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Lipophilic Prodrug Charge Masking strategy for novel oxytocin prodrugs production

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Introduction

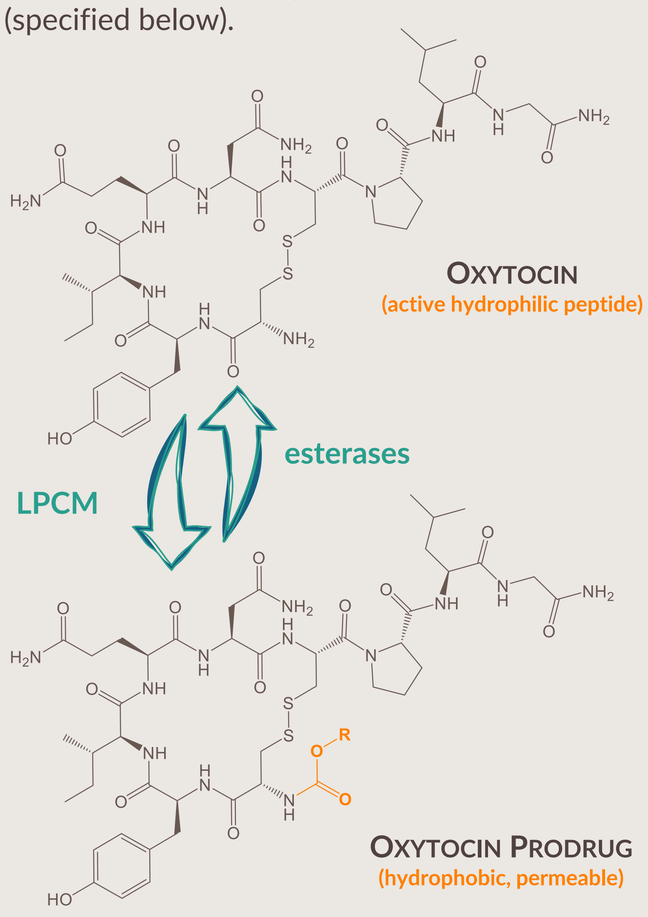
Lipophilic Prodrug Charge Masking strategy[1] is based on the introduction of transient charge masking group that would increse lipophilicity, and thus enhance peptide drug permeability. After permeation, enzymes (e.g. esterases) present throughout the body would bioconvert the prodrug to parent peptide with its original hydrophilic properties and activity. Masking groups used in this project are alkoxycarbonyl groups with general formula —R-O-C(=O)— . They vary in the alkyl chain lenghth, i.e. from 2 do 12 C atoms.

Oxytocin (OT) is a nonapeptide neurohormone produced in the hypothalamus, well characterized for its involvement in labor and lactation, but also social behavior, as well as affection. Its improper level was found in people suffering Autism Spectrum Disorders (ASD). ASD is a group of neurodevelopmental symptoms with specific pattern of abnormalities in social manners, manifested in problems with expressing emotions, which further leads to difficulties in communication and dealing with different social situations. Existing treatments focus mainly on behavioral therapy and there is no specific and effective drug treatment for the core dysfunctions of ASD reported. Those that are currently in use treat some symptoms like irritability, repetitive behavior but they cause many side effects and patients with autism may respond atypically[2].

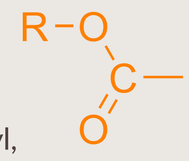
Treatment with OT may improve social abilities and emotions recognition of autistic individuals [2,3]. Thus, the suggestion to deliver it directly to cerebrospinal fluid emerged. Intranasal route of drug administration, allowing to skip blood-brain barrier and deliver therapeutics directly to the central nervous system, seemed to be an answer for this need. Some studies show that daily administration of intranasal OT contributes to improvement ASD symptoms and increases social abilities while the other group showed no efficacy in administering OT intranasally to autistic adolescents [2,3]. These differences are not necessarily caused by therapeutic inefficiencies of OT, but the route of administration, imperfectness of used devices, resulting in dose and efficacy diversity. Additionally, ASD children, with communication problems and social dysfunctions, have significant difficulty to tolerate nasal sprays. Thus, the oral administration seems to be more convenient conserving good effectiveness of therapy.

Objectives

We synthesized series of OT prodrugs where charge on the *N*-terminus was masked with alkoxycarbonyl groups with various alkyl chain lengths from 2 to 12 carbon atoms (specified below).



Following alkoxycarbonyl groups were introduced:

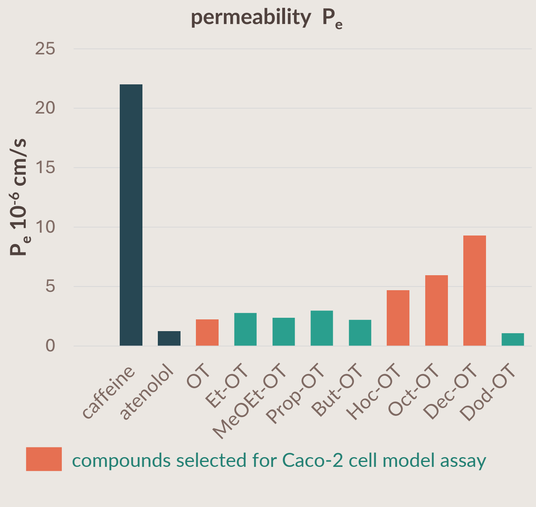


Et-etyloxycarbonyl,
Prop-propyloxycarbonyl,
MeOEt-2-methoxyethyloxycarbonyl,
But-butyloxycarbonyl,
Hoc-hexyloxycarbonyl,
Oct-octyloxycarbonyl,
Dec- decyloxycarbonyl,
Dod-dodecyloxycarbonyl;

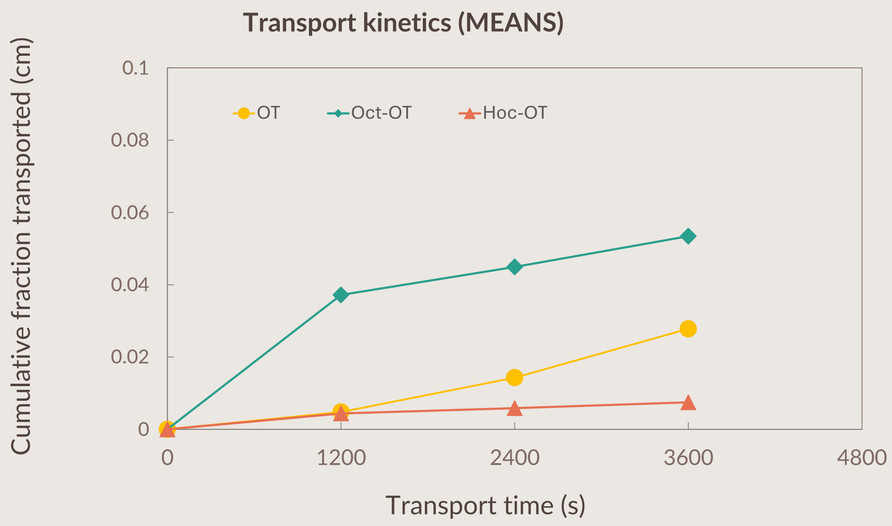
Results

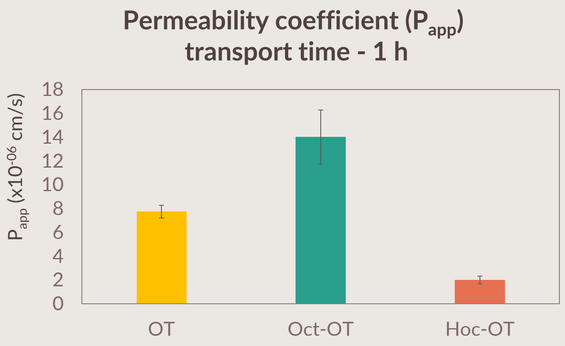
PAMPA test

Compound	Permeability P_e (10^{-6} cm/s)	Log P_e	Mass retention
caffeine	22	-5.01	26%
atenolol	1.24	-6.22	12%
OT	2.22	-5.65	19%
Et-OT	2.78	-5.56	16%
MeOEt-OT	2.37	-5.63	15%
Prop-OT	2.97	-5.53	33%
But-OT	2.2	-5.66	30%
Hoc-OT	4.67	-5.33	43%
Oct-OT	5.93	-5.23	33%
Dec-OT	9.28	-5.13	32%
Dod-OT	1.08	-5.79	67%



Caco-2 intestine epithelial model





P_{app} (×10^{-6})	OT	Oct-OT	Hoc-OT
Mean	7.74	14	1.99
SD	0.53	2.27	0.33

**Dec-OT was excluded due to analytical dificulties*

Conclusions

- ✓ Most OT analogues showed higher permeability (P_e) in compare to unmodified OT in PAMPA test;
- ✓ Three OT analogues: **Hoc-OT, Oct-OT, Dec-OT showed high permeability in PAMPA test;**
- ✓ Dod-OT PAMPA permebaility was surprisingly low; however **mass retention was almost 70%;**
- ✓ OT and its derivatives were able to cross the Caco-2 monolayer in the absorptive direction, and significant differences in their permeability were observed
- ✓ **CFT** (cumulative fraction transported**) of Oct-OT was significantly higher than the CFT of OT**, Hoc-OT was characterized by a lower CFT than OT;
- ✓ **P_{app} value calculated for Oct-OT was approximately 1.8-fold higher than that obtained for OT,** suggesting relatively high Oct-OT oral availability;

Methods

Synthesis

OT was **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 various 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 and TBTU/HOBt/NMM (molar ratio 1:1:1:2). **Masking groups** vere introduced using corresponding alkyl chloroformates on resin after *N*-terminal cysteine Fmoc deprotection. In short, alkyl chloroformate and DIPEA (molar ratio 1:2) in DMF were added to the peptidyl resin, reaction time was 1-20h. Coupling was repeated until negativ chloranil test. After completing the synthesis, peptide was 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.
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 test

PAMPA test was performed according to provider (**Corning®**) instructions, following Caco-2 convention, where the top well is a donor well. **Experimental conditions:**
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.

Caco-2 intestine eptihelial model

Caco-2 cells (ATCC HTB-37) were cultured at 37 °C under a 5% CO_2 atmosphere using Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 20% (v/v) fetal bovine serum, non-essential amino acids 100× and gentamycin (50 mg/ml). For experiments, the Caco-2 cells were plated at a density of 4×10^5 cells per cm^2 on polyethylene terephthalate (PET) capillary pore membranes with a pore size of 0.4 µm (Greiner PET hanging, 1.13 cm^2). The growth medium of Caco-2 cultures was replaced every 2 days. All experiments were performed on differentiated Caco-2 cell monolayers 21 days post-seeding. To check the integrity of the Caco-2 monolayer, transepithelial electrical resistance (TEER) was measured by employing the Millicell Electrical Resistance System (Millipore, Merck). Moreover, fluorescein-5-(and-6)-sulfonic acid was used as a transport marker, added to the apical side of the Caco-2 cultures at 200 µg/mL concentration. Fluoresceinated sulfonic acid, with a molecular weight of 478 Da, is a hydrophilic, charged molecule at physiological pH and is considered to be cell impermeable.

Transepithelial transport experiment

The analyzed compounds at 100 µg/mL concentration were prepared in transport medium (Hank's balanced salt solution – HBSS). Before the experiments, the Caco-2 monolayers were washed twice with HBSS and pre-incubated at 37 °C for 30 min. Transport was initiated by adding HBSS to the acceptor (basolateral) side and the analyzed compounds to the donor (apical) side. The Caco-2 cell cultures were placed in the incubator (37 °C) and agitated using a plate shaker (120 rpm). At 20-minute intervals, a sample was taken from the acceptor compartment, and HPLC analyzed the concentration of the transported compound. Each sample volume was replaced with fresh pre-heated (37 °C) HBSS. To quantify the transport of compound across the Caco-2 cell monolayer, the apparent permeability coefficient (Papp) was determined according to the protocol previously described by Tavelin et al.

Tavelin S., Gråsjö J., Taipalensuu J., Ocklind G. and Artursson P., in Methods in Molecular Biology, Epithelial Cell Culture Protocols, ed. C. Wise, Humana Press Inc., Totowa, New Jersey, 2002, vol. 188, pp. 233–272

LC-MS

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).

References

LPCM

1. Schumacher-Klinger A., et al., Mol. Pharm., 2018, 15, 3468–3477
2. Anagnostou E., et al., Brain Res., 2014, 1580, 188–198.
3. Guastella A. J., et al., J. Child Psychol. Psychiatry, 2015, 56, 444–452.

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