https://doi.org/10.17952/37EPS.2024.P1230

Lipophilic Prodrug Charge Masking- insights into new method of peptide prodrugs synthesis conception

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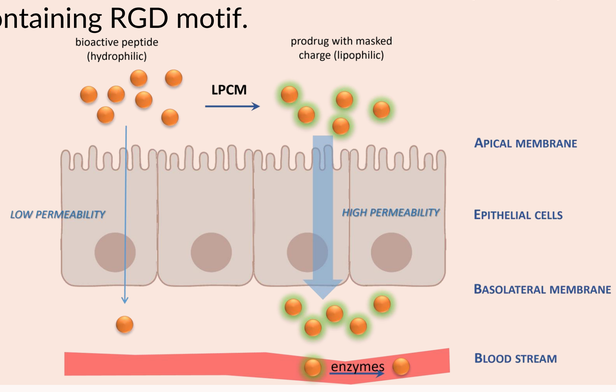
Background

Lipophilic Prodrug Charge Masking (**LPCM**) strategy was first described in 2018 by Schumacher et al.[1] as an answer to a growing need of peptide drugs oral delivery. In short, LPCM assumes transitional masking of hydrophilic peptides' charge (especially those having positively charged residues such as Arg, Lys, and free amino terminus, as well as His or Trp in their sequence) by lipophilic moieties (alkoxycarbonyl) connected by a cleavable bond. The resultant prodrug displays improved intestinal permeability in compare to mother peptide. Once it gets into the systemic blood circulation the lipophilic moiety is cleaved by the serum esterases (mainly CES), and a free active peptide is released. Initial studies proved better permeability and, thus, enhanced oral bioavailability of cyclic model prodrug hexapeptides containing RGD motif.

This outcome encouraged us to look closer to LPCM as a method to transform peptides into bioavailable drugs. We intended to verify its application to commonly known and applied peptide drugs with proved biological activity, i.e. vasopressin and desmopressin



Objectives

We synthesized series of vasopressin (AVP) and desmopressin (DVP) prodrugs with masked charged in the *N*-terminus (in case of AVP) or on Arg/D-Arg side chain. Masking groups were various alkoxycarbonyl moieties differing in the alkyl chain length, bearing from 1 to 12 C atoms. The challenging modification of guanidinium group was described in our paper [2].

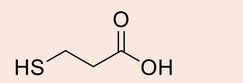
Obtained analogues permeability was examined using PAMPA test. We also incubated one of the DVP prudrugs (DVP(Hoc)) in rat serum to confirm its tranformation to unmodified peptide (DVP).

AVP

Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2

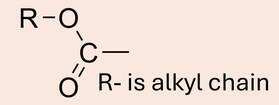
DVP

Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-D-Arg-Gly-NH_2



Mpa, desaminocysteine

Masking group



Following alkoxycarbonyl groups were introduced:

Et-etyloxycarbonyl,
Prop-propyloxycarbonyl,
MeOEt-2-methoxyethyloxycarbonyl,
But-butyloxycarbonyl,
Hoc-hexyloxycarbonyl,
Oct-octyloxycarbonyl,
Dec- decyloxycarbonyl,
Dod-dodecyloxycarbonyl

Results

PAMPA tests

compound	permeability P_e (10^{-6} cm/s)	Log Pe	Mass retention
caffeine	22	-5.01	26%
atenolol	1.24	-6.22	12%
AVP	5.15	-5.86	4%
Et-AVP	3.62	-5.67	58%
Prop-AVP	2.48	-5.84	41%
But-AVP	5.36	-5.63	57%
Hoc-AVP	4.26	-5.32	40%
Oct-AVP	0*	-	58%*
Dec-AVP	14.76	-4.88	80%
Dod-AVP	53.10	-4.32	81%
$AVP(Prop)_2$	1.27*	-5.99	37%*
$AVP(But)_2$	0.41*	-6.39	53%

*results unreliable; compound in the acceptor at the limit of qauntification, although its concentration decreased in the donnor side;

compound	permeability P_e (10^{-6} cm/s)	Log Pe	Mass retention
caffeine	33.80	-4.48	18.69%
atenolol	2.80	-5.66	0.10%
DVP	1.24	-5.95	2.73%
DVP(Et)	4.21	-3.48	9.77%
DVP(Prop)	4.77	-5.32	10.37%
DVP(But)	7.78	-5.11	9.32%
DVP(Hoc)	4.21	-4.97	23%
$DVP(Hoc)_2$	116*	-3.88	82.92%
DVP(Oct)	53.5*	-4.28	61.15%

caffeine- high permeability marker;
atenolol– low permeability marker;

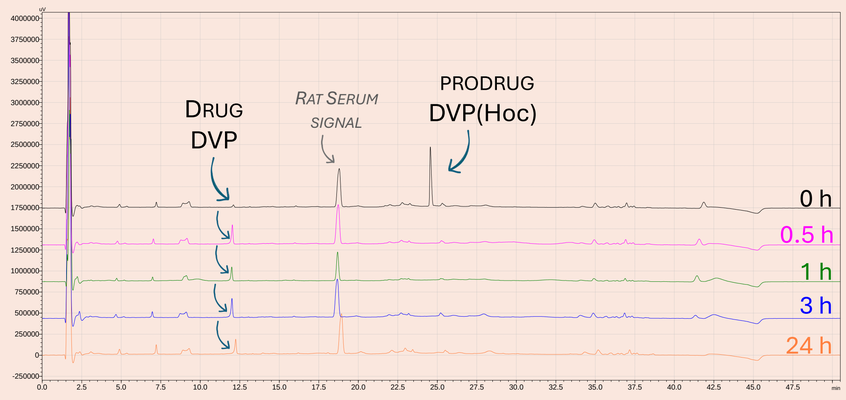
High permeability > 4.0 × 10^{-6} cm/s; (in Caco-2 convention, according to Corning®)

DVP(Hoc) prodrug to drug DVP conversion in rat serum

Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-D-**Arg**-Gly-NH_2 —Rat Serum→ Mpa-Tyr-Phe-Gln-Asn-Cys-Pro-D-**Arg**-Gly-NH_2

prodrug (Hoc) → **active drug**

- Rapid, within 30min, conversion of DVP prodrug into mother peptide.
- DVP stable for at least 24 h



Methods

Synthesis

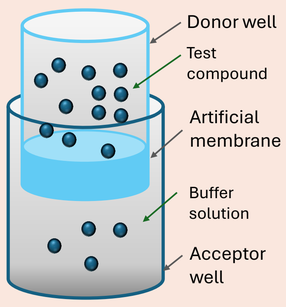
Peptides were synthesized using Fmoc chemistry on Prelude Peptide Synthesizer (Protein Technology, Inc., USA). Each peptide was synthesized on Rink Amide resin (loading 0.646 mmol/g, GL Biochem Shanghai). The protected derivatives of all Fmoc-amino acids were purchased from different commercial sources. The peptide chain was elongated in the consecutive cycles of deprotection and coupling. Deprotection was performed with 20% piperidine in DMF, and peptide chains elongation was performed using 3-fold molar excess of each Fmoc-protected amino acid, TBTU/HOBt/NMM (molar ratio 1:1:1:2). After completing the synthesis, peptides were cleaved from the resin and the protecting groups were removed in a one-step procedure using a mixture of TFA:phenol:triisopropylsilane:H_2O (88:5:2:5, v/v/v/v). Subsequently, the disulfide bridge was formed using a 0.1 M methanolic iodine solution.
Masking groups were introduced using corresponding alkyl chloroformates. In case of masking ***N*-terminal amine group** protection was intorduced on resin, using alkyl chloroformate and DIPEA (molar ratio 1:2) in DMF, reaction time 1-20h. Coupling was repeated until negativ chloranil test. **D-Arg side chain** was masked in solution. In short, eptide with formed disulphide bridge was dissolved in ACN/H_2O, 2.4 eq of DIPEA was added and cooled down to 0°C, later 1.2 eq of alkyl chloroformate in ACN was added dropwise. Reaction was monitored on HPLC and completed within 1h. Introduction of **two masking groups on Arg/D-Arg** side chain was more challanging and required preparation of Arg buidlduing blocks, as described in [2].
Obtained crude compunds were purified by reverse-phase high performance liquid chromatography (RP-HPLC) on PLC 2050 Gilson HPLC with Gilson Glider Prep. Software (Gilson, France), equipped with Grace Vydac C18 (218TP) HPLC column (22 × 250 mm, 10 µm, 300 Å, Resolution Systems). The solvent systems were 0.1% TFA (A) and 80% acetonitrile in A (B). The purity of each peptide was checked by RP-HPLC on a Shimadzu Prominence-I LC-2050C 3D equipped with a Kinetex XB-C18 column (150 × 4.6 mm, 5 µm) and a UV-Vis detector. A linear gradient from 10% to 90% B for 20 min, flow rate 1 mL min^{-1}, and detection at 214 nm was used. The mass spectrometry analysis of the synthesized compounds was carried out on MALDI MS (Autoflex maX MALDI-TOF spectrometer, Bruker Daltonics, Germany) using an α-cyano-4-hydroxycinnamic acid and/or 2,5-dihydroxybenzoic acid matrix.

PAMPA

PAMPA test was performed according to provider (**Corning®**) instructions.
Experiment conditions:
Caco-2 convention (top well is donor well)
Buffer: PBS pH 7.4
Incubation: 25 °C, 22h,
Sample concentration: 10 µM (AVP assay) and 50 µM (DVP assay)
Plates: **Corning® Gentest Pre-coated PAMPA Plate System**

- membrane construction: **lipid-oil-lipid trilayer**; hexane was used to disperse the contents of layers; The **oil layer** mimics the hydrophobic interior of the biological membrane and allows the amphiphilic lipids to anchor on it and is crucial for maintaining a robust and stable artificial membrane**. Lipid layer** is mixture of phospholipids.

PAMPA Caco-2 convention



The concentrations of the tested compounds in each well of the acceptor and donor plates were determined by LC-MS technique. The analysis was conducted by UHPLC-MS system (Shimadzu Nexera X2 with LCMS-2020 detector) using a ReproSil Pure 120 ODS-3 column (Dr. Maisch GmbH, 100×2 mm, 2.4 µm particle size) with several gradients of ACN with 0.1% FA and 0.05% TFA at a flow rate of 0.3 mL/min. The eluted solution was monitored by ESI-MS detector operated in the positive ionization mode with the use of the select ion monitoring mode (SIM).

Conclusions and future perspectives

- ✓ Unmodified AVP shows good permeability P_e 5.15 x 10^{-6} cm/s
- ✓ But-AVP P_e was only slightly better than that of AVP, but mass retention was 14 times higher;
- ✓ P_e for AVP analogues with longest alikyl chains (**Dec- and Dod-AVP**) are very high (almost **3 and 10 times higher than for AVP**), but their concentration in the acceptor well was very low, on the detection limit;
- ✓ **Most compounds are characterized by high mass retention**, which suggests that they stay attached to the membrane, as their concentration in the donor well decreases;
- ✓ We improved DVP permeability (about 3-fold) when Et, Prop or Hoc groups were introduced on D-Arg side chain, while over **6 –fold** improvement was observed in case of **DVP(But)** prodrug;
- ✓ We proved that prodrug (DVP(Hoc)) is rapidly transformed in Rat Serum to active, unmodified peptide drug DVP within 30 min of incubation;

- ➢ DVP and its most promising prodrugs (DVP(Et)), (DVP(Prop)), (DVP(But)), (DVP(Hoc)), will be tested on Caco-2 cell model;
- ➢ Activity of DVP prodrug showing the highest P_{app} will be examined and compared to unmodified DVP in *in vivo* assay;

References

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LPCM

Arg-builduing blocks

Acknowledgements

NATIONAL SCIENCE CENTRE POLAND

National Science Centre Poland Grant *Lipophilic Prodrug Charge Masking (LPCM) as a novel strategy to enhance oral bioavailability of charged active peptides. Optimization and generalization of the method*, **UMO-2019/35/D/NZ7/00174**