions in OEShape Toolkit.[21] The overall flow of the SHAPE4 program involves the following steps: (1) the α-shape program is used to detect potential binding site atoms and the Delauney tetrahedra formed by these atoms for a given protein structure; (2) a program is then developed to calculate orthogonal centers defined by the vertices of the above Delauney tetrahedra, and generate inner spheres around each orthogonal center; (3) the overall shape of this collection of spheres is then represented

by GAUSSIAN functions; and (4) the shape representation is then used by SHAPE4 to query a database of molecules whose conformers are pre-generated. To allow for more detailed information to be used, pharmacophore group information is derived from the ferritin complex structures (with isoflurane and halothane), and added in the shape pharmacophore models. As reported in the SHAPE4 publication,[19] it implements an efficient, structure-based shape matching technology for virtual screening.

In this work, since a known ligand molecule (i.e., isoflurane in 1xz3 or halothane in 1xz1) is bound to the binding pocket of ferritin, we skipped the step of α-shape analysis. Instead, we directly used the coordinates of the bound ligand as the reference to conduct Delauney tessellation so that binding pocket residues form a set of Delauney tetrahedra with the ligand atoms. The space occupied by this set of tetrahedra fully characterizes the binding pocket of ferritin.

2.3. Application of the shape pharmacophore models to virtual screening

As described above, the overall workflow of SHAPE4 involves (1) creating the binding pocket shape model that incorporates both the shape and pharmacophore information, and then (2) applying the shape pharmacophore models to search multi-conformer database using the SHAPE4 program. Omega[22] was used to generate the multi-conformer molecular database. Kirchmair parameters were adopted in this work due to its reported effectiveness in generating bioactive conformers.[23] We increased the maximum number of allowed conformers from 500 to 2000. Our own studies indicated that conformer generation with a maximum number of 2000 could enumerate conformers that were much more similar to the experimental ligand conformations in crystal structures when compared to either the default OMEGA setting, or those recommended by Kirchmair. The whole set of database molecules are virtually screened, that is, scored with the shape pharmacophore models, and are then ranked according to the scores, which characterizes the fitness of shape matching as well as the fitness based on combined shape and pharmacophore matching.

2.4. Validation of shape pharmacophore models via retrospective discovery of known anesthetics

One of the standard approaches to validating computational model(s) is to conduct retrospective analysis based on virtual screening results. To this end, we constructed a database of molecules consisting of known anesthetic molecules and a set of randomly sampled molecules. The randomly sampled set was obtained from a collection offered by Asinex.[24] The known anesthetic compounds include propofol analogs,[25] isoflurane and halothane. With this database, we can conduct virtual screening (as described above) using the SHAPE4 models, and examine the enrichment curves. The enrichment curves are obtained by calculating the percentage of known anesthetics found at different fraction levels of the SHAPE4-ranked database. Figure 1 has summarized the overall workflow from binding site detection to virtual screening using SHAPE4. As described in previous two sections, the binding pocket is first defined by applying the Delauney tessellation procedure to the X-ray structure of ferritin–ligand complex (1xz3 or 1xz1). This procedure defines the space formed by both the ligand atoms and the ferritin atoms, hence the binding pocket space. To allow for SHAPE4 to use this information, this space is further approximated by a grid representation, which is used by OEShape ToolKit functions inside the SHAPE4 program. The ferritin–ligand structure is also used to define pharmacophore centers that are complementary to the binding pocket characteristics in terms of hydrogen bond acceptor/donor, hydrophobic, or charged centers.
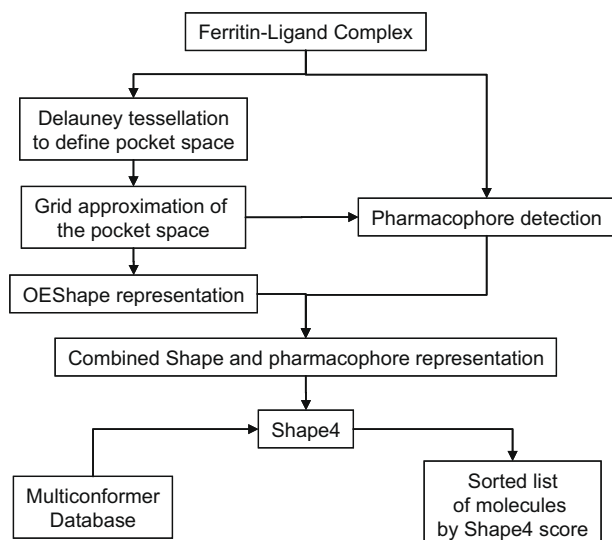
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Figure 1. Flowchart for virtual screening using the SHAPE4 method.

Table 2
EC$_{50}$ values of 14 compounds[25]

Compounds	EC$_{50}$
Phenol	3.0
2,6-Dimethylphenol	3.9
2-Isopropylphenol	4.4
2,6-Diethylphenol	4.9
2-tert-Butyl-6-methylphenol	6.3
2,6-Diethyphenylbromide	4.7
2,6-Diethyphenylisocyanate	4.6
2,6-Diethyphenylisothiocyanate	4.8
2,6-Diisopropylphenol(propofol)	5.7
3,5-Diisopropylcatechol	4.4
3,5-Di-tert-butylphenol	4.0
4-Iodo-2,6-diisopropylphenol	5.0
2,6-Di-sec-butylphenol	5.6
2,4-Di-sec-butylphenol	4.5

The combined shape and pharmacophore information is used by the SHAPE4 program to score potential hits against the ferritin target. The sorted list of molecules based on the SHAPE4 score is then used to generate the aforementioned enrichment curves. Since the whole methodology has been published, we refer interested readers to the original article for more details.[19]

2.5. Binding data of known anesthetic molecules with ferritin

We have experimentally obtained the dissociation constants (K_D) for a set of known anesthetic agents with apoferritin measured by the ITC (Isothermal Titration Calorimetry) technique (Table 1). Our goal is to obtain new experimentally determined data to test the shape pharmacophore models quantitatively, by examining the correlations between the K_D values and the calculated shape pharmacophore scores. We note that these K_D values have been obtained as a totally separate experimental endeavor from the computational modeling. No retrospective fitting has been performed. Thus, they should be considered as an experimental validation of the SHAPE4 methodology.

2.6. EC$_{50}$ data of known anesthetic molecules

The EC$_{50}$ data of a group of 14 anesthetic active molecules have been published by Krasowski et al.[25] for GABA$_A$ current enhancement. This is considered to be an important in vitro feature for a large group of general anesthetics, and may contribute to the observed in vivo effect (anesthesia). A correlation between the shape pharmacophore model scores and these data would therefore test not only the ability of the in silico approach to model apoferritin

binding, but the relevance of the template itself to proteins of in vivo significance (Table 2).

3. Results and discussion

Here, we report the results of a computational geometry analysis of the X-ray structures of ferritin bound to halothane and isoflurane. We also describe several shape pharmacophore models that can be derived by focusing on different portions of the ferritin binding pocket. We then show the enrichment curves obtained from virtual screening experiments with each of these pharmacophore models. We will explore the relationship between the binding energetic data (K_D) of a few anesthetic molecules as well as that between the known GABA$_A$ EC$_{50}$ data and the shape pharmacophore scores.

3.1. Delauney triangulation of the 3D structures of ferritin

An important feature emerging from a computational geometry analysis[26–28] of the apoferritin structure is the prominent 'U'-shaped concave binding pocket located at the dimer interface of two 4-helix bundles, which accommodates halothane, isoflurane and propofol analogs. The molecular graphics is shown in Figure 2, in which the binding pocket located at the dimer interface is shown.

3.2. Shape pharmacophore models

We generated the 3D negative image of the apoferritin binding pocket, which faithfully reflects the shape of the binding pocket. Three most reasonable strategies to derive the shape pharmacophore models include (A) the model derived from the deepest 'base' portion of the ferritin-dimer binding pocket; (B) model derived from about half of the ferritin-dimer binding pocket; and (C) the model derived from the entire ferritin-dimer binding pocket. The derived shape pharmacophore models are shown in Figure 3.

3.3. Virtual screening experiments found known anesthetics at the top of the rank

Using the three shape pharmacophore models described above (Fig. 3), we have conducted three virtual screening experiments. Strategy A focuses on the binding pocket for known anesthetic agents, halothane and isoflurane, bound to ferritin. These experiments should demonstrate that SHAPE4 can discover/recover halothane, isoflurane and other related compounds from among a set of randomly sampled molecules. Due to the strict constraints put

Table 1
K_D data for five known anesthetic compounds

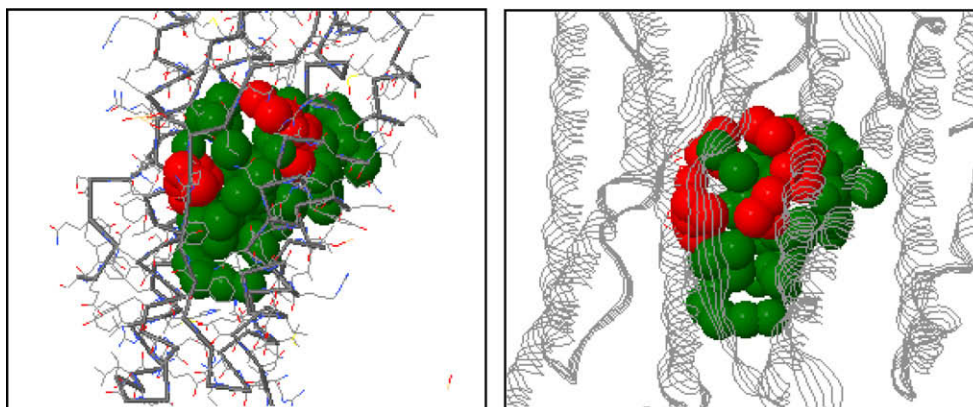|	MW	Concn (mM) in buffer	Binding	K_a
Propofol (2,6-diisopropylphenol)	178.28	0.23	Yes	2.5E+05
Diethyphenol	150	0.31	Yes	7.1E+04
2-Isopropylphenol	136.19	0.50	Yes	6.7E+04
2,6-Dimethyl phenol	122.17	0.36	Yes	1.7E+04
Phenol	94	3.8	Yes	1600

Figure 2. The anesthetic binding pockets located on the dimer interface between two 4-helix bundles of apoferritin molecules. The same binding pocket can accommodate (left) halothane, and (right) isoflurane. Residues colored in red are those that directly interact with anesthetics.
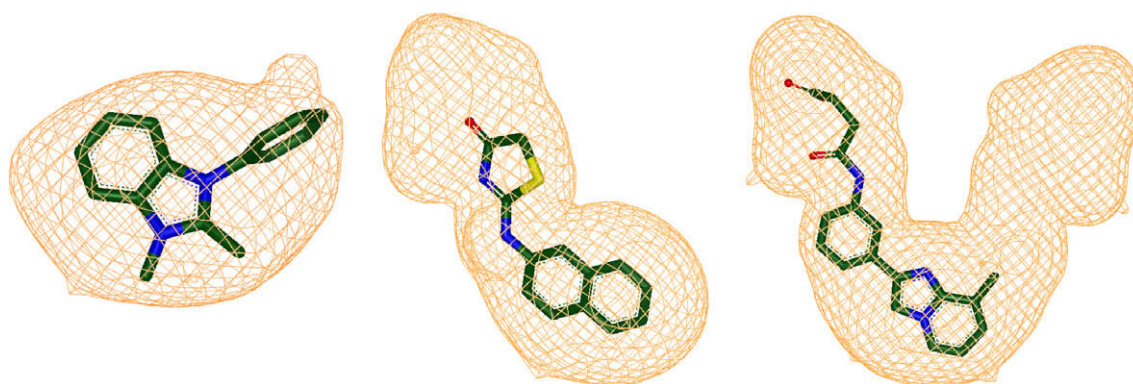

Figure 3. Three strategies to create the shape pharmacophore models. Strategy A uses only the base portion of the binding pocket (left), strategy B uses one side of the dimer binding pocket (middle), and strategy C uses the whole binding pocket formed by the dimer (right). In each case, we show one of the molecules that can fit to the model.

on the query, this model should prevent us from finding a more diverse set of candidate compounds that may also bind to ferritin. Strategy B uses half of the ferritin-dimer binding pocket to create the SHAPE4 query. This strategy captures additional space and pharmacophore features available in the ferritin binding pocket, and thus, potentially affords an opportunity to find more diverse set of candidate compounds. Strategy C uses the whole binding pocket formed by the ferritin-dimer structure. This last strategy should afford the most opportunity to find additional diverse set of compounds. These points are demonstrated in the enrichment curves (Figs. 4–6).

Enrichment curves were generated to demonstrate the effectiveness of the virtual screening experiments. As described in Section 2, we used a diverse subset of compounds extracted from the Asinex database as 'decoys' and mixed them with known anesthetic agents (halothane, isoflurane and propofol analogs). We then test if SHAPE4 can discover known anesthetic molecules from this set of mixed molecules with each of the three shape pharmacophore models.

For strategy A, the enrichment curves have the steepest ascent at around 1% of the total database. This means that all the known agents are found at the very top of the list. However, the constraints
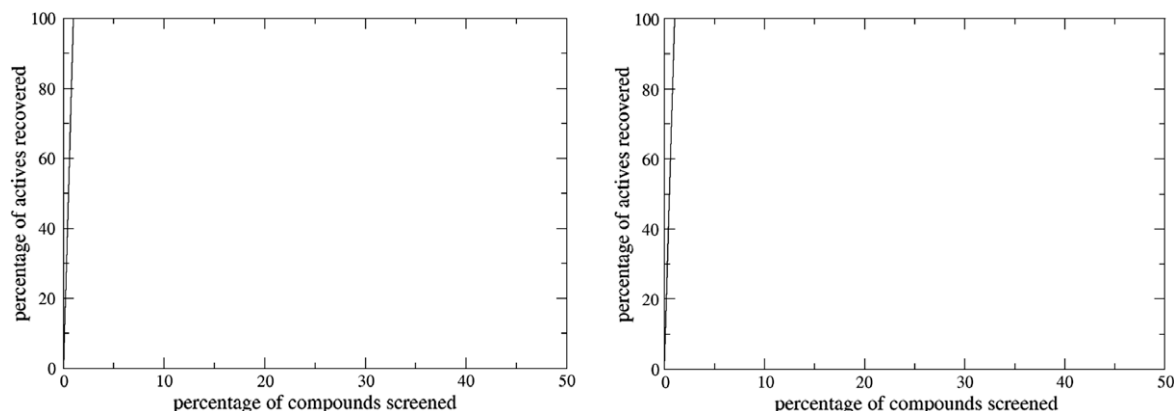

Figure 4. Enrichment curves obtained from virtual screening using strategy-A derived shape pharmacophore models. Left: halothane–ferritin-dimer derived model. Right: isofluorane–ferritin-dimer derived model.
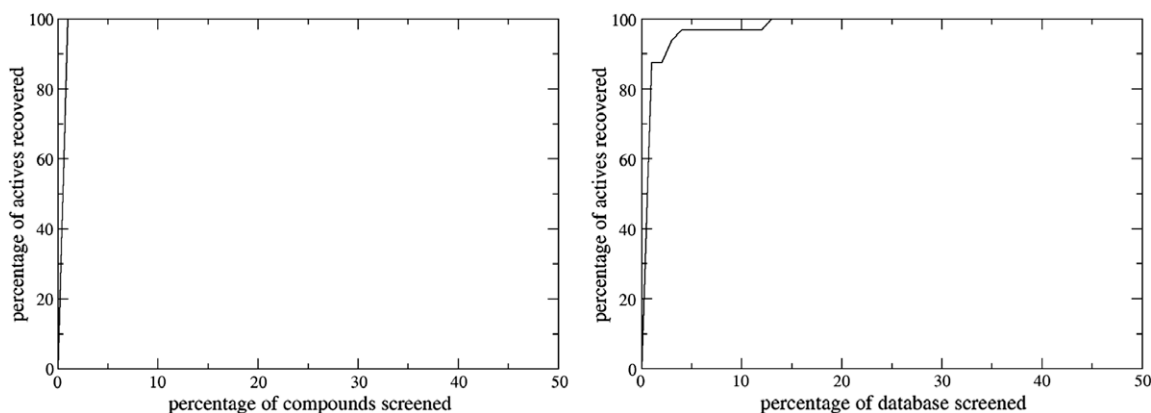
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Figure 5. Enrichment curves obtained from virtual screening using strategy-B derived shape pharmacophore models. Left: halothane–ferritin-dimer derived model. Right: isofluorane–ferritin-dimer derived model.
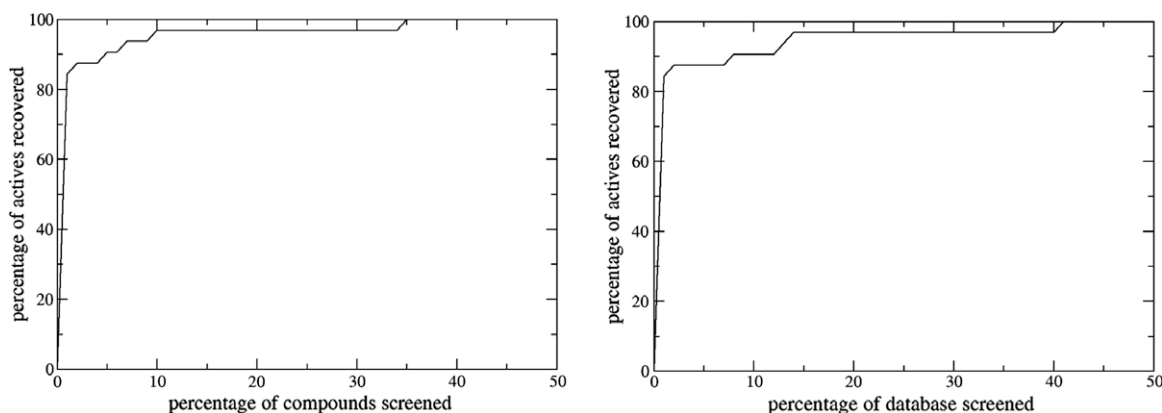

Figure 6. Enrichment curves obtained from virtual screening using strategy-C. Left: halothane–ferritin-dimer derived model. Right: isofluorane–ferritin-dimer derived model.

underlying this strategy may limit our ability to discover new molecules. For strategy B, we were able to find novel molecules and yet retain the ability to find known binders. In strategy C, we found 100% of the known anesthetic agents only after screening 40% of the whole database. This indicates that we are now finding much more diverse molecules that score better than these known molecules in terms of their ability to match the whole binding pocket of ferritin.

Thus, all three shape pharmacophore models seem to be able to recover known anesthetic compounds from the mist of decoy molecular structures. This observation indicates that we may be able to use these models either individually, or in concert, to search compound databases for potential anesthetic agents. Such virtual screening approaches can be combined with experimental HTS (high throughput screening) to effectively search for new anesthetics.

3.4. Shape pharmacophore scores of known anesthetics correlate well with binding data

We have examined if SHAPE4 can distinguish not only actives from inactives, but can also correlate with the energetics of compound binding to apoferritin, and most importantly, the degree of GABAergic activity. Based on compound availability, we have determined 5 of the 14 compounds for their binding to apoferritin with Isothermal Titration Calorimetry (ITC) experimental technique, and this relationship is shown below as Figure 7. As noted

before, we emphasize that the ITC data have been obtained as a totally separate experimental endeavor from the computational modeling. No retrospective fitting has been performed. Thus, Figure 7 should be considered as an experimental validation of our methodology. We found, somewhat surprisingly, that the shape pharmacophore scores calculated with SHAPE4 correlate exceedingly well with the experimental K_D measurement using either strategy A or B. We note that the correlation between the scores obtained by strategy C and K_D is less good. Fundamentally, strategy C includes more space in the binding pocket, and fewer molecules can simultaneously fit the whole pocket due to the U-shape nature
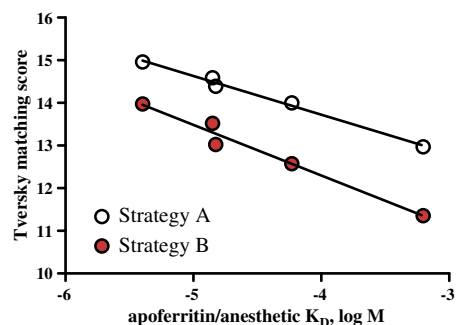

Figure 7. Correlation between shape pharmacophore scores and binding data (K_D). Two strategies have been shown here.
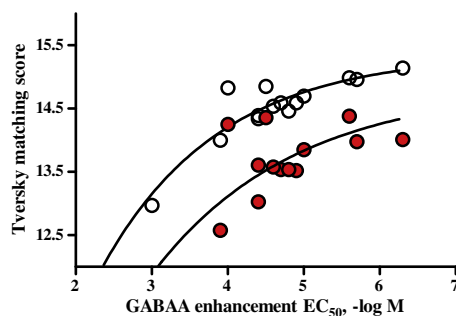
Figure 8. Correlation between the shape pharmacophore-based scores and the EC_{50} data. Scores from strategy-A and strategy-B are shown.

of the space. As a result, this additional volume adds more noise to the scoring when quantitative correlation is sought.

For GABAergic activity, we took advantage of the EC_{50} data published by Krasowski et al.[25] for $GABA_A$ current enhancement of each of 14 active compounds. Figure 8 below shows an excellent, but non-linear relationship for the 14 compounds. The basis for the non-linearity is not clear, but is not surprising because shape pharmacophore only measures the similarity between the anesthetic molecule and ferritin binding pocket shapes, and the score itself has limits that force non-linearity. Since the EC50 data have never been used to calibrate our SHAPE4 scoring function, Figure 8 should be considered as an external validation of the SHAPE4 methodology applied to the ferritin target. In Figures 7 and 8, strategy B provides lower absolute scores than strategy A, but still a significant correlation to GABAergic activity. This is remarkable given that the score derives from a shape match to an unrelated protein, and gives confidence that the new surrogate target approach may yield novel GABAergic compounds. Further, it is important to note that physicochemical properties have not yet been included, and are expected to significantly enhance the relationship.

4. Conclusions and perspectives

We have introduced a surrogate protein target, apoferritin, as the structural template for structure-based design of potential anesthetic compounds. A shape pharmacophore modeling method, introduced recently by our group,[19] captures the essential features of the ferritin binding pocket. The derived shape pharmacophore models can recover known anesthetic molecules from a diverse set of decoys randomly sampled from the Asinex database. Thus, shape pharmacophore models can qualitatively distinguish anesthetic molecules from other unrelated compounds. This is remarkable since no molecular energetic terms are included in the scoring function. However, this is consistent with the fact that the ferritin binding pocket is largely hydrophobic, and therefore, shape and size play a dominant role in binding.

Further analysis also revealed significant quantitative relationships between the shape pharmacophore model scores and experimental K_D values of a group of related anesthetic molecules. This is unexpected due to the lack of energetic calibration of the scoring function. Even more surprising is the finding that shape pharmacophore model scores of known anesthetic compounds are nicely correlated, in a nonlinear fashion, with the functional $GABA_A$ data (EC_{50}) of 14 compounds. This finding again probably arises from the central importance of shape and size in anesthetic molecules,

and strengthens our confidence in applying these shape pharmacophore models to virtual screening for novel anesthetic agents.

Thus far, we have not trained the shape pharmacophore modeling method with known binding (K_D) or EC_{50} data. It is conceivable that novel structure-based QSAR modeling can be employed when more data is obtained for ferritin binding molecules. The trained models may better encode the different contributions of shape matching and pharmacophore matching, so that the models can better quantitatively predict the activities of new molecules.

As pointed out by one of the reviewers, the ultimate validation of any in silico approach would be by new experimental validation, preferably by newly discovered molecules predicted by the computational method. This is indeed our ultimate goal. The compounds recommended by SHAPE4 will be obtained and experimentally tested in a HTS screening project. The results will be reported in the next phase of this project.

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