

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Charged cyclic hexapeptides: Updating molecular descriptors for permeability purposes

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1683578> since 2018-12-05T12:20:11Z

Published version:

DOI:10.1016/j.ejps.2018.06.021

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1 Charged cyclic hexapeptides: updating 2 molecular descriptors for permeability 3 purposes

4 G. Ermondi ¹, M. Vallaro ¹, M.P. Camacho Leal ¹, T. Potter ², S. Visentin ¹ and G. Caron ¹ *

5 ¹ Molecular Biotechnology and Health Sciences Dept., Università degli Studi di Torino, via Quarello 15,
6 10135 Torino, Italy.

7 ² Cyprotex Discovery Limited, No. 24 Mereside, Alderley Park, Macclesfield, Cheshire, UK

8 *E-mail: giulia.caron@unito.it, telephone: +39 011 6708337*

9

10 **1. Introduction**

11 Peptides have recently gained renewed attention for development as therapeutics since compared to small
12 molecules they can interact along the flat surfaces of proteins, which could lead to greater specificity and
13 potency for drug targets. Because of this property they are expected to be ligands for difficult target classes
14 (Arkin et al., 2014). However, peptides as drugs suffer from major limitations from a pharmacokinetic (PK)
15 point of view, with a limited ability to cross the gut wall and a short plasma half-life (Rand et al., 2012). Cyclic
16 peptides are potentially more convincing candidates than linear analogues (Okumu et al., 1997),(Caron and
17 Ermondi, 2017),(Nielsen et al., 2017) since cyclization minimizes degradation by removing cleavable N- and
18 C-termini and by shielding components from metabolic enzymes. Unfortunately, cyclic peptides often show
19 poor oral bioavailability (F) and many studies have been recently reported in the literature exemplifying
20 strategies to design permeable derivatives (Qian et al., 2017). Although progress has been made (Rezai et al.,
21 2006b), (White et al., 2011), (Beck et al., 2012), (Nielsen et al., 2014), (Hewitt et al., 2015), (Thansandote et al.,
22 2015) (their review is beyond the scope of the work), a general approach to obtain oral highly bioavailable
23 cyclic peptides has not been demonstrated. The result is that most cyclic peptides on the market and under
24 clinical development show modest permeability and bioavailability (F) <1% (Nielsen et al., 2017). Therefore,
25 an insight into the physicochemical properties normally considered to affect oral bioavailability would be of
26 benefit in drug discovery optimization strategies.

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

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

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

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

27 **2. Materials and Methods**

28 **2.1. Physicochemical descriptors**

29 2D structures of the peptides were used to calculate the molecular weight (MW), the topological polar
30 surface area (TPSA), the number of hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA) with
31 Marvin suite (Marvin Suite, ver. 17.21.0, <https://www.chemaxon.com/>).

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

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

9 **2.2. MD simulations**

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

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

19 Water molecules were described using the TIP3P model (Jorgensen et al., 1983) as implemented in
20 GROMACS, whereas GAFF (Wang et al., 2004) topologies and equilibrated boxes for chloroform and DMSO
21 were taken from the Web site at <http://virtualchemistry.org> (Caleman et al., 2012). In all cases, the simulated
22 system consisted of solvent molecules surrounding a single hexapeptide in a cubic box with side length of
23 size 40 Å with periodic boundary conditions. The number of solvent molecules was fixed to reproduce the
24 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
25 DMSO respectively).

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

1 The setup of the simulations in a DMPC bilayer was done using the Ligand Reader & Modeler and the
2 Membrane Builder tools available on the CHARMM-GUI web server (<http://www.charmm-gui.org/>). The
3 ligand structures, in their neutral form, were firstly submitted to the Ligand Reader & Modeler (Jo et al.,
4 2008) to obtain the topology files and then the outputs were used in the Membrane Builder tool to generate
5 the DMPC bilayer (Jo et al. 2007). Both the top and bottom leaflets contain 40 lipid molecules, water and KCl
6 (0.15 M) were also added to hydrate and neutralize the system (7 K⁺, 7 Cl⁻, and ~2300 waters), resulting in a
7 simulation system of 50.6 × 50.6 × 69 Å³. MD were carried out using GROMACS with CHARMM36 force field
8 following the protocol suggested by the Membrane Builder tool. After 375-ps equilibration, each system was
9 subjected to 10-ns production. All the simulations were carried out in NPT ensemble (P=1 atm and
10 T=303.15K). Trajectories were generated with a 2-fs time step and bonds with hydrogen were constrained
11 with the LINCS algorithm.

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

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

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

19 **2.3. Synthesis**

20 The three cyclic hexapeptides were prepared according to standard procedures reported in the literature
21 (Chatterjee et al., 2012). Linear peptides (Ala, X, Gly, Gly, Ala, Trp, where X is Asn, Asp, and Lys, respectively)
22 were prepared by solid-phase peptide synthesis (SPPS) on an automated peptide synthesizer (CEM Liberty)
23 equipped with a microwave reactor. Fmoc-Trp(Boc)-OH was linked to a 2-Chlorotriyl Chloride (2-ClTrt) resin,
24 loading 1.12meq/g. Amino acids (side chains of the amino acids used in the synthesis were protected as
25 follows: Boc (Lys, Trp), OtBu (Asp), TrT (Asn)) were attached stepwise to the Trp moiety using the standard
26 Fmoc chemistry to obtain linear protected derivatives. PyBOP/DIPEA in DMF was used as coupling agents and
27 20% piperidine in DMF was used to release the Fmoc protection groups. Cleavage from the resin was
28 accomplished by a mixture of CH₃COOH, 2,2,2-trifluoroethanol and DCM (1:1:3). The assembled linear
29 peptides were cyclized in solution using DMF as solvent and HATU/HOBT and sym-collidine as the base.
30 Purification was performed on a Shimadzu HPLC instrument equipped with a SCL-10AVP controller, a double
31 beam SPD-10AVP UV detector, a LC-8A pump, a SIL-10AP injection system and a FRC-10 fraction collector.
32 Cyclized peptides were purified by RP chromatography (Phenomenex Synergy 4u FUSION-RP column (80A
33 150x10mm 4micron) with isocratic conditions using mixtures of CH₃CN 10-35%/H₂O as the eluents. After
34 purification of the cyclic peptides, they were flash-frozen in liquid nitrogen and then lyophilized overnight.
35 The purity and identity of the compounds were assessed by HPLC-HRMS (LTQ-Orbitrap, see Fig S1. in the

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

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

6 **2.4. Stability**

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

10 **2.5. Cell culture, transport studies and permeability calculations**

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

16 Transport experiments were performed in either apical to basolateral (A-B) direction or the basolateral to
17 apical (B-A) direction. Final volumes in the assay were 0.4ml apically and 1.4 ml basolaterally. These volumes
18 have been chosen as they give the same surface levels of the solutions in the two chambers so that no effect
19 of hydrostatic pressure difference is present. Samples were removed from the receiver compartment every
20 10 minutes over a 1 h time-course.

21 Transport experiments from AP to BL side as well as from the BL to AP side were performed in duplicate at
22 37°C. An HPLC instrument equipped with a UV detector was used to quantify the concentrations in the apical
23 and basolateral compartments to calculate flux. Propranolol (highly permeable) and hydrochlorothiazide
24 (low permeable) were used as controls.

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

$$27 \quad P_{app} = \left(\frac{dC}{dt} \right) \left(\frac{V_R}{AC_0} \right) \quad Eq. 1$$

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

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

$$32 \quad ER = \frac{P_{app}^{BA}}{P_{app}^{AB}} \quad Eq. 2$$

33 Standard polycarbonate Transwell® Permeable supports from Corning Costar (12 mm Insert Diameter,
34 1.12cm² membrane growth area, 12 well plate format, 3µm pore density) were used to perform experiments.

1 P_{app} data were submitted to ANOVA analysis.

2 **2.6. pK_a measurements**

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

9 **2.7. Lipophilicity in the octanol/water system**

10 To measure the log P a shake flask method was used. 2.5mg of compound were dissolved in 3mL of buffer
11 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 12.0 (KOH) with 0.15M KCl). 1mL of this solution was put in a separate vial to which 1mL of octanol was added
13 for **1** (previously saturated with H₂O 0.15M) and 10 mL of octanol for **2** and **3**. The vials were vortexed for 10
14 minutes and the two phases were separated and analyzed by HPLC using a Supelcosil™ LC-ABZ alkylamide
15 column (Supelco, 5 cm x 4.6 mm, 5 mm packing, 120 °Å pore size) and a mobile phase 30acetonitrile:70buffer
16 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**
17 and **3**. Measurements were performed in triplicate.

18 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)
19 assuming that at pH = 7.0, **2** and **3** are completely ionized and thus log D^{7.0} is the lipophilicity of the ionized
20 species. Briefly, this equation is based on the experimental observation that the difference of lipophilicity
21 between the neutral and ionized species of a monoprotic substance in the octanol–water system (but not in
22 other biphasic systems) is about 3.

23 **2.8. Chromatographic indexes**

24 The retention times (t_R) were measured on three columns: (1) IAM.PC.DD.2 column: its surface formed by
25 covalently bonding the membrane-forming phospholipids to silica (Regis, 10cmx4.6cm 10um packing 300Å
26 pore size); (2) ZIC-HILIC column (sulfoalkylbetaine zwitterionic phase on a silica gel support, 10 cmx4.6 mm,
27 5 mm packing, 200 Å pore size) from SeQuant (Umeå, Sweden) and (3) ZIC-cHILIC column (phosphorylcholine
28 zwitterionic phase on a silica gel support, 10 cmx4.6 mm, 3 mm packing, 100 Å pore size) from SeQuant
29 (Umeå, Sweden).

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

$$33 \log k' = \log ((t_R - t_0)/t_0) \quad Eq. 3$$

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

1 Direct measurements of $\log k'_{IAM}$ values in fully aqueous mobile phases ($\log K_w^{IAM}$) were only possible for the
 2 compounds eluting within 30 minutes. For the solutes requiring the addition of acetonitrile in the eluent, the
 3 $\log K_w^{IAM}$ values were calculated by an extrapolation method. Log k values were determined at a minimum of
 4 three different acetonitrile percentages (φ) in the mobile phases (from 10 to 50%, v/v) and the intercept
 5 values of the linear relationships ($R^2 \geq 0.99$) between $\log k$ and φ values were assumed as $\log K_w^{IAM}$ values.
 6 Citric acid was used to measure IAM t_0 , toluene was used to determine t_0 under HILIC conditions.
 7 A HPLC Varian ProStar instrument equipped with a 410 autosampler, a PDA 335 LC Detector and Galaxie
 8 Chromatography Data System Version 1.9.302.952 was used.

9 **2.9. NMR**

10 The temperature dependence of amide proton chemical shifts was derived from 1D spectra recorded on a
 11 JEOL ECZR600. Spectra were measured in DMSO- d_6 (0.75mL ampules, 99.9 atom % D) between 298 K and
 12 313 K, in 5K increments. All the spectra at 298K are reported in the Fig. S3 of the Supporting Material.

14 **3. Results and Discussion**

15 **3.1. Synthesis**

16 Linear peptides were prepared on a solid support using standard solid-phase peptide synthesis. To avoid
 17 intermolecular reactions, final cyclization was performed in solution as in most cases described in literature
 18 it resulted in higher yields of the final peptide (see Materials and Methods)(Okumu et al., 1997). Protected
 19 cyclic peptides were purified by semi-preparative HPLC and after a quantitative deprotection step, **1**, **2** and
 20 **3** were obtained with a yield around 45-50%. The purity of the final compounds was >90% (see Fig. S1 in the
 21 S.I.). Stability studies showed that the peptides degradation under physiological conditions was <5% in 8
 22 hours (data not shown).

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

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

Name	Residues position						MW	Log P	HBD	HBA	rotB	TPSA [\AA^2]
	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

29

1 3.2. Permeability

2 Although not completely understood, the transport mechanism of cyclic polar hexapeptides seems to occur
3 via the paracellular and the transcellular route (Okumu et al., 1997). Therefore, we set-up a cellular system
4 utilizing MDCK cells to measure passive permeability. A manual methodology based on replacement
5 experiments was applied and validated with reference compounds (see Materials and Methods). Efflux ratios
6 ($P_{app} B-A/P_{app} A-B$) were <2 and thus confirmed the absence of P-gp-mediated transport processes. Results
7 are shown in Fig. 1 in which data were normalized using propranolol (a permeable drug) as a reference. Figure
8 1 shows that the three peptides were more permeable than hydrochlorothiazide and less than propranolol
9 (relative $P_{app} = 1$). Interestingly, **3** (positively charged) is more permeable than **1** (neutral) and **2** (negatively
10 charged) (ANOVA p -value 0.013, P_{app} significantly different).

11

12 *Figure 1. MDCK permeability data normalized using propranolol (for which relative $P_{app} = 1$).*

13 *Hydrochlorothiazide (a very poorly permeable drug) value is shown for comparison*

14

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

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

22 3.3. Ionization

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

27

28 *Table 2. Predicted and experimental pK_a s 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)

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

30

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).

11

12 *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***

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

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

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

16

17 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).

25 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.

31

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

4 **3.5. Chromatographic indexes**

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

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

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

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

19
20 *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

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

23 **log k'_{80} HILIC and log k'_{80} c-HILIC were obtained using a mobile phase containing 20% buffer (pH = 7.0
24 and 80% CH₃CN, see Materials and Methods)

25
26 The IAM data showed that the presence of a positive charge (**3**) produced a strong increase in the retention
27 when compared to neutral **1**, whereas the opposite is true for **2** that carries a negative charge (Fig. 2B).

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

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

4 **3.6. Structural analysis**

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

14

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

16

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

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

34

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

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

9
10 *Figure 5. The frames taken every 2.5 ps from MD trajectory of 3 in the three systems were superimposed*
11 *over all heavy atoms of the cyclic portion of the molecule: A) water, B) chloroform; C) lipid bilayer. The lysine*
12 *side chain shows different conformations: in cyan extended conformations; in yellow folded conformations*
13 *due to IMHB formation; in green folded conformations without IMHB formation.*

14
15 Other authors studied lysine containing cyclic peptides (Biron et al., 2008)(Rand et al., 2012) to explore the
16 impact of a positive charged residue on permeability. Previous reports show that cyclic peptides with
17 different amino-acid composition and different degrees of methylation are difficult to compare. That said,
18 we observed that our data are roughly in line with results provided by Biron and coworkers (Biron et al.,
19 2008) which support the favorable impact of the presence of a Lys residue on permeability.

20 21 **4. Conclusions**

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

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

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

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

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

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

9

10 **Acknowledgement**

11 The authors thanks Compagnia di San Paolo for financial support (LIMPET project) and NVidia which
12 provided the GPU Nvidia Titan XP.

1 **References**

- 2 Arkin, M.R., Tang, Y., Wells, J.A., 2014. Small-molecule inhibitors of protein-protein interactions:
3 Progressing toward the reality. *Chem. Biol.* 21, 1102–1114.
- 4 Beck, J.G., Chatterjee, J., Laufer, B., Kiran, M.U., Frank, A.O., Neubauer, S., Ovadia, O., Greenberg, S., Gilon,
5 C., Ho, A., Kessler, H., 2012. Intestinal Permeability of Cyclic Peptides: Common Key Backbone Motifs
6 Identified. *J. Am. Chem. Soc.* 134, 12125-12133.
- 7 Biron, E., Chatterjee, J., Ovadia, O., Langenegger, D., Brueggen, J., Hoyer, D., Schmid, H. a, Jelinek, R., Gilon,
8 C., Hoffman, A., Kessler, H., 2008. Improving oral bioavailability of peptides by multiple N-methylation:
9 somatostatin analogues. *Angew. Chem. Int. Ed. Engl.* 47, 2595–9.
- 10 Bussi, G., Donadio, D., Parrinello, M., 2007. Canonical sampling through velocity rescaling. *J. Chem. Phys.*
11 126.
- 12 Caleman, C., van Maaren, P.J., Hong, M. et al., 2012, Force Field Benchmark of Organic Liquids: Density,
13 Enthalpy of Vaporization, Heat Capacities, Surface Tension, Isothermal Compressibility, Volumetric
14 Expansion Coefficient, and Dielectric Constant, *J. Chem. Theor. Comput.* 8, 61-74 (2012); David van
15 der Spoel and Paul J. van Maaren and Carl Caleman GROMACS Molecule & Liquid Database,
16 *Bioinformatics* 28, 752-753.
- 17 Caron, G., Ermondi, G., 2017. Updating molecular properties during early drug discovery. *Drug Discov.*
18 *Today* 22, 835–840.
- 19 Caron, G., Ermondi, G., Scherrer, R.A., 2006. Lipophilicity, polarity, and hydrophobicity, Vol. 5 in
20 *Comprehensive Medicinal Chemistry II* ed. Taylor, J.B., Triggles, D. J. Eds. Elsevier, Oxford,425-452.
- 21 Caron, G., Reymond, F., Carrupt, P.-A., Girault, H.H., Testa, B., 1999. Combined molecular lipophilicity
22 descriptors and their role in understanding intramolecular effects. *Pharm. Sci. Technol. Today* 2, 327-
23 335.
- 24 Charifson, P.S., Walters, W.P., 2014. Acidic and Basic Drugs in Medicinal Chemistry: A Perspective 57, 9701-
25 9717.
- 26 Chatterjee, J., Laufer, B., Kessler, H., 2012. Synthesis of N-methylated cyclic peptides. *Nat. Protoc.* 7, 432–
27 44.
- 28 Cierpicki, T., Otlewski, J., 2001. Amide proton temperature coefficients as hydrogen bond indicators in
29 proteins. *J. Biomol. NMR* 21, 249–261.
- 30 Di, L.I., Whitney-pickett, C., Umland, J.P., Zhang, H.U.I., Zhang, X.U.N., Gebhard, D.F., Lai, Y., Iii, J.J.F.,
31 Davidson, R.E., Smith, R., Reyner, E.L., Lee, C., Feng, B.O., Rotter, C., Varma, M. V, Kempshall, S.,
32 Fenner, K., El-kattan, A.F., Liston, T.E., Troutman, M.D., 2011. Development of a New Permeability
33 Assay Using Low-Efflux 100, 4974–4985.
- 34 Di Palma, S., Boersema, P.J., Heck, A.J.R., Mohammed, S., 2011. Zwitterionic hydrophilic interaction liquid
35 chromatography (ZIC-HILIC and ZIC-CHILIC) provide high resolution separation and increase sensitivity

1 in proteome analysis. *Anal. Chem.* 83, 3440–3447.

2 Ermondi, G., Vallaro, M., Caron, G., 2018. Learning how to use IAM chromatography for predicting
3 permeability. *Eur. J. Pharm. Sci.* 114, 385-390.

4 Hewitt, W.M., Leung, S.S.F., Pye, C.R., Ponkey, A.R., Bednarek, M., Jacobson, M.P., Lokey, R.S., 2015. Cell-
5 permeable cyclic peptides from synthetic libraries inspired by natural products. *J. Am. Chem. Soc.* 137,
6 715–721.

7 Hubatsch, I., Ragnarsson, E.G.E., Artursson, P., 2007. Determination of drug permeability and prediction of
8 drug absorption in Caco-2 monolayers. *Nat. Protoc.* 2, 2111–9.

9 Jakalian, A., Bush, B.L., Jack, D.B., Bayly, C.I., 2000. Fast, efficient generation of high-quality atomic charges.
10 AM1-BCC model: I. Method. *J. Comput. Chem.* 21, 132–146.

11 Jo, S., Kim, T., Iyer, V.G., Im, W., 2008. CHARMM-GUI: A Web-based Graphical User Interface for CHARMM.
12 *J. Comput. Chem.* 29, 1859-1865

13 Jo, S., Kim, T., Im, W., 2007, Automated Builder and Database of Protein/Membrane Complexes for
14 Molecular Dynamics Simulations. *PLoS ONE* 2(9):e880.

15 Jorgensen, W.L., Chandrasekhar, J., Madura, J.D., Impey, R.W., Klein, M.L., 1983. Comparison of simple
16 potential functions for simulating liquid water. *J. Chem. Phys.* 79, 926–935.

17 Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 1997. Experimental and computational approaches
18 to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv.*
19 *Rev* 23, 3–26.

20 Liu, X., Testa, B., Fahr, A., 2011. Lipophilicity and its relationship with passive drug permeation. *Pharm. Res.*
21 28, 962–77.

22 Maier, J.A., Martinez, C., Kasavajhala, K., Wickstrom, L., Hauser, K.E., Simmerling, C., 2015. ff14SB:
23 Improving the Accuracy of Protein Side Chain and Backbone Parameters from ff99SB. *J. Chem. Theory*
24 *Comput.* 11, 3696–3713.

25 Milletti, F., 2012. Cell-penetrating peptides: classes, origin, and current landscape. *Drug Discov. Today.* 17,
26 850-860.

27 Nielsen, D.S., Hoang, H.N., Lohman, R.-J., Hill, T. a, Lucke, A.J., Craik, D.J., Edmonds, D.J., Griffith, D. a,
28 Rotter, C.J., Ruggeri, R.B., Price, D. a, Liras, S., Fairlie, D.P., 2014. Improving on nature: making a cyclic
29 heptapeptide orally bioavailable. *Angew. Chem. Int. Ed. Engl.* 53, 12059–63.

30 Nielsen, D.S., Shepherd, N.E., Xu, W., Lucke, A.J., Stoermer, M.J., Fairlie, D.P., 2017. Orally Absorbed Cyclic
31 Peptides. *Chem. Rev.* 117, 8094–8128.

32 Noga, S., Bocian, S., Buszewski, B., 2013. Hydrophilic interaction liquid chromatography columns
33 classification by effect of solvation and chemometric methods. *J. Chromatogr. A* 1278, 89–97.

34 Okumu, F.W., Pauletti, G.M., D.G., V.V., T.J., S., Borchardt, R.T., 1997. Effect of restricted conformational
35 flexibility on the permeation of model hexapeptides across Caco-2 cell monolayers. *Pharm. Res* 14,

1 169–175.

2 Qian, Z., Dougherty, P.G., Pei, D., 2017. ScienceDirect Targeting intracellular protein – protein interactions
3 with cell-permeable cyclic peptides. *Curr. Opin. Chem. Biol.* 38, 80–86.

4 Rand, A.C., Leung, S.S.F., Eng, H., Rotter, C.J., Sharma, R., Kalgutkar, A.S., Zhang, Y., Varma, M. V, Farley,
5 K.A., Khunte, B., Limberakis, C., Price, D.A., Liras, S., Mathiowetz, A.M., Jacobson, M.P., Lokey, R.S.,
6 2012. Optimizing PK properties of cyclic peptides: the effect of side chain substitutions on
7 permeability and clearance. *Medchemcomm* 3, 1282–1289.

8 Rezai, T., Bock J.E., Zhou M.V., Kalyanaraman C., Lokey R.S., Jacobson M.P., 2006. Conformational flexibility,
9 internal hydrogen bonding, and passive membrane permeability: successful in silico prediction of the
10 relative permeabilities of cyclic peptides. *J. Am. Chem. Soc.* 128, 14073-80

11 Rezai, T., Yu, B., Millhauser, G.L., Jacobson, M.P., Lokey, R.S., 2006. Testing the conformational hypothesis
12 of passive membrane permeability using synthetic cyclic peptide diastereomers. *J. Am. Chem. Soc.*
13 128, 2510–1.

14 Schoenherr, D., Wollatz, U., Haznar-Garbacz, D., Hanke, U., Box, K.J., Taylor, R., Ruiz, R., Beato, S., Becker,
15 D., Weitschies, W., 2015. Characterisation of selected active agents regarding pKa values, solubility
16 concentrations and pH profiles by SiriusT3. *Eur. J. Pharm. Biopharm.* 92, 155–170.

17 Taillardat-Bertschinger, A., Carrupt, P.-A., Barbato, F., Testa, B., 2003. Immobilized artificial membrane
18 HPLC in drug research. *J. Med. Chem.* 46, 655–65.

19 Thansandote, P., Harris, R.M., Dexter, H.L., Simpson, G.L., Pal, S., Upton, R.J., Valko, K., 2015. Improving the
20 passive permeability of macrocyclic peptides: Balancing permeability with other physicochemical
21 properties. *Bioorg. Med. Chem.* 23, 322–327.

22 Tréhin, R., Merkle, H.P., 2004. Chances and pitfalls of cell penetrating peptides for cellular drug delivery.
23 *Eur J Pharm Biopharm.* 58, 209-23.

24 Tsopelas, F., Vallianatou, T., Tsantili-Kakoulidou, A., 2016. Advances in immobilized artificial membrane
25 (IAM) chromatography for novel drug discovery. *Expert Opin. Drug Discov.* 441, 1–16.

26 Wang, J., Wolf, R. M., Caldwell, J. W., Kollman, P. A., Case, D. A., 2004, Development and testing of a
27 general AMBER force field, *J. Comp. Chem.* 25, 1157-1174.

28 White, T.R., Renzelman, C.M., Rand, A.C., Rezai, T., McEwen, C.M., Gelev, V.M., Turner, R. a, Linington, R.G.,
29 Leung, S.S.F., Kalgutkar, A.S., Bauman, J.N., Zhang, Y., Liras, S., Price, D. a, Mathiowetz, A.M., Jacobson,
30 M.P., Lokey, R.S., 2011. On-resin N-methylation of cyclic peptides for discovery of orally bioavailable
31 scaffolds. *Nat. Chem. Biol.* 7, 810–7.

32