Photolabeling strategies to study membranotropic peptides interacting with lipids and proteins in membranes

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Antimicrobial peptides, cell-penetrating peptides, fusion peptides, transmembrane peptides, affinity photolabeling.

ABSTRACT: Membranotropic peptides is a class of peptides that exert their biological action at the level of cell membranes. Understanding how they interact with their different membrane binding partners (lipids, proteins and/or glycoconjugates) is important to decipher their mechanism of action. Affinity photolabeling is a powerful method to study non-covalent interactions and provide a sub-molecular picture of the contacts between two interacting partners. In this review, we give a panorama of photolabeling-based studies of the interactions between membranotropic peptides and membranes using either photoreactive lipids or peptides.

INTRODUCTION

Membranotropic peptides is a broad class of natural or synthetic peptides which act at the level of cell membranes.[1] There is a large diversity in terms of biological action of these peptides. Generally, it encompasses antimicrobial peptides (AMPs), cell-penetrating peptides (CPPs), fusion peptides, amyloid peptides and short trans-membrane segments.

Membranotropic peptides present a very broad diversity in terms of primary and secondary structures. They are enriched in hydrophobic residues, even though some CPPs are purely hydrophilic. CPPs and most AMPs usually have a net positive charge and are enriched in Lys and Arg residues. In terms of secondary structures, α-helices are often encountered, but β-structures or even random-coil can also be found. Structural plasticity, with a coil-to-secondary structure transition in the presence of a membrane, is also often observed. Despite this structural diversity, and whether their role is to destroy, cross or anchor proteins at the membrane, membranotropic peptides all share a distinctive feature: they are able to interact with membrane components, generally lipids, and also possibly proteins or surface glycoconjugates. Because they can play an important role in diseases (fusion peptides, amyloids) or because they present high therapeutic interest (AMPs, CPPs), their mechanisms of action have been widely studied for several decades. The first step through which they exert their biological action is the attachment at the membrane through the interaction with specific binding partners. It is thus critical to get a clear molecular picture of this initial interaction to understand the way it triggers the downstream steps of their different mechanisms of action.

There are many experimental approaches to study the interactions of membranotropic peptides with membrane components [1–3]. Amongst the most commonly used, one can cite calorimetry (ITC and DSC), spectroscopic approaches (NMR, fluorescence, SPR, IR, CD…), molecular dynamics simulations and general biochemical approaches such as co-sedimentation, pull-down assays or affinity photolabeling.

Affinity photolabeling, also called affinity photocrosslinking or photoaffinity labeling is a well-established method to study non-covalent interactions between biomolecules. It is a simple, and direct way to "transform" non-covalent interactions into a covalent bond by irradiation of the sample, thus stabilizing the interaction and simplifying the downstream sample handling and analysis. In principle, it gives the possibility to identify close atomic contacts even in the context of a complex biological environment such as a cell membrane.

Photolabeling relies on the unique properties of essentially three classes of photoreactive labels: arylazides, diazirines and benzophenone, that are presented in Table 1. When irradiated at an appropriate wavelength, the stable precursors generate highly reactive species, which very quickly react with other molecules in their immediate environment. Compared to the other approaches cited above, photolabeling appears as a very powerful method. It relies on a methodology that requires no specific or expensive instrument and that can be implemented easily in any standard chemistry or biochemistry laboratory. This approach also provides very comprehensive information on the studied interaction, *i.e.* partner identification and binding site characterization, in a single experiment. Another strength resides in the fact that it can be implemented in a native biological context. The situation is unfortunately not always as simple,

and numerous difficulties can be encountered, as the examples cited in this review will illustrate. Affinity photocrosslinking remains, nevertheless, a very powerful weapon in the arsenal of the biophysical chemist interested in peptide/membrane interactions.

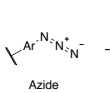
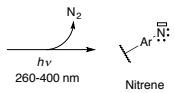
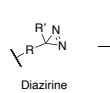
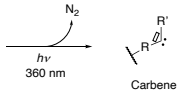
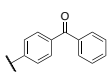
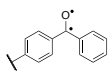
Stable precursor	Highly reactive species	Reactivity	Remarks
 Azide	 Nitrene	Insert in C-H bonds and heteroatom-H bonds More reactive towards electron-rich amino acids May undergo a rearrangement	Wide variety of aryl azide derivatives Irradiation wavelength (260 nm) may cause damage to biomoelcules N-C or N-heteroatom bonds may be less robust
 Diazirine	 Carbene	Insert in C-H bonds and heteroatom-H bonds Increased reactivity compared to nitrenes, especially in a hydrophobic environment More reactive towards electron-rich amino acids	Form stable C-C or C-heteroatom bonds Alkyldiazirine (R=CH_2) are small, but less stable and generate less reactive carbenes because of rapid isomerization of the carbene Aryldiazirine (R=Ar) are bulkier, but more stable and generate more reactive carbenes
 Benzophenone	 Benzophenone biradical	Insert in C-H bonds Low reactivity with water Increased reactivity on doubly allylic and allylic positions *vs.* secondary hydrogens Increased reactivity with methionines	Form stable C-C bonds Increased chemical and light stability Bulky

Table 1: reactivities, advantages and disadvantages of classical photoreactive labels used to study membrane-peptide interactions.

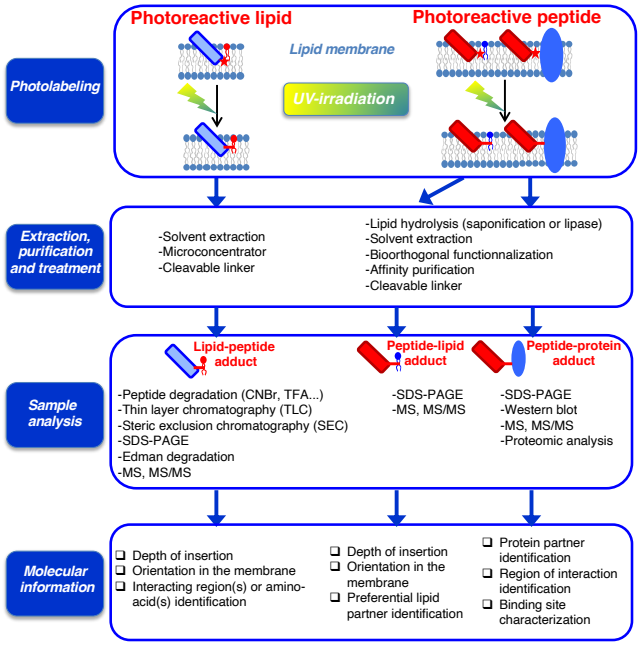


Figure 1: Two photolabeling strategies and downstream sample analysis to study peptide/membrane interactions. The star represents the photoreactive label. The photoprobe is represented in red, the covalently bound specific partner to be identified in blue. MS: mass spectrometry, MS/MS: tandem mass spectrometry.

In this review, we will focus on photolabeling applied to the study of the interactions between membranotropic peptides and membranes. Two approaches can be implemented, with the photoreactive label being carried either by a lipid (lipid photoprobe) or the peptide (peptide photoprobe) (Figure 1). In both cases, a covalent bond will be created between the photoprobe and the interacting partner. A major difficulty that can be encountered in both cases is the fact that the generated photoadduct is formed by two moieties with very different chemical-physical properties. This can usually be overcome by proper sample preparation, a finely tuned analytical method or a smartly designed photoprobe as will be illustrated in the developed examples.

In the following paragraphs, we will present several studies that used either approaches to study the interaction of membrane active peptides with membranes and show how it provided valuable molecular information such as peptide insertion in the membrane, identification of membrane interacting regions, lipid or membrane protein partner identification.

PHOTOLABELING USING PHOTOREACTIVE LIPIDS

In this section we focus on the study of the interactions of membrane-active peptides with membranes incorporating photoreactive lipids. Photoreactive lipids were introduced in the mid-70's as a general approach to probe hydrophobic interactions between lipids and proteins in membranes [4]. Since then, many lipid probes have been developed to study such interactions. There are many good reviews describing the different existing probes and how they were used to study protein-lipid interactions [5–9]. Theoretically, this approach should be able to yield precious geometrical information on the system composed of the interacting peptide and the membrane incorporating the photoreactive lipid. In particular, it should be possible to pinpoint exactly which amino-acid(s) are in close proximity with the photoreactive label on the lipid. The situation, however, is often more complex, as the following examples will illustrate.

Sometimes, technical limitations do not allow to get access to amino-acid sequence after crosslinking, thus preventing the precise determination of the labeled residues. The different reactivities of photolabels used in the literature towards chemical functions present on the interacting partner (Table 1) can also lead to a biased picture of the exact position of the peptide in the bilayer. For example, benzophenone presents an increased reactivity towards methionines [10,11] and allylic positions [12] whereas diazirines and arylazides prefer electron-rich amino-acids [13], provided these functions are in direct proximity to the photolabel. Issues about the exact position of the photolabel within the bilayer due to thermal fluctuations, the so-called 'looping back' of the probe, occur when the photolabel is fixed at the end of a long lipid acyl-chain, thus leading to a somewhat broad distribution of accessible positions for the photocrosslinking (roughly C6 to the center of the bilayer) [14–16].

Another point of concern is the fact that photoreactive lipids naturally tend to react primarily with the most abundant molecules in their immediate surrounding, *i.e.* other lipids [4,6,17], thus greatly reducing the yield of the peptide/lipid photoreaction. Detection of the photoadduct of interest thereby requires highly sensitive methods such as radiolabels coupled to efficient sample enrichment or purification steps. Finally, photolabels are bulky moieties that can potentially alter membrane properties such as packing or rigidity. They are usually introduced at low molar ratio (5 % or less) thus minimizing unwanted effects on side reactivity and/or membrane packing. Interestingly, lipids modified with a photolabel can still be metabolically incorporated in bacterial or eucaryotic membranes [18,19]. They are also

recognized by phospholipid-exchange proteins [17] or phospholipases [4,17,20], indicating that their modified structure does not impair their recognition by enzymes.

Examples will cover the cases of three emblematic antimicrobial peptides: Alamethicin, Gramicidin A and Magainin, as well as the short transmembrane protein glycophorin A, and the protein involved in the fusion of Influenza virus Hemaglutinin (HA) and in particular its fusion peptide. The sequences of all the studied peptides are given in Table 2.

Peptide	Sequence	Ref
Alamethicin	Ac-^{1}Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-^{20}Phl-[^{3}H]	[21]
Ala$_{19}$-Magainin	^{1}Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Ala-Ile-Met-Asn-^{23}Ser-NH$_2$	[22]
Gramicidin A	[^{14}C-Formyl]-^{1}Val-Gly-Ala-DLeu-Ala-DVal-Val-DVal-Trp-DLeu-Trp-DLeu-Trp-DLeu-^{15}Trp-NH(CH$_2$)$_2$OH	[17,20]
Section of TC peptide (transmembrane and C-terminus domain of Glycophorin A)	^{67}His-Phe-Ser-Glu-Pro-Glu-Ile-Thr-Leu-Ile-Ile-Phe-Gly-Val-Met-Ala-Gly-Val-Ile-Gly-Thr-Ile-Leu-Leu-Ile-Ser-Tyr-Gly-Ile-Arg^{96}	[23–25]
HA2 fusion peptide	^{1}Gly-Leu-Phe-Gly-Ala-Ile-Ala-Gly-Phe-Ile-Glu-Gly-Gly-Trp-Thr-Gly-Met-Ile-Asp-Gly-Trp-Tyr-Gly-Tyr-His^{25}	[26–29]

Table 2: sequences of the different membranotropic peptides studied using photoreactive lipids. The labeled residues or regions are written in red and underlined. Ac: acetyl, Aib: 2-aminoisobutyric acid, Phl: phenylalaninol.

Alamethicin, a proof of concept to probe peptide insertion

Alamethicin is one of the first examples of a study of the interaction of an AMP with membranes using hydrophobic photolabeling [21]. Alamethicin is a non-ribosomally synthesized peptide extracted from the fungus *Trychoderma viride,* containing the non-proteinogenic aminoacid Aib (2-aminoisobutyric acid) and a phenylalaninol (Phl) at its C-terminus. Its sequence is given in Table 2. Four to six Alamethicin monomers can self-assemble on membranes to form a voltage-dependent, "barrel-stave" type pore [30]. In 1981, Latorre and coworkers studied the interaction of Alamethicin, tritiated on its C-terminus, with a lipid bilayer formed entirely with the aryl diazirine-modified phosphatidylcholine **1** (Figure 2). After irradiation of the sample, the photoproduct was treated with trifluoroacetic acid (TFA) to yield hydrolyzed peptide fragments at the level of proline secondary amide bonds, thus creating three peptide fragments: 2-20, 14-20 and 2-13. Separation of these fragments by steric-exclusion chromatography and detection of radioactivity and phosphate content in each fragment showed that Alamethicin was crosslinked to the lipid on the 2-13 N-terminus stretch. Unfortunately, the authors could not determine on which residue(s) the crosslink actually occurred. They also showed that photocrosslinking occured independently of an applied electric field and that the photocrosslink product was still able to induce voltage-gated conductance, similar to uncrosslinked Alamethicin. Taken together, these results were one of the first direct evidence showing that Alamethicin is at least partially inserted within a lipid bilayer through its N-terminus even in the absence of an applied voltage.

Magainin 2 and depth-dependent probes, to study peptide orientation in the membrane

Another example of the study of the insertion of an AMP in a lipid bilayer is that of Magainin 2, published in 1998 [22]. Magainins are isolated from the frog *Xenopus laevis* and have broad spectrum antimicrobial activity. They are thought to assemble on and permeabilize membranes by forming toroidal pores [30]. Toroidal pores are formed of associated peptides and lipids, with peptides and lipid head groups lining the lumen of the pore. No discontinuity in the bilayer is observed, contrary to barrel-stave pores. However, two pools of Magainin 2 are possibly present at the membrane. These two pools have different orientations relative to the membrane: a majoritarian pool of Magainin 2 lying flat on the membrane (parallel) and a minoritarian pool of Magainin 2 forming pores and positioned perpendicular to the membrane, or obliquely. Hydrophobic photolabeling using depth-dependent probes thus appeared as a technique of choice to distinguish between the two populations. Indeed, the geometry of toroidal pores would allow the side-chains of certain residues of Magainin 2 to be closer to the center of the lipid-bilayer. Aryl nitrene-based, tritiated galactosylceramide photolipids GalCer-PL **2** (shallow probe) and GalCer-C8-PL **3** (deep probe) (Figure 2) were incorporated in PC or PC/PG vesicles to study the insertion of Ala$_{19}$-Magainin (Table 2), an analogue of Magainin 2. The choice of galactosylceramides as photoprobes is actually quite puzzling, as they are not particularly representative of bacterial lipids. As they were incorporated in small amounts in PC or PG lipid bilayers, they could be regarded as "inert" probes, but the authors did not discuss this point. Magainin did not run properly on a gel in the presence of lipids, and thus had to be purified with a microconcentrator equipped with a cation-exchange membrane. Photolabeling yield was then evaluated by running the purified Magainin on an SDS-PAGE gel and counting the radioactivity in the peptide band (containing both crosslinked and free Magainin). Throughout the study, the authors compared the yield of labeling with the two probes, "shallow" GalCer-PL **2** and "deep" GalCer-C8-PL **3** using various experimental conditions. Overall, the yield of labeling was always slightly higher with **2** than with **3**. They also showed that photolabeling was always more efficient in liposomes containing 20 % of negatively charged PG compared to pure PC liposomes, which is consistent with what is known on the selectivity of Magainin and other AMPs towards negatively charged membranes. They also showed that increasing the peptide to lipid ratio led to an increase of photolabeling yield using both probes, suggesting concentration-dependent interaction with and insertion within PG-containing membranes. Finally, a time-course experiment over 30 min using GalCer-C8-PL showed rapid (less than 30 s) and stable (over 30 min) insertion of the peptide. At least, the system reached an equilibrium by 30 s, with a constant proportion of peptide inserted deeper in the membrane.

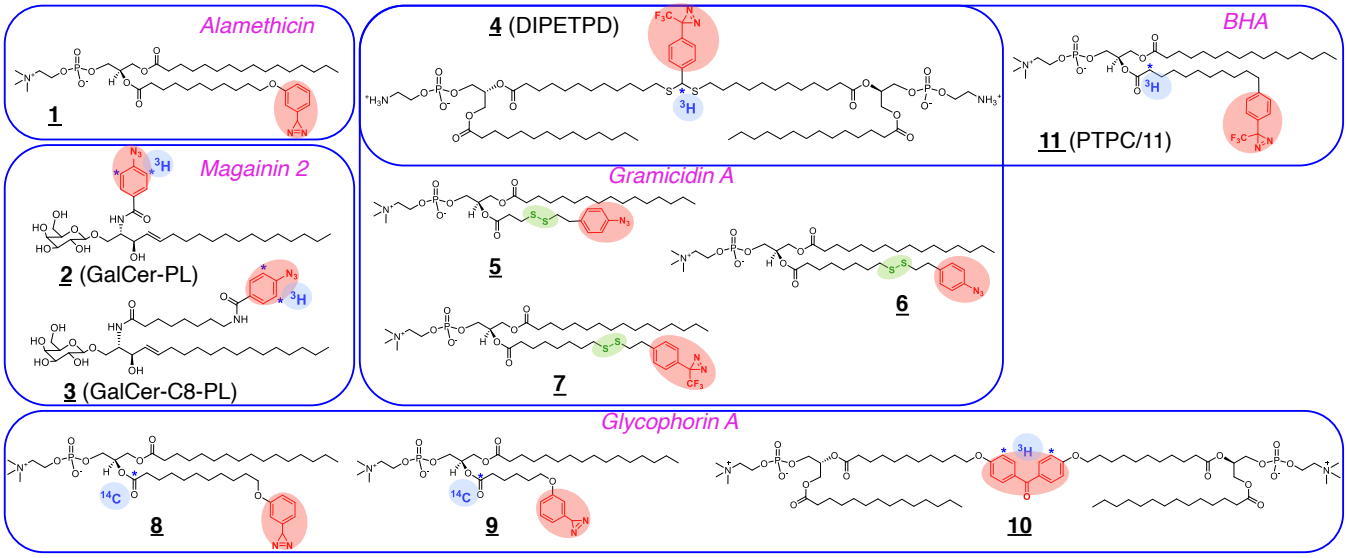


Figure 2: photoreactive lipid probes used to study the interaction of different membrane-active peptides (magenta italics) with lipid membranes. The photolabels are indicated in red. In the case of isotopic labeling on the lipid, the position (*) and nature of isotopes are indicated in blue. Cleavable S-S bonds are indicated in green.

As a control, the authors checked that the probe did not label water-soluble lipid-binding myelin basic protein, thus ensuring the probe was correctly inserted in the lipid bilayer. The question of the looping-back of the probe was discussed, but the authors always tracked the ratio of labeling with the deep and shallow probe. If significant looping-back was occurring, then the yield of labeling with the deep probe should be higher, which was never the case. Finally, the position(s) at which photolabeling occurs was not determined in this study thus providing an incomplete picture on the way Magainin inserts into membranes. The authors pointed out however, that Magainin has a good number of amino acids with high reactivities towards nitrenes and that they are well distributed along the sequence.

Gramicidin A, a model system for the development of smartly designed probes

A solution to overcome the issue of looping back of the probe was proposed in 1993 with the design of a photoactivatable membrane spanning probe [20]. It consists in a bola-phospholipid where two acyl chains are linked by a photoreactive trifluoromethylphenyldiazirine, DIPETPD **4** (Figure 2). This guarantees that the photolabel remains in the hydrophobic core of the bilayer. The authors studied the incorporation of the probe in lipid bilayers using TNBS-labeling of free amines. They showed that the addition of the bipolar probe did not modify the permeability of model membrane towards TNBS. Electron microscopy also showed that liposomes incorporating the bipolar probe were uniform and unilamellar. This new probe was tested, using [^{14}C]-Gramicidin A as a case study. Gramicidin A (Table 2) is a hydrophobic, non-ribosomally synthesized, 15 residues peptide, containing many D amino acids. Gramicidin A is thought to create an ion channel in membranes by forming a head-to-head membrane-spanning dimer, the two N-termini being located very close to the center of the bilayer. Irradiation of a membrane incorporating the bola-lipid probe and Gramicidin A led to the formation of a photoadduct, as observed by thin layer chromatography (TLC) and autoradiography [20]. The valine residue located at the N-terminus of Gramicidin A, is poorly reactive towards carbenes. Substitution of this valine by an electron-rich tryptophan, highly reactive towards carbenes, led to a two-fold increase of the photolabeling yield, thus confirming that the photolabel was indeed located in the center of the bilayer. Again, the authors stated that further analysis at the level of individual amino-acids was extremely difficult.

Such an issue had already been uncovered several years previously by the same group and an elegant design had been proposed to make sample handling downstream of crosslinking easier [17]. In the nitrene (**5** and **6**) and carbene-based (**7**) photoreactive labels used in this study, a cleavable disulfide bridge (in green, Figure 2) was introduced between the photolabel and the end of the acyl chain. Photolabeling followed by treatment with reductive conditions would release the target molecule carrying a free reactive thiol, which could be further exploited for separation or functionalization. Again, [^{14}C]-Gramicidin A was used as a case study. Photolabeling was observed with extremely good yields, that could not be accounted for if only aliphatic amino-acid side chains, which are less reactive towards carbenes or nitrenes, were labeled. After treatment with reducing conditions, the labeled peptides were covalently attached *via* a stable thioether bond to glass beads coated with *N*-substituted maleimide arms. This was exploited for solid-phase Edman degradation of crosslinked Gramicidin A, conventional methods being unsuccessful. Edman degradation showed that crosslinking occurred essentially on the tryptophans in position 9, 11 and 13. As the authors claimed, this did not necessarily reflect the exact position of the label in the membrane but rather the relative reactivities of the amino-acid side chains on Gramicidin A.

Positioning of Glycophorin A transmembrane domain and reactivity bias

Another interesting example showing the issues related to the looping-back of the probe and the differential reactivities of

phoreactive labels is the study of Glycophorin A position in the membrane. Glycophorin A is a 131 residues polypeptide and is a major sialoglycoprotein of the human erythrocyte membrane. In 1982, the group of Khorana tried to obtain a finer picture of the insertion of this peptide in membranes by using diazirine-based lipid probes (**8** and **9**, Figure 2), the diazirine being attached at the ω position of the *sn-2* acyl chain[23]. They observed that most of the cross-linking involved the carboxyl group of Glu[70], thus suggesting that this residue was located within the bilayer [23]. However, twenty years later the group of Nakatani used the same approach with a different lipid probe and obtained very different results [24,25]. They used a benzophenone-based bola-phospholipid probe (**10**, Figure 2), thus guaranteeing that the label was located at the center of bilayer, with no possibility for looping back. DSC characterization of the probe **10** showed complete miscibility with DMPC in the fluid phase [31]. Whereas carbenes are more reactive towards nucleophilic amino acids such as glutamate, benzophenone is more prone to react with aliphatic amino-acids as the benzophenone biradical is more reactive towards inert C-H bonds [12]. Using this setup, they showed that the cross-linking occurred almost exclusively on Val[80] and Met[81] to a lesser extent, suggesting that these residues are the ones closer to the center of the bilayer. One has to be aware however that benzophenone can also show reactivity bias, notably and quite interestingly towards methionines [10,11].

Identification of membrane-interacting regions, the example of Hemaglutinin

Hydrophobic photolabeling can give us a better molecular picture of a known interacting system as the examples above show. It can also be used to identify proteins, or sections of proteins interacting with membranes. For example, it was used by the group of Josef Brunner to identify the region of the soluble ectodomain of the influenza protein hemaglutinin (BHA) responsible for the hydrophobic binding to the target membrane [26,27]. Hemaglutinin is a heterodimer composed of two glycopeptide subunits, HA1 and HA2, linked by a disulfide bridge. BHA corresponds to the water soluble ectodomain, released by bromelain cleavage. In 1988, the group of Josef Brunner first showed that only BHA2 was labeled by a diazirine-based probe PTPC/11 (**11**, Figure 2), suggesting that the region responsible for membrane binding is solely located on this subunit [26]. Further analysis revealed that only the hydrophobic N-terminus 1-21 fragment is responsible for membrane binding [27]. Crosslinking essentially occurred on residues Phe[3], Ile[6], Phe[9], Trp[14], Met[17] and Trp[21]. This 3-4 residues periodicity in labeling strongly suggested that this "fusion" peptide adopts a helical conformation in the membrane. Interestingly, a few years later, the same group extended the method to the study of intact influenza viruses interaction with model membranes incorporating the photolabeling probe [28]. At the same time, the group of Frederic Richards compared the photocrosslinking of HA2 and BHA2 with two probes[29], the one used by the group of Josef Brunner (probe **11**)[26] and the bolalipid-based DIPETPD **4** [20]. Interestingly, they showed that the fusion peptide of BHA2 was labeled by probe **11**, as described by the group of Josef Brunner [26], but not by DIPETPD **4**, suggesting a shallow parallel insertion of the helical fusion peptide [29]. However, when performing the same experiment with full HA2, rather than the water soluble BHA2, they observed labeling with both probes, thus showing that the fusion peptide of HA2 inserted deeper, and probably in a more perpendicular manner in the membrane [29].

PHOTOREACTIVE PEPTIDES TO STUDY INTERACTIONS WITH LIPIDS AND MEMBRANE PROTEINS

In the following section we will focus on peptide-membrane interactions probed with photoreactive peptides. There are few photolabeling studies involving peptide probes to study interactions with lipids or membrane components. As already demonstrated in the previous section, photolabeling studies are not straightforward and several difficulties have to be envisaged. Incorporation of a photoprobe in a peptide sequence can have a strong effect on the peptide properties, due to the relative important contribution of the mass and chemical nature of the photolabel. Therefore, designing a photoreactive peptide requires validation of the photoreactive analogue as a good model to mimic the natural membrane-active peptide. The yield of photolabeling is often low, whatever the system and nature of the photoreactive label and efficient purification steps are critical, especially when working in complex systems. To this end, a specific tag, such as biotin, can be added to the peptide sequence during peptide synthesis or afterwards by post-functionalization using biocompatible click-chemistry for example. Appropriate controls are needed to avoid false positive identifications due to non-specific non covalent interactions. Finally, and as already illustrated in the previous section, the formed covalent adducts can be difficult to detect and/or characterize due to their relatively high molecular weight and the different nature of the crosslinked partners. These points will be discussed throughout the following paragraphs. Examples will cover three types of membrane-active peptides: the transmembrane peptide WALP23 designed to mimic transmembrane proteins to study the hydrophobic mismatch phenomenon, the emblematic CPPs Penetratin and poly-Arg derivatives and finally peptidomimetic antibiotics derived from the well-known AMPs polymyxin B and protegrin-I.

Interactions with lipids: Photoreactive WALP23 to study hydrophobic mismatch

In order to avoid the exposure of hydrophobic regions of membrane spanning proteins, the thickness of the lipid bilayer around the protein should match the thickness of the transmembrane section of the protein. A difference of thickness between the bilayer and the protein is called hydrophobic mismatch. There are several processes that can help counteract hydrophobic mismatch, such as protein tilting, or lipid sorting [32]. To investigate preferential sorting based on hydrophobic matching, Ridder *et al.* [33] designed and synthesized a model peptide of 23 residues (**WALP23**) which mimics the transmembrane domain of membrane-spanning proteins with a hydrophobic segment flanked by two Trp at both ends, Trp residues being known to reside preferentially at membrane/water interfaces. The photolabel used is the 3-(trifluoromethyl-*3H*-diazirin-3-yl)-phenylalanine (TMD-Phe) inserted either at position 6 (**WALP23-TMD6**) or 8 (**WALP23-TMD8**) (Figure 3), allowing probing the depth of insertion and the orientation of the peptide probe in the membrane.

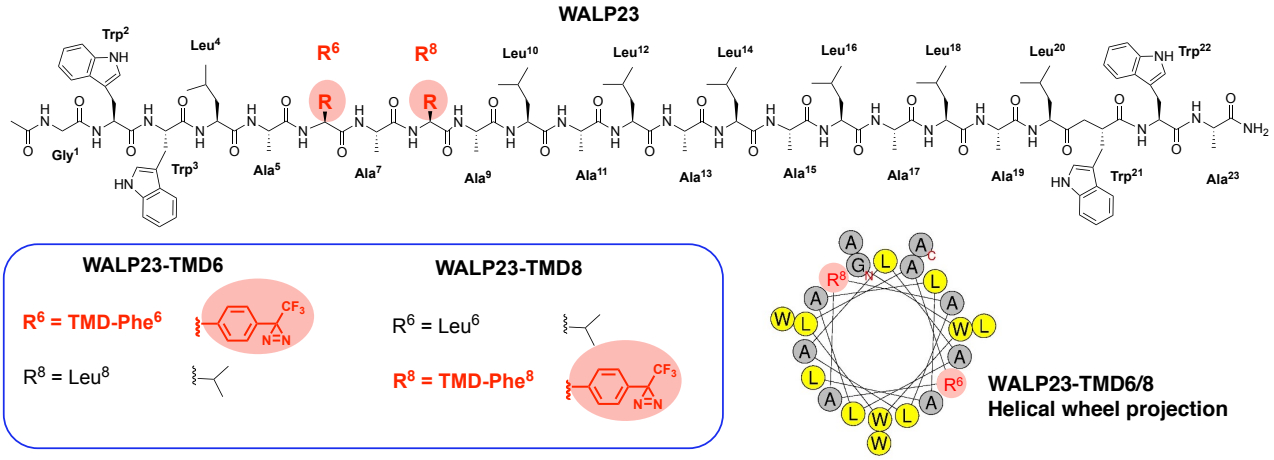


Figure 3: developed structure of the WALP23 peptide. Photoreactive probe TMD-Phe in red substitutes Leu in position 6 in WALP23-TMD6 or 8 in WALP23-TMD8 [33]. The helical wheel projection of WALP23-TMD6/ 8 is presented. This projection was generated with Heliquest [34]. The position of the photolabel is in red. N and C-termini are indicated with red N and C, respectively.

In **WALP23-TMD 6** and **8**, TMD-Phe is located on either side of the α-helix structure adopted by the peptide in lipid membranes (Figure 3, see helical projection). Mixed multilammelar vesicules (MLVs) containing **WALP23-TMD 6** or **8** and PC lipid mixtures with different acyl chains were prepared to mimic a complex membrane environment. The photolabeling experiment was performed by UV-irradiation of MLVs of different lipids compositions with a peptide/lipid ratio ranging from 1:25 to 1:100, for 1 to 10 min, at temperatures avoiding phase separation for mixed lipid compositions. Control experiments were carried out in the same conditions without irradiation. With a diazirine-based photolabel, the non-irradiated peptide should be observed at its theoretical mass whereas the irradiated one would lose a N_2 molecule leading to a mass default of 28 Da, easily detected by MS. In this workflow, the purification step was a simple peptide/lipid two-phase extraction. No specific purification tag was added to the **WALP23** sequence. Detection of the photoadducts was performed by gel electrophoresis (SDS-PAGE) with a smart staining method. Silver staining was used for the peptide and subsequent Coomassie blue staining for the lipids allowed the detection of the photoadducts as two superimposed bands. Peptide/lipid photoadducts were identified by MS and fragmented by MS/MS, giving a direct evidence of the covalent bond between the peptide and the lipid. Further characterization of the site of photocrosslinking required a treatment of the irradiated samples with phospholipase C or phospholipase A2. Analysis of the resulting products by ESI-MS revealed that the peptide could crosslink either acyl chain of the phospholipids. Using PC lipids with different chain length, the authors hoped to highlight a preference of **WALP23** for lipids with C18:1 fatty acid chains but unfortunately the amount of different crosslinked species only reflected the lipid composition of the MLVs.

Taken together, these results did not allow the authors to observe molecular sorting of lipids, but this elegant work was the first example of such a study and constitutes a very good starting point to discuss the following cases.

Interaction with lipids: Identification of privileged lipid binding partners of the CPP Penetratin

CPPs are a class of membrane-active peptides with short sequences of amino acids, mainly cationic and capable of penetrating cells. Two independent pathways have been described for their internalization: direct translocation and endocytosis. Photoaffinity labeling studies from two different laboratories have been carried out to investigate CPPs interactions with lipids, proteins and proteoglycans with the aim to understand these internalization mechanisms.

The first studies discussed herein are focused on the interactions of Penetratin with membrane lipids. Penetratin is a 16 residues peptide corresponding to the third helix of the homeodomain of the Antennapedia homeoprotein. It is one of the first discovered natural CPP, and it can enter cells by directly crossing the plasma membrane [35]. A first methodological study [36] compared lipid photo-crosslinking efficacy of Penetratin analogues bearing three different photolabels: benzophenone (Bzp), dithienylketone (DTK) and TMD with the analogues **Pen(Lys48Bzp)**, **Pen(Lys48DTK)** and **Pen(Lys48TMD)** (Figure 4A). Substitution of the Trp^{48} was chosen since this residue is crucial for the internalization properties of Penetratin. A biotin was added at the N-terminus, separated from the Penetratin sequence by a spacer composed of four glycine residues, which allowed biotin/streptavidin affinity purification of the photoadducts before MALDI-TOF MS analysis. DSC validated **Pen(Lys48Bzp)**, **Pen(Lys48DTK)**, and **Pen(Lys48TMD)** as good models of the natural Penetratin and they were further used for photolabeling with DMPG MLVs. The same experimental conditions but whithout UV-irradiation were used for controls. Only Bzp and TMD labels led to covalent adducts identifiable by MALDI-TOF MS. Contrarily to **WALP23,** entire photoadducts could not be observed and a saponification step was required to reduce the size of the lipid moiety of the adducts and thus allow MS detection. The harsh conditions of the saponification led to abundant oxidation of the photoadducts, particularly on the sulfur atoms found in biotin and methionine. These oxidations can be a real issue when working with low amount of material, as it “dilutes” the MS signal on several peaks.

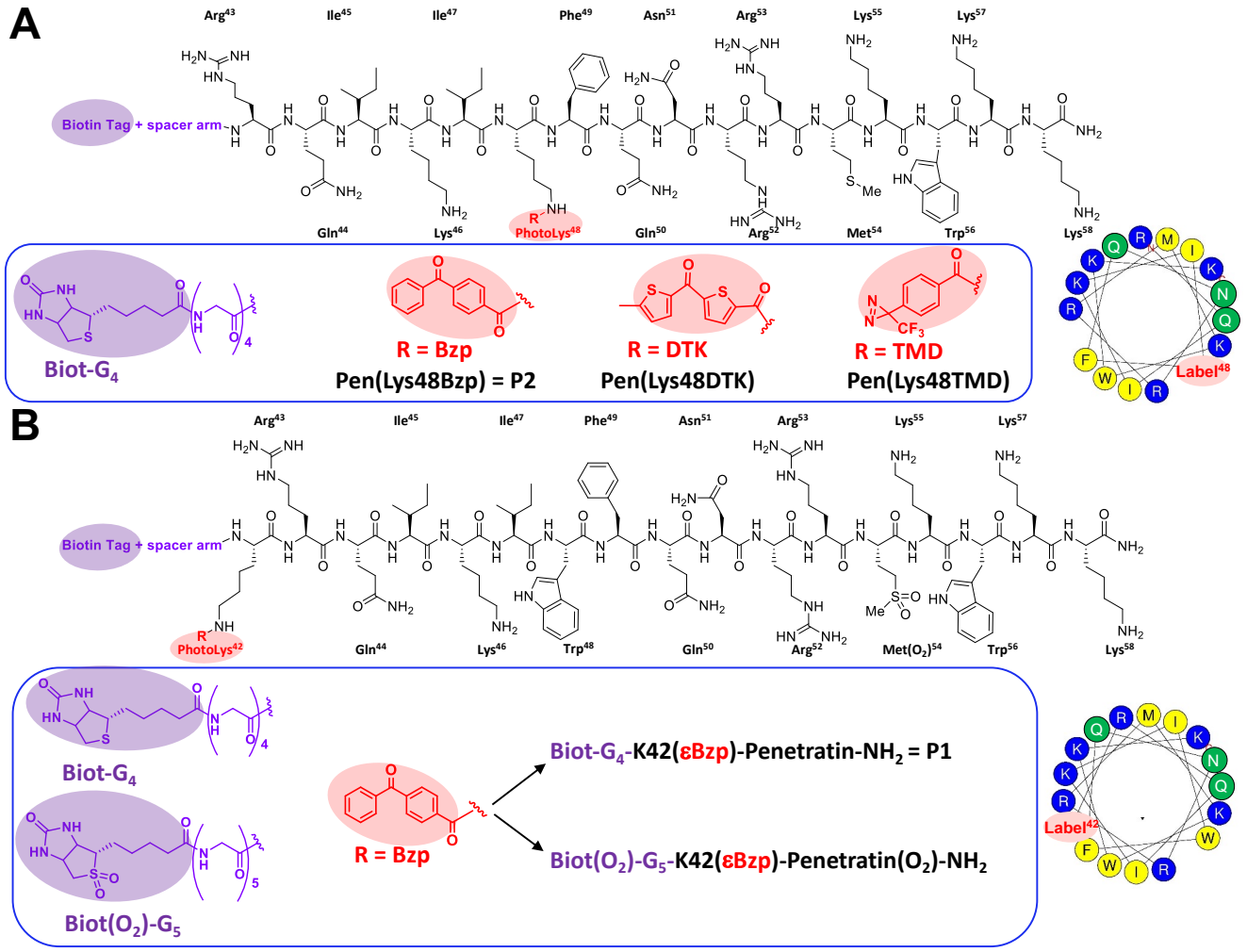


Figure 4: developed structures of photoreactive analogues derived from Penetratin (helix III of Antennapedia homeodomain, residues 43 to 58) [36–38]. Photolabels (R) are in red, affinity purification biotin tags are in purple. Bzp: benzophenone, DTK: dithienylketone and TMD: trifluoromethylaryldiazirine. Penetratin(O_2) is for Penetratin with a Met sulfone (Met(O_2)), Biot is for biotin and Biot(O_2) is for biotin sulfone. The helical wheel projections, generated with Heliquest [34], are presented. Basic residues are colored in blue, polar residues in green, apolar residues in yellow and photolabels in red.

Bzp-photolabeling was pursued to evaluate the phospholipid preference of Penetratin to better understand the early stages of direct translocation [37]. **Pen(Lys48Bzp)** also called **P2** by the authors, and **Biot-G_4-[K42(εBzp)]Penetratin** (or **P1**) with the Bzp located at the N-terminus of the Penetratin sequence were used. Efficacies of internalization of **P1** and **P2** were comparable to that of natural Penetratin, showing that the photolabel did not disrupt the CPP properties of the analogues. MLVs of various phospholipids compositions were prepared and photolabeling performed as previously described, with a peptide/lipid ratio of 1:10. Bzp photoreactivity was exploited to probe phospholipid environment and insertion depth of **P1** and **P2**. This study demonstrated that Penetratin shows a preference for negatively charged (*vs* zwitterionic) polar heads and for unsaturated (*vs* saturated) and short (*vs* long) saturated phospholipids. Bzp has a better reactivity for doubly allylic than for allylic and for secondary hydrogens [12] (Table 1), which could partly explain the increased yield when using lipids with unsaturated chains. The depth of Bzp insertion in anionic vesicles for **P1** and **P2** was estimated to be centered around the carbon atoms C8-C10, as it was able to crosslink at the level of the allylic position of Δ^9 unsaturated fatty acids, even though no direct evidence was given.

To further exploit this system, Bechtella *et al.* [38] improved the overall strategy by eliminating oxidation reactions. For this, fully oxidized biotin sulfone (Biot(O_2)) and methionine sulfone were used (**Biot(O_2)-G_5-K42(εBzp)-Penetratin(O_2)-NH_2**, Figure 4B). Saponification time was shortened, and performed at a lower temperature, with a higher concentration of NaOH. The resulting MS signal was enhanced by a factor of 10 to 100 allowing authors to exploit the side products of the reaction of the UV-activated biradical carbonyl Bzp (triplet state). The so-called Paternò-Büchi reaction, corresponding to the formation of an oxetane ring with C=C bonds of unsaturated lipids led to the formation of relatively unstable adducts at the same m/z than conventional adducts. The tension exerted on the oxetane linking the bulky peptide and fatty acid can weaken the adduct

which leads to its spontaneous fragmentation by the retro Paternò-Büchi reaction. Resulting diagnostic ions gave valuable information on the depth of insertion of the Bzp label directly in the MS level, without the need of further MS/MS fragmentation. This was a real advantage when working with small amounts of material. The authors also uncovered an unexpected Bzp-induced photo-oxidation reaction leading to the formation of a double-bond on saturated lipids, as previously described in solution by Breslow *et al*. on photoreactive cholesterol analogues [39]. Breslow *et al*. showed that this particular photoreactivity of Bzp was directed by the position of Bzp relative to cholesterol and by the stiffness of the linker between the two[39]. When transposed to the study of Penetratin/lipids interactions, this could be indicative of the fluidity of the membrane around the probe [38]. Additionally, Bechtella *et al.* showed that a further crosslinking reaction can occur on this newly formed unsaturated lipid and informative retro Paternò-Büchi products can thus be produced [38].

Analysis of the MS spectra in the higher m/z regions of the MS spectra revealed no peptide dimerization, but low intensity peaks corresponding to Penetratin crosslinked to a fatty acid dimer were detected. This was interpreted as Bzp-induced photosensitization, forming radicals on unsaturated fatty acids which can further react with their closer neighbors.

The saponification step however remained a limitation of the method. This step was required to reduce the size and complexity of the photoadduct for MS analysis. Because of this step, no direct information concerning the polar head of the crosslinked lipid could be obtained, which remains a limitation of this method when applied to lipid partner identification from a complex natural lipid mixture. Finally, it has to be noted that crosslinking on the glycerol backbone or phosphate headgroup was never observed.

Interactions with membrane proteins: identification of membrane receptors for the internalization of poly-Arg CPPs

The following studies focus on poly-Arg derived CPPs. The group of Futaki has a long history of deciphering the internalization mechanisms of poly-Arg CPPs [40]. They previously evidenced the involvement of endocytosis pathways in poly-Arg cellular uptake and suggested an important role for proteoglycans as endocytosis effectors. Photocrosslinking coupled to MALDI-TOF MS and peptide mass fingerprinting (PMF) were used to identify potential poly-Arg membrane protein partners. Contrarily to the previous examples, the authors worked *in cellulo* with HeLa or HEK293 cells, with a potassium-rich buffer to allow the peptide to reside longer on the plasma membranes favoring crosslinking of membrane partners. TMD-Phe was chosen as the photoreactive probe.

In a first study[41], Tanaka *et al.* used the Arg_{12} analogue **biotin-TMDPhe-R_{12}** (Figure 5) for their photolabeling experiments. **Biotin-TMDPhe-R_4**, an analogue of Arg_4 which shows no internalization was chosen as the control. After irradiation, membrane proteins were isolated, and biotinylated adducts were purified and separated using SDS-PAGE. Specific bands were analyzed by MALDI-TOF MS after trypsin in-gel digestion. Four protein bands were identified, which all derived from myosin 9. Based on these results and on the literature, the authors made the assumption that the $CXCR_4$ receptor and possibly syndecan-4 which is expressed as a membrane complex with $CXCR_4$, could be implicated in the cellular-uptake of Arg-rich CPPs. Co-localization experiments indicated the involvement of $CXCR_4$, which was further confirmed using $CXCR_4$-knockdown HeLa cells in which a significant decrease in R_{12} uptake was observed. No direct evidence of the photocrosslinking of **biotin-TMDPhe-R_{12}** with $CXCR_4$ or syndecan 4 was however observed in this study. Then, an Arg_8 analogue, **biotin-TMDPhe-R_8** (Figure 5) was used with a similar set-up [42]. Western blotting revealed a protein band at 37 kDa in the cells treated with **biotin-TMDPhe-R_8** compared with non-treated control. MALDI-TOF PMF suggested that the protein was a cytosolic, peripheral membrane protein, lanthionine synthetase component C-like protein 1 (LanCL1). Increased cellular-uptake of Arg_8 by LanCL1-overexpressing cells was observed.

Over the years, the same group greatly improved their purification system[43]. Biotin was separated from the CPP sequence by a cleavable diazobenzene linker (in green, Figure 5) which can be cleaved by reduction upon sodium dithionite $Na_2S_2O_4$ treatment. This smart design closely related to the one introduced by Brunner *et al*. [17], considerably reduced non-specific binding during purification. This optimized **PhotoR8CL** probe (Figure 5) was used in photolabeling experiments. Among the identified protein partners, authors focused on the proteoglycan syndecan-4. Knockdown and overexpression of syndecan-4 revealed its implication in the cellular-uptake of R_8. The fact that LanCL1 was not identified in this study was not discussed.

The strength of these studies lies in the fact that the authors always validated the biological relevance of the identified protein partners, even though photoreactive peptides were not used for these biological studies. Altogether they allow a better understanding of the endocytosis-mediated internalization pathway described for the cellular-uptake of poly-Arg derived CPPs.

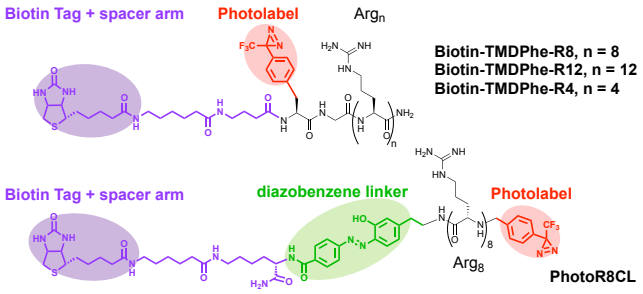


Figure 5: developed structures of the Biotin-TMDPhe-R8, R12, R4 and photoR8CL photoreactive analogues [41–43]. Biotin tag is in purple, photocleavable linker in green and photolabel in red.

Interactions with membrane proteins: binding partner identification for peptidomimetic antibiotics

The last type of membrane-active peptides that will be discussed are peptidomimetic antibiotics derived from the antimicrobial peptides polymyxin B (PMB) and protegrin-I (PG-I).

Polymyxins is a family of macrocyclic cationic and hydrophobic decapeptides with an N-terminal fatty acid chain, naturally produced by B*acillus sp* [44]. Their selective antimicrobial activity against Gram-negative bacteria comes from electrostatic interactions with negatively charged lipopolysaccharide (LPS), a major component of outer membrane (OM) of the Gram-negative bacteria [45], and further disruption of outer and inner membranes [46,47]. Structures of polymyxin B1, B2 and B3 (PMB1, 2 and 3) are presented in Figure 6.

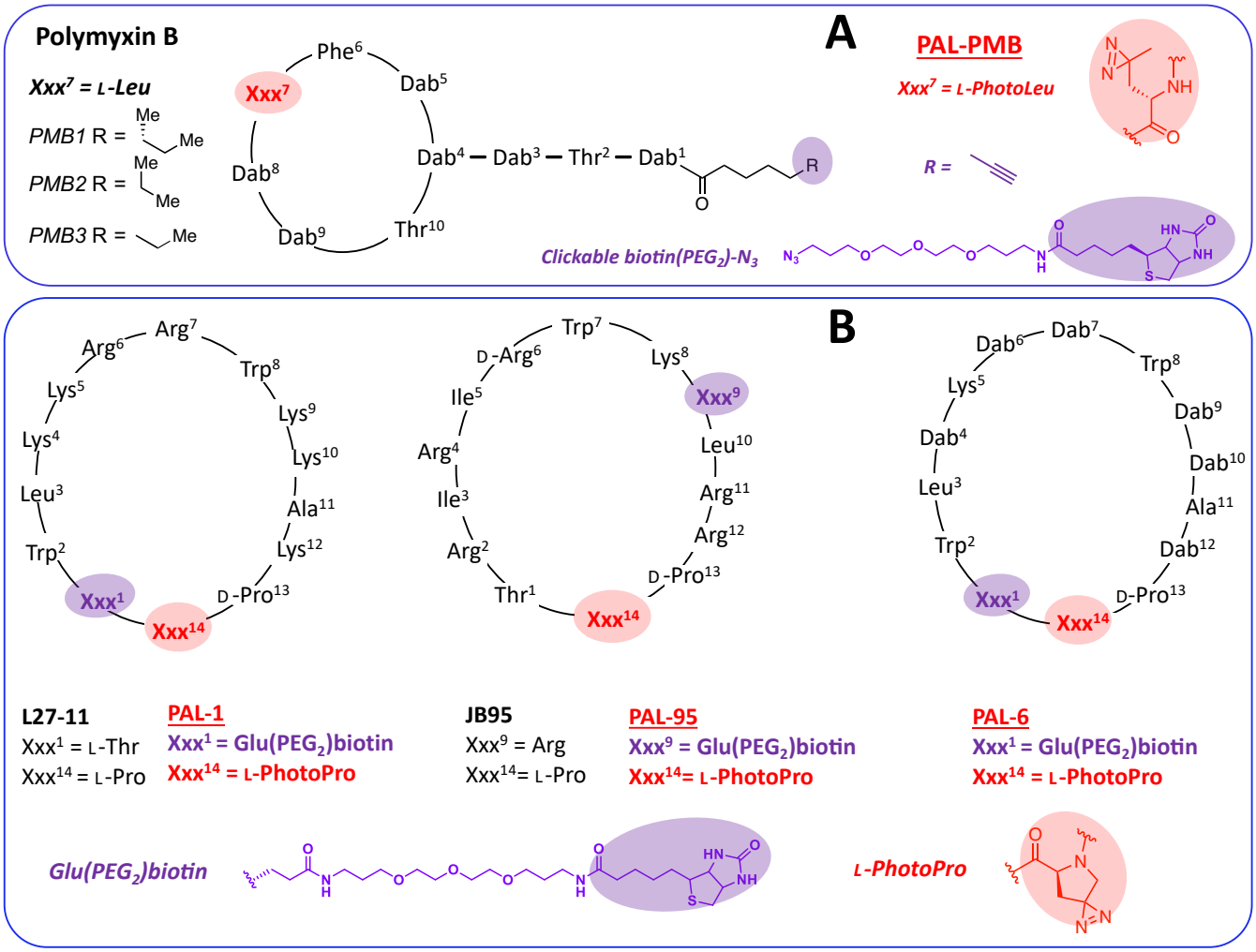


Figure 6: developed structures of macrocyclic peptidomimetic antibiotics PMB, L27-11 and JB95 and their derived photoreactive analogues PAL-PMB, PAL-1, PAL-95 and PAL-6[48–51]. Dab: diaminobutyric acid. Affinity purification tags are in purple and photolabels in red.

In 2000, Tsubery *et al.*[52] showed that the enantiomeric form of the PMB nonapeptide (D-PMBN) could bind LPS as efficiently as native L-PMBN, but poorly sensitized bacteria to other antibiotics, unlike L-PMBN. Based on this work, Robinson *et al*. suggested that an interaction between a chiral bacterial receptor and polymyxin in bacteria could likely exist. To investigate this hypothesis, they synthesized a photoreactive polymyxin B analogue, **PAL-PMB** (Figure 6A), to detect interactions between polymyxins and a potential OM protein receptor using affinity photolabeling [48]. In **PAL-PMB**, the Leu^7 residue of PMB was replaced by a diazirine-based L-photoLeu^7 and an alkyne moiety was added at the N-terminus *via* an oct-7-ynoyl fatty acyl, in order to introduce a biotin tag for affinity purification, *via* a post-labeling strategy using a click-chemistry reaction with a biotin-PEG$_2$-N$_3$. **PAL-PMB** retained potent antimicrobial activity against *E. coli* and *P. aeruginosa (PA)* and was used in photocrosslinking experiments with *E. coli* to identify potential **PAL-PMB** receptors. Several crosslinked OM proteins were isolated after carbonate extraction and labeled with biotin-PEG$_2$-N$_3$, and were further identified after SDS-PAGE electrophoresis and Western blotting. Identified proteins included OmpA/C/F, the maltose and fatty acids transporters LamB and FadL, and another band around 100 kDa that could correspond to OM transporters and OM protein folding catalysts involved in the biogenesis of the OM. A negative control experiment was performed without **PAL-PMB**.

Further proteomic analysis to characterize these proteins were not performed in this work but were developed in the following articles in which the same group investigated peptidomimetic antibiotics derived from PG-I. Among them, the **L27-11** analogue possessed interesting MICs in the nanomolar range towards *PA* strains and other *Pseudomonas spp* [49]. The enantiomeric form of **L27-11** was essentially inactive against *PA* suggesting that the antimicrobial target of **L27-11** was chiral and not the achiral lipids of the cell membrane targeted by the original peptide PG-I.

To gain insight on the mechanism of action of **L27-11**, a forward genetic screen was established on the wild-type *PA* PAO1 (PAO1^{WT}), to characterize the genetic basis for the resistance to **L27-11**. The *LptD1* gene appeared as conferring the resistant phenotype, PAO1^{RES1}. Mutation in *LptD1* impaired the binding of **L27-11** to LptD. LptD, together with LptE, is involved in the assembly of LPS at the surface of the OM in Gram-negative bacteria. LptD is composed of a periplasmic N-terminal domain

that adopts a β-jellyroll fold and a C-terminal β-barrel domain. In the LptD from *Pseudomonas* and some other Gram-negative bacteria, the periplasmic domain comprises an additional insert domain. The LptE core resides within the lumen of the LptD β-barrel. LptD depletion in *E. coli* causes inhibition of the LPS transport function in OM biogenesis [53]. Photoaffinity labeling experiments were performed to determine whether **L27-11** binds to LptD in intact cells (*PA* PAO1^{WT})[49]. For this task, a photoreactive analogue of **L27-11**, **PAL-1,** was synthesized [49,54].

PAL-1 contains a L-4,4-diazarinylproline (L-photoPro^{14}) and a biotin tag at position 1 (Figure 6B). Photoaffinity labeling of *PA* PAO1^{WT} cells with **PAL-1** consistently revealed a major photolabeled protein by SDS-PAGE with an apparent mass close to that expected for *PA* LptD. As a well-designed control, the same experiment was repeated in the presence of 100 folds excess of inert **L27-11** and the labeled band disappeared. Incubation of cells without peptide addition was also carried out as a negative control. Interestingly, when photolabeling was performed on the resistant mutant *PA* PAO1^{RES1}, the LptD protein was no longer able to bind the antibiotic with comparable affinity. In these experiments, a specific shift to higher pI of the LptD spot (2D-gel) due to seven additional positively charged residues of **PAL-1** undoubtedly further evidenced the formation of a covalent adduct between **PAL-1** and LptD. No crosslinked peptides were detected by MS.

Another conformationally constrained β-hairpin peptidomimetic **JB-95** (Figure 6B) was discovered by the same group [50]. **JB-95** presents potent antimicrobial activity against a panel of Gram-positive and Gram-negative bacteria and, in particular, against *E. coli*. Its enantiomer showed reduced activity against all-tested organisms. In this work, Urfer *et al.* first looked at the effect of the incubation of *E. coli* cells with **JB-95** on the OM proteome. SDS-PAGE revealed a decrease in the relative amount of some membrane proteins compared with untreated control. A semi-quantitative study by ESI-LC-MS/MS identified many OM β-barrel proteins that were differentially expressed in the presence of **JB-95**. Photolabeling experiments were conducted to validate these identifications, using the photoreactive analogue of **JB-95**, **PAL-95** which contains L-photoPro^{14} and a Glu(PEG$_2$)-biotin^{9} as shown in Figure 6B. **PAL-95** retained a high antimicrobial activity against *E. coli*. After photolabeling, the OM proteome was extracted and analyzed by 2D-gel electrophoresis. Photolabeled biotinylated proteins were detected by immunoblotting. These results evidenced that **JB-95** interacts with many β-barrel proteins in OM of *E. coli*, including the essential proteins LptD and BamA required for biogenesis of the OM.

Finally, the same authors designed a very smart quantitative proteomic approach in which they could better characterize the region of interaction between **L27-11** and LptD using the **PAL-6** photoreactive analogue (Figure 6B)[51]. **PAL-6** contains a L-photoPro^{14} residue with a Glu(PEG$_2$)-biotin^{1} and multiple substitutions of Arg/Lys for diaminobutyric acid (Dab). The position of the biotin and the stereochemical configuration of the residue 9 were changed compared to **PAL-1** but no specific reasons were given. The Dab substitution is commonly used to prevent protease recognition while retaining the number of positively charged residues which are important for solubility issues and molecular recognition. **PAL-6** retained potent antimicrobial activity against *PA* PAO1^{WT}.

The quantitative proteomic approach developed in this last study arose from the assumption that **PAL-6**-labeled peptides should be less likely to be detected by MS due to altered mass of the precursor, fragmentation pattern and ionization efficiency. Therefore, comparison of peptide abundance of the non-crosslinked peptide between **PAL-6**-labeled and unlabeled LptD/LptE$_{His}$ digests was performed to identify **PAL-6** modified regions. Once proteolytic digestion was optimized, authors analyzed the **PAL-6**-labeled and unlabeled samples by LC-ESI-MS/MS in data-independent acquisition mode for label-free quantification of LptD/LptE$_{His}$ derived peptides. Peptides were quantified by integration of associated fragment-ion traces. These results identified a preferential binding to the periplasmic region of recombinant LptD/LptE$_{His}$ *in vitro,* which is close to the site of the mutation observed in the resistant mutant *PA* PAO1^{RES1}.

In these last examples, alkyldiazirine analogues of natural amino-acids, namely photoLeu and photoPro (Figure 6) were used. These derivatives are extremely close to the natural amino-acids, in terms of chemical structure and properties. Incorporation of these photoreactive amino acids had no impact on the antimicrobial properties of the different peptidomimetic probes [48–51,54]. On the other hand, alkyldiazirine labels tend to be less stable and generate less reactive carbenes than aryldiazirines [55].

CONCLUSION

Affinity photocrosslinking is a powerful and unique method to study the non-covalent interactions between membrane active peptides and membrane components, as illustrated by the various examples detailed herein, and between interacting partners in general. It was first introduced for such applications in the early 80's with photoreactive lipids. This approach, however is not always easy to implement and many difficulties can be encountered, as illustrated is this review. These difficulties are related to incorporation of the probe in membranes, positioning and reactivity of the photoreactive label, photoadduct isolation and sample analysis.

More recently, the technique has re-attracted researchers' attention with the use of photoreactive peptides, which are convenient to obtain as it is easy to introduce modified amino-acids carrying a photolabel by solid-phase peptide synthesis. This revival comes together with the development of highly sensitive (sub-picomolar) analytical methods such as MS, MS/MS and proteomic analysis, knowing that photolabeling yields usually remain low regardless of the chosen photoreactive label. At the same time, the emergence of performant biorthogonal reactions for sample functionalization such as click-chemistry will greatly improve issues related to sample recovery and enrichment, as illustrated herein with the PMB photoreactive and clickable analogue **PAL-PMB** [48]. In that perspective, the development of new photoreactive and clickable bifunctional lipids, with additional functionalities and/or that can be metabolized by cells for the biosynthesis of membrane phospholipids [56] are opening new exciting opportunities for the study of the interactions of membrane proteins with lipids in the complex environment of a live cell membrane and will certainly bring new information on the mode of action of membrane active peptides.

The success of photolabeling studies is tightly linked with the design of the probe. The choice of an appropriate photolabel is critical. There are many diazirine derivatives closely mimicking

natural amino-acids, which is not the case for Bzp derivatives. Developing new Bzp analogues of amino acids would be a great benefit to the field. In terms of biological activity, smaller labels like alkyldiazirines are probably better tolerated, but bulkier labels like Bzp or TMD are not necessarily problematic, provided they are introduced at the right position, especially because of their better reactivity and stability. Structural information obtained by alternative methods like NMR or molecular modelling can also provide valuable insight for the successful design of the probe. All things considered, a critical point is to ensure the biological activity of the probe is maintained. Another promising development in the design of the probe is the use of cleavable linker between the biomolecule of interest (peptide or lipid) and the photolabel. These linkers help simplify the downstream analytical workflow by reducing the size of the adduct and its chemical complexity.

As demonstrated in this review, affinity photocrosslinking allows molecular characterization of the site of interaction between membranotropic peptides and their binding partner(s), which is a real asset for the design of peptides with interesting biological properties such as cell delivery or antimicrobial activity, or for the understanding of molecular mechanisms underlying peptide-mediated cell processes. However, it has to be kept in mind that complementary experiments using more classical biochemistry/biology tools are still required to validate the photolabeling data in order to get a global and fully integrated understanding of the interaction networks both at the molecular and cellular level.

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Author Contributions

The manuscript was written through contributions of both authors.

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ABBREVIATIONS

Aib, 2-aminoisobutyric acid; AMP, antimicrobial peptide; BHA, bromelain-cleaved hemagglutinin; Biot(O_2), Biotin sulfone; Bzp, benzophenone; CPP, cell-penetrating peptide; Dab, diaminobutyric acid; DSC, differential scanning calorimetry; DTK, dithioketone; MS, mass spectrometry; MS/MS, tandem mass spectrometry; LPS, lipopolysaccharide; MIC, minimal inhibitory concentration; OM, outer membrane; PC, phosphatidylcholine; PG, phosphatidylglycerol; PG-I, protegrin-I; Phl, phenylalaninol; PMB, polymyxin B; PMF, peptide mass fingerprinting; TLC, thin layer chromatography; TMD, trifluoromethyldiazirine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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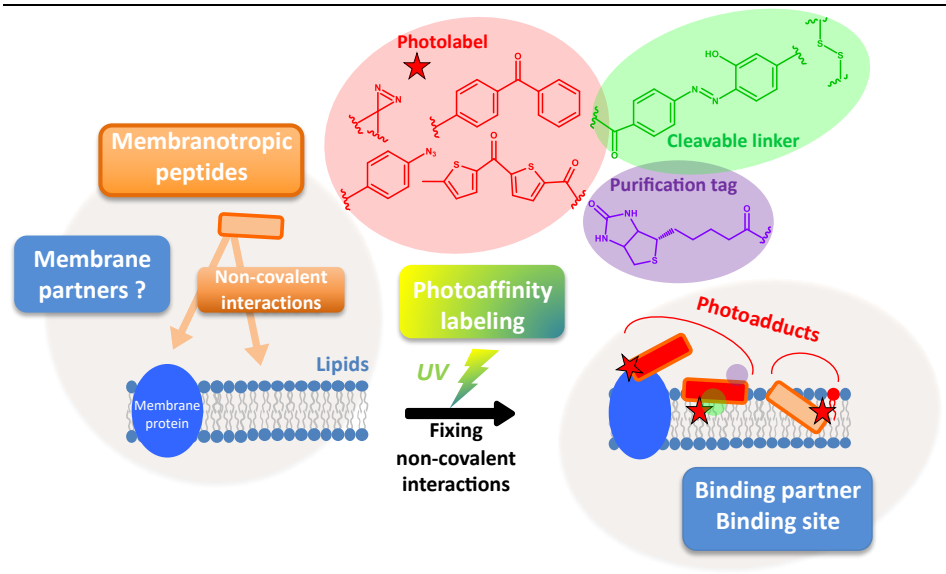
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