The Role of Electrostatic Interactions in Human Serum Albumin Binding and Stabilization by Halothane*

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Electrostatic interactions have been proposed as a potentially important force for anesthetics and protein binding but have not yet been tested directly. In the present study, we used wild-type human serum albumin (HSA) and specific site-directed mutants as a native protein model to investigate the role of electrostatic interactions in halothane binding. Structural geometry analysis of the HSA-halothane complex predicted an absence of significant electrostatic interactions, and direct binding (tryptophan fluorescence and zonal elution chromatography) and stability experiments (hydrogen exchange) confirmed that loss of charge in the binding sites, by charged to uncharged mutations and by changing ionic strength of the buffer, generally increased both regional (tryptophan region) and global halothane/HSA affinity. The results indicate that electrostatic interactions (full charges) either do not contribute or diminish halothane binding to HSA, leaving only the more general hydrophobic and van der Waals forces as the major contributors to the binding interaction.

Inhalational anesthetics can alter the activity of a wide variety of proteins, but the molecular nature of the interactions underlying the functional effect is still poorly understood. Guided by the Meyer-Overton correlation between anesthetic potency and solubility in a lipid-like environment, studies in the past three decades have concluded that anesthetics must bind to hydrophobic regions within target protein (especially membrane protein, i.e. ion channels) through weak van der Waals interactions and the hydrophobic effect (1–5). Electrostatic interactions were proposed recently as potentially important binding forces between anesthetics and target proteins (1, 6, 7).

Halogen atoms, especially fluorine, are more electronegative than carbon atoms, and therefore the C-halogen bond is polarized in inhalational anesthetics. Ab initio calculations indicated that halothane has a small permanent dipole moment (8), which may contribute binding to relevant targets. Similar compounds with less dipole than halothane are poor anesthetics, although this might be partially due to much lower solubility in water (1). Because polarity appears to be an important feature of anesthetics, it is reasonable to speculate that anesthetic binding sites contain polar moieties. Charged residues such as arginine and lysine and polar but uncharged aromatic groups with a partial negative charge in the center of the ring (9, 10) may contribute to the polarity of anesthetic binding sites. The weakly polar anesthetics might therefore interact with the charged residues directly and/or form dipole-quadrupole (a form of weak cation–π) interactions with the aromatic side chains. The latter may be strengthened by the positively charged residues coordinating the more electronegative end of the anesthetic molecule.

Because functionally important anesthetic targets remain unidentified, we have made use of surrogate proteins with appropriate binding character (1, 11–14). Designed peptides, for example, have been used to investigate the halothane binding site and its characteristics. In a synthetic four-helix-bundle protein, substitution of tyrosine for tryptophan decreased anesthetic binding affinity by about 5-fold, suggesting that the less dense electron cloud of tyrosine coordinates the relatively positive end of the anesthetic molecule less well (14). In the present study, we have used human serum albumin (HSA) as a native protein model to investigate electrostatic interactions directly. HSA is useful because it satisfies major pharmacodynamic criteria for simulating the anesthetic targets (11) and has a binding affinity for halothane within 10-fold of its clinical EC50, and a high resolution structure of HSA alone and in complex with halothane is now available (15). We focused on a large interdomain cavity containing the only tryptophan in HSA in this study. Previous work has confirmed that anesthetics bind to this region (12, 16), which also contains many charged residues (15, 17). If the relatively positive end of halothane coordinates with the π system of the tryptophan indole ring in a weak cation–π interaction, then it is possible that nearby positively charged residues may coordinate the relatively negative trifluoromethyl end of halothane (1). We predict that loss of positively charged side chains near Trp will eliminate an electrostatic contribution to halothane binding and therefore weaken halothane-HSA binding constants. To test this, we performed geometrical analysis of HSA (1E78) and the HSA-halothane complex structures (1E7B and 1E7C), expressed nine site-directed HSA mutants, and tested these for altered halothane binding using fluorescence spectroscopy, zonal elution chromatography, and amide hydrogen exchange combined with ionic strength experiments.

EXPERIMENTAL PROCEDURES

Materials
Halothane (1-bromo-1-chloro-2,2,2-trifluoroethane) was obtained from Halocarbon Laboratories (Hackensack, NJ). The thymol preserv-

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¶ The abbreviations used are: HSA, human serum albumin; wtHSA, wild-type HSA.
Expression and Purification of Recombinant HSA

Recombinant wild-type HSA (wtHSA) and nine HSA mutants were expressed in a yeast expression system. Seven single mutants (K195M, K199M, R218M, R218P, R218H, R222M, and R257M) of HSA replaced positively charged residues with uncharged ones, one single mutant (R242V) replaced an uncharged polar residue with an uncharged nonpolar residue, and one single mutant (F211V) replaced an aromatic residue with an uncharged, nonpolar residue. All of the replaced residues are within 15 Å from C3 of W214 in 1E78 (Table I; Fig. 1A) and the known halothane binding sites (halothane 2005 and halothane 2006) in 1E7C (Fig. 1B) (15). We chose these residues because each line the tryptophan cavity (15), and our previous fluorescence and photolabeling data showed that halothane binds in close proximity to W214 (12, 13, 16).

Cloning of HSA Coding Region—With human liver cDNA as a template, the entire coding region of the HSA gene, including the native signal sequence, was amplified by the polymerase chain reaction using Vent DNA polymerase (New England Biolabs). The resulting DNA fragment was inserted into the plasmid vector pHiL-D2 (Invitrogen) using standard cloning techniques. pHiL-D2 is a shuttle vector that can introduce genes into the yeast species Pichia pastoris using standard cloning techniques. Each pHiL-D2 expression plasmid contains a methanol-inducible promoter located upstream of the HSA homologous recombination. Specific mutations were introduced into the expression cassette stably integrated into the yeast chromosomal DNA from each clone used to produce a particular HSA mutants.

Regional Binding Measurements

Quenching of Trp fluorescence was used to study halothane-HSA regional binding as described previously (16). Briefly, halothane was dissolved in the phosphate buffer to a concentration of 10 mM and loaded into a gas-tight Hamilton syringe. Different concentrations of halothane were equilibrated with 1 mM wtHSA (or mutants) in a quartz cell during fluorescence measurements. The point of 50% inhibition (IC50) of the fluorescence maxima in halothane-free species was calculated (12, 16) with Hill plots. The buffer used for fluorescence experimentation was 130 mM sodium chloride, 20 mM sodium phosphate, pH 7.0.

Fluorescence measurements were performed with a Fluorescence Spectrophotometer RF-5301PC (Shimadzu, Columbia, MD). A 10-mm-pathlength quartz cell (1.5 ml) with a polystyrene membrane stopper was used without much difference in air pockets. All fluorescence measurements were made at room temperature (25–1 °C). Excitation and emission slit widths were 3 and 5 nm, respectively. The background fluorescence was subtracted from each emission spectrum. All HSA mutants have Trp at the 214 position, which is located in subdomain IIA, one of the binding sites for aromatic and heterocyclic compounds. The single tryptophan was excited at a wavelength of 295 nm, and emission spectra were recorded from 310 nm to 450 nm for all HSA mutants.

In addition to the above halothane titrations, we also examined the influence of ionic strength on halothane binding to wtHSA. Thus, a halothane IC50 was calculated for titrations performed in 20 mM sodium phosphate buffer containing 0, 130, or 500 mM sodium chloride.

Global Binding Measurements

Zonal elution chromatography was used to study halothane-HSA global binding as we described previously (19). Briefly, 3 ml of Af-fGel 10 was washed in cold distilled water and transferred to a graduated cylinder. Excess water was removed, and coupling was accomplished by adding 2.5 ml of the HSA solution (50 mg/ml) and gently mixing. After coupling for 2 h at room temperature, the gel was allowed to settle, and the supernatant was removed. To block all remaining unreacted groups, the gel was then incubated with 0.3 mM glycine, pH 7.0, for 30 min. A control gel was prepared by reacting 3.0 ml of 0.3 mM glycine, pH 7.0, with 3.0 ml of gel for 2 h as described above. Aliquots of the pre- and postcoupling solutions were saved to determine the immobilized mass. Protein assays were performed with the Bio-Rad reagent. The coupled gel was washed with degassed mobile phase (10 mM NaPO4, pH 7.0) and packed into Bio-Rad MT-2 columns (holding about 2.0 ml of gel). Columns were connected to a Shimadzu LC-600 Liquid Chromato-
Graph pump and then flushed with mobile phase at 0.4 ml/min until a steady baseline, as detected by UV absorbance at 210 nm (Shimadzu SPD-6AV), was established.

With the apparatus running at 0.4 ml/min, the column was equilibrated with 10 mM sodium phosphate buffer containing either 0, 130, or 500 mM NaCl. Small (50 µl) aliquots of 10 mM halothane in the same mobile phase were injected (t = 0), and absorbance (210 nm) was monitored for 20 min. Retention times for the halothane peaks from the different sodium chloride concentrations and for the different columns (HSA and glycine) were compared.

### Hydrogen-Tritium Exchange

Changes in ligand binding should be reflected in changes in protein stability, and amide hydrogen exchange is the most sensitive method of measuring these changes (55). Protein solutions (1 mg/ml) were incubated with 5–10 nCi of [3H]OH in 1 M GdnCl, 0.1 M NaH2PO4 buffer, pH 8.5, for at least 18 h at room temperature for exchange-in. Free [3H]OH (HSA and glycine) were compared.

### Proteins

- **HSA**
- **Halothane**

### Table II

**Energetically significant cation-π interactions in HSA model**

| Protein Data Bank ID | HLT 2005 | HLT 2006 |
|----------------------|----------|----------|
| From C1 From C2 | From C1 From C2 |
| IE78 | R144-Y140 | R222-F223 | R160-F156 |
| R197-Y148 | R144-Y140 | R144-Y140 |
| R257-Y150 | R197-Y148 | R257-Y150 |
| R336-Y332 | R336-Y332 | R336-Y332 |
| K106-Y148 | K106-Y148 | R410-Y411 |
| K233-Y263 | K233-Y263 | K106-Y30 |
| K144-Y111 | K144-Y111 | K235-Y263 |
| K199-W214 | K199-W214 | K325-Y319 |

### Table III

**Protein Data Bank ID**

| HLT 2005 | From C1 | From C2 | Difference |
|----------|---------|---------|------------|
| IE78 | 10.6 ± 1.0 | 11.2 ± 1.0 | 0.6 |
| R144-Y140 | 8.1 ± 0.9 | 7.9 ± 0.8 | 1.4 |

**Hydrogen-Tritium Exchange**

The distance (Å) from halothane C1 and C2 to the aromatic ring of Trp

| Protein Data Bank file | From C1 | From C2 | Difference |
|------------------------|---------|---------|------------|
| IE78 | 10.6 ± 1.0 | 11.2 ± 1.0 | 0.6 |
| R144-Y140 | 8.1 ± 0.9 | 7.9 ± 0.8 | 1.4 |

**Results**

### Structural Geometry Analysis

We focused on halothane 2005 and 2006 in 1E7C because these two molecules are the closest to W214. Using distance analysis, we found that halothane does not have any orientation preference to any of the mutated residues (Table II). The closest charged to uncharged mutations (K199M, K233V, R218M, and R257M) generally decreased the IC50 of halothane by 10–35%, indicating improvement in halothane binding in this W214 binding site. H242V also decreased IC50.

Because the deletion of one aromatic ring-containing residue (F211V) for halothane quenching of wtHSA fluorescence was 2.9 kcal/mol, these single mutations produced only small or no changes in halothane 2005 and 2006 in 1E7C because these two molecules are the closest to W214. Using distance analysis, we found that halothane 2005 is R257. The nearest atoms are the O of arginine and the Cl of halothane (3.6 Å). The closest charged residues from halothane 2005 is R257. The nearest atoms are the O of arginine and the Cl of halothane (3.6 Å). The closest charged residues from halothane 2006 are R218 and R222. In this case, the nearest atoms are C and Cl (3.1 Å) between R218 and halothane, respectively, and N and F (3.7 Å) between R222 and halothane, respectively. Only this latter N-F (3.7 Å) interaction between R222 and halothane 2006 could indicate an attractive electrostatic interaction.

CaPTURE detected eight energetically significant cation-π interactions in 1E78, 1E7B (with 3 halothane molecules), and 1E7C (with 8 halothane molecules) (Table III). None of these were between halothane and HSA. Both 2005 and 2006 halothane molecules point to the tryptophan ring with their relatively negative end (trifluoromethyl end; Table IV). Although the total number of cation-π interactions was preserved in the HSA-halothane complex, the distribution was different than in HSA alone. For example, the K199-W214 interaction is not present in 1E7C.

### Effect on Fluorescence Quenching of Mutations—With 295 nm excitation, the wavelength of maximum fluorescence emission is 339 nm in wtHSA, and ranges from 338 to 340 nm in all mutants except K195M (341 nm) and R218F (343 nm). The IC50 for halothane quenching of wtHSA fluorescence was 2.9 ± 0.1. The charged to uncharged mutations (K195M, K199M, R218M, R222M, and R257M) generally decreased the IC50 of halothane by 10–35%, indicating improvement in halothane binding in this W214 binding site. H242V also decreased IC50.

The deletion of one aromatic ring-containing residue slows down hydrogen exchange for global protein stability. Consistent with the changes in ligand binding, the distribution is different than in HSA alone. For example, the K199-W214 interaction is not present in 1E7C.

### Effect on Hydrogen Exchange of Mutations—Because the mutation may change native state stability, which may affect binding independently of changes in the local environment, we performed hydrogen exchange to global protein stability. These single mutations produced only small or no changes in baseline protein stability as indicated by hydrogen exchange, and the changes are limited to the mutations at position 218. ΔΔG values are 1.4 ± 0.3 kcal/mol in R218P, −0.4 ± 0.2 kcal/mol in R218H, and −0.5 ± 0.2 kcal/mol in R218M.

Tryptophan fluorescence reports only a local or regional binding interaction of halothane, and it is not clear whether or how this contributes to global stability. Consistent with the...
tryptophan fluorescence data, hydrogen exchange shows that halothane generally enhances the global protein stability of the charged to uncharged mutants more than that of wtHSA (Fig. 4), suggesting improved specific binding and protein stabilization. Better stabilization also was observed in F211V. Despite consistency of the direction of change, there was no significant correlation between halothane IC50 and ΔG (Fig. 5).

**FIG. 2.** Loss of charge generally enhances HSA affinity for halothane. IC50, or the halothane concentration resulting in 50% inhibition of the maximum fluorescence emission, decreased in most of the charged to uncharged mutations. Loss of one aromatic ring (F211V) also enhanced halothane binding. Data are the mean ± S.E., n = 2.

**FIG. 3.** Cavity volume plays a role in halothane binding. Residue volume difference reflects the wild-type residue volume minus the mutant residue volume. Because all the mutations in the present study are large to small, we assumed cavity volume increased after mutation. A significant correlation (r2 = 0.5, p = 0.03) between residue volume difference and the affinity was revealed by linear regression analysis. IC50 is the halothane concentration resulting in a 50% inhibition of the maximum fluorescence emission.

**FIG. 4.** Loss of charge results in better stabilization by halothane. Only in R257M did halothane cause a slight decrease in stability relative to wtHSA. ΔG is the change in free energy change produced by 7 mM halothane. Data are the mean ± S.E., n = 2.

**FIG. 5.** No significant correlation between regional (domain IIA) binding (IC50) of halothane and the global stability change (ΔG) by halothane was found. IC50 is the halothane concentration resulting in a 50% of inhibition of the maximum fluorescence emission. ΔG is the change in free energy change produced by 7 mM halothane. The line represents a least squares linear regression.

**DISCUSSION**

The polar aspect of anesthetic binding sites has been stressed many times previously (1, 6, 7, 20–23) but has not been the subject of direct testing. Contrary to our initial prediction, the charged to uncharged mutation in the W214 region of HSA generally strengthened halothane binding and stabil-
The retention time for halothane was prolonged with increasing NaCl concentration: 6.27 min in 0 mM NaCl (HSA0 curve), 6.43 min in 130 mM NaCl (HSA130 curve), and 6.77 min in 500 mM NaCl (HSA500 curve). The retention time for halothane in the control column was significantly shorter (5.3 min) than that in the HSA column and did not change with increasing NaCl concentration. For simplicity, only one curve from the glycine control column is presented because the curves from different salt concentration overlap.

**Fig. 7. The effect of ionic strength on regional binding of wtHSA to halothane.** The retention time for halothane was prolonged with increasing NaCl concentration: 6.27 min in 0 mM NaCl (HSA0 curve), 6.43 min in 130 mM NaCl (HSA130 curve), and 6.77 min in 500 mM NaCl (HSA500 curve). The retention time for halothane in the control column was significantly shorter (5.3 min) than that in the HSA column and did not change with increasing NaCl concentration. For simplicity, only one curve from the glycine control column is presented because the curves from different salt concentration overlap.

Furthermore, the increase in ionic strength also enhanced both regional and global binding, and, finally, geometric analysis of high resolution structures of the halothane-HSA complex failed to provide evidence of significant electrostatic interactions in the tryptophan binding site. These results are consistent in indicating that electrostatic interactions do not enhance HSA-halothane binding. In fact, these data would suggest that electrostatic features in this cavity actually hinder halothane binding. If this binding site shares similarity with sites on functionally relevant proteins, our results suggest that binding of halothane and probably other inhaled anesthetics is controlled primarily by the more general hydrophobic and van der Waals forces.

**Selectivity/Specificity**

Specificity to ligand protein interactions is achieved by a multiplicity of features, namely electrostatic, van der Waals, hydrophobic, and steric effects. These factors contribute toward selective and energetic binding interactions. In a folded protein, the arrangement of positively and negatively charged residues causes a considerable variation in the electrostatic potential throughout the protein. This provides, at specific sites, significant electrostatic contributions to the free energy of binding of the ligand. For example, a major binding force between indomethacin and HSA is electrostatic in nature (24). Our finding that electrostatic interactions do not contribute to halothane binding in a natural and pharmacodynamically relevant protein model leaves only the other noncovalent interactions to contribute to the binding free energy. This results in low binding affinity, as shown here, and also reduces the specificity and selectivity of binding sites. This paucity of interactions suggests that many targets of comparable anesthetic binding affinity are more likely than a few high affinity targets.

**Electrostatic Interactions**

**Full Charge Separation**—Structure geometrical analysis revealed an absence of direct positive and negative electrostatic interactions between halothane and the charged residues of HSA. Consistent with this, our fluorescence results showed that halothane affinity is enhanced by charged to uncharged mutations, suggesting that electrostatic interactions did not contribute to HSA-halothane binding but actually hindered the binding interaction. This is also consistent with previous potency correlative analysis (21). However, this conclusion must be tempered by the fact that the investigated site is complex and has several fully charged lining residues. Thus, we cannot be certain that removing a single charge at a time will not strengthen a potential electrostatic interaction with another, unaltered residue or enhance the cavity polarity in a favorable way. We think this is unlikely because a charged to uncharged mutation enhanced binding in every one of the seven positions and because geometric analysis of the HSA-halothane complex did not reveal any significant electrostatic pairs. Nevertheless, this does not eliminate a role for weak polar effects (partial charges and polarizable atoms). Small organic molecules that have anesthetic properties generally have a modest dipole moment or are polarizable; thus, it is likely that the binding environments for these compounds have a complementary polar nature.

Although a charged to uncharged mutation enhanced halothane binding at every position investigated, not all such mutations had this effect. For example, whereas R218M enhanced binding, R218H and R218P decreased halothane binding some-
what. This may be explained by other effects of these less common residues. For example, whereas histidine is essentially uncharged at physiologic pH, it is nevertheless polar, bulky, and less flexible than arginine. Similarly, proline is rigid and is known to distort secondary and perhaps tertiary structure. This is consistent with the large effect of R218P on native state stability and a small red shift of the maximum fluorescence emission. Thus, it is likely that the apparent inconsistency of these two mutations at position 218 is due to unintended steric effects. Without crystal structures for each mutant, this conclusion must remain tentative.

Increases in ionic strength should “screen” charges in water-accessible sites and reduce their effect on ligand binding in a competitive way. Thus, if electrostatic interactions were important, an increase in ionic strength should reduce their contribution to binding free energy and thus weaken binding. Instead, we found that an increase in ionic strength strengthened binding. Increased ionic strength also stabilized HSA as shown by hydrogen exchange, but this did not alter the degree of stabilization produced by halothane. Taken together, the ionic strength experiments also suggest that electrostatic interactions do not contribute to halothane binding energetics.

**Cation-π Interactions**—Structure geometry analysis shows that cation-π interactions among amino acid residues exist in HSA, and the distribution of cation-π interactions is altered in the HSA-halothane complex. Although this might be due to competitive interactions between native cation-π and halothane, our inability to detect any cation-π interaction between any halothane atom and a protein atom makes this unlikely. Furthermore, replacing one aromatic ring in this binding cavity with an aliphatic residue (F211V) also increased halothane binding affinity. Thus, the change in cation-π distribution probably reflects a subtle change in protein conformation in the HSA-halothane complex. Our inability to detect an anesthetic-protein cation-π interaction in this HSA binding site does not negate the potential importance of such interactions in other sites. Indeed, recent work in designed four-helix bundles suggested a cation-π interaction between anesthetics and proteins (14). It might be relevant to note that bulky aromatic residues are more commonly found in internal protein cavities where anesthetics might be expected to bind (25), and thus it is attractive to speculate that cation-π interactions contribute to binding in some protein targets.

**Other Effects of Mutation**

In addition to effects on polarity and charge, the mutations introduce volume and steric effects. If internal protein cavities are attractive binding sites for anesthetics, then volume is important; if the volume is too small, the molecule cannot be accommodated, and if it is too large, van der Waals London forces (which fall off rapidly as a function of distance) will be insufficient to produce binding. Indeed, we found a significant relationship between change in residue volume and IC₅₀, whereby mutations producing little change in cavity volume enhanced affinity, and those increasing cavity volume reduced affinity. Thus, loss of charge appears to improve halothane binding when the change in cavity volume is small, but this is probably offset by loss of van der Waals contacts when volume change is large.

Steric and conformational effects also need to be considered. For example, the slight decrease in halothane affinity in the proline and histidine mutations may relate not only to volume effects but also to the more rigid nature of their side chains. Methionine has considerable conformational entropy and thus may be able to accommodate the ligand in this tryptophan cavity at a lower free energy expense. The same would be true for the smaller aliphatic side chain of valine. Thus, the basis for alterations in affinity with these mutations is likely to be multifactorial.

**Global Binding and Stability**

Halothane binds to at least 7–10 sites in HSA (12, 15, 16), so clearly mutagenesis of all possible interacting residues would have been prohibitive. Zonal elution chromatography provides an alternative tool to investigate the global weak interaction between anesthetics and proteins (19). Consistent with the mutagenesis and regional binding study, the result indicated that HSA affinity to halothane strengthened with the increase of the salt concentration. These experiments suggest a generality of the binding sites; electrostatic interactions do not appear to be important for binding in the other binding sites of HSA either.

Most proteins are only marginally stable in order to allow the conformational changes that underlie their function. Thus, small changes in native stability may have considerable implications to protein activity and biologic function. A drawback of mutagenesis is that the mutations might disturb the global protein stability significantly and unpredictably. Thus, we considered it important to evaluate the effects of these mutations on native state stability using amide hydrogen exchange kinetics. Fortunately, the stability change of the mutants in the present study is generally very small and is limited to mutations at the 218 position. R218P, for example, destabilized HSA by over 1 kcal/mol, which alone may have considerable effects on ligand binding and function independent of any regional effects. It is even possible that this large effect on global stability is responsible for the decrease in halothane binding affinity noted with this mutation.

We have previously suggested that anesthetics exert their effects on proteins at the molecular level by regional specific binding and global alterations in conformational stability (11, 12, 26, 27). In agreement with the fluorescence results, the present hydrogen exchange results also indicated that halothane stabilizes almost all the charged to uncharged mutants better than wtHSA, except R257M. The lack of correlation between halothane stabilization (ΔΔG) and fluorescence quenching (IC₅₀) with the various mutants most likely reflects that the W214 site is only one of many halothane binding sites in this protein (12, 15, 16).

In summary, we used HSA and specific site-directed mutants as a native protein model to investigate the role of electrostatic interactions in halothane binding. Structural geometry analysis of the HSA-halothane complex predicted an absence of significant electrostatic interactions, and direct binding and stability experiments confirmed that loss of charge in the binding site generally increased halothane/HSA affinity. The results all point to the same conclusion: electrostatic (positive and negative) interactions either do not contribute to or diminish halothane binding to HSA, leaving only the more general hydrophobic and van der Waals forces as the major contributors to the binding interaction. An important implication of this study is that if HSA binding sites are similar to functionally relevant targets, then the likelihood of finding highly specific and selective inhalational anesthetic binding sites or targets is low.

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