



# Stereoselectivity of Isoflurane in Adhesion Molecule Leukocyte Function-Associated Antigen-1

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## Abstract

**Background:** Isoflurane in clinical use is a racemate of S- and R-isoflurane. Previous studies have demonstrated that the effects of S-isoflurane on relevant anesthetic targets might be modestly stronger (less than 2-fold) than R-isoflurane. The X-ray crystallographic structure of the immunological target, leukocyte function-associated antigen-1 (LFA-1) with racemic isoflurane suggested that only S-isoflurane bound specifically to this protein. If so, the use of specific isoflurane enantiomers may have advantage in the surgical settings where a wide range of inflammatory responses is expected to occur. Here, we have further tested the hypothesis that isoflurane enantioselectivity is apparent in solution binding and functional studies.

**Methods:** First, binding of isoflurane enantiomers to LFA-1 was studied using 1-aminoanthracene (1-AMA) displacement assays. The binding site of each enantiomer on LFA-1 was studied using the docking program *GLIDE*. Functional studies employed the flow-cytometry based ICAM binding assay.

**Results:** Both enantiomers decreased 1-AMA fluorescence signal (at 520 nm), indicating that both competed with 1-AMA and bound to the  $\alpha$ L I domain. The docking simulation demonstrated that both enantiomers bound to the LFA-1 "lovastatin site." ICAM binding assays showed that S-isoflurane inhibited more potently than R-isoflurane, consistent with the result of 1-AMA competition assay.

**Conclusions:** In contrast with the x-ray crystallography, both enantiomers bound to and inhibited LFA-1. S-isoflurane showed slight preference over R-isoflurane.

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## Introduction

Many biologically active molecules, including drugs, exist in different chiral forms, and it is possible that each specific enantiomer can interact with their targets differently, thereby potentially eliciting different biological responses. Thus, the potential exists for a racemic mixture of a compound to cause diverse, and perhaps unwanted, side effects. Regardless, racemic drugs are used commonly in clinical medicine, as the purification or synthesis of one specific enantiomer can be costly. Novel drug discovery can be even more costly, so there has been recent interest in exploiting enantioselectivity in the last two decades. While the majority of drugs on the market were sold as racemic mixtures in early 1990s, about 40% of drugs were marketed as single enantiomers by 2002 [1]. Some of these single enantiomers were newly developed, while some were isolated from the previously marketed racemic mixtures [2]. The advantages of using specific enantiomers over the racemic mixture might include their less complex and more selective pharmacodynamic profiles, and the potential for improved therapeutic index and the reduction of side effects. In extreme cases, one enantiomer may

act as an antagonist (such as R(-)-PN 202-791; calcium channel antagonist), while the other may be an agonist (such as S(+)-PN 202-791; calcium channel agonist) [3].

The interest in single enantiomers has also reached the anesthesia field, as demonstrated by the introduction of ropivacaine and levo-bupivacaine [3]. Further, even the inhalational general anesthetics have chiral centers. For example, isoflurane has been successfully separated into its enantiomers by Huang et al. and others in 1990s [4], allowing investigators to test the potency of each separately. The S- enantiomer of isoflurane was shown to be approximately 2-times more potent *in vitro* and *in vivo* than R- enantiomer isoflurane (**Table 1**). However, the structural basis for the differential potency of isoflurane enantiomers has yet to emerge, but it has been reported that S-isoflurane binds with higher affinity to model proteins, like human serum albumin [5].

Previously we demonstrated that isoflurane interacted with the adhesion molecule leukocyte adhesion-associated antigen-1 (LFA-1). This protein is expressed ubiquitously on leukocytes and is involved in various immunological actions such as leukocyte arrest on the endothelium [6] and immunological synapse formation [7]. This heterodimeric molecule consisting of  $\alpha$ - and  $\beta$  subunits is

**Table 1.** The previous studies using isoflurane enantiomers.

Type of experiment	Study results	Reference
<i>In vivo</i>	The lipid emulsion of isoflurane injection to rats. S(+) was 40+/-8% more potent than R(-) at producing loss of righting reflex.	[35]
<i>In vivo</i>	The inhalation of isoflurane enantiomers to rats. Minimum alveolar concentration MAC was S(+); 1.06%, R(-); 1.62%, suggesting S(+) was about 50% more potent.	[36]
<i>In vivo</i>	The determination of MAC using rats. Inhalational route. S(+): 0.0144 +/- 0.0012 atm, R(-); 0.0169 +/- 0.0020 atm. Not statistically significant.	[37]
<i>In vivo</i>	Intraperitoneal injection of isoflurane enantiomers to mice. Sleep time was longer with S(+) over R(-).	[38]
<i>In vitro</i>	S(+) was more potent and efficacious than R(-) in enhancing [ <sup>3</sup> H] flunitrazepam binding to GABA <sub>A</sub> receptor complex.	[13]
<i>In vitro</i>	S(+) had greater increase in GABA receptor mediated IPSCs than R(-)	[14]
<i>In vitro</i>	S(+) was 1.6 times as potent as R(-) in augmenting GABA gated Cl flux.	[15]
<i>In vitro</i>	Cl flux induced by GABA was potentiated by isoflurane. The maximum stereoselectivity occurred at 0.3 mM isoflurane (S > R), about 2 times.	[16]
<i>In vitro</i>	No stereospecific effects of isoflurane in isolated guinea pig hearts (the effect on LVP, AV conduction, coronary flow)	[48]
<i>In vitro</i>	No stereoselectivity of isoflurane seen to inhibit isradipine binding to L-type calcium channel	[49]
<i>In vitro</i>	S(+) bound bovine serum albumin with slower association and dissociation rates than R(-). No difference in static condition.	[50]
<i>In vitro</i>	S(+) was twofold more effective than R(-) both in eliciting the anesthetic-activated potassium current and in inhibiting a current mediated by neuronal nicotinic acetylcholine receptors	[17]

The reported studies of isoflurane enantiomers are summarized in the table.

MAC; minimum alveolar concentration, GABA; gamma-aminobutyric acid, LVP; left ventricular pressure, AV; atrio-ventricular, IPSC; inhibitory postsynaptic current.  
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activated through the intracellular activation signals called the “inside-out” signal and undergoes dynamic conformational changes [8,9]. The  $\alpha 7$  helix of the ligand binding domain (the  $\alpha L$  I domain) of the  $\alpha$  subunit is extremely flexible [10]. In a resting (inactive) state, the  $\alpha 7$  helix remains helical, forming a pocket called the “lovastatin site.” In a high-affinity (active) state, however, an unwinding, downward movement of the  $\alpha 7$  helix occurs causing a loss of the “lovastatin site”, which allows the  $\alpha L$  I domain to bind ligands at the metal-ion dependent adhesion site (MIDAS) (**Figure 1**). The X-ray crystallization of racemic isoflurane complexed with the  $\alpha L$  I domain demonstrated that isoflurane bound to this “lovastatin site” [11], and the functional relevance of this binding in LFA-1 blockade by isoflurane was shown by the mutagenesis experiment [12]. Interestingly, further refinement of this complex suggested that only S-isoflurane, fit into the electron density map of this region; the R-isomer produced a steric clash with lining residues. This seemingly extreme example of isoflurane stereoselectivity on LFA-1 suggested by the X-ray crystallographic structure is striking as compared to the modest, functional stereoselectivity suggested by its central nervous system effects [13,14,15,16,17]. Our previous study demonstrated that the interaction of racemic isoflurane with LFA-1 had functional relevance *in vivo* [18]. Thus, if the suggested selectivity on LFA-1 can be demonstrated in solution, it may open the door to more selective application of these drugs in the perioperative setting.

In this study, we tested the hypothesis that the enantioselectivity of isoflurane suggested by the crystal structures of LFA-1-isoflurane complex, is also observed in solution binding and functional studies.

## Methods

### Protein expression and purification

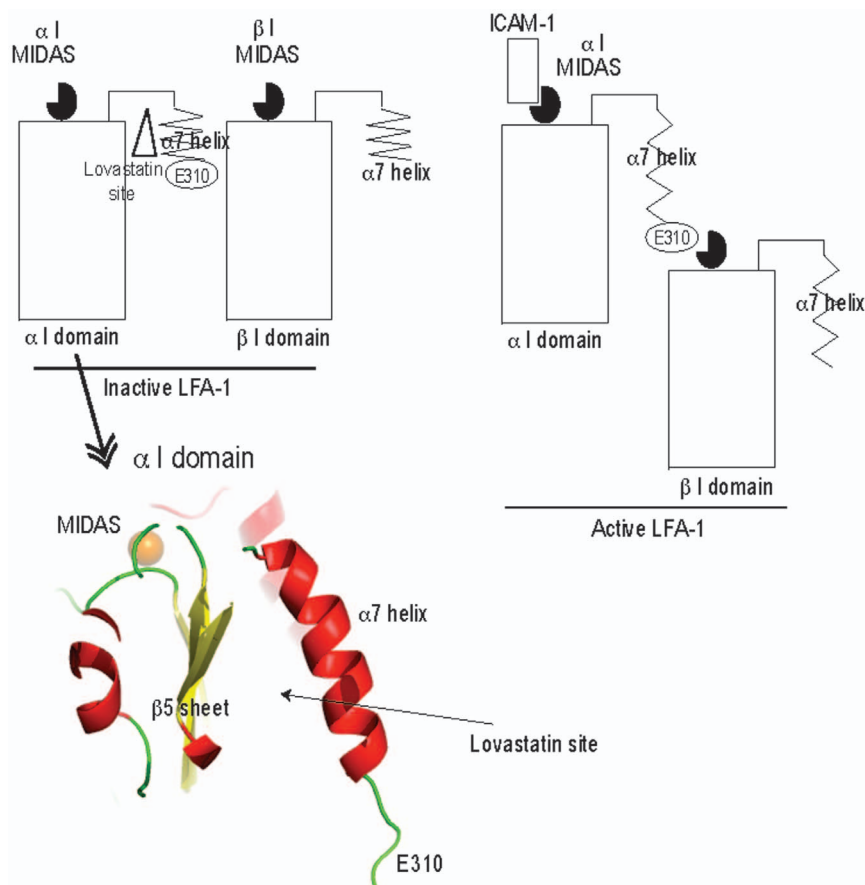
The  $\alpha L$  I domain wild type (WT, residues 128-307 of the integrin  $\alpha L$  subunit) (low affinity conformer) of LFA-1 were expressed in BL21 (DE3) cells. The  $\alpha I$  domains were expressed as inclusion bodies, which were solubilized and refolded as previously described [19]. Soluble full ectodomain LFA-1 protein was expressed from CHO Lec 3.2.8.1 cells stably transfected with LFA-1 plasmids and purified as previously described [20].

### Cell-free LFA-1: ICAM-1 binding assays

LFA-1: ICAM-1 binding assays were performed as previously described with minor modifications [21]. Briefly, soluble LFA-1 protein (10  $\mu$ g/mL) was immobilized on the capturing antibody named anti-Velcro antibody (Immune Disease Institute, Boston, MA) on 96 well plates. Following blocking, human ICAM-1-Fc $\alpha$  fusion protein (5  $\mu$ g/mL) was added to wells with HEPES-buffered saline (HBS) containing 1 mM MnCl<sub>2</sub> and in some cases, 10-25  $\mu$ M 1-aminoanthracene (1-AMA) (Sigma; St. Louis, MO, USA). After incubation for 1 hour at room temperature, unbound ICAM-1 was washed off and bound ICAM-1 was detected using peroxidase-labeled goat anti-human IgA and substrate (BD, Franklin Lakes, NJ, USA). Absorbance was measured at 405 nm. ICAM-1 binding % was defined as [(optimal density (OD) of 1-AMA)/OD of mock-treated sample]  $\times$  100%.

### Transient transfection of LFA-1 in 293 T cells

293 T cells were cultured in HEPES modified - Dulbecco's modified Eagle medium (DMEM)/10% fetal bovine serum (FBS) at 37°C, 5% CO<sub>2</sub>. Wild type LFA-1 plasmid was transfected using Lipofectamine 2000 (Invitrogen; Carlsbad, CA, USA).



**Figure 1. Schematic description of leukocyte function-associated antigen-1 (LFA-1) structure and “lovastatin site”.** Inactive and active forms of LFA-1 are shown in cartoon. The  $\alpha 7$  helix is very flexible as shown. In an inactive form, there is a cavity called “lovastatin site” underneath the  $\alpha 7$  helix of the  $\alpha L$  I domain. There is no active signal communication from the  $\beta I$  domain to the  $\alpha I$  domain. However, in an active form, the  $\alpha 7$  helix of the  $\alpha I$  domain gets unwound and Glu-310, which has a negative charge, interacts with metal-ion dependent adhesion site (MIDAS) (positive charge) in the  $\beta I$  domain. This is how the  $\beta I$  domain communicates with the  $\alpha I$  domain. And MIDAS in the  $\alpha I$  domain binds to the ligand.  
doi:10.1371/journal.pone.0096649.g001

### Fluorescence microscopy

293 T cells were transiently transfected with LFA-1 on glass bottom dishes (MatTek Corporation; Ashland, MA, USA). LFA-1 on the cells was stained with TS1/12 antibody (Immune Disease Institute, Boston, MA, USA) followed by anti-mouse Cy5 antibody (Life Technologies; Grand Island, NY, USA). 1-AMA was added to medium, incubated for 10 minutes, and then the cells were examined with fluorescence microscopy.

### Chiral gas chromatography

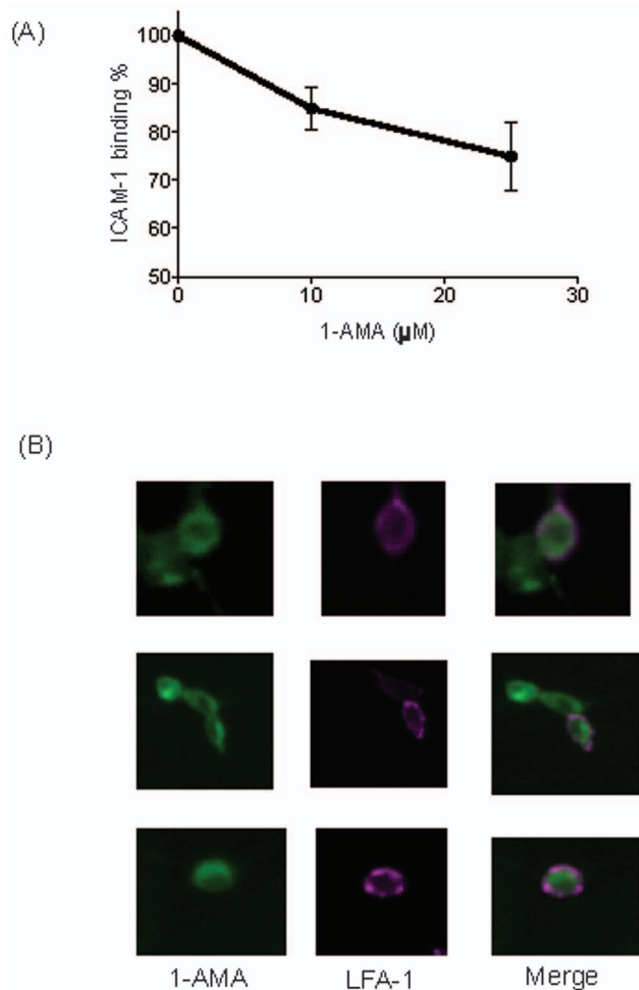
Isoflurane enantiomers (S-isoflurane and R-isoflurane) were kindly provided by Baxter (Cambridge, MA, USA). Racemic isoflurane was from Abbott Laboratories (Abbott Park, IL, USA). We first used chiral gas chromatography with mass spectrometry detection (GC/MS) to evaluate the purity of each optical enantiomer. Samples of isoflurane were diluted in heptane (Sigma) and injected into the gas chromatography (Model G1540N-6410N, Agilent Technologies; Santa Clara, CA, USA). We used a 30-m, 0.25-mm, 0.25  $\mu\text{m}$  20% permethylated  $\beta$ -cyclodextrin chiral 20B column (Agilent Technologies). The parameters were a split ratio of 1: 20, injector temperature of 70m°C, oven temperature of 60°C, and carrier gas hydrogen at 1 mL/min. Mass spectrometry quantification was conducted in single ion model at  $m/z$  51, 117 and 141.

### 1-AMA competition assay

The competition of 1-AMA binding with the isoflurane enantiomers was performed using the  $\alpha L$  I domain. The  $\alpha L$  I domain (0.2  $\mu\text{M}$ ) was first pre-equilibrated with 10  $\mu\text{M}$  1-AMA (Sigma; St. Louis, MO, USA). After addition of 0.1  $\mu\text{M}$  to 4 mM of S-, or R- isoflurane, samples were excited at 380 nm and emission spectra were collected from the range of 400 nm to 700 nm. The reduction of the fluorescence signal at 540 nm was corrected by subtracting the baseline fluorescence curves of 1-AMA and the  $\alpha L$  I domain, and plotted against isoflurane enantiomer concentration. Both competition curves were fitted to variable slope Hill curves. Analysis was performed using PRISM 5 software (Graph Pad Software, La Jolla, CA, USA).

### Docking simulation of S- and R-isoflurane

The structure of the  $\alpha L$  I domain was obtained from the Protein Data Bank (PDB) 1ZOO [22]. The program *GLIDE* (Schrodinger; Cambridge, MA, USA) was used to perform rigid molecular docking of isoflurane enantiomers with the  $\alpha L$  I domain, as previously described [20]. The structures of the isoflurane enantiomers were obtained through PubChem (<http://pubchem.ncbi.nlm.nih.gov/>). Isoflurane binding position was sought with the grid size of 25  $\times$  25  $\times$  25  $\text{\AA}^3$  and the centroid grid residue of Tyr-257. No positional constraint was applied. *GLIDE* has a

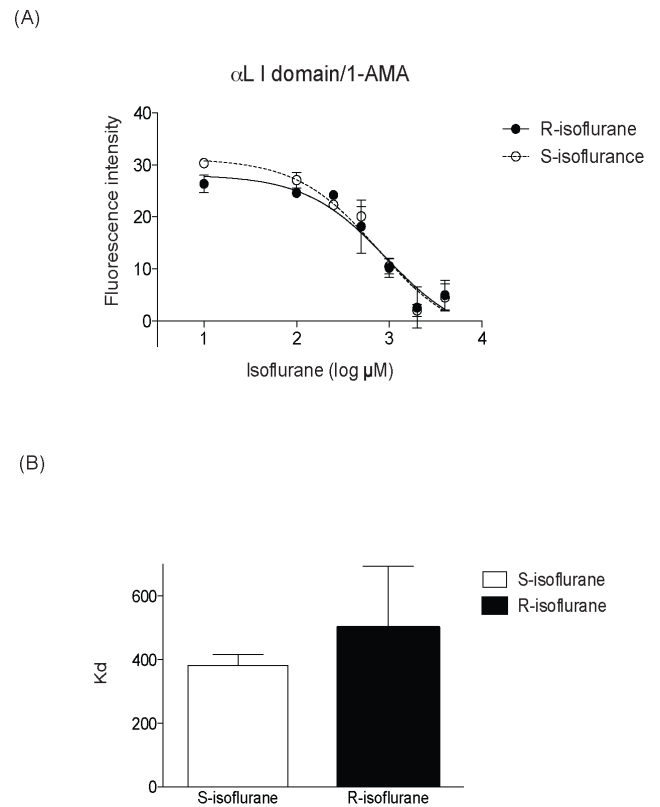


**Figure 2. The interaction of 1-aminoanthracene (1-AMA) with leukocyte function-associated antigen-1 (LFA-1).** (A) LFA-1: ICAM-1 binding was tested in cell-free system in the presence of 1-AMA. 1-AMA impaired ICAM-1 binding. (B) Localization of 1-AMA was tested in transient LFA-1 transfectants. In Figure, green represents 1-AMA, and red represents LFA-1. doi:10.1371/journal.pone.0096649.g002

scoring system (“glidescore”) that ranks docked pairs based on the predicted interaction free-energy. The pair with the most negative glidescore is considered to have the highest affinity, thus we selected the docked pair with the most negative glidescore.

#### Cell-based Intercellular adhesion molecule-1 (ICAM-1) binding assay

ICAM-1 is the major endogenous ligand for LFA-1. A cell-based LFA-1: ICAM-1 binding assay was performed using flow cytometry as previously described [23]. Briefly, 293 T cells transiently transfected with LFA-1 were harvested in HBS containing 10 mM EDTA 48 hours after transfection. Cells were washed with HBS, and then resuspended in HBS. Cells were aliquoted to polymerase chain reaction tubes (Axygen; Union City, CA, USA) and then centrifuged. Cell pellets were given 150  $\mu\text{l}$  aliquot of HBS, 2 mM  $\text{MnCl}_2$  containing isoflurane at  $2 \times$  final concentrations, and another 150  $\mu\text{l}$  aliquot of HBS containing 10  $\mu\text{g}/\text{mL}$  ICAM-1-Fc $\alpha$  fusion protein. Tubes were immediately capped, mixed, and incubated for 30 minutes at room temperature. Following wash, cells were incubated with anti-human IgA-



**Figure 3. The determination of isoflurane enantiomers' binding to the  $\alpha\text{L I}$  domain using 1-aminoanthracene (1-AMA).** (A) The reduction of fluorescence intensity versus isoflurane concentration after displacement of 1-AMA from the  $\alpha\text{L I}$  domain. Error bars indicate standard deviation (S.D.) of means of three independent experiments. (B) First,  $K_d$  values of S- and R-isoflurane in each independent experiment was obtained. Then the average  $K_d$  values of S- and R-isoflurane of three independent experiments were calculated (S-isoflurane 374.8  $\mu\text{M}$ , R-isoflurane 460.2  $\mu\text{M}$ ). Data represent mean  $\pm$  S.D. F test was used for statistical analysis. No statistical significance was observed ( $p = 0.0629$ ). doi:10.1371/journal.pone.0096649.g003

FITC (Invitrogen) as a secondary antibody for 30 minutes. The cells were then washed and subjected to flow cytometry analysis using a FACScan instrument (BD Bioscience; San Jose, CA, USA). ICAM-1 binding % was defined as [mean fluorescence intensity (MFI) of samples at various concentrations of isoflurane divided by mean fluorescence intensity of mock-treated sample]  $\times$  100%.

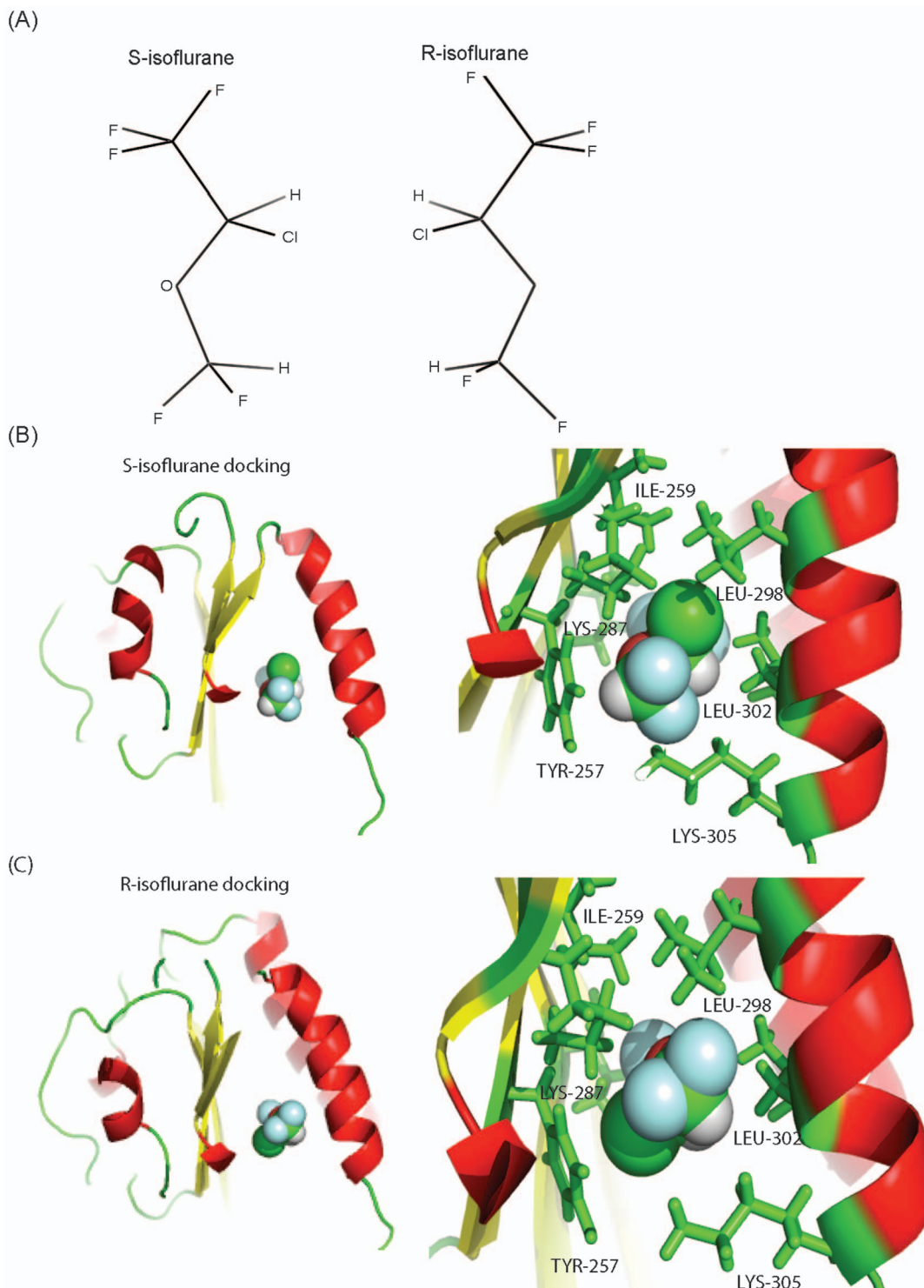
#### Statistical analysis

All the statistical analyses were performed using PRISM 5 software. The details of statistical analysis were described in the corresponding figure legends.  $P < 0.05$  was considered statistically significant.

#### Results

##### The interaction of 1-AMA with LFA-1

1-AMA is a small molecule with environment-dependent fluorescence and general anesthetic properties as demonstrated by the potentiation of gamma-aminobutyric acid (GABA)-ergic transmission [24,25,26]. It has been used to explore protein cavities, and binds to a well-characterized “general anesthetic site” in horse spleen apoferritin (HSAF) [26,27]. We previously



**Figure 4. The docking of isoflurane enantiomers at “lovastatin site”.** (A) The scheme of S- and R-isoflurane is shown. (B, C) The docking result of isoflurane enantiomers onto the  $\alpha$ L I domain is shown. Right panels showed blowup of the docking site. A part of amino acid residues within  $4 \text{ \AA}^3$  from isoflurane were shown with side chains in green. In isoflurane: red, oxygen; green, chlorine; light blue, fluoride. doi:10.1371/journal.pone.0096649.g004

demonstrated that 1-AMA exhibited fluorescence shift in the presence of  $\alpha$ L I domain, suggesting that 1-AMA interacted with LFA-1 [21]. Our previous direct photolabeling work showed that propofol bound the “lovastatin site.” In addition propofol displaced 1-AMA from LFA-1. We explored the size of available

cavities on the surface of  $\alpha$ L I domain and found that only the “lovastatin site” was a larger cavity than 1-AMA. Thus we concluded that 1-AMA bound the “lovastatin site.” This site is also the binding site of LFA-1 allosteric antagonists, and therefore we hypothesized that 1-AMA would block LFA-1. As shown in



**Table 2.** The distance of near-by amino acid residues from isoflurane (< 4 Å).

Amino acid	R-isoflurane (Å)	S-isoflurane (Å)
Leu-132	3.0	3.2
Phe-134	3.9	3.6
Phe-153	3.4	2.8
Val-157	2.6	2.4
Ile-235	2.5	2.5
Tyr-257	3.9	2.9
Ile-259	3.5	3.6
Leu-198	2.8	3.5
Leu-302	2.4	2.4
Lys-305	3.8	3.9

doi:10.1371/journal.pone.0096649.t002

**Figure 2A**, 1-AMA inhibited the binding of LFA-1 to its ligand ICAM-1.

### The binding of 1-AMA to LFA-1 on the cell surface

The interaction of 1-AMA with LFA-1 was studied in a cell-based system as well. Using microscopy, we asked if 1-AMA binds to LFA-1 on the surface of transfected cells. 1-AMA fluorescence was clearly co-localized with LFA-1 (**Figure 2B**), however, it was also seen in non-transfected cells, indicating that it has other binding targets. Because there are many proteins with hydrophobic cavities that can accommodate 1-AMA (~500 Å<sup>3</sup>)[28], this was predicted. Our previous work showed that isoflurane bound LFA-1 at the “lovastatin site” [12,20], so we hypothesized that isoflurane and 1-AMA would compete at this site, with the S-enantiomer being more potent than R-isoflurane. Because 1-AMA displacement requires a small amount of reagents and the available amount of isoflurane enantiomers is limited, we opted to use this method in a cell-free system.

### The purity of S- and R- isoflurane enantiomers

We validated that the S- and R-enantiomers as supplied by the company were greater than 99% pure, and that the racemic mixture consisted of 50% (±2%) of each. (**Figure S1A-C**).

### Interactions of the αL I domain with two isoflurane enantiomers

Our hypothesis was that only S-isoflurane binds to the “lovastatin site”. In contrast, however, *both* enantiomers reduced fluorescence intensity, suggesting that they both interact with the lovastatin site on the αL I domain (**Figure 3A**). The dissociation constants (K<sub>d</sub>) for S-isoflurane and R-isoflurane (374.8 μM and 460.2 μM, resp.) were not statistically different (**Figure 3B**).

### Docking of S- and R-isoflurane into the αL I domain

Both enantiomers were successfully docked to the “lovastatin site” using GLIDE (**Figure 4B and 4C**). The neighboring amino acid residues are listed in **Table 2**. In both S- and R- isoflurane docking models, the trifluoromethyl heads were in the same orientation and formed hydrophobic interactions with Ile-235 and Leu-302. However, the difluoromethyl groups assumed different orientations. The difluoromethyl group in R-isoflurane had hydrophobic interactions with Leu-298 and Leu-302, while in S-isoflurane it interacted with Tyr-257 and Leu-302. Consistent with the 1-AMA experiments, the comparison of the docking scores indicated an inability to detect a significant difference of affinity to the αL I domain between S- and R-isoflurane (**Table 3**).

### Inhibition of LFA-1 by S- and R- isoflurane

The above results indicate that both isoflurane enantiomers bind to the “lovastatin site,” although subtle differences may exist which might be detectable using functional assays. Thus, we tested if both enantiomers could inhibit ligand (ICAM) binding. We used Mn<sup>2+</sup> to activate LFA-1, which mimics inside-out signaling as described above. Also we confirmed that concentrations of S-, R- and racemic isoflurane remained steady during the incubation for 30 minutes. Both S- and R-isoflurane inhibited the binding of ICAM-1 to LFA-1 (**Figure 5**). Interestingly, S-isoflurane inhibited ICAM-1 binding ~50% more potently than R-isoflurane, indicating the presence of functional enantioselectivity of isoflurane enantiomers to LFA-1.

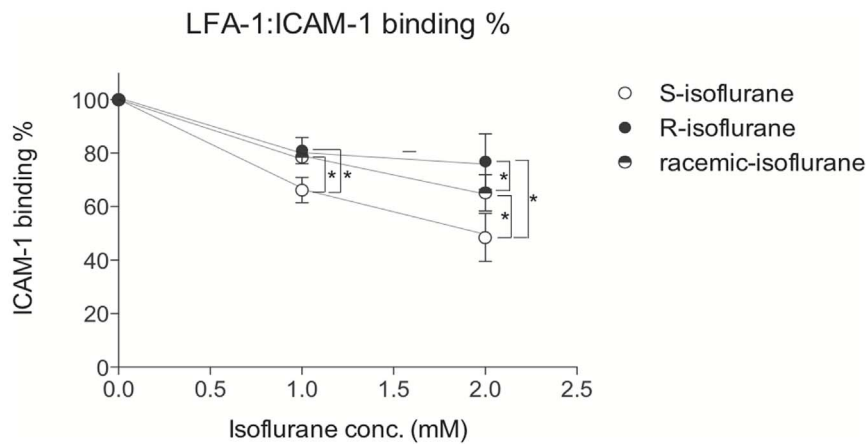
### Discussion

Here we have demonstrated that both S- and R-isoflurane interact with LFA-1 at the lovastatin site, and impair its ligand binding. 1-AMA displacement assay and docking simulations were unable to detect a statistically significant difference in the interaction of the αL I domain with isoflurane enantiomers. Both enantiomers inhibited the binding of LFA-1 to ICAM-1 in cell-

**Table 3.** GLIDE docking score.

	S-isoflurane	R-isoflurane
GLIDE score	-4.495	-4.321

doi:10.1371/journal.pone.0096649.t003



**Figure 5. The ligand binding assay of leukocyte function-associated antigen-1 (LFA-1) using isoflurane enantiomers.** The binding of LFA-1 to ICAM-1 in the presence of isoflurane (racemate, enantiomers) was tested using 293T cells overexpressing LFA-1 using flow cytometry as described in the method. Data represent mean  $\pm$  S.D. of triplicates. To compare any difference in ICAM-1 binding % in the presence of racemic, S- and R- isoflurane, statistical analysis was performed using two-way ANOVA with Bonferroni *post hoc* analysis. \* denotes  $p < 0.05$ . doi:10.1371/journal.pone.0096649.g005

based assays, with the S-enantiomer being more potent. The enantioselectivity of isoflurane on anesthetic targets in the CNS has been documented as in **Table 1**, and in general indicate a very modest effect. This study is the first to investigate the enantioselectivity of targets relevant to immune function.

Our experimental results were inconsistent with structural analysis of previous x-ray crystallography experiments, which indicated that R-isoflurane could not bind this pocket due to steric clash with Tyr-257 [11]. While X ray crystallography is a very useful tool to provide detailed structural information, it may not reflect subtle changes in protein dynamics that allow accommodation of ligands (“induced fit”). Also, numerous lattice contacts at the protein-protein interfaces can subtly alter the crystal structure from the solution structure. Examples are alpha-lactalbumin [29], myoglobin [30] and aspartate transcarbamylase [31]. Finally, it is known that crystallization may not demonstrate structural variability as in the case of intestinal fatty-acid-binding apoprotein where X-ray crystallization did not demonstrate the variability of backbone structure [32]. Both 1-AMA displacement and cell-based ICAM-1 binding assays, were performed in a solution setting where dynamics should be retained. The “lovastatin site” underneath the C-terminal  $\alpha 7$ -helix was demonstrated to be the most variable region in reported I-domain X-ray crystallographic structures [33], as demonstrated by the difference of cavity size depending on the crystallization conditions [21]. The region was quite flexible in solution nuclear magnetic resonance structures [10]. Therefore, the probable explanation of the discrepancy between the structural and our data is a loss of normal protein flexibility or dynamics [10] that might normally result in “induced fit”, and which are reduced in the crystal due to multiple lattice contacts, leading into the altered features of small molecule binding sites. Our study further stresses the importance of solution-based assay for the validation of crystallographic studies.

Pfeiffer’s rule states that the stereoselectivity of a drug is a function of the mass of drug necessary to produce its pharmacological effects [34]. Given that isoflurane’s plasma concentration that produces general anesthetic effects is relatively high ( $\sim$  mM range), it is expected that isoflurane would demonstrate only modest stereoselectivity. Accordingly, previous *in vivo* studies of isoflurane enantiomers did not demonstrate more than a 20-50% difference in anesthetic effect [35,36,37,38]. Our cell-based

functional results showed that a similar magnitude of functional stereoselectivity was the case for LFA-1 as well. Previously, we demonstrated that isoflurane also bound the top domain of the  $\beta 2$  subunit called the  $\beta I$  domain, which is also an important regulator of ICAM-1 binding [20]. There could also be a difference of affinity against this domain between S- and R- isoflurane, which may explain the small difference of the results between 1-AMA displacement assay (or docking simulation) using only the  $\alpha L$  I domain and cell-based ligand binding assay. Unfortunately, the fact that the isolated  $\beta 2$  I domain protein has not been successfully expressed [39] prevented us from testing this hypothesis.

LFA-1 antagonism by isoflurane has a few intriguing clinical implications. For example, LFA-1 knockout mice had a higher mortality than wild-type mice after intraperitoneal *S. pneumoniae* infection [40]. However, Emoto et al. demonstrated that LFA-1 knockout mice were *resistant* to lipopolysaccharide-induced liver injury [41]. Further, Miyamoto et al. showed that LFA-1 knockout mice had resistance to *Listeria monocytogenes* infection [42]. Blockade of LFA-1 has been shown to be beneficial in graft survival in various transplantation models [43,44,45]. A clinical pilot study of allo-islet transplantation recipients treated with the humanized anti-LFA-1 antibody efalizumab showed a favorable outcome [46]. In addition, efalizumab has been used to treat psoriasis [47]. Unfortunately, this drug has been withdrawn from the market due to a risk of fatal brain infections. These studies demonstrate that LFA-1 blockade could be beneficial or detrimental depending on circumstances. Also it has to be noted that many of these studies have been done with permanent LFA-1 blockade (knockout mice), or antibody blockade which usually has a prolonged effect. In our previous studies, we demonstrated that a short exposure of isoflurane (2–4 hours) attenuated neutrophil recruitment in the setting of skin inflammation, a process in which LFA-1 plays a large role. In the future, the impact of transient LFA-1 blockade by anesthetics needs to be examined. The answer to this question will allow us to consider redesigning, or reformulating our anesthetic drugs to mitigate this LFA-1 functional alteration.

Additional finding in this study is that 1-AMA, which is a general anesthetic binding site probe, possessing general anesthetic activity, is an LFA-1 antagonist and bound LFA-1 on the cell

surface. However, the existence of other targets is likely, which clouds interpretation.

In conclusion, we have demonstrated that both isoflurane enantiomers interact with LFA-1, an important component of immunologic cascades, with evidence for subtle enantioselectivity. The clinical significance of this interaction remains to be determined in the future.

## Supporting Information

**Figure S1 Chiral gas chromatography of isoflurane.** The gas chromatographic traces were shown. (A) racemic isoflurane, (B) S- or (C) R- isoflurane. The area under the curve was used to calculate the composition of S- and R-isoflurane in each solution.

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