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The effect of anesthetics on toll like receptor 9

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Abstract

Toll like receptors (TLRs) are critical receptors to respond to danger signals, and their functions are relevant in the perioperative period. We previously reported that volatile anesthetics directly bound to TLR2 and TLR4 and attenuated their functions. Given that TLR9 can respond to mitochondrial DNA, a danger signal that is released upon tissue injury, we examined the role of anesthetics on TLR9 function. Our reporter assay showed that volatile anesthetics isoflurane and sevoflurane increased the activation of TLR9, while propofol attenuated it. TLR9 activation occurs via its dimerization. The dimerization is facilitated by unmethylated cytosine-phosphate-guanine (CpG) DNA as well as DNA containing cytosine at the second position from 5'-end (5'-xCx DNA). Our structural analysis using photoactivable anesthetics and rigid docking simulation showed that isoflurane and sevoflurane bound to both TLR9 dimer interface and 5'-xCx DNA binding site. Propofol bound to the TLR9 antagonist binding site. This is the first illustration that anesthetics can affect the binding of nucleic acids to their receptor. This study sets the foundation for the effect of anesthetics on TLR9 and will pave the way for future studies to determine the significance of such interactions in the clinical setting.

Keywords

electrostatic potential; nucleic acid; toll-like receptor; volatile anesthetics

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AUTHOR CONTRIBUTIONS

K. Yuki designed research; S. Koutsogiannaki, W. Bu, H. Ishida, and K. Yuki performed experiments; W. Bu, U. Ohto, and R.G. Eckenhoff analyzed data; S. Koutsogiannaki, W. Bu, L. Hou, M. Shibamura-Fujiogi and K. Yuki wrote the paper.

CONFLICT OF INTEREST

All the authors do not have any conflict of interest.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

1 | INTRODUCTION

While surgery has revolutionized our medical care, a surgical insult can pose a “trauma” to patients, causing an array of immunological changes.¹ Pro-inflammatory responses are characteristic early-stage responses, which are orchestrated by the host immune system. Immune cells are mounted with a variety of pattern recognition receptors (PRRs) that recognize endogenous danger signals from injured tissues and foreign pathogens. Toll-like receptors (TLRs) are members of the PRR family for innate host defense and evolutionarily ancient.² Ten human TLRs (TLR1-TLR10) and 12 mouse TLRs (TLR1-9, TLR11-13) have been identified thus far and bind to a wide range of redundant and overlapping ligands. TLR2 and TLR4 respond to the largest number of ligands among the TLR family; both of them recognize not only bacterial cell wall components, but also an array of endogenous danger signals from injured tissues such as high mobility group box 1 (HMGB-1) and heat shock proteins (HSPs). They are responsible for perioperative pathophysiology such as ischemia reperfusion injury.^{3,4} Anesthesia is a requisite component for surgical procedures. General anesthesia, characterized by analgesia, amnesia, and immobility, is a type of anesthesia frequently used, and provided by volatile and intravenous anesthetics. In line with our growing understanding that anesthetics can be immunomodulatory,⁵⁻⁹ we previously demonstrated that commonly used volatile anesthetics isoflurane and sevoflurane directly bound to TLR2 and TLR4 and attenuated their function.^{10,11}

The ectodomain of TLRs have 19-25 tandem leucine-rich-repeats (LRRs) with horseshoe-shaped solenoid.^{12,13} Due to this structural similarity, we hypothesized that anesthetics could affect other TLRs as well. TLR9 serves as a receptor for single stranded DNA having a cytosine-phosphate-guanine (CpG) dideoxynucleotide motif¹⁴ and is well known to bind to bacterial DNA¹⁵ as well as DNA virus such as herpes simplex virus-1, adenovirus and poxvirus.¹⁶ In addition to these microbial DNA, TLR9 also recognizes mitochondrial DNA (mtDNA).¹⁷ mtDNA is released by damaged cells and can be also detected in the setting of surgery.^{18,19}

Because TLR9 activation by mtDNA induces inflammatory responses,^{20,21} it is important to understand whether or not anesthetics affect TLR9 function. In this study, we examined the role of anesthetics in TLR9 function. We also performed structural analysis to understand the mode of interaction between anesthetics and TLR9.

2 | MATERIALS AND METHODS

2.1 | TLR9 reporter assay

Human TLR9 reporter cell line HEK-TLR9 cells containing an nuclear factor κ -light-chain-enhancer of activated B cells (NF κ B)-inducible secreted embryonic alkaline phosphate (SEAP) reporter (InvivoGen; San Diego, CA, USA) were used to assess the effect of anesthetics on TLR9 activation as we previously reported.¹⁰ The cells were cultured per the company's protocol. They were stimulated with TLR9 agonist ODN2006 (5 μ g/mL) (InvivoGen) for 6 hours. In some experiments, cells were placed in an air-tight chamber with inflow and outflow ports and exposed to isoflurane (1% and 2%) or sevoflurane (1% and 2%) introduced from the inflow port using a vaporizer for 6 hours as we previously

described.²² The concentration of volatile anesthetics was measured using infrared spectroscopy (Ultima; Datex Instrument Corp, Helsinki, Finland). Intravenous anesthetics (propofol, dexmedetomidine, and ketamine) were added to corresponding wells for 6 hours at a concentration of 10 and/or 100 μ M. Stock solutions for propofol, dexmedetomidine, and ketamine were dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO was kept the same (0.1%) for propofol and dexmedetomidine experiments. The degree of NF- κ B activation was assessed by quantitating SEAP in the medium per the company protocol. Samples were subjected to a spectrophotometer analysis at 655 nm.

2.2 | Cytokine analysis of human whole blood stimulated with TLR9 agonist

We stimulated whole blood with ODN2006 (5 μ g/mL) for 6 hours. Serum was subjected to cytokine profiling using Proteome profiler human cytokine array kit (AYR005B, R&D systems; Minneapolis, MN, USA). Interleukin (IL)-8 was among the upregulated cytokines by TLR9 ligand stimulation. The effect of isoflurane, sevoflurane, and propofol on IL-8 production in whole blood was examined by IL-8 enzyme-linked immunosorbent assay (ELISA) kit (R&D systems).

2.3 | Immunoprecipitation

The interaction between TLR9 and downstream molecule myeloid differentiation 88 (MyD88) was examined by immunoprecipitation. After HEK-TLR9 cells were stimulated with ODN2006 for 3 hours with or without isoflurane (2%), sevoflurane (2%), or propofol (100 μ M), they were subjected to lysis with RIPA buffer containing protease inhibitor cocktail Complete. Following centrifugation, supernatants were subjected to immunoprecipitation using rabbit anti-human TLR9 antibody (Cell Signaling Technology; Danvers, MA, USA) and Dynabeads Protein A immunoprecipitation kit (ThermoFisher Scientific; Waltham, MA, USA). Bead pellets were subjected to western blot analysis and probed with mouse anti-human MyD88 antibody (Santa Cruz Biotechnology Inc; Dallas, TX, USA) followed by antimouse IgG-horseradish peroxidase (HRP) antibody (Cell Signaling). Immunoreactive proteins were visualized by enhanced chemiluminescence.

2.4 | Horse and mouse TLR9 protein purification

The expression vector pMT/BiP/V5-His encoding the extracellular domain of horse TLR9 (residues 21-817) and mouse TLR9 (residues 26-818) was stably transfected into the Drosophila S2 cells and the proteins were purified with IgG Sepharose 6 Fast flow, Superdex 200 gel filtration chromatography, and HiTrap SP cation exchange chromatography (GE Healthcare; Chicago, IL, USA) as we previously described.²³

2.5 | TLR9 photolabeling experiments using azi-anesthetics

Azi-isoflurane, azi-sevoflurane, and m-azipropofol (AziPm) are photoactivatable analogues of isoflurane, sevoflurane and propofol, respectively.^{22,24-26} Azi-isoflurane (30 μ M), azi-sevoflurane (50 μ M), or aziPm (10 μ M) was equilibrated with TLR9 protein (1 mg/mL) in a reaction volume of 300 μ L for 10 minutes. Samples equilibrated with azi-isoflurane or azi-sevoflurane were exposed to 300 nm light using Rayonet RPR-3000 lamp (Southern New England Ultraviolet, Branford, CT, USA). Samples equilibrated with AziPm were exposed

to 350 nm light under a Rayonet RPR-3500 lamp. The protein was separated by SDS-PAGE gel and stained with Coomassie G-250 (Bio-Rad, Hercules, CA, USA). The protein gel band was excised for liquid chromatography-mass spectrometry (MS). After trypsin digestion, samples were injected into a C18 nano-liquid chromatography column with online electrospray into a Thermo LTQ Orbitrap XL Mass Spectrometer (Thermo Fischer Scientific). Raw data were acquired with XCalibur (Thermo Fischer Scientific), and MaxQuant was used to search b and y ions against the sequence of TLR9. Search parameters were 1 amu parent ion tolerance, 1 amu fragment ion tolerance, full tryptic digest, 1 missed cleavage, methionine oxidation (+15.9941), and cysteine alkylation (+57.02146) as a fixed modification. Filter parameters were Xcorr scores (+1 ion) 1.5, (+2 ion) 2.0, (+3 ion) 2.5, delta Cn 0.08, and peptide probability >.05. Photolabeled peptides were searched with the additional dynamic azi-isoflurane (+195.97143 *m/z*), azi-sevoflurane (+230.0559 *m/z*), or aziPm (+216.1996 *m/z*) modifications. MS work was performed at the Proteomics Core Facility of the Wistar Institute (University of Pennsylvania, Philadelphia, PA, USA).

2.6 | Rigid docking analysis

The protein structure was obtained from the Protein Data Bank (PDB; <https://www.pdb.org/>). We used PDB 3PWB, 3PWC, 3PWD, and 5Y3J for horse TLR9 and PDB 3PWF for mouse TLR9. Rigid docking was performed using Autodock Vina (Scripps Laboratories, San Diego, CA, USA). Isoflurane, sevoflurane, or propofol binding site was sought within the grid size of 40 x 40 x 40 cubic angstroms using each adducted residue from photolabeling experiment as a grid center. No positional constraint was applied. We selected the docking position with the highest predicted affinity.

2.7 | Statistics

Data were analyzed as indicated in the corresponding figure legends. Statistical significance was defined as $P < .05$. All the statistical calculations were performed using PRISM 5 software (GraphPad Software, La Jolla, CA, USA).

3 | RESULTS

3.1 | Isoflurane and sevoflurane increased TLR9 activation, while propofol attenuated it

We have tested the effect of various anesthetics on human TLR9 activation by ODN2006, CpG rich 24 mer oligonucleotides (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'). ODN2006 stimulates NF κ B by binding to TLR9. Volatile anesthetics isoflurane and sevoflurane significantly increased the activation of TLR9 (Figure 1A). Intravenous anesthetic propofol reduced the activation of TLR9 (Figure 1A). Intravenous anesthetics dexmedetomidine and ketamine did not affect TLR9 activation (Figure 1A). Because TLR9 ligand can be circulating in blood stream during surgery,^{18,19} understanding the effect of anesthetics on cytokine production induced by TLR9 ligand can be clinically relevant. First we determined cytokine profiles in whole blood stimulated by ODN2006. IL1- α , IL-6, IL-8, C-X-C motif ligand 10 (CXCL10), CXCL11a, and macrophage inflammatory protein (MIP)-1a/MIP-1b levels were upregulated by ODN2006 stimulation, while IL-16 level was downregulated (Figure 1B). We also tested cytokine expression in HEK-TLR9 cells stimulated with ODN2006 and found that IL-1 α , IL-8, and CXCL11a were detected in culture supernatant

(data shown). Based on these results, we examined the effect of anesthetics on ODN2006 mediated IL-8 production in whole blood. While isoflurane and sevoflurane increased IL-8 production, while propofol decreased it (Figure 1C). In line with our data of NF κ B activation and IL-8 production, isoflurane, and sevoflurane-treated samples demonstrated more association between TLR9 and MyD88 than no anesthetic exposure sample (Figure 1D). Propofol did not affect the interaction between them significantly.

Because we previously showed that isoflurane and sevoflurane directly bound to TLR2 and TLR4 and attenuated their functions,^{10,11} we hypothesized that they would directly bind to TLR9 and affect its function. Propofol at the higher dose attenuated TLR4 function.¹¹ Although we did not examine the direct interaction between propofol and TLR4, we hypothesize that propofol would bind to TLR9. Currently horse and mouse TLR9 structures have been elucidated structurally,²³ but human structure has not. The homology between human and horse TLR9 amino acid sequences is 84%, and the homology between human and mouse TLR9 is 75% (Supporting Figure S1). TLR9 has two binding sites for DNA; One site for CpG DNA and another one for 5'-xCx DNA. The mechanism of TLR9 activation has been elucidated mainly from horse TLR9 structure analysis^{23,27}; CpG DNA helps TLR9 to dimerize (TLR9/TLR9*) for activation, and 5'-xCx DNA motif further augments the dimerization and activation^{23,27} (Figure 2A). Respective residues in horse and mouse TLR9 that directly interact with CpG DNA 1668 (5'-TCCATGACGTTTCCTGATGCT-3') and 5'-xCx DNA (5'-TCGCCC) were summarized in Supporting Tables S1 and S2. TLR9 antagonist DNA4084 binds to the inner circle of TLR9 (Figure 2B). Amino acid residues that interact with DNA4084 were also summarized in Supporting Tables S1 and S2. K181, H260, R262, N263, and F343 are located at the interface between TLR9 and TLR9* in the presence of CpG DNA. In the presence of TLR9 antagonist DNA4084, they directly interact with it instead (Figure 2C). In Figure 2D, the direct clash between DNA4084 and TLR9* at the loop is shown.

3.2 | Isoflurane directly bound to horse TLR9 at the dimer interface and the 5'-xCx DNA binding site

The photolabeling experiment using azi-isoflurane showed a number of adducted residues, indicating that isoflurane directly bound to horse TLR9 (Table 1, Figure 3). Rigid docking showed four isoflurane binding pockets (Figure 4A). Nearby amino acid residues for each docked isoflurane were shown in Table 2. Among the isoflurane binding sites, the binding of isoflurane at the pocket 2 involved D259, H260, A261, F343, and Y345 (Figure 4B, Table 2). H260, A261, and F343 are involved in dimer formation at the interface between TLR9 and TLR9* (Supporting Tables S1). D259, H260, A261, and Y345 are residues serving for binding to 5'-xCx DNA. Isoflurane's electrostatic potential map showed a large negative charge potential surface (Figure 4C), which could lead to charge interaction between isoflurane and positively charged residues of TLR9 (Figure 4B). DNA is negatively charged in general, and H260 and 5'-xCx DNA can form charge interactions. ODN2006 presumably binds to the CpG DNA binding site on TLR9 for activation. It may also bind at 5'-xCx DNA site. The binding of isoflurane to the pocket 2 might augment the interaction between TLR9 and TLR9* by itself or together with 5'-xCx DNA. As previously described,²⁷ 5'-xCx DNA alone cannot induce TLR9 dimer formation for its activation. As in line with our finding that

isoflurane bound to 5'-xCx DNA binding site, not to CpG DNA binding site, isoflurane alone did not activate TLR9 (Figure 1A). The presence of 5'-xCx DNA together with CpG DNA would enhance dimerization and activation in human, horse, and cow TLR9 compared to CpG DNA alone.^{23,27,28} In contrast, the degree of dimerization and activation of mouse TLR9 by CpG DNA alone is not different from by the combination of CpG DNA and 5'-xCx DNA.²⁹ Thus, we did not examine the role of isoflurane sites on mouse TLR9 despite we observed adducted residues (Table 1, Supporting Figure S2).

3.3 | Sevoflurane directly bound to horse TLR9 at the dimer interface and the 5'-xCx DNA binding site

Sevoflurane exposure led to the activation of TLR9 as isoflurane did. We also observed sevoflurane binding to horse TLR9 as the presence of adducted residues by azi-sevoflurane (Table 1, Figure 3). Two sevoflurane binding sites were identified by docking simulation (Figure 5A). The sevoflurane binding pocket 2 (Figure 5B) overlapped with the isoflurane binding site (Figure 4B). Residues that interacted with sevoflurane included R257, D259, H260, A261, E317, F343, and Y345. The electrostatic potential map of sevoflurane showed a large area of negatively charged component (Figure 5C) similar to that of isoflurane. Although we observed adducted residues on mouse TLR9 by azi-sevoflurane (Table 1, Supporting Figure S2), we did not examine the role of sevoflurane binding sites on mouse TLR9 due to the reason described in isoflurane section.

3.4 | Propofol directly bound to horse and mouse TLR9 at the TLR9 antagonist binding site

In our functional assay, propofol attenuated TLR9 activation. Multiple residues adducted in the photolabeling experiment on horse TLR9 (Table 1, Figure 3) were examined using docking simulation. Propofol bound to horse TLR9 at the three different locations (Figure 6A). Propofol interacted with R256, H260, A261, R262, E317, and F343 at the pocket 2 (Figure 6B). DNA4084 also inhibits mouse TLR9.²³ Mouse TLR9 residues that interacted with DNA4084 were quite homologous to the ones in human TLR9 (Supporting Table S2). Mouse TLR9 residues were adducted by aziPm, indicating that propofol also bound to mouse TLR9 (Table 1, Supporting Figure S2). Docking simulation showed that propofol bound to the region of mouse TLR9 homologous to the pocket 2 in horse TLR9 (Figure 6C). The electrostatic potential map of propofol demonstrated that a large area of propofol was positively charged (Figure 6D). The presence of propofol might reduce the interaction between negatively charged DNA and TLR9. This may be in part responsible for the reduction of TLR9 activation by propofol.

4 | DISCUSSION

In our study, we showed that isoflurane and sevoflurane increased TLR9 activation in the presence of CpG DNA, while propofol attenuated TLR9 activation. Structural analysis suggested that isoflurane and sevoflurane, largely negatively charged, bound to the TLR9 dimer interface as well as to the 5'-xCx DNA binding site. Propofol, largely positively charged, bound to TLR9 at the DNA4084 binding site, which likely attenuated dimer formation.

TLR9 is expressed on a number of cells including neutrophils, monocytes, dendritic cells, lymphocytes and epithelial cells. The activation of TLR9 by microbial DNA would enhance type I immune responses characterized by enhanced interferon (IFN)- γ production.³⁰ Surgical procedures are often postponed in the setting of active infection unless procedures require urgency/emergency or surgical control of infection is required. TLR9 can also be stimulated by endogenous danger signal mtDNA. During surgical procedures, traumatized tissues by surgical procedures and tissues undergoing ischemia can undergo cell death, with resultant release of mtDNA. mtDNA has been detected in circulating blood in a subset of patients undergoing surgical procedures.^{18,19,31} In the study of trauma patients, their plasma activated TLR9.³² It is worthwhile mentioning that mtDNA itself does not bind to TLR9. mtDNA needs to be digested by DNase to interact with TLR9.³³ Because TLR9 may interact with its ligands during surgical procedures, the effect of surgical anesthetics on TLR9 activation can be a clinically important information. In this study, we have shown that volatile anesthetics enhanced TLR9 activation. In a preclinical model of venous thrombosis, TLR9^{-/-} mice showed increased size in venous thrombosis.³⁴ Depletion of neutrophils from TLR9^{-/-} mice showed reduced size in thrombosis.³⁵ The impact of thrombosis development and resolution in TLR9 activation at different levels has not been examined yet. Its identification would be critical to speculate the clinical role of anesthetics on perioperative TLR9 function for further investigation.

We and others have described the immunomodulatory role of anesthetics.^{5,6} Leukocyte function-associated antigen-1 (LFA-1), macrophage-1 antigen (Mac-1), TLR2, TLR4, Ras-related protein 1 (Rap1), and Fas pathway have been described.^{7,11,22,25,26,36-40} This study adds TLR9 to the list of anesthetic target molecules. Anesthesia studies so far have described that anesthetics primarily affect protein-protein interaction. Our study suggested that anesthetics could affect the interaction between nucleic acid and its receptor. The importance of electrostatic potential for anesthetic interaction with receptors has been previously described in the case of albumin.⁴¹ Here, we described the difference and similarity of the electrostatic potential among isoflurane, sevoflurane, and propofol. Our electrostatic potential map analysis showed quite a difference between volatile anesthetics and propofol, which could at least in part explain the difference in their behaviors to TLR9. Although we showed the interaction between anesthetics and TLR9, TLR9 activation is a very complex process involving posttranslational modifications. The investigation of anesthetic effects on a cascade of TLR9 activation process would be the subject for future investigation.

In conclusion, we have shown that volatile and intravenous anesthetics affected TLR9 function differently. Our structural analysis also suggested that anesthetics directly bound to TLR9 to affect the interaction between nucleic acid and TLR9.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

5'-xCx DNA	DNA containing cytosine at the second position from 5' - end
AziPm	m-azipropofol
CpG	cytosine-phosphate-guanine
CXCL	C-X-C motif ligand
DEX	dexmedetomidine
DMSO	dimethylsulfoxide
ELISA	enzyme-linked immunosorbent assay
HMGB-1	high mobility group box 1
HSP	heat shock protein
IFN	interferon
IL	interleukin
ISF	isoflurane
KET	ketamine
LFA-1	leukocyte function-associated antigen-1
LRR	leucine-rich-repeat
Mac-1	macrophage 1 antigen
MIP	macrophage inflammatory protein
MS	mass spectrometry
mtDNA	mitochondrial DNA
MyD88	myeloid differentiation 88
NFκB	nuclear factor κ-light-chain-enhancer of activated B cells
PDB	Protein Data Bank
PFL	propofol
PRR	pattern recognition receptor
Rap1	Ras-related protein 1

SEAP	secreted embryonic alkaline phosphate
SVF	sevoflurane
TLR	toll like receptor

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FIGURE 1.

The effect of anesthetics on TLR9 activation. A, The effect of volatile anesthetics isoflurane (ISF, 1 and 2%) and sevoflurane (SVF, 1 and 2%) on TLR9 activation was examined using HEK-TLR9 reporter cells stimulated with ODN2006 as described in the Methods. We also examined the effect of intravenous anesthetics propofol (PFL, 10 and 100 μ M), ketamine (KET, 100 μ M), and dexmedetomidine (DEX, 100 μ M) on TLR9 activation. Data were shown as mean $\pm$ SD of six replicates. Statistical analysis was performed using one-way ANOVA with Bonferroni post hoc analysis. * P < .05 and *** P < .001 vs ODN2006 stimulated group without anesthetics, respectively. B, The profile of cytokines produced by ODN2006 stimulation in whole blood was tested in duplicates using Proteome Profiler human cytokine array kit. The result of membrane blot was shown on the left. The expression level of cytokines tested analyzed by Image J software was shown on the right. C, The effect of isoflurane (ISF, 2%), sevoflurane (SVF, 2%), and propofol (PFL, 100 μ M) on IL-8 production in whole blood was examined using IL-8 ELISA assay. Data were shown as mean $\pm$ SD of triplicate. * P < .05 and ** P < .01 vs ODN2006 stimulated samples without anesthetics, respectively. D, The effect of isoflurane (ISF, 2%), sevoflurane (SVF, 2%), and propofol (PFL, 100 μ M) on TLR9-MyD88 interaction was examined using immunoprecipitation. The image shown was representative of two independent experiments. The expression level of MyD88 was determined by Image J software

FIGURE 2.

TLR9 activation and inactivation mechanisms. A, The mechanism of TLR9 activation by DNA agonists. (left) Unliganded TLR9 (PDB: 3WPB) are subjected to CpG DNA ligand ODN1668. Two TLR9 are shown in green (TLR9) and orange (TLR9*). (middle) TLR9 becomes dimerized and activated with ODN1668 (cyan) at the interface (PDB: 3WPC). (right) The degree of TLR9 activation is further enhanced by 2nd DNA ligand 5'-xCx DNA (purple-blue). (PDB: 5Y3J). B, The binding of TLR9 antagonist DNA4084 (hot pink) to TLR9 is shown (PDB: 3WPD). TLR9 remains a monomer in the presence of DNA4084. C, Amino acids that serve for dimer formation between TLR9 and TLR9* in the presence of TLR9 agonist are shown in blue on the TLR9 complexed with DNA4084. Blue amino acids in red circle bind to DNA4084. D, TLR9 complexed with DNA4084 (PDB: 3WPD) was aligned together with the TLR9 dimerized with TLR9* in the presence of TLR9 agonist (PDB: 5Y3J). The loop that clashes between TLR9* and DNA4084 is shown in circle

(A) azi-isoflurane

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31      41      51      61      71      81
RSPWQGTLPF FLPCQLQPHG LVNCHMLFLK SVPHFSAAAP RNNVTSLSLL SNRIHHHLS
91      101     111     121     131     141
DFAQLSNLQK LNLKWNCPFA GLSPMHFFCH MTIEPWIFLA VPTIEELNLS YNGITVPAL
151     161     171     181     191     201
PSSLVSLILS RTNIIQLDPT SLTGLHARF LYMDGNCYK NPGRALEVA PGALLGLGNL
211     221     231     241     251     261
THLSLKYNNL TIVPRSLPFS LEYLLLSYNH IVTLAPEDIA NLTAIRVLDV GNCRCRCDHA
271     281     291     301     311     321
RNPCEVCPHK FPOLHSDFTS HLSRLEGVL KDSSLYQLNF RWFRLGNLT VLDLSENFY
331     341     351     361     371     381
DCITTKAFQ GLAQLRRNL SPNYHKVYSF AHLTIAPSGF SLLSLQELDM HGIFFRSLSQ
391     401     411     421     431     441
KTIQPLARLF MLQRLYLQN FINQAQLGIF KDFPGLRYID LSNRISGAV EPVATTGEVD
451     461     471     481     491     501
GSKKWLISR DLTPGFLDTP SSEDPMPSCK NLSFTLDLSR NNLTVPQPM FAQLSRQLCL
511     521     531     541     551     561
RLSHNSISQA VNGSQFVPLT SLQVLDLSHN KLDLYHGRSF TELPREALD LSYNSQFFSM
571     581     591     601     611     621
RGVGHNLSPV AQLPTIRYLS LAHNGHSRV SQQLCSTSLW ALDFSGNLS QMAEGDLYL
631     641     651     661     671     681
RFFQGLRSLI RLDLSQNRHL TLTPCTLGNL PKSLQLRLR NNYLAFFNWS SLTLLPNLET
691     701     711     721     731     741
LDLAGNQLKA LNSGSLPSGT QLQRLDSRN SIIFVVGFF ALATRLRELN LSNALRTVE
751     761     771     781     791     801
PSWFGFLAGS LEVLDSANP LHCACGAFFV DFLIQQAQV PGLPSRVKCG SPQQLQGRSI
811     821
FAQDLRLCLD ESLSDWEFLV PR

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(B) azi-sevoflurane

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31      41      51      61      71      81
RSPWQGTLPF FLPCQLQPHG LVNCHMLFLK SVPHFSAAAP RNNVTSLSLL SNRIHHHLS
91      101     111     121     131     141
DFAQLSNLQK LNLKWNCPFA GLSPMHFFCH MTIEPWIFLA VPTIEELNLS YNGITVPAL
151     161     171     181     191     201
PSSLVSLILS RTNIIQLDPT SLTGLHARF LYMDGNCYK NPGRALEVA PGALLGLGNL
211     221     231     241     251     261
THLSLKYNNL TIVPRSLPFS LEYLLLSYNH IVTLAPEDIA NLTAIRVLDV GNCRCRCDHA
271     281     291     301     311     321
RNPCEVCPHK FPOLHSDFTS HLSRLEGVL KDSSLYQLNF RWFRLGNLT VLDLSENFY
331     341     351     361     371     381
DCITTKAFQ GLAQLRRNL SPNYHKVYSF AHLTIAPSGF SLLSLQELDM HGIFFRSLSQ
391     401     411     421     431     441
KTIQPLARLF MLQRLYLQN FINQAQLGIF KDFPGLRYID LSNRISGAV EPVATTGEVD
451     461     471     481     491     501
GSKKWLISR DLTPGFLDTP SSEDPMPSCK NLSFTLDLSR NNLTVPQPM FAQLSRQLCL
511     521     531     541     551     561
RLSHNSISQA VNGSQFVPLT SLQVLDLSHN KLDLYHGRSF TELPREALD LSYNSQFFSM
571     581     591     601     611     621
RGVGHNLSPV AQLPTIRYLS LAHNGHSRV SQQLCSTSLW ALDFSGNLS QMAEGDLYL
631     641     651     661     671     681
RFFQGLRSLI RLDLSQNRHL TLTPCTLGNL PKSLQLRLR NNYLAFFNWS SLTLLPNLET
691     701     711     721     731     741
LDLAGNQLKA LNSGSLPSGT QLQRLDSRN SIIFVVGFF ALATRLRELN LSNALRTVE
751     761     771     781     791     801
PSWFGFLAGS LEVLDSANP LHCACGAFFV DFLIQQAQV PGLPSRVKCG SPQQLQGRSI
811     821
FAQDLRLCLD ESLSDWEFLV PR

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(C) aziPm

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31      41      51      61      71      81
RSPWQGTLPF FLPCQLQPHG LVNCHMLFLK SVPHFSAAAP RNNVTSLSLL SNRIHHHLS
91      101     111     121     131     141
DFAQLSNLQK LNLKWNCPFA GLSPMHFFCH MTIEPWIFLA VPTIEELNLS YNGITVPAL
151     161     171     181     191     201
PSSLVSLILS RTNIIQLDPT SLTGLHARF LYMDGNCYK NPGRALEVA PGALLGLGNL
211     221     231     241     251     261
THLSLKYNNL TIVPRSLPFS LEYLLLSYNH IVTLAPEDIA NLTAIRVLDV GNCRCRCDHA
271     281     291     301     311     321
RNPCEVCPHK FPOLHSDFTS HLSRLEGVL KDSSLYQLNF RWFRLGNLT VLDLSENFY
331     341     351     361     371     381
DCITTKAFQ GLAQLRRNL SPNYHKVYSF AHLTIAPSGF SLLSLQELDM HGIFFRSLSQ
391     401     411     421     431     441
KTIQPLARLF MLQRLYLQN FINQAQLGIF KDFPGLRYID LSNRISGAV EPVATTGEVD
451     461     471     481     491     501
GSKKWLISR DLTPGFLDTP SSEDPMPSCK NLSFTLDLSR NNLTVPQPM FAQLSRQLCL
511     521     531     541     551     561
RLSHNSISQA VNGSQFVPLT SLQVLDLSHN KLDLYHGRSF TELPREALD LSYNSQFFSM
571     581     591     601     611     621
RGVGHNLSPV AQLPTIRYLS LAHNGHSRV SQQLCSTSLW ALDFSGNLS QMAEGDLYL
631     641     651     661     671     681
RFFQGLRSLI RLDLSQNRHL TLTPCTLGNL PKSLQLRLR NNYLAFFNWS SLTLLPNLET
691     701     711     721     731     741
LDLAGNQLKA LNSGSLPSGT QLQRLDSRN SIIFVVGFF ALATRLRELN LSNALRTVE
751     761     771     781     791     801
PSWFGFLAGS LEVLDSANP LHCACGAFFV DFLIQQAQV PGLPSRVKCG SPQQLQGRSI
811     821
FAQDLRLCLD ESLSDWEFLV PR

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FIGURE 3.

Coverage map for horse TLR9 mass spectrometry analysis of photolabeled samples with isoflurane, sevoflurane, and propofol. Sequence of the purified horse TLR9 with high-confidence coverage in the mass spectrometry analysis denoted as highlighted residues. Adducted residues are shown in asterisk

FIGURE 4.

The interaction of isoflurane with horse TLR9. A, Isoflurane binding sites on TLR9 dimer (TLR9/TLR9*) with CpG DNA (DNA1668) (PDB: 3WPC) are shown. Based on the photolabeling and rigid docking experiments, four isoflurane binding pockets were identified. B, Isoflurane pocket 2 is located at the interface between TLR9 and TLR9*. Blowout image of the isoflurane binding site is shown. Amino acid residues that interact with isoflurane are shown with stick images. In the isoflurane molecule, green; Cl, red; O, cyan; C and light blue; F. Polar interaction is shown in dotted yellow line. Positive charge residues are highlighted in gray. C, The electrostatic potential map of isoflurane is shown. The figure was made from <http://www.webmo.net/demoserver>. Different charged distribution on the molecular surface is shown by color scale. Red is the most negative and blue is the most positive. D, Amino acid residues that interact with isoflurane at the pocket 2 are shown in TLR9 dimer complexed with CpG DNA + 5'-xCx DNA (PDB: 5Y3J)

FIGURE 5.

The interaction of sevoflurane with horse TLR9. A, Sevoflurane binding sites on TLR9 dimer (TLR9/TLR9*) with CpG DNA (DNA1668) (PDB: 3WPC) are shown. Based on the photolabeling and rigid docking experiments, two isoflurane binding pockets were identified. B, Sevoflurane pocket 2 is located at the interface between TLR9 and TLR9*. Blowout image of the isoflurane binding site is shown. Amino acid residues that interact with isoflurane are shown with stick images. In the sevoflurane molecule, red; O, cyan; C and light blue; F. C, The electrostatic potential map of sevoflurane is shown. The figure was made from <http://www.webmo.net/demoserver>. Different charged distribution on the molecular surface is shown by color scale. Red is the most negative and blue is the most positive

FIGURE 6.

The interaction of propofol with TLR9. A, Propofol binding sites on horse TLR9 monomer with DNA4084 (PDB: 3WPD) are shown. Based on the photolabeling and rigid docking experiments, three propofol binding pockets were identified. B, Propofol pocket 2 in horse TLR9 is located at the binding site of DNA4084. Blowout image of the propofol binding site is shown. Amino acid residues that interact with propofol are shown with stick images. In the propofol molecule, red; O, cyan; C and light gray; H. C, Propofol binding site on mouse TLR9 is located at the binding site of DNA4084. Blowout image of the propofol binding site is shown. Amino acid residues that interact with propofol are shown with stick images. D, The electrostatic potential map of propofol is shown. The figure was made from <http://www.webmo.net/demoserver>. Different charged distribution on the molecular surface is shown by color scale. Red is the most negative and blue is the most positive

TABLE 1
Adducted residues of horse and mouse TLR9 by azi-isoflurane, azi-sevoflurane, and AziPm

Azi-anesthetic	Adducted residues	Sequence coverage (%)
Horse TLR9		
Azi-isoflurane	H167, V290, I420, S496, L643	84.2
Azi-sevoflurane	L166, L495	75.3
AziPm	L169, D293, S342, Y397, Y419, S496, L643, Y664	91.6
Mouse TLR9		
Azi-isoflurane	S199, R645	79.5
Azi-sevoflurane	L422, L644	80.0
AziPm	S199, Y299, Y345, D421, P646	70.0

TABLE 2

Nearby amino acids of horse TLR9 from docked isoflurane

Isoflurane binding site	Nearby amino acids
Pocket 1	S67, L68, S69, K91, L92, N93, H111, M112, T113, I114, E127, L128, N129, I135, T136, T137, V138, P139, A140, L141, T161, S162, L163, L166
Pocket 2	Y179, Y180, H231, R257, D259, H260, A261, P264, C265, V266, D293, F317, E343, Y345
Pocket 3	A406, Q407, L408, G409, G429, A430, V431, D434, P468, S469, N472, L473, E490, M491, A493, Q494, Q524, V525, E548, A549, Q656, L657, E680, T681, R705
Pocket 4	T681, L682, D683, R705, L706, D707, E729, L730, N731, V754, D756
Sevoflurane binding site	Nearby amino acids
Pocket 1	H111, M112, T113, I114, I135, T136, T137, V138, P139, A140, L141, T161, S162, L163, T164, L166
Pocket 2	R257, D259, H260, A261, E317, F343, Y345, V349, S350, F351, F375, F402,
Propofol binding site	Nearby amino acids
Pocket 1	L42, N44, N46, S67, L68, S69, K91, L92, N93, H111, M112, T113, I114, P116, E127, T136, T137, V138, P139, I155, L156, Q157, D159, T161, S162, R186, A187, L188, E189, V190, L211, T212, T213, P215
Pocket 2	R256, R257, C258, D259, H260, A261, R262, C268, P269, H270, F272, P273, S295, L296, Y297, Q298, W303, E317, F343, Y345, V349, S350, F351, F375, F402, R426
Pocket 3	R661, T681, L682, D683, R705, L706, D707, E729, L730, N731