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Inhalational Anesthetic Photolabeling

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Abstract

Photolabeling has allowed considerable progress in the understanding of anesthetic binding to proteins, of target identity, and of site localization. There are, however, few groups doing this work, so this article is an attempt to demystify the method. We will discuss the theory, method, and limitations of this useful experimental approach.

Keywords

Inhaled anesthetics; Photolabeling; Halothane

1. Introduction

The inhaled anesthetics are a particularly troublesome group of drugs for investigators. Their small size, volatility, and few interactive features result in weak binding constants making it difficult to discover targets and binding sites for further drug refinement (1). Photolabeling allows conversion of these rapid binding constants into stable covalent bonds for subsequent identification with conventional biochemistry. Initially applied with the drug halothane, the toolbox of probes now contains several novel, small volatile diazirine compounds.

1.1. Theory

Halothane (1-Bromo-1-Chloro-2,2,2-trifluoroethane)—This halogenated ethane has a weakened C-Br bond because of the adjacent trifluoromethyl group. Cleaved by 254 nm illumination, a carbon-centered-free radical is generated, which then reacts with adjacent

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¹*Warning note:* The diazirines are relatively unstable molecules and need to be handled with some caution. Phase changes, impact, and pressure spikes can cause an explosion. For example, Hamilton microsyringes can generate large hydraulic forces, and if containing a diazirine, can trigger an explosion. Handle only limited quantities and wear appropriate protection when dealing with the neat compound. Further, all such novel and/or radiolabeled volatile compounds should be handled in a well-functioning fume hood.

²For determining concentrations, the diazirine compounds have a characteristic double peak of absorbance at 300–340 nm, with extinction coefficients between 100 and 150 M⁻¹.cm⁻¹.

³Photolysis is occasionally divided into two or more sessions in order to switch cuvette sides for greater penetrance or invert cuvettes for greater mixing. This results in somewhat greater efficiency of label incorporation.

⁴With the diazirines, successful photolysis can be verified by the generation of small, readily observed bubbles (released nitrogen) in the cuvette.

⁵Glass fiber filters equilibrate (become saturated) with scintillant very slowly. Equilibration can be accelerated by vigorous shaking to break the filter apart, but still requires more than 12–24 h.

⁶After proteolysis, some fragments in the UV plus ligand sample “disappear” from the mass spectrum, rather than just shift in mass, as compared to the UV alone sample. This is probably due to increased adherence (MALDI) or hydrophobicity (LC/MS) as a result of an adduction event. The matrix used for MALDI may have to be altered, or in the case of LC/MS, the elution gradient changed for the labeled samples.

electrophilic groups to form a stable adduct. By virtue of a radiolabeled carbon, the adduct can be detected and monitored through any subsequent chemistry (2, 3). The carbon-centered radical is not reactive enough to label nonselectively; aromatics are preferred (3). Halothane photolysis is complex, however, in that the predicted reaction produces two reactive moieties (chlorotrifluoroethyl radical and free Br); the potential for subsequent reactions is high. In addition, 254 nm illumination causes direct photolytic damage to the target, and combined with the chain reaction initiators from halothane photolysis, considerable cross-linking occurs. This limits the subsequent identification approach to fairly crude techniques such as gel and column chromatography, limited proteolysis, and if lucky, the laborious Edman degradation (3, 4). Edman chemistry can be blocked by backbone atom addition by this small molecule. The principal advantage of halothane photolabeling, is, of course, that it is an unmodified general inhalational anesthetic with an extensive literature and clinical history.

Anesthetic/diazirine compounds—Because of the above technical problems with halothane photolabeling, compounds that retain anesthetic character, yet include the diazirine group, have been designed, synthesized, and tested. Both halogenated alkanes (5) and ethers (6) have now been validated. These compounds have the large advantage of requiring longer UV photolysis (320–350 nm), inducing less target damage and the release of only a single and very reactive intermediate, a halogenated carbene. The carbene is predicted to react with target sites more quickly than the carbon-centered radical and demonstrates considerably less photoselectivity toward amino acids, both important features in interpreting the adduction site. Because of the somewhat cleaner chemistry, more sophisticated detection and analysis approaches like mass spectroscopy can be employed. This considerably reduces the cost and increases the safety of the below approaches. Nevertheless, appropriate design of the compound can include an exchangeable hydrogen atom, so that tritiation can occur through base-catalyzed exchange.

2. Materials

2.1. Photolabels

It is important to note at the outset that *not a single photoaffinity anesthetic label* is commercially available, limiting the current implementation of this approach to custom synthesis or collaboration with one of only two or three academic groups. Detailed description of the synthetic routes to these compounds is considered beyond the scope of this paper; the methods for synthesis of these novel compounds are contained in the original references for those courageous investigators with organic synthesis capability.

1. *Halothane* – (1-bromo-1-chloro-2,2,2-trifluoroethane). This can be custom synthesized with a ^{14}C on the trifluoromethyl, but is extremely expensive. Further, there is a risk of β -degradation; the β -particle liberated by decay of the ^{14}C atom has enough energy to occasionally cleave the C-Br bond, and initiate chain reactions.
2. *Halothane mimic*. (3-(2-bromo-2-chloro-1,1-difluoroethyl)-3H-diazirine)(Fig. 1a) (5).

3. *H-diaziflurane*. (3-(difluoro(2,2,2-trifluoroethoxy)methyl)-3H-diazirine)(Fig. 1b) (6).
4. *Azi-isoflurane*. (3-((1-chloro-2,2,2-trifluoroethoxy)difluoromethyl)-3H-diazirine) (Fig 1c)(7).

2.2. Other Components

1. *Buffers*. All buffers have not been tested, but the inorganic buffers are generally preferred (e.g., phosphate) to avoid scavenging reactions (see below). The labels are tolerant of pH values in the biologic range.
2. *Target* (tissues, proteins, membranes, etc.). To achieve high labeling efficiency, reasonable UV penetrance is required. Thus, protein concentration of tissue homogenates is generally kept below 1 mg/ml, and samples are continuously mixed during photolysis.
3. *Nitrogen/Argon source* with air stone or equivalent means of deoxygenating buffers.
4. *UV source*. 254 nm – Oriel 6035 Low Pressure Hg(Ar) Calibration lamp driven at 18 mA.
300–350 nm – Rayonet reactor with appropriate bulbs (300 or 350 nm).
5. *Hamilton Gas Tight syringes* of various sizes, with removable blunt needles and Teflon seals. Extra Teflon washers. These are wonderful vessels for handling hydrophobic, volatile chemicals like the anesthetics. Plasticware should be avoided.
6. *Quartz cuvettes*. The Teflon-stoppered varieties are preferred, and path length should be limited to 5 mm or less owing to poor penetrance of UV. The 1 × 0.5 × 4 cm cuvettes hold 2 ml, and accommodate microstir bars for mixing during photolysis.
7. *Filters and centrifuges*. This will depend on the desired separations, proteolysis and biochemistry to be employed postphotolysis.

3. Methods

1. Buffers for suspension of the protein or tissue should be deoxygenated with either argon or nitrogen bubbling. This reduces reactions between molecular oxygen and the radical or carbene, and effectively increases efficiency of the desired adduction. The deoxygenated buffer is drawn up into Hamilton gas-tight syringes that contain Teflon microstir bars.
2. The anesthetic photolabel is drawn up into Hamilton microsyringes and injected into the larger gas-tight syringe containing buffer through the removable needle port. The extra Teflon washer under the well-tightened knurled nut is necessary to get a good seal around the injecting needle. The syringe is then placed on a magnetic stirrer and allowed to stir for several hours in the dark. Solubilization of the label can be facilitated by connecting two syringes via the removable needle (as

above) and passing the buffer back and forth through the narrow needle lumen. In such cases, adequate anesthetic dissolution can be achieved in less than 15 min. At this point, the anesthetic solution can be aliquoted into Teflon (or better yet, foil) - sealed glass vials and stored at -80°C for later use, or used immediately.

3. If using radioactivity, a small aliquot (1–2 μl) of the solubilized label is injected into scintillation vials filled with cocktail for counting to determine anesthetic concentration from the known specific activity. If not using radioactivity, this step can be ignored.
4. We use 2 ml $10 \times 5 \times 40$ mm Teflon-stoppered quartz cuvettes for most experiments. These easily accommodate a microstir bar for mixing during photolysis if the apparatus is held at about a 15° angle from horizontal. A shuttered housing provides exact illumination times, and close proximity between lamp and cuvette. In our system, the lamp is about 1 cm from the cuvette surface. For very small volume samples due to scarcity of either ligand or target, cuvettes as small as 300 μl are commercially available, and smaller can be designed. We have also photolabeled slide-mounted brain sections by fabricating a thin aluminum and quartz cuvette into which is inserted the entire slide (8). Of greatest concern here, however, is the homogeneity of illumination, which has proven difficult for some light sources (notably the Oriel calibration lamp).
5. If labeling tissue homogenate or protein solutions, a predetermined amount is added to quartz cuvettes, with or without added buffer, to bring the protein to 1 mg/ml or less (to facilitate UV penetrance) or to introduce other components (like agonists, antagonists, etc.) leaving just enough head-space for the final addition of volatile photolabel. Cuvettes are tightly sealed with Teflon stoppers, and then mixed by inversion several times and immediately exposed to the appropriate wavelength (300–350 nm for diazirines, 254 nm for halothane). Distances are kept small to maximize illumination amplitude. Illumination times for 254 nm light are kept to less than 60 s because of target damage, while we often expose to 350 nm illumination for 5–10 min with little evidence of target damage.
6. In some cases, it is useful to determine specificity of labeling by adding potential competitor ligands to the cuvette. Further, estimates of the IC_{50} can be achieved by varying the concentration of competitor in the cuvette, as long as it is understood by the investigator that the system is nonequilibrium and the results, therefore, are only a rough (generally low) estimate of the IC_{50} .
7. We generally do not include scavenging molecules, such as reduced glutathione, in the photolysis mixture because this unexpectedly reduces specific binding with little effect on nonspecific binding. In the case of halothane, photolysis of the molecule in the bulk, unbound aqueous phase creates two species that are thought to recombine to reform halothane. On the other hand, if bound in a site with a photochemical partner, then the desired adduction (labeling) occurs. Thus, providing a photochemical partner in solution, such as glutathione, reduces the mass of halothane capable of entering and labeling sites of interest during the photolysis period (which is very long relative to binding and unbinding rates for

these molecules). In the case of the carbene, since it can react with water (albeit at lower rates), the inclusion of glutathione makes little difference.

8. After photolysis, the labeled material is separated from unbound aqueous phase by any of several approaches: dialysis, centrifugation, filtration, column chromatography, SDS-PAGE, 2D-electrophoresis (9), etc., depending on the subsequent approach used for identification of labeling magnitude, labeled target, or labeled site. For example, if simply measuring incorporated radioactive label in a protein sample, we precipitate the protein with 10–20% trichloroacetic acid (TCA), filter through glass fiber filters (Whatman GFB), and wash with dilute (1%) TCA. The filter is then equilibrated with scintillant and counted. Although the free ligand was initially volatile, one cannot rely on vacuum extraction to rid the labeled mixture of free label. The photolysis often creates a variety of small water soluble by products.
9. To determine radiolabel incorporation into gel bands (intact proteins or proteolytic fragments), the band of interest is excised with a scalpel blade, placed in scintillation vials with 30% H₂O₂, capped tightly and heated to 60°C for several hours (or until the gel slice liquefies). Scintillation fluid is then added and the sample counted. The β radiation from either ¹⁴C or ³H lacks sufficient energy to escape the gel – even if cut or ground into small pieces.
10. For samples that are expected to be analyzed for label incorporation by mass spectroscopy, it is critical to purify to near homogeneity to avoid contaminating sequences. Recent progress with LC/MS approaches reduces this need somewhat. Further, it is important to compare proteins and peptides to samples that have been exposed to UV alone.
11. We have found that anesthetic photolabeling is of reasonably high efficiency – demonstrated by the unprecedented ability to observe electron density of the adduct within an anesthetic binding site by X-ray diffraction (6).

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Fig. 1.

Volatile anesthetic photolabels. Shown are the stick structures for the halothane mimic (a), the H-diazirithane (b) and azisoflurane (c)