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Shedding Light on Anesthetic Mechanisms: Application of Photoaffinity Ligands

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Abstract

Anesthetic photoaffinity ligands have had an increasing presence within anesthesiology research. These ligands mimic parent general anesthetics, and allow investigators to study anesthetic interactions with receptors and enzymes; identify novel targets; and determine distribution within biological systems. To date nearly all general anesthetics used in medicine have a corresponding photoaffinity ligand represented in the literature. In this review we examine all aspects of the current methodologies, including ligand design, characterization and deployment. Finally we offer

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points of consideration and highlight the future outlook as more photoaffinity ligands emerge within the field.

Introduction

Studying the interactions between general anesthetics and their macromolecular targets is crucial to the understanding of the biochemistry for these drugs. In recent years the application of photoaffinity labeling for this purpose has increased. Traditionally the objectives of this method are to allow researchers to identify the binding site(s) within established targets (micro-level) and, to a lesser extant, determine novel binding molecules and distribution within a given biological system (macro-level). Within anesthesia research there has been significant advancement in the development of photoaffinity ligands (PALs), from the initial use of neat halothane [1] to complex syntheses of bifunctional PALs allowing for affinity-based protein profiling[2]. The primary objective of this review is to feature current technologies and to discuss the application and results of anesthetic PALs target identifications.

Photoaffinity Ligand

In the current process of photoaffinity labeling, the chemical structure of the drug of interest (parent ligand) is modified to incorporate a photoreactive group that, upon ultraviolet (UV) irradiation, generates a highly reactive chemical intermediate. This intermediate then chemically “adducts” or “labels,” by covalent insertion to a bond in close proximity, such as solvent components or an occupied macromolecule. In the instance of a PAL bound to a macromolecule, the ligand adduct acts as a traceable modification that can be used to investigate ligand-target interactions and/or ligand distribution within the organ or tissue. A critical advantage that PALs provide for general anesthetics is that they mitigate the relative lower affinities, generally micromolar, associated with this particular class of drugs. They accomplish this by dramatically prolonging drug unbinding rates, thereby providing an irreversible snapshot of these otherwise transient interaction(s). On the other hand, the inherent high dissociation constants of anesthetics remains an experimental hurdle chiefly by adding to the difficultly in validation of specific binding sites in photoaffinity labeling studies. These concepts and current methodologies for validation of anesthetic-specific sites will be discussed later in the review. The following sections are devoted to the initial design and physicochemical characterization of an anesthetic PAL, both critical to the interpretation of anesthetic photoaffinity labeling results.

Anesthetic Photoaffinity Ligand

Design

Several factors are considered during the design of an anesthetic PAL; most notably that introduction of the photoreactive group into the anesthetic chemical structure does not significantly alter the biochemical and pharmacological properties of the parent drug. Diazirines are nearly universal as the photoreactive group incorpated within anesthetic PALs (Figure 1). With UV irradiation, diazirines undergo photoactivation, resulting in the release

of an inert dinitrogen molecule and the generation of carbene chemical species that indiscriminately insert into the nearest molecule. The popularity of this particular photoreactive group can be largely attributed to the less damaging UV wavelength needed for photoactiviation, its relative stability and smaller size, and highly reactive intermediate product, the carbene. In combination, these attributes allow for less deviation of binding properties compared to the parent anesthetic. We direct the interested reader to previous reviews for further detailed discussion on the development and use of PALs and photoreactive groups [3–6].

In practice, the design and synthesis of a PAL to effectively mimic a general anesthetic can be arguably the most difficult process due to the challenging synthesis of stable molecules containing photoreactive groups, and the lack of in depth knowledge of molecular recognition elements between anesthetics and their important macromolecular targets. The small size and relatively featureless nature of most general anesthetics make any chemical modification, even a diazirine, a relatively large perturbation. As it stands, even the best-designed PAL requires considerable chemical deviation from the parent anesthetic structure (exception being halothane[1]) and alone cannot definitively determine a binding target and/or site for the parent drug.

Anesthetic Photoaffinity Ligand

Physicochemical Properties

After incorporation of the diazirine into the anesthetic chemical structure, the resulting changes in physicochemical properties require careful characterization before experimental deployment of the PAL. The stability and photactivation efficiency of the diazirine (or any incorporated photoreactive group) are unique to each developed PAL. The diazirine moeity should produce a distinctive and pronounced UV absorbance [4,7] that decays with increasing exposure to the appropriate UV wavelength [8,9]. The rate of decay provides an estimate of photoactivation efficiency within the given buffer. A basic equation (Eq. 1) [10] can give the half-life of photoactivation ($T_{1/2}$) with relationship to the intensity of the lamp (I_0) applied, molar extinction coefficient (ε) and quantum yield of photoactivation (Ψ).

$$T_{1/2}=0.3/(\Psi I_0 \varepsilon)10 \quad (1)$$

The chemical nature of the subsequent reactive intermediate generated by photoactivation is also unique to each PAL. The reactivity of the intermediate directly influences the propensity to covalently insert into nearby molecules or, depending on ligand chemical structure, itself (e.g. *intra*molecular reactions). For example, studies of 6-azipregnanolone, a PAL derivative for pregnanolone, reported that major photoactivation products were likely generated from internal rearrangement or bimolecular insertion reactions [11]. As a result, some macromolecular sites would not be sufficiently represented to allow for detection due to low to zero yield of adducted intermolecular products. It could be argued that a propensity for intermolecular reactions decreases the probability for nonspecific labeling, but this ultimately depends on the relative reaction rates.

Assuming that a photoactivated ligand intermediate demonstrates sufficient probability for intermolecular interactions, it is generally considered that most carbenes do not demonstrate significant preference for covalent insertion, being capable of both nucleophilic and electrophilic attack [4]. This includes to the solvent molecules, which under usual conditions are in far greater abundance than a target macromolecule. For example, the singlet carbene intermediate is readily quenched by covalent insertion into adjacent water molecules [12], and therefore presents another mechanism that deters nonspecific labeling. Similarly, amphipathic molecules, such as lipids and/or detergents, have also been shown to be readily adducted [6]. Weiser et al. demonstrated that the tritiated propofol dervative [^{3}H]*meta*-azipropofol, while showing preference towards synaptic dense regions (and therefore presumably protein), ubiquitously labeled whole rat brain, implying that lipids also act as a considerable photoadducted product [13].

Increasing evidence has suggested that proteins are a significant contributor to anesthetic mechanisms. As such, PALs have been applied toward understanding protein binding sites of anesthetics. Table 1 gives the list of photoadducted residues of halothane and diazirine-containing anesthetic PALs with protein targets. To our knowledge, there has not been a systematic investigation of an anesthetic PAL that conclusively demonstrates preferential insertion into selected amino acids within a protein or polypeptide. Indeed previous work has demonstrated that, while the efficiency (defined as mole of PAL per mole of stationary pure amino acid) of labeling of specific amino acids may be greater for a given PAL (e.g. Cys, Trp, His, Phe), a carbene can covalently insert into all amino acids even within a transmembrane domain [6,14]. It should be noted that backbone atoms within a protein, not just side chains, can act as potential insertion sites. Indeed, previous evidence with aziisoflurane modification of apoferritin implies that backbone atoms (such as carbonyl oxygens) are suitable photoadduction sites [15].

An exception to preferential residue labeling arises with aliphatic-diazirines and their increased predisposition to undergo diazo isomerization, an alternative *intra*molecular rearrangement. The diazo isomerization of the diazirine can lead to the generation of a carbocation, rather than a carbene, an intermediate that preferentially undergoes electrophilic attack of electron dense (nucleophilic) residues [7]. The fraction of the PAL and its resulting products generated by this unwanted isomerization is specific to the chemical properties of the PAL with the event being debated for some aliphatic-diazirines [16]. Regardless, the propensity of labeling Asp, Glu, His, and Tyr by azietomidate [17,18] led to the development of the etomidate derivatives pTFD-etomidate [19] and TDBzl-etomidate [20], both of which contain trifluoromethyl diazirine and trifluoromethylaryl diazirine respectively. These additional chemical groups chemically favor carbene generation, rather than diazo isomerization, leading to the desired indiscriminate covalent insertions [4,7,21]. The synthetic changes of etomidate PALs resulted in a broader range of labeled residues, including hydrophobic residues [20,22,23]. However whether these modifications were due to changes in photochemistry and/or to altered equilibrium binding due to the changes in chemical structure is unclear.

Anesthetic Photoaffinity Ligand

Biochemical and Pharmacological Activity

It is universally agreed that any novel anesthetic derivative, including PALs, requires a thorough investigation of biochemical and pharmacological activities to assure retention of parent drug characteristics. These studies can be placed in three basic groups; 1) equilibrium binding to anesthetic protein models, 2) isolated functional studies and 3) *in vivo* demonstration of pharmacological endpoints.

Similar equilibrium binding of the parent anesthetic and the PAL derivative to model proteins provides some evidence towards the retention of basic molecular recognition elements (hydrophobic forces, hydrogen bonding, van der Waals and electrostatic forces, etc.) between the parent drug and developed PAL. Well characterized anesthetic protein models previously crystalized in complex with the parent anesthetic at high resolution, such as apo-ferritin[24,25] and human serum albumin [26,27], are often used.

The PAL should demonstrate similar functional effect(s) within an established protein target as the parent drug. For many micro-level studies, the targets considered include the cys-loop pentameric ligand gated ion channels, such as the nicotinic acetylcholine receptor (nAChR) and γ-amino butyric acid type A ($GABA_A$) receptor. Functional characterization therefore requires electrophysiology. Within these studies investigators often observe changes in potency and/or efficacy dependent on the type and position of chemical modifications made to turn the parent ligand into a PAL, as observed with the *meta*- and *ortho*- trifluoromethyl-diazrine substitutions on propofol PALs [28,29]. Similarly the different PAL derivatives of etomidate PALs show altered activity on nAChR, including approximately 5-fold differences in potency between azietomidate and pTFD-etomidate for inhibition [19,30]. Because the contributions of these individual targets to the desired effect (anesthesia) is not known, it is not clear *a priori* how important these subtle changes in potency are for the interpretation of photoaffinity labeling data. Regardless, these studies aim to demonstrate that the ligand retains functional activity similar to the parent anesthetic. However this demonstration, while necessary, is not sufficient to indicate a common binding site in that the PAL may act as a functionally active ligand but at other site(s).

In addition to retention of activity *in vitro*, retention of *in vivo* activity is also a necessary form of validation of the anesthetic PAL. These studies usually include tadpole immobility assays or rodent loss of righting reflex within wild type and/or mutant models [31]. Since the total mechanism by which any of the general anesthetics produce their *in vivo* endpoints remain unclear, the retention of *in vivo* activity alone is insufficient evidence to support binding and action at specific protein targets. An interesting demonstration of this is TDBzl-etomidate, an etomidate PAL derivative. TDBzl-etomidate demonstrates comparable potency for tadpole immobility and more potent potentiation of the $GABA_A$ receptor relative to the parent anesthetic [20]. However unlike etomidate, this PAL acts as a positive modulator of nAChR, [19] indicating that caution is required when attempting to correlate *in vivo* potency and a shared binding site within a specific target macromolecule.

Each of the biochemical and pharmacological investigations can be extended to provide evidence to confirm the PALs' capabilities to successfully insert into the bound targets after photoactivation. For example photoaffinity labeling of model proteins with halothane [1], isoflurane [15], or propofol [28,29] PAL derivatives have demonstrated insertion into residues lining the crystallographically confirmed site(s) of serum albumin or apo-ferritin. Irreversable enhancement of $GABA_A$ receptor gating and desensitization has been reported upon azietomidate photoactivation [32]. *Meta*-azipropofol photoactivation *in vivo* has shown significant prolongation of emergence, nearly 10-fold, of tadpole immobility [33] suggesting successful covalent insertion into a sufficient mass or number of targets that contribute to an anesthetic endpoint. While the absence of this "optoanesthesia" feature does not negate the validity of a PAL, its presence is strong evidence for validity and utility. Evidence for reliable photoadduction can be considered just as important as the studies demonstrating retention of biochemical and pharmacological activity in that this characteristic is directly responsible for the identification of novel binding partners.

Micro-level Photoaffinity Labeling

The majority of studies in the current literature use anesthetic PALs to determine anesthetic binding sites within a specific preselected protein target, an approach we term micro-level studies. The identification and characterization of drug binding sites promotes increased understanding of the molecular mechanism(s) within pharmacological targets and, potentially, drug modifications to improve the drug safety profile.

Micro-level Target Detection

Methodologies

Two different methodologies of protein microsequencing are commonly used to identify protein binding sites photolabeled by a PAL. These include Edman degradation (ED) and mass spectrometry (MS), both of which determine binding sites to the amino acid level. Simplified schematics for both methods are shown in Figure 2a–b. ED and MS have been used extensively for multiple types of potential protein targets, soluble and insoluble, over the entire range of anesthetic PALs (Table 1) and much has been learned in terms of binding site location, specificity and mechanism of protein modulation. Depending on the characteristics and size of the investigated protein target, both ED and MS can become complex. For example, larger multimeric membrane proteins may require additional steps of precipitation, separation and protease digestions in order to achieve sufficient coverage of the sequence to confidently reveal or exclude adduction sites.

While the fundamental endpoint results of each method are identical (e.g. identities of the adducted amino acids), the final means to gather the results are notably different. For ED, individual amino acids are sequentially and chemically cleaved (e.g. the Edman reaction) from pools of purified peptide fragments from the digested target protein. The resulting pool of the cleaved amino acid is then separated into two smaller pools; one used for the identification of the amino acid using high-performance liquid chromatography, the other to detect whether this amino acid contains a radiolabeled PAL adduct. It should be noted that when a backbone atom is labeled by the radiolabeled PAL, the Edman reaction (or prior

peptide cleavages) may be retarded. Although this has not been systematically studied, evidence in support of the possibility is the frequent observation that subsequent amino acid yield decreases after a radiolabeled amino acid.

In contrast, in the MS approach, the PAL adduct is detected by the change the label imparts to the molecular mass-to-charge (*m/z*) ratio of a peptide fragment. For amino acid level localization of the adduct, second level (MS/MS) or higher order data are required. Most often ion traps, quadrupole mass filters and mass analyzers are combined to the electrospray source for detection. The recent development of orbitrap and Fourier Transform Ion Cyclotron Resonance has resulted in very high mass accuracy, resolution, and dynamic range of detection for the MS method [34–36]. These recent advances in MS technology and the non-dependence on radioactivity have contributed to the increased use of MS as a method for detection in photoaffinity labeling studies.

Micro-level Photoaffinity Labeling

Anesthestic-Specific Binding Site Validation

The protein binding sites identified by PAL labeling require several levels of validation to have confidence that the revealed site is the same as that of the parent anesthetic. According to pharmacological convention, *specific* photoaffinity labeling would be irreversible labeling by the PAL, within a *saturable* protein binding site. For the purposed of this review, we define *anesthetic-specific* photoaffinity labeling as when this same labeled saturable site would be shared by the parent anesthetic. In contrast, *nonspecific* photoaffinity labeling is a result of random adduction, such as to peripheral, solvent accessible, or lipid exposed regions of a protein. Nonspecific labeling could also occur with the migration of the reactive intermediate after photoactivation in solvent, lipid or protein matrix to a random and remote site. All these forms of labeling will occur over the course of a single experiment, but in general nonspecific labeling is challenging to detect in most conditions due to the large number of potential adduction sites, generally lower affinity and therefore lower occupancy, and random nature. Regardless, nonspecific labeling should have low reproducibility, so to categorize sites as “specific” requires several experiments.

Validation of an *anesthetic*-specific over a *PAL*-specific binding site is also required for a target protein. Notably, it should be recognized that both instances may contribute to a mechanism or component of “anesthesia,” however only parent anesthetic-specific adducted sites are of interest and relevance to clinical medicine. Generally the initial step includes reconciling the labeled residues against existing crystal structures or developed models to identify potential localization within a protein cavity, interface or pore site. The characteristics of these sites may indicate potential mechanisms as well as the likelihood for shared binding by the parent anesthetic. For example, the adduct site of aziisoflurane in platelet receptor integrin αIIbβ3 resided near a calcium binding site, a critical region for regulation of the protein. This proximity immediately suggests a potential mechanism for isoflurane-induced attenuation of platelet aggregation [37].

Another common form of validation is by mutagenesis of the adducted residue(s), followed by functional studies and perhaps repeat photoaffinity labeling experiments. These studies,

while indirect, allow association of the photoadducted residue and site to functional activity of the parent anesthetic within the protein. However, common to all mutagenesis investigations, the change in residue might also alter protein structure or dynamics, altering the protein's response to the anesthetic instead of changing the affinity for the binding site or altering protein structure preventing access to a regionally distinct binding site. Furthermore, since the photoadduction, and therefore site might be side chain independent, mutagenesis studies might not provide a clear interpretation.

Finally direct evidence of a parent anesthetic-specific adduct site would be represented by successful inhibition of PAL labeling through competitive binding, or "protection," by the parent anesthetic [10]. Although intuitive, protection from photolabeling is complicated by the non-equilibrium nature of most photolabeling experiments. A kinetic mechanism for protection of a parent anesthetic-specific site from photoadduction is described in Figure 3, and is similar to that previously described for photoaffinity agents and other protection experiments for non-equilibrium systems [10,38]. The model has simultaneous dependence on the two different affinities of the ligands (e.g. protecting ligand and PAL) for the protein target, and the photoreactivity of the PAL (which is highly dependent on the experimental conditions noted in section 2.2). Since the photolabeling event is irreversible, in contrast to the parent ligand, binding sites will be gradually depleted. Further, the PAL will also be gradually depleted by solvent labeling. Therefore, multiple consecutive and competing rates, unique to the target protein, protecting ligand and PAL, are present within a typical protection experiment. As a result, protection experiments require careful attention, otherwise, results can be misinterpreted.

Some physicochemical limitations of the PAL or the method for label detection may prevent quantitative assessment of protection. For example, a relatively high molar ratio of the parent anesthetic to PAL is generally required for protection (>100:1). However, such a high concentration may not be possible due to limitations in parent anesthetic solubility as reported previously [39], making interpretation of protection experiments a challenge. Quantitative detection by MS also poses limitations, particularly for label-free quantification that requires considerable protection for significance under most experimental conditions [40]. However with continuing advancement of the technique, such as isobaric labeling methods [41,42], quantification of protection experiments by MS will be possible.

Micro-level Target Detection

Significant Findings

The potential for in-depth knowledge of the molecular mechanisms of anesthesia and/or drug side effects is reflected by the wide range of developed anesthetic PALs and their numerous micro-level investigations. In this section we will focus on selected significant findings for anesthetic PALs as examples of insights given by the technique. We direct the reader to our previous review, as well as to Table 1, [3] that provide a comprehensive overview of all developed anesthetic PALs and studied protein targets.

To date, two volatile anesthetic PALs have been deployed in micro-level investigations; neat halothane and aziisoflurane. Permitted by ^{14}C-labeled halothane, ED identified halothane

modification of extracellular and transmembrane domains within *Torpedo* nAChR [43]. In particular, isoflurane protected halothane labeling of Tyr-228 within the δ subunit in a state-dependent manner. This finding highlighted a potential pocket formed within the receptor's desensitized state that can accommodate either anesthetic and, through stabilization of the state, may contribute to the functional inhibition of nAChR. A similar trend of potential state-dependent binding was suggested by aziisoflurane labeling of lymphocyte function associated antigen-1 [44]. The labeling of Ile-135 and Glu-137 within the lymphocyte function associated antigen-1β1 domain by aziisoflurane suggested a closed state-dependent binding. The stabilization of this conformation by the volatile anesthetic may contribute to the impaired lymphocyte arrest and the antiinflammatory actions displayed by isoflurane [45–47].

The PAL derivative of mephobarbital, (R)-(−)-*m*TFD-mephobarbital, was shown to act as a particularly potent positive modulator of the $GABA_A$ receptor; approaching the potencies of etomidate and propofol [48]. As such (R)-(−)-*m*TFD-mephobarbital was used to uncover binding sites of barbiturate anesthetics. When compared to azietomidate, photoaffinity labeling studies suggested distinct sets of sites within the $GABA_A$ receptor for the two anesthetics [49]. Both (R)-(−)-*m*TFD-mephobarbital and azietomidate labeled intersubunit sites, and at a similar depth within the transmembrane region; however, the different anesthetics targeted different subunit interfaces. *m*TFD-mephobarbital selectively labeled α+/β- and γ+/β- while azietomidate selectively labeled the two β+/α-intersubunit sites with the $GABA_A$ receptors[49]. These studies demonstrate that the heterogeneity of anesthetic chemical structures may be reflected by different site locations within the same target. Indeed, with the likely joint use of multiple PALs, a progressive understanding of the molecular mechanisms, including state-dependent binding and structure-function relationships, for anesthetics within complex target proteins can be achieved.

Macro-level Photoaffinity Labeling

Macro-level photoaffinity labeling is a much less prevalent method compared to the application of micro-level photoaffinity labeling within anesthesiology research. Despite this, macro-level investigations are becoming recognized as a useful tool within chemical biology due to the increased need to uncover druggable targets, matched with the growing awareness of how promiscuous many drugs are. Anesthetics can be considered a prime example of the current paradox in drug development in that a there is an urgent need for optimized chemical designs in order to improve potency or decrease toxicity yet the lack of mechanistic knowledge prevents educated design. The following section presents an overview of selected studies and perspectives of anesthetic PALs in macro-level studies.

Macro-level Photoaffinity Labeling

Significant Findings

Previously the labeling of rat brain membranes with [^{3}H]6-azipregnanolone provided an unbiased, affinity-based picture of neurosteroid targets within a complex biological system [50]. Based on radioactivity of slices from a subsequent SDS-PAGE separation of proteins, specific labeling of a few gel bands was observed. Two proteins, tubulin and voltage-dependent anion channel-1 (VDAC-1), were subsequently identified and further

investigated as potential protein targets. Micro-level studies found that tubulin was at Cys-354 [11], a residue within the colchicine binding site, and consistent with the ability of 6-azipregnanolone and pregnanolone to inhibit tubulin polymerization [11]. Other work using the non-clinical anesthetic PAL azidoanthracene has also implicated tubulin as a potential anesthetic target [51]. Similarly further work on VDAC-1 found that it was unlikely to be an important target in the VDAC-1/$GABA_A$ receptor interaction pathway [52]. VDAC-1 might however be responsible for alternative pathways and/or anesthetic side effects.

VDAC-1 was also identified as a specific anesthetic binding protein target in a macro-level investigation using [^{3}H]*meta*-azipropofol [33]. The subsequent micro-level investigation found that VDAC gating was modulated by propofol, and two binding sites were identified [53]. In addition to VDAC-1, synaptosomal-associated protein- 25kDa (SNAP-25) was identified as a labeled protein within the macro-level investigation [54]. The SNARE complex has potential as a target for anesthesia, with volatile anesthetics and propofol inhibiting neurotransmitter release by interactions with the complex [55,56]. These few examples demonstrate how photoaffinity labeling has provided unexpected molecular targets that should provide opportunities for drug improvement when the associated physiology is understood.

Macro-level Photoaffinity Labeling

Perspectives

It is anticipated that many protein targets involved in anesthesia are low abundance integral membrane proteins such as ion channels and receptors. Both characteristics result in complications for macro-level detection using classical PAL techniques [50,54]. However, over the past decade advancements in the photoaffinity labeling field have shown successful coupling of photoaffinity labeling and bioorthogonal reactions; for an excellent discussion of these developments we direct the reader to a 2012 review [57]. Tandem anesthetic photoaffinity- click chemistry conjugation involves the additional incorporation of a biologically inert alkyne into the anesthetic PAL structure [2]. The additional chemical group allows for affinity-based protein profiling of the anesthetic. This technology has numerous powerful applications from micro- to macro-level protein profiling and imaging investigations within complex systems.

General anesthetics are often considered to have low affinity for their targets relative to most other drugs. As such, the simplified view of “one drug, one target” for these drugs is exceedingly improbable. While the diversity of targets opens up new avenues for further development, it also presents significant challenges with respect to characterization and validation. Challenges associated with these macro-level investigations are similar to, if not greater than, micro-level investigations: sufficient PAL development and validation of identified protein targets. As the science moves towards systems biology and personalized medicine, the precise target-interaction profiles for each general anesthetic become increasingly necessary.

Conclusions

An ultimate goal of anesthesiology research is to understand the biochemistry that leads to pharmacological phenotypes displayed by anesthetics. The objective of this review was to present an overview of advancements and considerations in the use of anesthetic photoaffinity labeling as an experimental tool. Thus far anesthetic PALs have helped refine hypotheses and present new directions with respect to molecular targets. This knowledge should provide for educated improvements in drug design and allow for selective administration of an anesthetic to distinct populations (e.g., “personalized” anesthesia). The mechanisms of anesthesia have remained elusive for nearly two centuries and increasing evidence has suggested a highly complex process. In combination with other advancing techniques, we expect the use of anesthetic photoaffinity PALs to continue to be a significant tool in shedding light on this puzzle in medicine.

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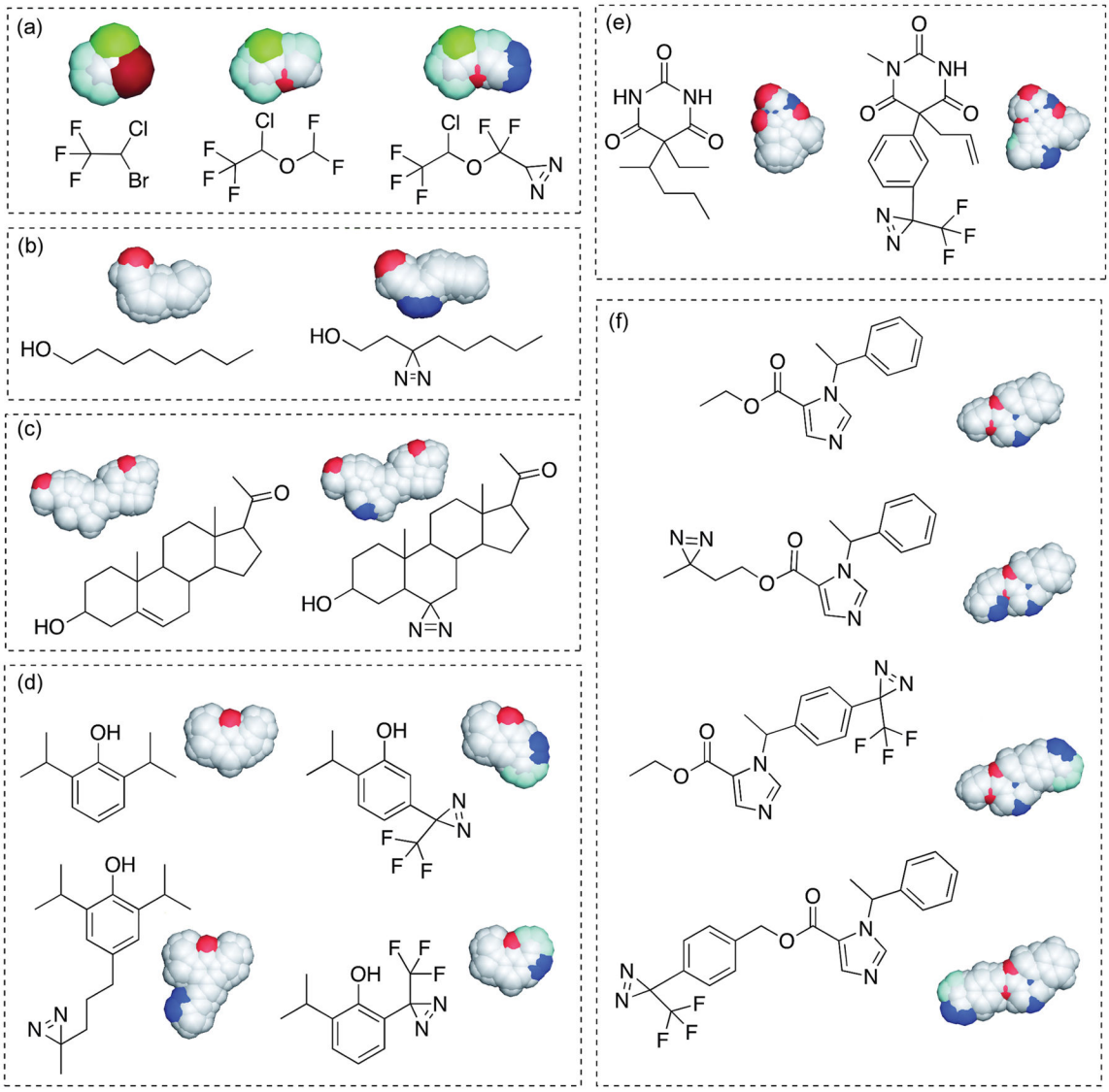

Figure 1.

Chemical and space-filling structures of general anesthetics and corresponding photoaffinity ligands. With the exception of halothane, a diazirine serves as the photoactive group. Halothane and Haloethers (a); halothane (left), isoflurane (middle), aziisoflurane (right). Alcohols (b): octanol (left), 3-azioctanol (right). Neurosteriods (c); pregnenolone (left), 6-azipregnenolone (right). Alkylphenols (d); propofol (top left), *meta*-azipropofol (top right), *para*-4-aziC5-propofol (bottom left), *ortho*-propofol diazirine (bottom right). Barbiturates (e); pentobarbital (left), *m*TFD-mephobarbital (right). Imidazoles (f); etomidate (top), azietomidate (upper middle), *p*TFD-etomidate (lower middle), TDBzl-etomidate (bottom)

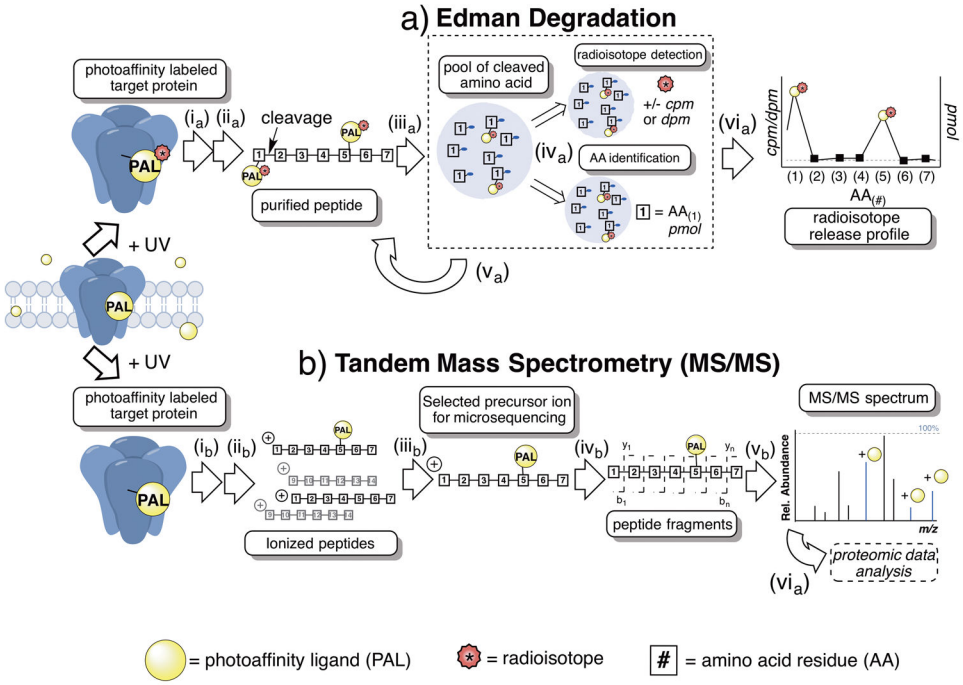

Figure 2.

Simplified schemes for the major methods used in micro-level anesthetic photoaffinity labeling; a) Edman degradation b) Tandem mass spectrometry (MS/MS). a) Edman degradation; i_a) enriched target protein is photoaffinity labeled with radiolabeled (*) anesthetic photoaffinity ligand (PAL) and protein(s) are separated and digested into peptides; ii_a) peptides are isolated and rigorously purified; iii_a) the first amino acid from the N-termini is cleaved from purified peptide via Edman reaction; iv_a) the cleaved amino acid is isolated, quantified and separated into two pools for (*top*) radioisotope detection by scintillation counting and (*bottom*) amino acid identification by chromatography; v_a) the cycle is repeated, gradually sequencing the entire purified peptide; vi_a) the amino acid sequence is plotted against radioactivity (cpm, counts per minute, or dpm, disintegrations per minute) resulting in radioisotope release profile. b) Tandem mass spectrometry (MS/MS); i_b) enriched target protein is photoaffinity labeled with anesthetic photoaffinity ligand (PAL) and protein(s) are separated and digested into peptides that are further separated by on- and/or offline chromatography methods; ii_b) peptides undergo ionization generally by electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI); iii_b) precursor ions are separated by mass/charge (*m/z*) within MS1 (aka. MS1 precursor ion); iv_b) MS1 precursor ion undergoes mass fragmentation often by collision-induced dissociation (CID) resulting in largely b or y fragment ions; v_b) mass spectrometry software are used for data acquisition, database search analysis and representation.

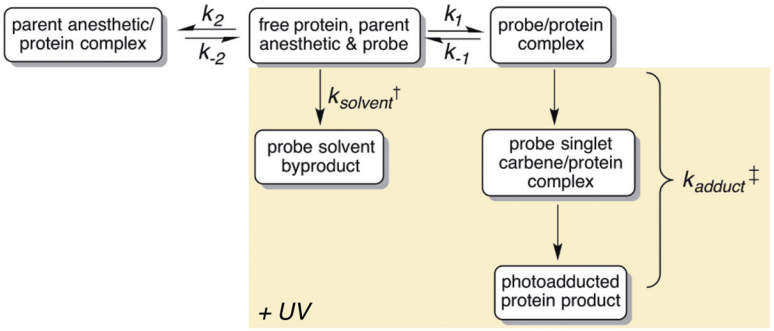

Figure 3.

Schematic representation of the steps involved in the most basic mechanism of parent anesthetic protection of anesthetic photoaffinity labeling experiments. Highlighted region denotes the non-equilibrium reactions upon UV irradiation. †($k_{solvent}$) Rate of solvent quenching of the ligand, given that carbenes are readily quenched by water the rate is limited by photoactivation of the ligand and therefore a first order rate constant. ‡(k_{adduct}) Rate of photoadduction represents two consecutive first order reactions, the photoactivation of the ligand and the insertion by the singlet carbene into the protein.

Table 1

Summary of adducted residues by anesthetic photoaffinity ligandss using amino acid sequncing methods

Parent anesthetic	Anesthetic photoaffinity ligand	Amino acid sequencing method[a]	Protein	Residue	Ref.
halothane	halothane	Edman degradation(14C)	apo-ferritin	Trp-15	58
			nAChR	αTyr-213, γTyr-111, δPhe-206 δTyr-228	43
octanol	3-azioctanol	Edman degradation (3H, 1H)	nAChR	αTyr-190, αTyr-198, αGlu-262, αHis-408, αCys-412	18
		MS/MS	adenylate kinase	His-36	59
			neural cell adhesion molecule L1	Glu-33, Tyr-418	60
			protein kinase Cδ	Tyr-236, Lys-40, Glu-2	61
			protein kinase Cε	Tyr-176, Tyr-238, Tyr-250	62,63
			japanese firefly luciferase	Glu-313	36
isoflurane	aziisoflurane	MS/MS	apo-ferritin	Arg-59	15
			lymphocyte function associated antigen-1	Leu-135, Glu-137, Tyr-257, Leu-302, Lys-304, Lys-305	15,44
			platelet receptor integrin aIIbb3	Asp-158, Lys-159	37
etomidate	azietomidate	Edman degradation (3H)	nAChR	αTyr-98, αTyr-190, αGlu-262, αGlu-390, αCys-412, βAsp-268, δAsp-59, δSer-258, δCys-236, δSer-262, δGln-276	17,64
			$GABA_A$ receptor	α1Met-236, β1Met-286	65–67
	pTFD-etomidate	Edman degradation (3H)	nAChR	αLeu-251, αSer-252,αVal-255, αLeu-258, βLeu-257, βLeu-261, δLeu-265, δVal-269	19
	TDBzl-etomidate	Edman degradation (3H)	nAChR	αLeu-251, αSer-252, αVal-255, γMet-299, δLeu-265, δVal-269, δLeu-272, δLeu-273, δGln-276	68
			$GABA_A$ receptor	α1Cys-234, α1Met-236, β3Met-286, β3Cys-288, β3Val-290	69
barbituate	(R)-(−)-*m*TFD-mephobarbital	Edman degradation(3H)	nAChR	αIle-231, αMet-242, αCys-412, βMet-249, βSer-254 βLeu-257, βVal-261, βLeu-265, γCys-252, γMet-299, δMet-257, δSer-258, δSer-262, δLeu-265, δVal-269	70
			$GABA_A$ receptor	α1Ala-291, α1Tyr-294, β3Met-227, β3Met-227, γ2Ser-301	49
pregnanolone	6-azipregnanolone	MS/MS	tubulin	Cys-354	11
			$GABA_A$ receptor	β3Phe-301	39

Parent anesthetic	Anesthetic photoaffinity ligand	Amino acid sequencing method[a]	Protein	Residue	Ref.
propofol	*meta*-azipropofol	Edman degradation (3H, 1H)	nAChR	αSer-248, αSer-252, δArg-277, δPhe-232, δCys-236, δVal-269, δThr-274	71
			$GABA_A$ receptor	α1Met-236, α1Ile-239, β3Met-227, β3Met-286	72
			GLIC	Met-205, Tyr-254, Met-261, Asn-307	73
		MS/MS	apo-ferritin	Leu-24, Leu-81	28
			lymphocyte function associated antigen-1	Ile-254, Tyr-257, Ile-258, Lys-287, Leu-302, Lys-304	74
			SIRT2 deacetylase	Tyr-139, Phe-190, Met-206	75
			VDAC	Gly-56, Val-184	53
	para-4-aziC5-propofol [b]		nAChR		76
	ortho-propofol diazirine	MS/MS	human serum albumin	Lys-41, Trp-111, Lys-525, His-535, Lys-536	29
			$GABA_A$ receptor	βHis-267	29

(nAChR) nicotinic acetylcholine receptor; ($GABA_A$) γ-aminobutyric acid type A; (GLIC) gloeobacter ligand-gated ion channel; (SIRT2) sirtuin-2 ; (VDAC) voltage-dependent anion channel.

[a] Noted in ( ) with Edman degradation amino acid sequencing method are the isotopes used for photoaffinity ligand detection.

[b] para-4-aziC5-propofol was not applied in amino acid sequencing however displayed adduction to nAChR by gel electrophoresis and autoradiography