Charged cyclic hexapeptides: Updating molecular descriptors for permeability purposes

Charged cyclic hexapeptides: updating molecular descriptors for permeability purposes

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1. Introduction

Peptides have recently gained renewed attention for development as therapeutics since compared to small molecules they can interact along the flat surfaces of proteins, which could lead to greater specificity and potency for drug targets. Because of this property they are expected to be ligands for difficult target classes (Arkin et al., 2014). However, peptides as drugs suffer from major limitations from a pharmacokinetic (PK) point of view, with a limited ability to cross the gut wall and a short plasma half-life (Rand et al., 2012). Cyclic peptides are potentially more convincing candidates than linear analogues (Okumu et al., 1997),(Caron and Ermondi, 2017),(Nielsen et al., 2017) since cyclization minimizes degradation by removing cleavable N- and C-termini and by shielding components from metabolic enzymes. Unfortunately, cyclic peptides often show poor oral bioavailability (F) and many studies have been recently reported in the literature exemplifying strategies to design permeable derivatives (Qian et al., 2017). Although progress has been made (Rezai et al., 2006b), (White et al., 2011), (Beck et al., 2012), (Nielsen et al., 2014), (Hewitt et al., 2015), (Thansandote et al., 2015) (their review is beyond the scope of the work), a general approach to obtain oral highly bioavailable cyclic peptides has not been demonstrated. The result is that most cyclic peptides on the market and under clinical development show modest permeability and bioavailability (F) <1% (Nielsen et al., 2017). Therefore, an insight into the physicochemical properties normally considered to affect oral bioavailability would be of benefit in drug discovery optimization strategies.

Traditionally, the calculated molecular weight (MW), the predicted octanol−water partition coefficient (ClogP), the number of hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA), the number of rotatable bonds (RotB), and the topological polar surface area (TPSA) are used to characterize drug candidates. Since these descriptors were developed for characterizing small molecules (Lipinski et al., 1997)

an updated approach towards cyclic peptides and macrocycles is needed; for example, these descriptors do not take ionization into account.

The aim of this study was to use experimental and computational methods to quantify the impact of conformation and ionization on passive permeability for an *ad hoc* designed series of three cyclic hexapeptides. Conformational flexibility is a major determinant of the properties of macrocycles and other drugs in the beyond rule of 5 (bRo5) space (Rezai et al. 2006b). The relevance of ionization in passive permeability is supported by the evidence that all of the three mechanisms by which peptides can enter the cells (the inverted micelle model, direct penetration and endocytic uptake (Tréhin&Merkle, 2004)) require the peptide to have good affinity for the negatively charged membrane surface. To investigate this, we synthesized three model hydrophilic compounds with the general sequence c(Ala-*X*-Gly-Gly-Ala-Trp) expected to be differently charged at pH = 7.0: the neutral **1** c(Ala-*Asn*-Gly-Gly-Ala-Trp), the anionic **2,** c(Ala-*Asp*-Gly-Gly-Ala-Trp) and the cationic **3** c(Ala-*Lys*-Gly-Gly-Ala-Trp). To explore the uncontaminated contribution of ionization to permeability, we specifically designed cyclic peptides expected to show a modest permeability, e.g. hydrophobic amino acids were discarded, and the lipophilicity of the lateral chains was lowered as much as possible (i.e. we preferred Asp and Asn over Glu and Gln, respectively, and Lys over Arg).

For all compounds, we firstly measured permeability in a Madin-Darby Canine Kidney (MDCK) cellular system for which passive permeability is known to be the dominant permeation mechanism (Di et al. 2011). Secondly, we determined pK_a and lipophilicity in octanol/water (log P and log $D^{7.0}$). We also measured a range of chromatographic indexes using approaches appropriate to the ionization properties of solutes (Immobilized Artificial Membrane (IAM) (Taillardat-Bertschinger et al., 2003) and Hydrophilic Interaction (HILIC) (Noga et al., 2013) systems. Experimental amide NMR chemical shift temperature coefficients ($\Delta\sigma HN/\Delta T$) were used to monitor the formation of intramolecular hydrogen bonds (IMHBs) involving amidic backbone NH groups in water. Molecular dynamics (MD) simulations in water, chloroform and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) lipid bilayer were performed to assess how the three cyclic hexapeptides behave in different environments.

2. Materials and Methods

2.1. Physicochemical descriptors

2D structures of the peptides were used to calculate the molecular weight (MW), the topological polar surface area (TPSA), the number of hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA) with Marvin suite (Marvin Suite, ver. 17.21.0, https://www.chemaxon.com/).

The logarithm of the partition coefficient (log P) was calculated with different tools implementing different algorithms: Marvin ver. 17.21.0 (https://www.chemaxon.com/), MoKa v. 2.6.5 (www.moldiscovery.com), XLOGP3 (http://www.sioc-ccbg.ac.cn/skins/ccbgwebsite/software/xlogp3/) and MlogP (as implemented in http://www.swissadme.ch/index.php). Marvin assumes that the log P of a molecule is composed of the

increment of its atoms. To consider molecular complexity this algorithm takes into account electron delocalization and contributions of ionic forms and the effect of hydrogen bonds. MoKa uses an algorithm based on descriptors derived from GRID molecular interaction fields. XLOGP3 predicts the log P of a query compound by using the known log P value of a reference compound as a starting point. The difference in the log P values of the query compound and the reference compound is then estimated by an additive model. MLOGP uses the sum of lipophilic and hydrophilic atoms as two basic descriptors. These two descriptors were able to explain 73% of the variance in the experimental log P values for 1230 compounds. The use of 11 correction factors covered 91% of the variance.

2.2. MD simulations

Molecules were firstly minimized with semi-empirical PM6 Hamiltonian using MOPAC2012 (MOPAC2012, http:// http://openmopac.net/MOPAC2012.html) and then submitted to the final DFT minimization at the B3LYP/6-31G* level with ORCA software (ORCA, ver. 3.0.3, https://orcaforum.cec.mpg.de/).

All steps of MD simulations were set up using the BiKi Life Science software (default settings for plain MD simulations, ver. 1.3.5, http://www.bikitech.com/) which provides an intuitive GUI interface to GROMACS and Amber tools. In particular, molecules were parametrized with Antechamber software (ver. 14, http://ambermd.org/antechamber/ac.html) whereas MD simulations were done with GROMACS (ver.4.6.1, http://www.gromacs.org/) package using the Amber ff14 (Maier et al., 2015) force field and charges computed with the AM1-BCC method (Jakalian et al., 2000).

Water molecules were described using the TIP3P model (Jorgensen et al., 1983) as implemented in GROMACS, whereas GAFF (Wang et al., 2004) topologies and equilibrated boxes for chloroform and DMSO were taken from the Web site at http://virtualchemistry.org (Caleman et al., 2012). In all cases, the simulated system consisted of solvent molecules surrounding a single hexapeptide in a cubic box with side length of size 40 Å with periodic boundary conditions. The number of solvent molecules was fixed to reproduce the experimental density of the solvent at 298 K (996.7 g/l, 1479.1 g/l and 1095.0 g/l for water, chloroform and DMSO respectively).

For **2** and **3**, the water system was neutralized by adding an additional chloride and sodium ion respectively. The solvated systems were minimized using a steepest descent minimization (the maximum number of minimization cycles was set to 5000). Equilibration was carried out in four steps: firstly, three 100ps NVT simulations were performed to gradually increase the temperature up to the final 300 K, then one 1ns NPT step was carried out to allow the system to stably reach the pressure condition of 1 atm. Finally, one 50 ns MD production run was performed with a time step for integration equal to 0.002 ps and a number of steps equal to 25000000. Coordinates were saved every 10 ps; in total 5000 snapshots were obtained. The temperature coupling was done using a velocity rescaling with a stochastic term that ensures that a proper canonical ensemble is generated. (Bussi et al., 2007)

The setup of the simulations in a DMPC bilayer was done using the Ligand Reader & Modeler and the Membrane Builder tools available on the CHARMM-GUI web server (http://www.charmm-gui.org/). The ligand structures, in their neutral form, were firstly submitted to the Ligand Reader & Modeler (Jo et al., 2008) to obtain the topology files and then the outputs were used in the Membrane Builder tool to generate the DMPC bilayer (Jo et al. 2007). Both the top and bottom leaflets contain 40 lipid molecules, water and KCl (0.15 M) were also added to hydrate and neutralize the system (7 K^+, 7 Cl^-, and ~2300 waters), resulting in a simulation system of 50.6 × 50.6 × 69 $Å^3$. MD were carried out using GROMACS with CHARMM36 force field following the protocol suggested by the Membrane Builder tool. After 375-ps equilibration, each system was subjected to 10-ns production. All the simulations were carried out in NPT ensemble (P=1 atm and T=303.15K). Trajectories were generated with a 2-fs time step and bonds with hydrogen were constrained with the LINC algorithm.

The trajectories resulting from MD simulations in water were used to calculate the virtual log P from MLP implemented in VEGA (ver. 3.1.1.42, http://nova.colombo58.unimi.it/cms/index.php?Software_projects:VEGA_ZZ).

The intramolecular hydrogen bond network was analyzed using the Python script HBonanza (v. 1.01, http://rocce-vm0.ucsd.edu/data/sw/hosted/hbonanza/).

Processing was done on a two 8 cores Xeon E5 CPUs and 128GB of RAM and on a single 4 core Xeon EXA-W CPU equipped with a GPU Nvidia Titan XP.

2.3. Synthesis

The three cyclic hexapeptides were prepared according to standard procedures reported in the literature (Chatterjee et al., 2012). Linear peptides (Ala, X, Gly, Gly, Ala, Trp, where X is Asn, Asp, and Lys, respectively) were prepared by solid-phase peptide synthesis (SPPS) on an automated peptide synthesizer (CEM Liberty) equipped with a microwave reactor. Fmoc-Trp(Boc)-OH was linked to a 2-Chlortrityl Chloride (2-ClTrt) resin, loading 1.12meq/g. Amino acids (side chains of the amino acids used in the synthesis were protected as follows: Boc (Lys, Trp), OtBu (Asp), TrT (Asn)) were attached stepwise to the Trp moiety using the standard Fmoc chemistry to obtain linear protected derivatives. PyBOP/DIPEA in DMF was used as coupling agents and 20% piperidine in DMF was used to release the Fmoc protection groups. Cleavage from the resin was accomplished by a mixture of CH_3COOH, 2,2,2-trifluoroethanol and DCM (1:1:3). The assembled linear peptides were cyclized in solution using DMF as solvent and HATU/HOBT and sym–collidine as the base. Purification was performed on a Shimadzu HPLC instrument equipped with a SCL-10AVP controller, a double beam SPD-10AVP UV detector, a LC-8A pump, a SIL-10AP injection system and a FRC-10 fraction collector. Cyclized peptides were purified by RP chromatography (Phenomenex Synergy 4u FUSION-RP column (80A 150x10mm 4micron) with isocratic conditions using mixtures of CH_3CN 10-35%/H_2O as the eluents. After purification of the cyclic peptides, they were flash-frozen in liquid nitrogen and then lyophilized overnight. The purity and identity of the compounds were assessed by HPLC-HRMS (LTQ-Orbitrap, see Fig S1. in the

Supporting Material). Quantitative deprotection was performed re-suspending the protected cyclic peptides in a mixture of trifluoroacetic acid and triisopropylsilane producing **1**, **2** and **3**. The deprotected peptides were flash-frozen in liquid nitrogen and then lyophilized overnight.

All reagents and solvents were purchased from various commercial distributors without any further purification.

2.4. Stability

The peptide stability was determined by HPLC. The following experimental conditions were used: buffer = Hank's Balanced Salt Solution (HBSS), pH = 7.4, temperature = 37°C, concentration = 1mM (1%DMSO). Stability was monitored for 24h.

2.5. Cell culture, transport studies and permeability calculations

Preparation of MDCK cell cultures was followed as described in the literature (Di et al., 2011). MDCK cells were seeded onto each membrane of the transwell permeable support and following dosing samples were withdrawn from the receiver chamber at range of timepoints with dosing samples replaced by addition of buffer (Hubatsch et al., 2007). Assessment of cell monolayer integrity was always evaluated in parallel by assessment of Lucifer Yellow (LY) permeation.

Transport experiments were performed in either apical to basolateral (A-B) direction or the basolateral to apical (B-A) direction. Final volumes in the assay were 0.4ml apically and 1.4 ml basolaterally. These volumes have been chosen as they give the same surface levels of the solutions in the two chambers so that no effect of hydrostatic pressure difference is present. Samples were removed from the receiver compartment every 10 minutes over a 1 h time-course.

Transport experiments from AP to BL side as well as from the BL to AP side were performed in duplicate at 37°C. An HPLC instrument equipped with a UV detector was used to quantify the concentrations in the apical and basolateral compartments to calculate flux. Propranolol (highly permeable) and hydrochlorothiazide (low permeable) were used as controls.

The apparent permeability coefficient (P_{app}, unit: cm/s) was determined from the amount of compound transported per time, according to the following equation:

$$P_{app} = \left(\frac{dC}{dt}\right)\left(\frac{V_R}{AC_0}\right) \qquad \textit{Eq. 1}$$

where V_R is the volume of the receiver compartment, A is the surface area of the monolayer, C_0 is the initial donor concentration, and dC/dt is the slope of the plot of the cumulative receiver concentration with time.

Percent recovery was > 80%. Efflux ratios (ERs) were calculated from the permeabilities in the basolateral-to-apical (B-A) and apical-to-basolateral (A-B) directions as

$$ER = \frac{P_{app}\,BA}{P_{app}AB} \qquad \textit{Eq. 2}$$

Standard polycarbonate Transwell® Permeable supports from Corning Costar (12 mm Insert Diameter, 1.12cm^2 membrane growth area, 12 well plate format, 3µm pore density) were used to perform experiments.

P_{app} data were submitted to ANOVA analysis.

2.6. pK_a measurements

To measure the pK_a values we applied a potentiometric approach as described in the literature (Schoenherr et al., 2015). All measurements were performed using the SiriusT3 apparatus (Sirius Analytical Instruments Ltd., East Sussex, UK) equipped with an Ag/AgCl double junction reference pH electrode and a turbidity sensing device. The titration experiments were conducted in 0.15 M KCl solution under nitrogen atmosphere at a temperature of 25 ± 1 °C. All tests were performed using standardized 0.5 M KOH and 0.5 M HCl as titration reagents.

2.7. Lipophilicity in the octanol/water system

To measure the log P a shake flask method was used. 2.5mg of compound were dissolved in 3mL of buffer solution (**1** at pH 5.0 (ammonium formate buffer) with 0.15M KCl, **2** at pH 1.8 (HCl) with 0.15M KCl, **3** at pH 12.0 (KOH) with 0.15M KCl). 1mL of this solution was put in a separate vial to which 1mL of octanol was added for **1** (previously saturated with H_2O 0.15M) and 10 mL of octanol for **2** and **3**. The vials were vortexed for 10 minutes and the two phases were separated and analyzed by HPLC using a Supelcosil™ LC-ABZ alkylamide column (Supelco, 5 cm x 4.6 mm, 5 mm packing, 120 °A pore size) and a mobile phase 30acetonitrile:70buffer pH=7.0 for **1** and a PLRP-S column (100 Å, 5 μM, 50 × 4.6 mm from Agilent) and the same mobile phase for **2** and **3**. Measurements were performed in triplicate.

To obtain log $D^{7.0}$ from experimental log P, we used the relationship log $D^{7.0}$ = log P - 3 (Caron et al., 1999) assuming that at pH = 7.0, **2** and **3** are completely ionized and thus log $D^{7.0}$ is the lipophilicity of the ionized species. Briefly, this equation is based on the experimental observation that the difference of lipophilicity between the neutral and ionized species of a monoprotic substance in the octanol–water system (but not in other biphasic systems) is about 3.

2.8. Chromatographic indexes

The retention times (t_R) were measured on three columns: (1) IAM.PC.DD.2 column: its surface formed by covalently bonding the membrane-forming phospholipids to silica (Regis, 10cmx4.6cm 10um packing 300Å pore size); (2) ZIC-HILIC column (sulfoalkylbetaine zwitterionic phase on a silica gel support, 10 cmx4.6 mm, 5 mm packing, 200 Å pore size) from SeQuant (Umeå, Sweden) and (3) ZIC-cHILIC column (phosphorylcholine zwitterionic phase on a silica gel support, 10 cmx4.6 mm, 3 mm packing, 100 Å pore size) from SeQuant (Umeå, Sweden).

The mobile phase consisted of 20 mM ammonium acetate buffer at pH 6.9 and acetonitrile. Samples were dissolved in the mobile phase in a concentration range of 50-100 μg/mL. The flow rate was 1 mL/min. Measures were performed in triplicate. HILIC chromatographic indexes are expressed as log k' (Eq. 3)

$$log\ k' = log\ ((t_R - t_0)/t_0) \qquad Eq.\ 3$$

where k' is the retention factor, t_R is the retention time and t_0 is the dead time.

Direct measurements of log k'_{IAM} values in fully aqueous mobile phases (log K_w^{IAM}) were only possible for the compounds eluting within 30 minutes. For the solutes requiring the addition of acetonitrile in the eluent, the log K_w^{IAM} values were calculated by an extrapolation method. Log k values were determined at a minimum of three different acetonitrile percentages (φ) in the mobile phases (from 10 to 50%, v/v) and the intercept values of the linear relationships ($R^2 \geq 0.99$) between log k and φ values were assumed as log K_w^{IAM} values. Citric acid was used to measure IAM t_0, toluene was used to determine t_0 under HILIC conditions.

A HPLC Varian ProStar instrument equipped with a 410 autosampler, a PDA 335 LC Detector and Galaxie Chromatography Data System Version 1.9.302.952 was used.

2.9. NMR

The temperature dependence of amide proton chemical shifts was derived from 1D spectra recorded on a JEOL ECZR600. Spectra were measured in DMSO-d_6 (0.75mL ampules, 99.9 atom % D) between 298 K and 313 K, in 5K increments. All the spectra at 298K are reported in the Fig. S3 of the Supporting Material.

3. Results and Discussion

3.1. Synthesis

Linear peptides were prepared on a solid support using standard solid-phase peptide synthesis. To avoid intermolecular reactions, final cyclization was performed in solution as in most cases described in literature it resulted in higher yields of the final peptide (see Materials and Methods)(Okumu et al., 1997). Protected cyclic peptides were purified by semi-preparative HPLC and after a quantitative deprotection step, **1**, **2** and **3** were obtained with a yield around 45-50%. The purity of the final compounds was >90% (see Fig. S1 in the S.I.). Stability studies showed that the peptides degradation under physiological conditions was <5% in 8 hours (data not shown).

Table 1 shows five 2D descriptors traditionally used to characterize drugs and drug candidates, demonstrating that the three cyclic peptides are, as expected, beyond Ro5 (bRo5) compounds.

Table 1. Investigated peptides and molecular properties calculated with Marvin17.6.0.: molecular weight (MW), calculated log P, HBD and HBA counts (the number of hydrogen bond donor and acceptor groups), rotB (the number of rotatable bonds) and TPSA (the topological polar surface area).

Name	**Residues position**						**MW**	**Log P**	**HBD**	**HBA**	**rotB**	**TPSA [Å²]**
	1	*2*	3	4	5	6						
1	Ala	*Asn*	Gly	Gly	Ala	Trp	556.58	-4.01	9	14	4	233.48
2	Ala	*Asp*	Gly	Gly	Ala	Trp	557.56	-3.14	8	16	4	227.69
3	Ala	*Lys*	Gly	Gly	Ala	Trp	570.65	-3.30	9	13	6	216.41

3.2. Permeability

Although not completely understood, the transport mechanism of cyclic polar hexapeptides seems to occur via the paracellular and the transcellular route (Okumu et al., 1997). Therefore, we set-up a cellular system utilizing MDCK cells to measure passive permeability. A manual methodology based on replacement experiments was applied and validated with reference compounds (see Materials and Methods). Efflux ratios (P_{app} B-A/P_{app} A-B) were <2 and thus confirmed the absence of P-gp-mediated transport processes. Results are shown in Fig. 1 in which data were normalized using propranolol (a permeable drug) as a reference. Figure 1 shows that the three peptides were more permeable than hydrochlorothiazide and less than propranolol (relative P_{app} = 1). Interestingly, **3** (positively charged) is more permeable than **1** (neutral) and **2** (negatively charged) (ANOVA *p*-value 0.013, P_{app} significantly different).

Figure 1. MDCK permeability data normalized using propranolol (for which relative P_{app} = 1). Hydrochlorothiazide (a very poorly permeable drug) value is shown for comparison

Permeability was also measured in a Caco-2 (heterogeneous human epithelial colorectal adenocarcinoma) system (data not shown). Caco-2 data confirmed that **3**, the compound positively charged at pH = 7.0, is the most permeable cyclic hexapeptide.

Since **3** was more permeable than **2** and **1**, permeability data suggest that the presence of a positive charge could be favorable for increasing permeability. This finding is in line with the previous data associated with cell penetrating peptides (CPPs), a small group of short, natural and synthetic peptides that are often positively charged and able to cross cell membranes (Milletti, 2012).

3.3. Ionization

Predicted and measured pK_a values are reported in Table 2 together with the dominant species (> 50% (Charifson and Walters, 2014)) and the net charge at pH = 7.0. A good correlation between calculated and predicted pK_a was observed. Data confirmed that **1** was neutral at physiological pH, whereas **2** and **3** were negatively and positively charged, respectively.

Table 2. Predicted and experimental pK_as and electrical species dominant (>50%) at physiological pH.

Compound	calc pK_a*	exp pK_a	Species (net charge)
1	-	-	Neutral (0)
2	3.75	3.76	Anion (-1)
3	10.32	10.32	Cation (+1)

* calculated with MoKA ver. 2.6.5; Molecular Discovery, http://www.moldiscovery.com/

3.4. Lipophilicity in the octanol/water systems

The most common descriptor of lipophilicity is log P, which is the partition coefficient of the neutral form of a molecule between the aqueous and the octanol phases. Log P predictions were generated using different methods (i.e. Marvin, MoKa, XlogP3 and MlogP) and are reported in Table 3. Log P calculators implementing different theoretical approaches (see Materials and Methods) were selected to collect data. Although significant differences were observed between the predicted values, all data support that **3** is more lipophilic (= less hydrophilic) than **2** and **1**.

The unreliability of predicted data meant that log P was also determined experimentally at a pH at which only the neutral species is present (stability at acidic and basic pH was previously checked, data not shown). Data in Table 3 revealed that log P of **1** (-1.61) is lower than **3** (-0.92) and **2** (-0.65).

Table 3. Predicted and experimental log P and log $D^{7.0}$ values.

Compound	MoKa*	XLOGP3**	MLOGP**	log P_{oct} (exp)	log $D^{7.0}$
1	-3.2	-2.13	-3.05	-1.61	-1.61
2	-2.4	-1.47	-2.65	-0.65	-3.65***
3	-1.4	-0.84	-2.24	-0.92	-3.92***

*calculated with MoKa 2.6.5; Molecular Discovery (http://www.moldiscovery.com/)

**calculated with SwissAdme (http://www.swissadme.ch/)

***estimated by the equation log D = log P -3 (Caron et al., 1999)

Since **2** and **3** are charged at pH = 7.0 (see above) and log P describes the partition coefficient of neutral (uncharged) molecules, estimations of log $D^{7.0}$ were performed (the distribution constant, which is the ratio of the sum of the concentrations of all species of the compound in octanol to the sum of the concentrations of all species of the compound in buffer). **1** was neutral at all pH's and thus for this compound log $D^{7.0}$ = log P = -1.61. For **2** and **3** we determined a log $D^{7.0}$ value lower than -2 (according to the literature we could estimate -3.65 and -3.92, for **2** and **3,** respectively (Caron et al., 1999), see Materials and Methods). This means that, as expected, the three cyclic peptides at pH=7.0 were very hydrophilic, with **2** and **3** (fully ionized at pH = 7.0) significantly more hydrophilic than **1** (p-value < 0.01).

Overall, we found that the most permeable compound (**3**) was the most hydrophilic in the octanol/water system (Fig. 2A). This finding is not in line with most of the literature where it is often accepted that passive permeability increases with increasing lipophilicity (Liu et al., 2011). However, it is known that lipophilicity in octanol/water fails to take account of some important processes, such as ionic bonds, which are of particular importance when modeling the interaction of ionized compounds with biomembranes (Caron et al., 2006) which in turn are expected to be relevant in governing permeability of cyclic peptides.

Figure 2. Histograms showing physicochemical descriptors variation: A) log D_{oct}, B) log K_W^{IAM}, C) log k' 80 HILIC and D) log k' 80 c-HILIC

3.5. Chromatographic indexes

To obtain a physico-chemical characterization of cyclic peptides more relevant to permeability, we used chromatographic approaches. In particular, we measured chromatographic indexes in three systems in which retention is largely driven by ionization.

The first is IAM chromatography, a widely known system used to characterize the interaction with membranes (Taillardat-Bertschinger et al., 2003),(Tsopelas et al., 2016), for which retention of cations is generally favored over anions (Tsopelas et al., 2016),(Ermondi et al., 2018).

Besides IAM, we selected two HILIC systems characterized by zwitterionic stationary phases which differ in the spatial orientation of the positive and negative charged groups (Di Palma et al., 2011) and are thus expected to provide insights in the different ability of cyclic peptides to form ionic bonds. For clarity, IAM chromatography is a reversed-phase (RP) HPLC system, whereas HILIC is a variant of normal phase liquid chromatography (NP-LC) in which the stationary phase is more polar than the mobile phase (and thus polar analytes are more strongly retained than nonpolar ones).

Chromatographic indices are reported in Table 4 and in Fig. 2. The relationships between lipophilicity indexes and permeability data are reported in Fig. S2 (Supporting Material).

Table 4. Chromatographic indices for the three peptides (p-value < 0.01)

Compound	log K_W^{IAM}*	log k' 80 HILIC**	log k' 80 c-HILIC**
1	0.70	-0.17	-0.06
2	0.21	-0.40	0.19
3	1.29	0.94	0.87

*log K_W^{IAM} values were calculated by extrapolation of isocratic log k's against the mobile phase composition (pH buffer = 7.0) using linear regression (see Materials and Methods)

**log k' 80 HILIC and log k' 80 c-HILIC were obtained using a mobile phase containing 20% buffer (pH = 7.0 and 80% CH_3CN, see Materials and Methods)

The IAM data showed that the presence of a positive charge (**3**) produced a strong increase in the retention when compared to neutral **1,** whereas the opposite is true for **2** that carries a negative charge (Fig. 2B).

HILIC data provide a more complex picture. log k' values of **1** are similar in both HILIC systems. **2** which is negatively charged at the experimental pH, is less retained than the neutral analogue in the HILIC system, but more retained in the c-HILIC system (Fig. 2C-D). This is in line with the larger exposure of the positive charge exhibited by the c-HILIC stationary phase. **3**, present as a cation at pH =7.0, showed log k' values higher than those measured for **1** in both HILIC systems (Fig. 2C-D).

Overall **3** showed higher chromatographic indices in all approaches (Figure 2). This experimental evidence supports the observation that the cationic lysine present in **3** can easily establish intermolecular interactions with surrounding molecules.

3.6. Structural analysis

Structural information was needed to understand the ease to which **3** establishes intermolecular interactions with surrounding partners compared to **1** and **2**. To reach this aim we firstly measured the experimental amide NMR chemical shift temperature coefficients (ΔδNH/ΔT) to get insights into the IMHB network involving the backbone amides. These experiments are non-destructive and convenient measures of how much a given NH is solvent exposed. In particular, it has been proposed (Cierpicki and Otlewski, 2001) that backbone amides having very negative ΔδNH/ΔT (i.e., <−4.6 ppb/K) are water-exposed, whereas those having less-negative or slightly positive ΔδNH/ΔT (i.e., ≥−4.6 ppb/K) are involved in IMHBs. NMR data are in Fig. 3 and show a similar behavior for the three compounds, **3** being slightly different (i.e. the NH of the backbone are more involved in the formation of IMHBs).

*Figure 3. Dependence of chemical shift on temperature for the backbone amide protons: A) **1**, B) **2** and C) **3**.*

Following the recent literature (Rezai et al. 2006a), we decided to explore the conformational behavior of the compounds in different environments. Because of the limited solubility of the peptides, we could not perform NMR experiments in chloroform and therefore we moved to MD simulations using the following strategy: Firstly, we performed MD simulations in explicit water and monitored molecular motion over 50 ns after an initial equilibration period. To validate the procedure, we verified the stability of the systems by observing the modest fluctuations of the potential energy and temperature (Fig. S4 in the Supporting Material). In addition, it was checked that the virtual log P range calculated from **1** conformers included the experimental log P (Fig. S5 in the Supporting Material). Moreover, to validate the in silico procedures with experimental data, we verified that MD simulations in DMSO (the NMR solvent) provided results in line with the amide NMR chemical shift temperature coefficients. The results confirmed that MD gives the same information as NMR about the IMHB network inside the cycle (analysis of the trajectories for **1** are reported in Fig. S6 (Supporting Material)). MD simulations were then performed in chloroform and bilayer. For all the cyclopeptides, the occurrence of IMHBs in the backbone is generally slightly higher in chloroform and less in bilayer in comparison to water (data not shown).

Since experimental data suggest a critical role played by Lys2 of compounds **3** in the interaction with the environment, the involvement of the lateral chains of amino acids in position 2 in the formation in IMHBs is shown in Fig. 4.

Figure 4. IMHBs involving the lateral chains of the three amino acids in position 2. A) water, B) chloroform and C) membrane

In water, the IMHB network involves Asn (**1**) and Asp (**2**) but not Lys (**3**) which therefore is free to interact with surrounding molecules through its charged amino group. In chloroform, Lys contribute to the formation of IMHB because of the free doublet of the amino group (not protonated in the organic solvent). In a membrane environment, we did not register any evidence of the involvement of Lys2 in IMHB but on closer inspection (Fig. 5), we verified that in this environment the lateral chain is folded and not exposed as in water.

*Figure 5. The frames taken every 2.5 ps from MD trajectory of **3** in the three systems were superimposed over all heavy atoms of the cyclic portion of the molecule: A) water, B) chloroform; C) lipid bilayer. The lysine side chain shows different conformations: in cyan extended conformations; in yellow folded conformations due to IMHB formation; in green folded conformations without IMHB formation.*

Other authors studied lysine containing cyclic peptides (Biron et al., 2008)(Rand et al., 2012) to explore the impact of a positive charged residue on permeability. Previous reports show that cyclic peptides with different amino-acid composition and different degrees of methylation are difficult to compare. That said, we observed that our data are roughly in line with results provided by Biron and coworkers (Biron et al., 2008) which support the favorable impact of the presence of a Lys residue on permeability.

4. Conclusions

In this study, we synthesized three cyclic polar hexapeptides differing by one amino acid, which makes the three derivatives differently charged at physiological pH. We verified that cell permeability of the positively charged cyclic peptide is higher than that measured for the neutral and the anionic derivatives.

Physico-chemical data arising from chromatographic systems to evaluate ionization properties showed that the propensity of the cationic derivative to interact with the surrounding molecules through electrostatic interactions was mediated by the positively charged amino groups of the lysine moiety. This finding cannot be explained by standard log $D^{7.0}$ measured in the octanol/water system. Therefore, these results suggest that log K^{w}_{IAM} and the less common log k' HILIC and log k' c-HILIC descriptors deserve consideration as physico-chemical tools to rank permeability properties of polar cyclic hexapeptides.

Chromatographic data and MD simulations validated with amide NMR chemical shift temperature coefficients, highlighted how the positively charged amino group of the lysine moiety behaves differently from asparagine and aspartate.

Overall, all methods showed that the Lys derivative has a good affinity for a negatively charged membrane surface. Moreover, MD simulations in non-polar environments (chloroform and lipid bilayer) suggest that

compound **3** could also show a chameleonic effect and thus can adapt its conformation to suit the environment. These two findings potentially help explain the higher permeability of **3** when compared to the neutral and anionic derivatives.

Although the small number of compounds investigated prevents us from generalizing, taken together these data suggest that the introduction of a positive charge may be a viable strategy to improve the permeability of cyclic hexapeptides, but significant further work is necessary to confirm this. Moreover, we demonstrated how ionized compounds could be investigated using *ad hoc* molecular descriptors to characterize ionic interactions.

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