UNRAVELLING THE THERAPEUTIC POTENTIAL OF TRANSMEMBRANE PEPTIDES

ABSTRACT

In eukaryotic cells, membrane spanning proteins are tethered to the lipid bilayer mostly through α-helical transmembrane (TM) domains. These membrane-embedded sequences are capable of interacting with each other, driving both stable and dynamic supramolecular organization of TM proteins. Peptides derived from TM α-helical domains, known as TM peptides, can destabilize TM interactions and, as a result, modulate protein activity. In light of the pivotal role played in the cell by membrane proteins, agents modulating their functions are of great pharmaceutical interest.

In this review we will discuss general design requirements for TM peptides developed for therapeutic applications and present some examples of successful modulation of biologically relevant TM proteins. In addition, promising applications of TM peptides employing their membrane self-insertion properties are presented.

1. INTRODUCTION

Cell membranes are highly dynamic and complex systems composed of a lipid bilayer embedding a variety of integral proteins[1]. It was estimated that transmembrane (TM) proteins account for one third of the human genome, playing a pivotal role in a variety of biological processes as receptors, enzymes, channels, carriers and pumps[2]. Owing to the combination between their biological significance and their accessibility to exogenous agents, transmembrane proteins constitute the target of 60% of the FDA approved drugs[3].

Generally drugs target the soluble domains of membrane proteins, whereas the TM segment has long been considered a mere protein anchor. However, a growing body of evidences stresses the role played by the intra-membrane region in modulating the activity of TM proteins, affecting their conformation and oligomerization state[4]. In particular, the use of exogenous TM peptides to regulate lateral TM-TM protein interactions constitutes an interesting and promising strategy to modulate protein activity.

In the first part of the review, we summarize the structural charachteristics of α-helical membrane-spanning regions of TM proteins, which constitutes the basis for the development of TM peptide technology. In subsequent sections we focus on the design of TM peptides for therapeutic applications and we critically review the more relevant examples.

2. PROTEINS IN MEMBRANES – AN OVERVIEW

2.1 SEQUENCE REQUIREMENTS

Membranes exhibit a ~30 Å hydrophobic core, composed by variable-length lipid acyl-chains, framed by two chemically heterogeneous interfaces 15 Å thick, accommodating polar moieties (fig. 1)[5]. Given the necessity to cross the hydrophobic region of the bilayer, transmembrane proteins are equipped with a ~20 amino acid stretch naturally predisposed to fit in a non-polar environment. Although mainly composed of hydrophobic residues, protein TM regions were found to include around 20% of polar amino acids[4]. Insertion of a polar residue in a hydrophobic environment is energetically unfavorable, causing rearrangements and distortions inside the membrane bilayer to accommodate the polar side chain[6]. Much work has been devoted to calculate the energetic cost for bilayer-insertion of each aminoacid. In particular, Hessa et al. determined an energy scale for translocon-driven amino acid insertion in the TM region[7]. Results were in line with the experimental hydrophobicity scale obtained in a water/octanol membrane model system and statistical analysis of the TM region of known proteins[7]. As expected, hydrophobic residues are preferentially positioned in the central part of TM domains while polar residues are typically at the sides of the helix, interacting with charged head groups and water[7]. Although such distribution lowers the energetic cost of peptide side groups/acyl-chains interaction, peptidic amide backbone itself is inherently polar, containing hydrogen bond donors (amide protons) and acceptors (mainly carbonyl oxygens) that have to be stabilized in the core of lipid bilayer. Consequently, a secondary structure that satisfies H-bonds internally is needed, a requirement fulfilled by both α-helix and β-barrel structures [8]. Since β-barrels structures are mainly found in prokaryotic organisms[6], in this review we will focus on proteins displaying α-helical TM segments.

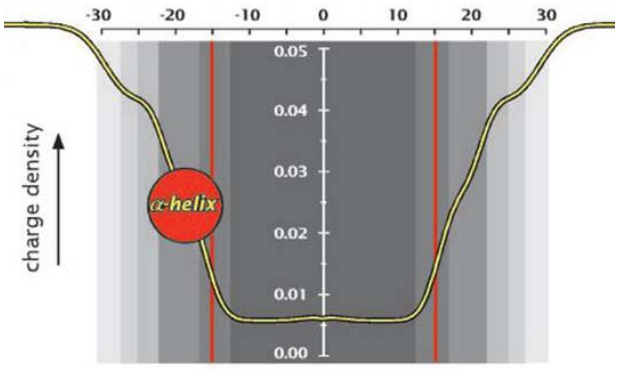

FIGURE 1. Polarity profile of a DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) lipid bilayer (5.4 water/lipid). The charge density was calculated for a time-averaged distribution of the principal lipid components in the membrane. Approximate interfacial location of an amphipatic α-helix is also indicated [5].

2.2 HELIX-HELIX INTERACTIONS IN THE MEMBRANE

Single pass membrane helices interact among each other either by dynamic, switchable contacts or via kinetically stable interactions, characteristic of protein complexes[9]. Permissive dynamic helix-helix interactions are mainly driven by low-energy van der Waals interactions, easily reversible, allowing the conformational flexibility required for biological function. Conversely, stable helix-helix contacts may also contain polar and electrostatic bonds[6].

The occurrence and the strength of the interactions depend mainly on the stability of the helices in the lipid environment. This property is closely related to the primary structure of the TM segments and the resulting specific geometric motif that can drive

inter helical association. In fact α-helix interactions are based on geometric complementarity: knobs-into-holes packing of helix side chains and characteristic hydrogen bond networks confer specificity and stability to the pair[6].
After analyzing a set of high-resolution crystal structures of TM proteins, DeGrado *et al*. made a classification of helical pairs based on their three-dimensional similarity and discovered the existence of a limited number of structures[10]. By grouping helical dimers that showed a super imposable structure, two thirds of the selected helical pairs were found to belong to few major clusters, characterized by a primary sequence motif and defined packing angles[10].
The most common packing motif found in TM segments is GXXXG, where glycine residues are separated by 3 random aminoacids. In the folded secondary structure, the two Gly (which can be substituted by other small amino acids, i.e. Ala, Ser) are positioned on the same side of the helix creating a shallow groove complemented by the second helix. This helix packing, also known as GAS motif (Gly, Ala, Ser) can be right- or left-handed depending on the angle formed by interface between TM helices[9].

2.3 BIOLOGICAL RELEVANCE OF HELIX-HELIX INTERACTIONS

The biological relevance of TM interactions is emphasized by manifestation of a disease phenotype as a result of mutations within helix TM domains [for a review see[4]]. Single amino acid substitutions can either affect correct protein folding and complex assembly or interfere with conformational changes associated with signal transduction. For instance, mutations of a glycine residue in the GXXXG motif can disrupt the helix-helix packing geometry and result in a looser TM interaction, perturbing both the tertiary and quaternary protein structure. Similarly, substitution

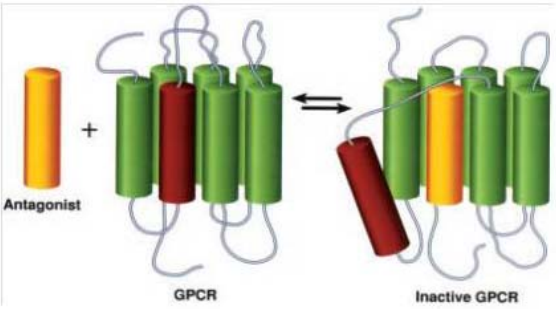

FIGURE 2: Proposed mechanism of action for TM peptides in a generic GPCR receptor [11]

of aminoacids participating in an intra-membrane H-bond or polar interaction is likely to alter the helices interplay either by weakening or strengthening it[4]. It was therefore postulated that, by controlling the folding and the oligomerization of transmembrane proteins, one can in turn modulate their activity. However, the development of agents able to modulate lateral intra-membrane protein-protein interactions have long been hampered by the inherent complexity of the surrounding hydrophobic environment.

3. TM PEPTIDES

3.1 MODELS FOR MEMBRANE PROTEIN STUDIES

Research on TM protein structure and functionality has long been slowed down by technical hurdles associated with producing, purifying and analyzing membrane-embedded proteins[12]. To circumvent these obstacles a "divide and conquer approach" was proposed in which a complex, multi span membrane protein is broken down into its TM components, easier to produce and to analyze[5]. The approach draws its justification from the well accepted "two stage model of membrane protein folding". According to this model, TM helices of multi-spanning membrane proteins are first independently inserted into the lipid bilayer and only subsequently they interact with each other, establishing the rationale for the independent TM segment study[13]. The resulting peptides derived from α- helical TM segments of single or multi-spanning membrane protein are generally indicated as TM peptides.
In the past years, a wealth of data have been generated using TM peptides as descriptors of membrane proteins, ranging from their structure to the interaction with biological membranes[14, 15].
However, applications of TM peptides are not only restricted to their role as membrane proteins equivalents. New functions as probes to determine membrane protein effective conformation as well as agents to stabilize protein folding intermediates have been suggested[124]. Yet one of the most intriguing application resides in the use of TM peptides as potential therapeutic agents.

3.2 POTENTIAL AS THERAPEUTICS

The first suggestion of a pharmacological application of TM peptides came in 1996, when Hebert *et al*. reported the ability of a peptide isolated from the TM region of β_2- adrenergic receptor (β_2AR) to inhibit both activation and dimerization of its "parental" protein *in vitro*[16]. The

TM peptide corresponded to receptor helix VI, indicated by molecular models as one of the most lipid-exposed regions, and likely involved in β_2AR dimerization. According to the suggested mechanism of action, the exogenous TM peptide bound the parental receptor TM region, thus masking the interface involved in inter molecular protein interactions causing suppression of the downstream signaling (fig.2)[16]. The result demonstrated that TM peptides can competitively bind lipid-buried helices of receptors, thus modulating their supramolecular organization and activity.
The study, originally aimed at clarifying functional and physical GPCR interactions, thus provided the rationale for developing a series of therapeutically active TM peptides.

3.3 DESIGN

TM peptide technology can be applied to regulate the activity of virtually every membrane protein, given it has multiple interacting membrane spanning helices and/or is involved in protein-protein TM interactions. However not all transmembrane proteins are suitable pharmaceutical targets. In general, targets are selected for their relevant and validated role in a biological process and for the therapeutic benefit deriving from their blockade (or activation)[17]. On the other hand, TM peptides have to satisfy a series of pharmacodynamics and pharmacokinetics criteria, before being considered as therapeutic candidates.
In general, the small size and amino acid composition of peptides account for their poor pharmacokinetics, leading to, respectively, short half lives and sensitivity to peptidases[18]. In addition, the highly hydrophobic character of TM peptides results in their poor solubility in aqueous media and constitute one of the major drawback in their *in vivo* application[19].
To overcome these obstacles, generation of a lead TM peptide is usually followed by an optimization process.

GENERATION OF LEAD TM PEPTIDE

TM peptides can be obtained using three strategies: (i) natural occurring TM regions, (ii) directed evolution and (iii) rational design.
"Borrowing" nature's design and using peptides corresponding to truncated native TM regions constituted the initial, more straightforward approach[20]. So far, this approach has been the most used for its simplicity, yielding TM peptides naturally able of inserting with opportune conformation in biological membranes. However "truncated" TM peptides are both numerically and conformationally limited to existing helices involved in native interactions.
Therefore, other strategies have been proposed to create a plethora of non-natural TM peptides, which can differently modulate TM protein activity.
Directed evolution can select TM-helix binders by creating libraries of virus proteins variants differing for the TM segment, as it was done with bovine papilloma virus protein E5[21]. E5 is composed of 44 amino acids and is inherently able to bind the TM domain of the platelet-derived growth factor β receptor (PDGFβR) . DiMaio and coworkers were able to create a 500 000 clones library containing random TM sequences and to isolate highly selective binders to two proteins completely unrelated to PDGFβR: human erythropoietin (hEPO) and CCR5receptor[21, 22]. This demonstrates the power of a directed evolution for isolation of multiple TM peptides for each target without previous knowledge of its sequence or structure. Besides, this approach allows the creation of TM peptides to target mutated/misfolded receptors.
De novo design of TM peptides using a computational method was proposed by Yin *et al.*[23]. The computed helical anti-membrane protein (CHAMP) is a cheap method to generate new TM peptide binders by *in silico* modeling using the target helix sequence as a starting point. The first step includes database mining and selection of a two-helix bundle. The target TM helix sequence is then threaded on one helix from the selected pair and a repacking algorithm used to generate an ideal binding sequence. The CHAMP method was used to design a TM peptide recognizing the TM region of αIIb integrin; the synthesized peptide sequence was then tested *in vitro* where it showed successful recognition of its cognate receptor [23].

OPTIMIZATION OF LEAD TM PEPTIDE

Design of TM peptides as modulators of biological functions has greatly benefited from the large amount of data obtained over the years using peptides as models for membrane proteins studies. Biophysical studies have given indications on how to tune peptide characteristics to achieve high solubility and partitioning into cell membrane (e.g. by modulating peptide length and adding flanking moieties). Meanwhile previous knowledge on peptides poor pharmacokinetics has promoted development of strategies to improve TM peptide stability by using amino acids with inverted stereochemistry.

LENGTH

A first indication of optimal peptide length comes from computational models of TM peptide membrane partitioning. A three step insertion process has been described comprising peptide adsorption, interfacial folding (IF) and folded TM insertion, where the thermodynamic penalty for peptide insertion is inversely proportional to the peptide length[24]. Ulmschneider *et al*. calculated the free energy profile for a set of poly-leucine peptides containing *n* residues as a function of peptide position along the membrane normal z and the tilt angle(α). Shorter peptides ($n \leq 7$) are preferentially located at the interface while longer sequences ($n > 8$) show increased preference for the TM state.

To decrease the energetic cost of TM insertion, peptide length should match the thickness of the cell membrane. Situations in which the length of TM peptides does not coincide with membrane span (defined as hydrophobic mismatch) require adaptation of the peptide and/or the bilayer. The energetic penalty is paid either by peptide tilting, kinking and redirecting its side chains or by stretching and disordering of the lipid bilayer[25]. Although some exceptions have been reported[26], in general TM peptides contain 20-30 aminoacids.

FLANKING MOIETIES

Like their parent proteins, TM peptides are mainly composed of hydrophobic residues and therefore exhibit, with few exceptions, a high tendency to aggregate and precipitate in aqueous buffer. While hydrophobic forces drive the folding of water-soluble proteins by burying apolar residues, in case of TM peptides, this spontaneous process dramatically decreases helices solubility. Strategies to reduce TM peptide aggregation mainly concern addition of flanking hydrophilic residues, although conjugation of sugars has also been reported[27]. In general, solubility effects of tag attachment are dependent on tag composition, location either at protein C or N-terminal and length[19].

Charged amino acids (mainly Lys but also Asp and Glu) are commonly overrepresented in flanking hydrophilic tags and have been shown to dramatically improve peptide solubility. A systematical study on the optimal length of hydrophilic tags used the glycophorin A TM segment as a model sequence, flanked by 2 to 10 lysine residues. Tags containing 3 to 6 Lys on each side were able to increase the solubility of TM fragment while retaining helicity and dimerization properties in detergent micelles[19].

Tomich *et al*. studied the solubility of TM helix 2 from glycine receptor α1 (M2GlyR) sfter a series of N- and C-terminal substitutions[28]. When compared to the wild type sequence, N-terminal addition of 4 Lys resulted in a 11-fold increase in TM peptide solubility (37.8 mg/ml compared to 3.3mg/ml) while the C-terminal tetra-lysine derivative of the peptide did not precipitate until a concentration of 77.4 mg/ml (23-fold solubility increase)[28].

Neutral tags, composed of Gly, His, Pro and N-methylglycine (Sar), have been proved useful, in combination with Lys-tags, in augmenting peptide solubility while leaving the other terminus uncharged, favoring membrane insertion and lowering the overall number of positive charges[19]. In fact, an elevated hydrophobic: charged amino acid ratio may cause a peptide-dependent membrane disruption as it happens for cationic antimicrobial peptides (CAPs). CAPs, as suggested by their name, are a class of naturally occurring Lys- and Arg-rich peptides harboring the ability to bind and eventually disrupt bacterial and eukaryotic membranes[29].

Poulsen *et al*. showed the bactericidal activity of a 6 lys-tagged TM peptide (K-TM4) derived from a small multidrug resistance (SMR) protein of *Halobacterium Salinarum,* while substitution of 3 of the Lys with a neutral Ala-Sar-Sar-Sar tag abolished bacterial toxicity up to the solubility limit of the TM peptide (i.e. 25 μM)[30].

On the other hand concomitant insertion in the tags of aromatic aminoacids, Trp in particular, can contribute to TM peptide biological activity. Aromatic residues, by locating at the membrane interface, promote proper TM peptide topology, limiting peptide perpendicular movement across the bilayer. Shank *et al*. demonstrated up to 4-fold increase in activity for an arginine-containing derivative of M2GlyR TM peptide upon insertion of a tryptophan residue[31]. However, it should be noted that aromatic residues are, with the exception of Tyr, also hydrophobic and their insertion may further hamper peptide solubility.

As a general rule, biological effects of addition of hydrophilic residues are protein-specific and require dedicated and case-specific testing to avoid unwanted effects such as tag-driven oligomerization and interaction with other charged medium components[12].

STEREOCHEMISTRY

TM peptides, like other therapeutic peptides and proteins, are subjected *in vivo* to the action of peptidases[18].

Degradation takes place mainly in the blood, the liver and the kidneys, determining a lifespan of few minutes for natural peptides with a molecular weight lower than 4KDa[32]. A common approach to render peptide-based therapeutics resistant to

proteolysis is the use D-amino acids. Substitution of natural residues with their diastereoisomers results in a mirror image of the original peptide, not recognized by the L-form of the peptidases[33]. The reciprocal chiral specificity of soluble protein-peptide interactions also implies that D-peptide ligands cannot recognize the L-form of their cognate receptors.

Yet the stereospecificity of the recognition process does not apply to proteins embedded in membranes. Using Glycophorin A (GPA) as a model protein, Shai and coworkers demonstrated the *in vivo* association of all-D GPA TM peptide with all-L GPA transmembrane domain[34]. By molecular dynamics studies the putative D/L-heterodimer structure was modeled on the available L-homodimer NMR data. Surprisingly, comparison of the two complex structures indicated a significant overlap in the contact surface area of every amino acid, sign of a structural similarity between the mixed enantiomer and the WT GPA dimer.

To satisfy helix-helix interactions, the D-peptide is forced to bend and tilt in unnatural, higher-energy conformations, with the energetic penalty being compensated by formation of H-bond (fig. 3)[35].

With regard to TM peptide design, chirality-independent protein TM domain interactions allow the straightforward development of more stable, D-analogues TM peptide therapeutics.

3.4 PRODUCTION

Production of TM peptides can occur either by recombinant expression or via chemical synthesis. Production of recombinant peptides poses technical challenges during expression and purification due to their instability and cell toxicity. To overcome these limitations, TM peptides can be expressed as fusion constructs with more soluble proteins and successively undergo chemical or proteolytic cleavage[36]. However chemical synthesis remains the preferred method of peptide synthesis. Using FMOC solid state peptide synthesis guarantees a more controlled end product allowing peptide manipulation (e.g. insertion of non-natural aminoacids, direct coupling of probes)[12].

4. EXAMPLES OF TM PEPTIDE APPLICATIONS

Although the idea of using TM peptides as regulators of TM proteins function dates back to 1996[16], since then, *in vitro* and/or *in vivo* testing of only a handful of TM peptides have been reported (for an exhaustive list see Supplementary Information from Bordag *et al.*[12]). In fact hurdles regarding TM peptides design in addition to their poor pharmacokinetic properties have slowed down the research. We are now going to describe some examples of TM peptides developed in the past 15 years to modulate cell functions and some new promising applications.

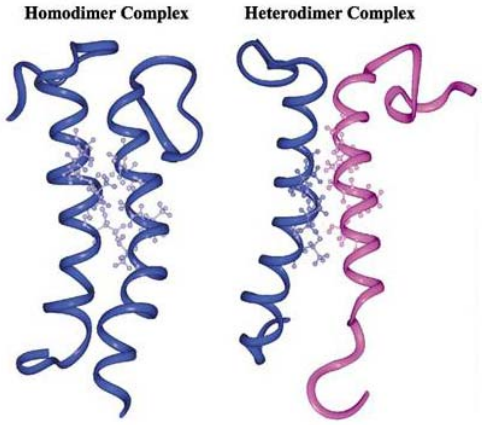

FIGURE 3: Similarity in the contact surface of GPA L/L homodimer (left) and L/D heterodimer (right). Side chains in the interacting region are shown at atomic detail [34]

4.1 DESTABILIZATION OF CD3/T-CELL ANTIGEN RECEPTOR COMPLEX

Located on the surface of T-lymphocytes, the CD3/T-cell receptor (CD3/TCR) complex interacts with the antigen-presenting Major Histocompatibility Complex, triggering T-cell activation. CD3/TCR is composed of a ligand-binding heterodimer lacking an intracellular domain (clonotypic αβ TCR) and a series of co-receptor proteins involved in the signaling (CD3 γ-, δ-, ε- and ζ-chains) (fig. 4a). Atypically, the TCR-α transmembrane region, highly conserved among mammals, includes 2 positive amino acids involved in polar intra-complex TM interactions, necessary for the correct receptor assembly[37].

Manolios *et al.* demonstrated that perturbation of the quaternary structure of the receptor by addition of a 9 aa peptide corresponding to the TCR-α core TM domain (NH_2-G-L-**R**-I-L-L-L-**K**-V-OH) inhibited T-cell activation *in vitro*[26]. Palmitic acid-conjugated core peptide (CP) at a concentration of 10μM reduced IL-2 production to almost background levels. However, while lipidation boosted CP activity favoring its interactions with cellular membranes, the derived highly-lipophilic peptide required suspension in squalene oil (2,6,10,15,19,23-hexametyl-tetracosane) for *in vivo* studies. Single injection of CP monopalmitate induced a protective effect in a rat model of adjuvant arthritis,

comparable to treatment with the immunosuppressant cyclosporine[26].
In an early clinical trial, topic application of 1mg palmytoylated core peptide/g cream on patients suffering from T-cell mediated dermatoses (psoriasis, atopic eczema, lichen planus) resulted in improvement in the conditions of almost all treated individuals, although the effect did not significantly differ from the corticosteroid-treated group (fig. 4b)[38]. Possibly, peptide activity *in vivo* can be improved by decreasing sensitivity to proteases by substituting L-amino acids with their D-enantiomers. According to this strategy, Gerber *et al.* demonstrated CP-TCR co-localization and anti-inflammatory activity was retained by a mirror peptide composed by D-amino acids, paving the way to development of immunotherapeutic D-peptides[39].

4.2 SHIFT IN ERBB1 DIMERIZATION BALANCE

Initially developed to study TM interactions between the tyrosine kinase receptors ErbB1-ErbB2, the ErbB1 TM peptide was only later considered as a potential therapeutic. Better known as, respectively, epidermal growth factor receptor (EGFR) and HER/c-neu, ErbB1 and ErbB2 play a pivotal role in cancer proliferation and differentiation and, as such, are well-known tumor targets. Upon binding with their cognate antigen ErbB receptors undergo a homo-/heterodimerization process driven by 2 GXXXG-like motifs contained in their TM domains. Gerber *et al.* demonstrated that the C-terminal GXXXG motif is involved in receptor homodimerization while the N-terminal is preferentially associated with heterodimerization[14].
To assess the potential of ErbB1 TM helix to inhibit parental receptor dimerization and signaling, the corresponding 25 amino acid peptide (NH_2-SIATGMVGALLLLLVVALGIGLFMR-OH) was synthesized and added to EGFR positive cells in detergent micelles. ErbB1 TM peptides co-localized with their "parental" receptor and at nM concentration they inhibit ErbB1 phosphorylation level to a maximum of 50% at nM concentration[41]. The limited effect can be explained by the multifactorial regulation of receptor dimerization: structural studies have in fact shown the extracellular domain also plays a role in ErbB homodimerization, complicating complex disassembly. Unfortunately, the experimental setting (incubation of cells in a detergent solution) makes it difficult to evaluate the real therapeutic potential of the ErbB1 TM peptide. Clearly a more soluble TM peptide form that allows testing in physiological conditions is needed.

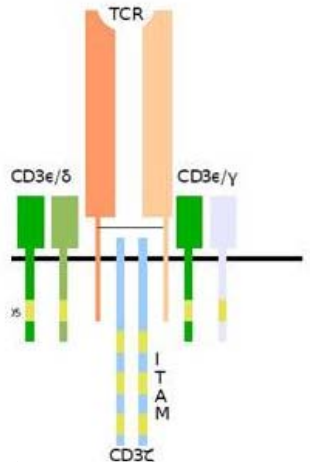

FIGURE 4: Schematic representation of TCR/CD3 complex (left) (modified from[40]). (b) Table indicating the outcome of TM core peptide treatment in patients suffering from T-cell dermatoses (P= psoriasis, AD = atopic dermatitis and L-P = lichen planus). TM core peptide was administered topically for 3 consecutive days in the form of a cream (1mg CP/g)[38].

Patient No.	*Diagnosis*	*Improvement*	*Cure*	*No effect*
1	AD	X		
2	AD		X	
3	AD		X	
4	AD		X	
5	AD	X		
6	P	X		
7	P			X
8	L-P	X		
9	L-P		X	

4.3 SPECIFIC *IN VIVO* BLOCKADE OF GPCR: THE CASE OF D2 DOPAMINE RECEPTOR

G-protein coupled receptors (GPCR) constitute the 4th largest family in the human proteome, ubiquitously involved in cell signaling and currently target of 50% of the drugs on the market [42]. In this scenario, the possibility of inactivating GPCRs by use of an exogenous peptide disrupting the natural interactions among the 7 receptor TM domains has attracted much interest.
In an extensive *in vivo* work George *et al*. tested the effect of administration of TM peptides derived from various GPC proteins and, among others, from the D2 dopamine receptor, a family subtype whose signaling abnormalities result in neuropsychiatric disorders[43].
The TM peptide derived from the helix 7 of dopamine receptor D2 (LYSAFTWLGYVNSAVNPIIY-NH_2 going under the name of D2-TM7) was injected in rat brains to evaluate binding to the D2 receptor and its structurally similar D1. Blockade of D2 dopamine receptor with D2-TM7 peptide was comparable to the effect exerted by the conventional antagonist (+)-butaclamol. Unlike this small molecule, the D2-TM7 peptide did not have any effect on the D1 receptor, proving the higher specificity of the peptide approach[43]. However, poor peptide solubility required D2-TM7 resuspension in buffer containing apolar compounds (8.3% DMSO and 0.4% digitonin) which would raise toxicity concerns in case of an human application. Therefore

an increase in TM peptide solubility before further testing is required.

4.4 REGULATION OF THE αIIbβ3 INTEGRIN "PUSH AND PULL" MECHANISM

Integrins are a family of membrane proteins mediating cell/cell and cell/extracellular matrix (ECM) adhesion. In particular, the fibrinogen receptor αIIbβ3 plays a paramount role in platelet aggregation a process implicated in hemostasis and thrombosis. Structurally, the αIIbβ3 heterodimer is held in its resting state by α-β subunits TM interactions. Activation is regulated by a "push and pull" mechanism: destabilization of the complex as a consequence of an internal (ADP) stimulus "pushes" the receptor to an active state whereas TM interactions pull it back to the resting conformation (fig. 5)[44]. Bennet and co-workers identified the pivotal role played in the TM dimerization process by a GXXXG motif on αIIb TM helix: mutation of the two glycine impaired αIIbβ3 complex formation[32]. Considering that the GXXXG motif can also drive a relative strong αIIb- αIIb TM interaction, αIIbβ3 activation can be achieved using an exogenous αIIb TM peptide that forms a αIIb- αIIb homodimer competing out the β3 TM domain. A 22-aa peptide, corresponding to αIIb-TM domain, was synthesized by Yin et al. and 2 lysine residues were appended at each terminus to enhance solubility and prevent aggregation (KKWVLVGVLGGLLLLTILVLAMWKKK) [15]. The resulting αIIb-TM peptide was soluble in aqueous buffer, acquired a α-helical conformation in phospholipid vesicles and specifically interacted with the TM domain on αIIb subunit[15]. In a size exclusion chromatography experiment , αIIb-TM peptide co-eluted with αIIbβ3 complex, confirming αIIb -TM agent binding to its receptor. Moreover, micromolar concentrations of the αIIb –TM peptide (1-3 μM) induced aggregation in gel-filtered platelets in an ADP independent mechanism[15].
However, the TM peptides targeting αIIbβ3 integrin have so far proven more useful in unraveling receptor activation mechanism than as therapeutics for bleeding disorders. This is partly due to the redundancy in αIIbβ3 regulation pathways, making it difficult to fully control its activation[44]. Conversely, by virtue of its unique mechanism of action, αIIbβ3 integrin constitutes a handy *in vitro* screening platform for newly designed TM peptides: in fact they have an agonistic activity, easier to quantify compared to an antagonistic one. This has made αIIbβ3 the system of choice for the development of computational -driven design of TM peptides[23].

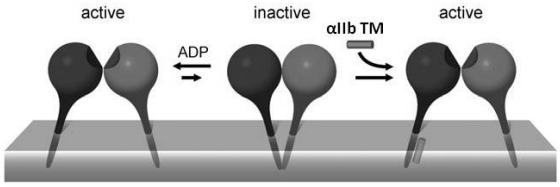

FIGURE 5: Schematic model of integrin αIIbβ3 activation mechanism. TM domains of integrins interact in the receptor inactive conformation. Integrin activation can be obtained by addition of an agonist, such as ADP or by addition of a αIIb TM peptide that destabilizes α and β chains TM interactions[23].

4.5 MULTIDRUG RESISTANCE (MDR) PROTEINS: PUTTING A HALT TO DRUG EFFLUX

P-GLYCOPROTEIN

Multidrug resistance (MDR) proteins are a class of membrane transporters responsible for the extrusion of a variety of drugs from the cell cytoplasm. Aberrantly expressed in cancer cells where they expel chemotherapic drugs, MDR proteins constitute themselves a druggable target. Given the lack of substrate specificity, development of an antagonist agent is out of the question and current strategies focus on an allosteric regulation of the receptor.
In this scenario the use of a TM peptide to destabilize the 12 TM-segment glycoprotein is extremely attractive. Tarasova *et al*. isolated all 12 TM segments from P-glycoprotein (P-gp, the most known MDR protein) and evaluated their inhibitory activity measuring the *in vitro* extrusion of the fluorescent substrate 3-ethyl-2-[3-ethyl-2(3H)-benzoxazolylidene)-1-propenyl]benzoxazolium iodide ($DiOC_2$)[45]. All the TM-derived peptides were active inhibitors of the parental protein, though with very different potency (IC_{50} ranging from 0.75 to more than 15 μM) indicating TM peptide capacity to destabilizing P-gp structural organization. The extremely poor solubility of the TM peptides was improved by addition of flanking Asp residues and/or extension to include natural-occurring extracellular loops increasing precipitation threshold up to 5 μM in culture medium.
Activity of the TM peptide could be affected by stereochemical and sequence modifications, giving indications for further improvement. For instance, addition of 2 terminal Asp residues caused a 5-fold increase in TM5-derived peptide potency (IC_{50} from 5 ± 0.5 μM to 1.1 ± 0.3 μM) while an all-D form of TM5 peptide with a N-terminal succinic moiety had an IC_{50} of 0.5±0.2 compared to 1.1±0.3 μM of natural L-form.

The activity of TM peptides was additionally tested *in vitro* on HCT15 cells: results showed a 5 fold reduction in doxorubicin IC50 in the presence of 1μM TM2 peptide after 96h incubation (fig. 6)[45].
Promises of TM peptides to antagonize P-gp-mediated drug efflux are heavily compromised by peptide aggregation in an aqueous environment, persistent even after tag addition. Overall this findings suggest that improvement in solubility is a *sine qua non* condition for further development of this approach.

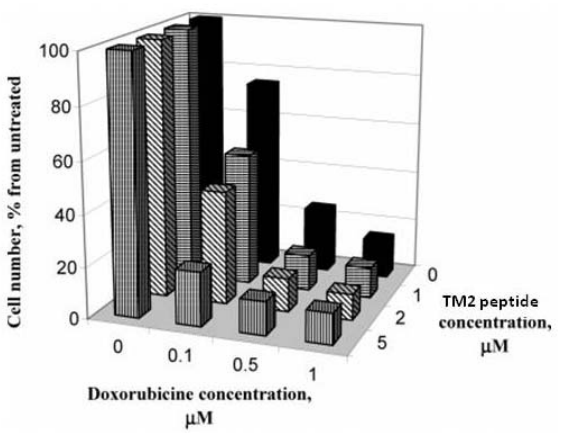

FIGURE 6: Effect of TM2 peptide over sensitization of HCT15 cells (human colonorectal carcinoma) towards doxorubicin after 96h treatment[45].

SMALL BACTERIAL MDR PROTEIN HSMR

Horizontal transfer of resistance genes and overexpression of membrane-bound multidrug efflux transporters are the two main causes of bacterial multidrug resistance[46]. Small multidrug resistance (SMR) proteins remove cationic cytotoxic compounds from bacterial cells with a mechanism involving the Glu residue in their TM region. Disruption of the active dimeric form of SMR proteins, driven by TM interaction of helices 4, is thought to affect drug efflux rate. In a recent study, down-regulation of SMR protein activity was obtained using a 21 aa peptide (VAGVVGLALIVAGVVVLNVAS) corresponding to TM4 of Hsmr SMR protein from *H.Salinarum*[30]. TM peptide solubility was raised above 6.5 mg/l by addition of a C-terminal poly-lysine chain and an acetylated Ala-$(Sar)_3$ tag at N-terminal. Extrusion rate of SMR substrate ethidium bromide by Hsmr-expressing *E.Coli* followed a first-order exponential decay, with a decay rate constant (*k)* of $10.1 \times 10^{-3} \pm 0.7 \times 10^{-3}$ s^{-1}. Bacteria treated with T4-TM peptide showed a dose-dependent reduction in the efflux rate with a saturation plateau at 1 μM ($k = 3.7 \times 10^{-3} \pm 0.2 \times 10^{-3}$ s^{-1} corresponding to a 60% efflux inhibition). Although promising, the application is still at the proof of concept level: the inhibitory effect of TM4 on cell growth is relatively poor and, according to the authors, may be due to L-peptide susceptibility to proteases[30]. However, use of a mirror peptide has not been successful: D-TM4 peptide did not bind Hsmr receptor, possibly because it failed to satisfy H-bonds requirements. In this perspective, computational studies applied to Hsmr might provide additional information for the synthesis of a functional D-TM4 peptide. On the other hand, advancements in the understanding of more complex bacterial MDR structures will provide the background knowledge for the development of a wider arsenal of "anti-bacterial" TM peptides.

4.6 A STEP TOWARD ONCOLOGY: BLOCKADE OF NEUROPILIN-1

Identified as the ligand binding subunit in the Sema3A membrane complex, neuropilin-1 (NRP1) receptor has been intensely studied for its involvement in cell migration and differentiation.
Implicated in CNS degeneration and in progression of some tumor types (e.g. glioblastoma), NRP1 constitutes an interesting pharmaceutical target. Upon activation, NRP1 has been shown to di- and oligomerize and act as a co-receptor for different membrane protein families (among the others tyrosine kinase receptors). Dimerization partially depends on the packing properties of GXXXGXXXG - containing TM helices, and is blocked by addition of a 27 aa peptide corresponding to NPR1 TM domain (identified as pTM-NRP1, sequence: ILITIIAMSAL**G**VLL**G**AVC**G**VVLYRKR)[47].
In a recent work, Nasarre *et al*. evaluated the effect of pTM-NRP1 *in vitro* and *in vivo* models of glioblastoma multiforme. Rat glioblastoma cells treated with 1 μM of pTM-NRP1 showed a lower proliferation when compared to PBS control and a significantly reduced angiogenesis in a tube formation assay. Orthopic brain tumor cells were pretreated with pTM-NRP1 for 2 hours before implantation in the subcortical region of male rats. Significant tumor shrinking (-80%) was observed after 8 days in all the treated animals (n=12). *In vivo* inhibition of pre-developed tumor was demonstrated in an heterotopic glioma model (tumor cell injected in the flank of athymic nude mice). Daily subcutaneous injection of 10^{-7} M pTM-NRP1 in PBS resulted in a consistent reduction of tumor volume, weight and newly formed blood vessels[47]. Absence of toxicity for different cell types and good stability (signal retained for 48h in the membrane) make pTM-NRP1 an interesting candidate for future development.

SYNTHETIC ANION CHANNEL-FORMING PEPTIDE FOR CFTR REPLACEMENT

An alternative application of TM peptides consists in their use as channel-forming units. In particular it has been proposed to restore normal cellular electrolyte balance in the epithelium of cystic fibrosis (CF) patients by generating synthetic anion-specific pores using channel-forming peptides (CFPs)[48].

CFPs can be *de novo* synthesized or derived from TM segments of native proteins. Tomich and coworkers identified the potential of the second TM helix (M2) of the glycine receptor (GlyR) to form a pentameric, Cl^- selective pore in epithelial monolayer[49]. However significant limitations concerning M2 peptide solubility, channel ion selectivity and lack of a gate- control mechanism had to be overcome.

In an attempt to improve peptide solubility while retaining ion transport activity, Shank *et al*. generated over 200 M2-derived sequences, redesigning the amino acid content of the TM region flanking loops[31]. Comparison of synthetic-channel activity between native M2 and derived NK_4-M2GlyR p22-T19R S22W peptide, showed a 2 fold increase in maximal ion flux, measured as maximal short circuit current (Isc_{MAX}). Moreover potency of NK_4-M2GlyR p22-T19R S22W in a planar bilayer was also improved with a $K_{1/2}$ of 71 μM compared to 210μM of the initial M2 sequence, a result possibly explained by the lower aggregation rate of the newly derived sequence[31]. Tests *in vitro* confirmed that NK_4-M2GlyR p22-T19R S22W showed an high permselectivity for Cl^-.

However, despite the advances in channel-forming peptide design, insertion of a regulatory mechanism still represents a formidable challenge that has to be tackled before *in vivo* testing.

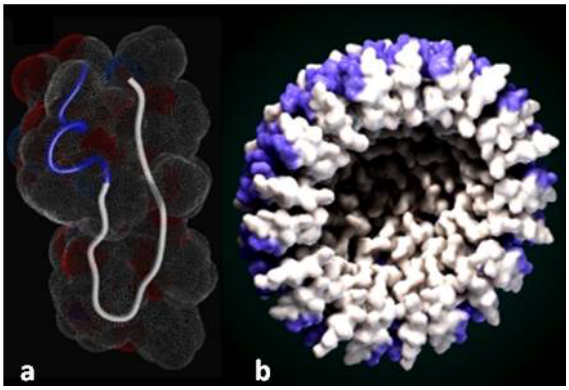

FIGURE 7. Space-filling model of hairpin conformation of CXCR4-2 in a monomeric form (a) and following self-assembly in a nanoparticle structure (b). Alpha-helical portion is highlighted in blue[50].

5. FUTURE PERSPECTIVES

Lately, new promising applications of TM peptides have been presented, solving TM peptide solubility hurdle while suggesting a new role as delivery systems.

CXCR4 NANOPARTICLES

More than 10 years ago, Tarasova *et al*. screened peptides corresponding to the 7 TM region of the chemokine receptor 4 (CXCR4) for their ability to inactivate the receptor. In disease settings CXCR4 acts as a co-receptor for HIV-1 infection and plays an essential role in lung and bone metastasis[11]. A 24 aa peptide, corresponding to the second helix of CXCR4 (CXCR4-2), able to spontaneously insert in cell membranes was selected. *In vitro* results indicated a complete block of CXCR4 downstream signaling at 0.2 μM peptide concentration and a marked reduction of HIV infection in a HIV-1 replication assay[11]. Although promising, the research line was suspended until recently, when Tarasov *et al*. showed the propensity of CXCR4-2 peptide, functionalized with a C-terminal 27 unit poly-ethylene glycol (PEG) tail, to self assemble in nanoparticles. The formed NP have a small and homogeneous size distribution (10-20 nm) and a surface negative charge (-20.8 ± 1.0 mV) and CD studies suggest a β-type conformation for CXCR4-2 peptides (fig. 7). Results obtained using both liposomes and *in vitro* testing indicate a spontaneous NP fusion with lipid bilayers accompanied by CXCR4-2 partial transition to an α-helical conformation[50].

TM peptide based NP hold promises in overcoming the poor pharmacokinetic of free peptides as demonstrated by considerable prolonged survival in a metastatic breast cancer mouse model treated with CXCR4-2-NP. Moreover, these peptide-based NP systems allow for encapsulation of hydrophobic drugs suggesting a future use as a bi-functional targeted delivery system.

PH (LOW) INSERTION PEPTIDE (PHLIP)

Derived from bacteriorhodopsin helix C , pH (Low) Insertion Peptide (pHLIP) was shown to spontaneously insert in membranes adopting a stable α-helix conformation[51]. The putative membrane inserting sequence is 22-aa long containing 2 aspartate and 2 tryptophan residues. Remarkably, pHLIP membrane insertion is driven by pH change: protonation of Asp side chains in the TM region (pK_a ~6) leads to transition from a weakly unstructured membrane-bound form to a bilayer spanning helix (fig. 8). Although derived

from a TM helix and sharing the common structural features of TM peptides, pHLIP differs in its activation mechanism, which is not based on direct modulation of a transmembrane protein. In fact, characterized by a relatively high solubility (almost 30μg/ml), absence of membrane leakage and no sign of toxicity *in vitro* (up to 10μM at pH 6.5) and *in vivo* (4mg/kg in mice), pHLIP constitutes an extremely promising candidate as a delivery system[52].

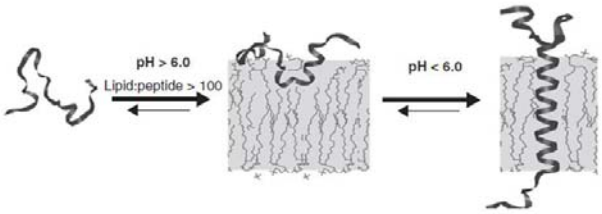

FIGURE 8: pHLIP pH dependent membrane interaction. At physiological pH unstructured pHLIP binds to the membrane in presence of a lipid: peptide ratio > 100. At low pH (<6) pHLIP forms a transmembrane α-helix[52]

The site-specific action of pHLIP derives from its ability to partition into membranes at a low pH (<6), a condition typically found in cancer but also arising from inflammation, ischemia, stroke and arthritis. Due to conserved orientation of pHLIP upon membrane insertion (N-terminus remains in the extracellular environment) is possible to decide whether the cargo molecule will be tethered to the cell surface (useful in diagnostics) or internalized (functional in cancer treatment)[52]. The energy released upon pHLIP folding and insertion is enough to translocate an uncharged cargo molecule with a logP ~2-3 and MW up to 5 kDa into the cell[53]. Significant suppression of cancer cell proliferation was obtained using 2-4 μM phalloidin-conjugated pHLIP at pH 6.2 (>90% in HeLa cells) while no toxic effect was noticeable at physiological pH[54].

pHLIP has lately proven effective *in vivo* conjugated with PET tracers for imaging, as a targeting moiety for nanoparticles delivery and tagged with fluorescent dyes for guiding tumor removal surgeries[55]. These encouraging results suggest potential applications of pHLIP in a variety of medical settings.

6. CONCLUSIONS

The efficacy of TM peptides in the modulation of the activity of transmembrane proteins has been proven using *in vitro* and *in vivo* systems. Nevertheless clinical translation still poses a number of challenges, primarily related to TM peptides poor stability and solubility, that remain challenging also after optimization.

Recently, strategies to improve poor pharmacokinetic properties of TM peptides have yielded promising result in the form of spontaneously-forming CXCR4 TM peptide nanoparticles. What is left to prove now, is the applicability of this approach to other TM peptides.

Future applications of TM peptides might be directed to rescuing the function of mutation-dependent misassembled proteins, by specifically substituting the altered helix. Besides, studies on the membrane protein interactome using a combination of computational and experimental methods can disclose a new set of druggable biologically relevant interactions. On the other hand, biophysical studies on spontaneous TM peptides partitioning in lipid bilayers can provide indications to further improve TM peptide pharmaceutical efficacy.

In our opinion, TM peptides harbor a yet undisclosed potential as therapeutics. Solving pharmacokinetic hurdles of existing TM peptides and/or presenting new more potent applications thereof, constitute the best way of unleashing that potential.

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