Synthesis and *in vitro* Cytotoxicity Testing of New Cyclen-Peptide Conjugations

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Summary
the new small peptide functionalized cyclen (1,4,7,10-tetraazacyclododecan) and L-DOPA (3,4-dihydroxyphenylalanine) derivatives were synthesized: cyclen-HisHis, cyclen-AspHis, cyclen-GluHis, DOPA-HisHis, as well as their Cu(II) and/or Zn(II) coordination compounds were prepared. The solid-phase synthesis strategy was used for preparation of new compounds. Synthesized cyclen- and DOPA-oligopeptide hybrid conjugations were purified by HPLC and analyzed using MS-ES spectrometer. The cytotoxicity assay showed that cyclen-dipeptide hybrids are non-toxic for cell line Hep G2 - ATCC® HB-8065™ (cells are derived from human liver) and HEK-293T - ATCC® CRL-11268™ (epithelial cells derived from kidney of human fetus).The antioxidant and anticancer activity studies will be next part of the ongoing project.

Abbreviations: *Cyclen - 1,4,7,10-tetraazacyclododecane, L-DOPA - 3,4-dihydroxyphenylalanine, Cyclen-HisHis-OH: 10-(carboxymethyl-histidyllhistidine)-1,4,7,10-tetraazacyclododecane; Cyclen-AspHis-OH: 10-(carboxymethyl-aspartylhistidine)-1,4,7,10-tetraazacyclododecane; Cyclen-GluHis-OH: 10-(carboxymethyl-glutamylhistidine)-1,4,7,10-tetraazacyclododecane; DOPA-HisHis-OH: 3,4-dihydroxyphenylalanine-6-histidylhistidine.*

Keywords: *peptide, cyclen, L-DOPA, macrocyclic, cytotoxicity, synthesize, derivative, anticancer, chromatogram.*

Introduction:

Macrocyclic polyamines have wide biological and medicinal applications. The new methodologies for their selective functionalization are of high interest due to their importance for a variety of diagnostic and therapeutic pharmaceuticals[1,2] and in the development of new MRI (Magnetic Resonance Imaging) contrast agents[3]. Recently, cyclen-based bifunctional chelators have attracted much interest in cancer therapy[4]. On the other hand, L-DOPA (3,4-dihydroxyphanylalanine) derivatives play a crucial role in the therapy of Parkinson disease (PD) as they increase the BBB penetration capacity of DOPA, which is well known medicine in the treatment of PD since 1960s. The DOPA peptidomimetics with amino acid cross-linked via oxygen atom were prepared and their antioxidant activities were studied[5].

In order to mimic biochemical processes, a number of artificial receptor molecules have been synthesized for many biologically vital molecules and enzymes. It is well known, that in such receptors, hydrogen bonding, hydrophobic or electrostatic interactions play significant role, as complementary features of the host-guest molecules. The Zinc-macrocyclic polyamine mode complexes with 2neN3 (cyclam) and ^{12}aneN4 (cyclen) showed the strong nucleophile Zn^{II} –OH^- is forms at physiological pH from Zn^{II}–H_2O species[6] and logK value (6.4) indicates that OH^- group is weakly bound to the metal ion and thus, can attack to CO_2 or carboesters. By complexation of ZnII with ^{12}aneN3 (cyclam) and ^{12}aneN4 (cyclen) the pKa is lowering from 9.0 (Zn^{II}–H_2O) to 7.3 and 7.9, respectively, at 25°C. The modification of macrocyclic polyamine receptor molecules with additional ligands (arms) enables to interact with nucleobase, sugar and other biomolecules moieties for a more efficient "multipoint" recognition, as well as for thermodynamic stabilization of the ternary complexes in aqueous solution[7]. Cyclen derivatives showed anti-HIV and anti-malarial activities[8], zinc(II)-cyclen-peptide hybrid compounds as potential inhibitor for Ras-Ras interactions[9], as well as, macrocyclic polyamines, their derivatives, and metal complexes as potential therapeutic agents in Alzheimer's disease treatment , were reported [10-13].

In our current works, the new small peptide functionalized cyclen and DOPA derivatives were synthesized: cyclen-HisHis (Fig.1), cyclen-AspHis, cyclen-GluHis (Fig.2), DOPA-HisHis, as well as their Cu(II) and/or Zn(II) coordination compounds were prepared. The solid-phase synthesis strategy was used for preparation of new compounds. Synthesized cyclen- and DOPA-oligopeptide hybrid conjugations were purified by HPLC and analyzed using MS-ES spectrometer. The His-rich cyclen conjugations could be serve as DNA, ATP and other biomolecules recognition models, as bifunctional molecules (protein interaction and metal chelation) in metal chelation therapy approach and polyphenolic DOPA derivatives, as metal chelators and radical scavengers.

Fig. 1. Cyclen-HisHis

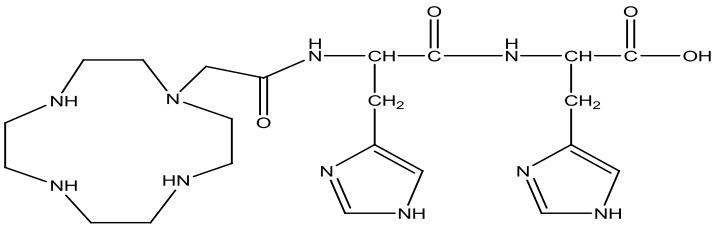
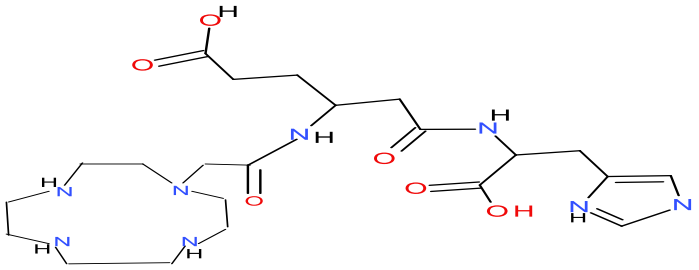


Fig. 2. Cyclen-GluHis



Methodology:

General: 2-chlorotrytilchloride resin, N,N'-diisoptopylcarbodiimide (DIC), piperidine (PIP) and Fmoc -protected amino acids: Fmoc-His(trt)-OH, Fmoc-Asp(tBu) -OH, Fmoc-Glu(tBu)-OH were purchased from Iris Biotech GmbH (Marktredwitz, DEU); triisopropylsilane (TIS) was from Novabiochem (Darmstadt, DEU); N,N-diisopropylethylamine (DIEA) was obtained from Merck (Darmstadt, DEU); 1-hydroxybenzotriazole (HOBT), 1,4,7,10-tetraazacyclododecane (cyclen), 1,4,7-tri-Boc-10-(carboxymethyl)-tetraazacyclododecane (3N-Boc-cyclen), Aβ (1-40), zinc chloride (unhydrous, $ZnCl_2$) and copper sulphate pentahydrate ($CuSO_4$) were purchased from Sigma -Aldrich (St. Louis, MO, USA); Aβ (1-16), and Fmoc-DOPA(acetonide)-OH were obtained from Bachem (Bubendorf, Switzerland). High-performance Liquid Chromatography (HPLC) grade acetonitrile (ACN), isopropyl alcohol (IPA) and dimethylformamide (DMF) were from Lachner (Neratovice, CZE); All other chemicals were of analytical or reagent grade.

Purification and Identification

After the synthesis, the crude products (dipeptides, cyclen-dipeptides, DOPA-dipeptides, DOPA-dipeptide-DOPA and cyclen-DOPA) were purified by RP-HPLC using an 100 min (3 ml/min) gradient from 0 to 100% CAN(Fig.3-4). For identification of synthesized compounds were analyzed by MALDI-TOF-MS spectrometer (Fig.5).

Fig. 3. Chromatogram of cyclen-HisHis

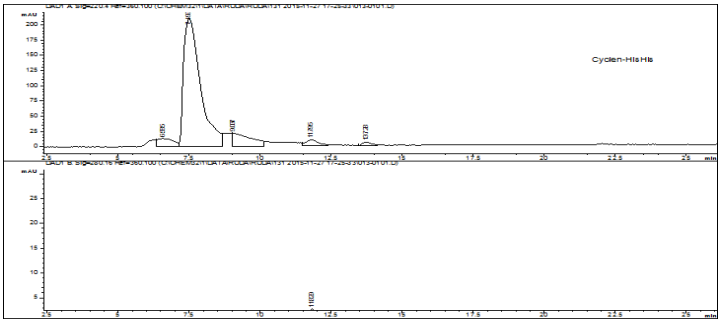


Fig. 4. Chromatogram of cyclen-AspHis

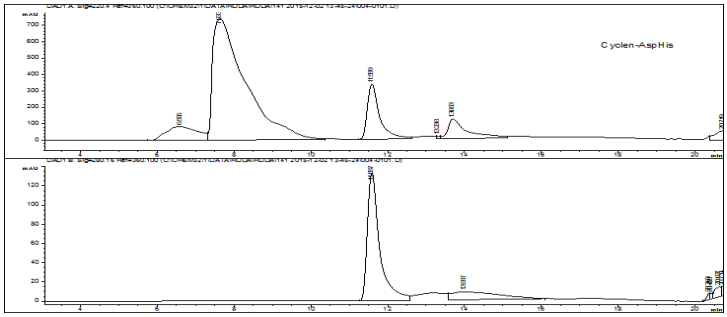
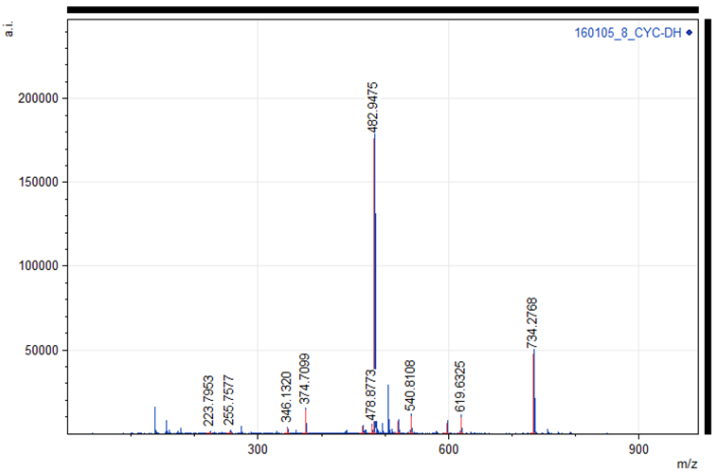


Fig. 5. MS-spectrum of cyclen-AspHis



Solid-phase conjugate synthesis of cyclen/DOPA-peptides

All peptide conjugations were synthesized manually by a stepwise strategy. The general procedure for each synthetic cycle, based on initial resin, was as follows: (1) attachment of Fmoc-His(trt)-OH or Fmoc-DOPA(ac)-OH on 2-chlorotrytilchloride resin (S = 1.55 mg/g), washing: DMF, i-PrOH, DMF, each 3x1 min; (2) Deprotection – 20% PIP in DMF for ~30 min; washing: DMF, i-PrOH, DMF, each 3x1 min; (3) Coupling: HOBT 4 eq in DMF, coupling reagent (Fmoc-AA, 3N-Boc-Cyclen-CH_2COOH, Fmoc-DOPA(ac)-OH), 2 eq in DMF; 2M DIC/DMF, 7 eq; Bromophenol blue (BB, 1% in DMF) – 26-31 µL; shaking the syringes at r.t. for at least 5 h, the reaction time was corrected depending upon the indicator (BB) colour; (4) washing: DMF, i-PrOH, DMF, each 3x1 min; (5) Cleavage of synthesized compounds from the resin and deprotection of side chains were performed in solution of 95% TFA, 2.5% TIS and 2.5% H2O, the reaction time was 3.5 h followed by 3-4 TFA washing; TFA was removed with the stream of nitrogen. The products were precipitated with tert-BuOMe and $(Et)_2O$ ethers and collected by centrifugation (2000xg, 2 min).

Cyclen-HisHis-OH: 3N-Boc-cyclen – 55 mg (102 µM, 1.0 eq) in 200 µL, Fmoc-His(trt)-® - 382 mg (204 µM, 2.0 eq), HOBT – 29.0 mg (214 µM, 2.1 eq) in 50 µL DMF, BB - 26 µL, DIC – 153 µL (306 µM, 3.0 eq) in 50 µL DMF. Yield: crude- 105 mg (81.1%), pure – 54 mg (41.7%); MS ($M + 4H^+$) 501.

Cyclen-AspHis-OH: 3N-Boc-cyclen – 55 mg (102 μM, 1.0 eq) in 200 μL, Fmoc-Asp(tBu)-® - 277 mg (208 μM, 1.0 eq), HOBT – 30.0 mg (218 μM, 2.1 eq) in 50 μL DMF, BB - 27 μL, DIC – 156 μL (312 μM, 3.0 eq) in 50 μL DMF. Yield: crude- 88.0 mg (70.6%), pure – 69 mg (55.3 %); MS (M + H^+) 483 .

Cyclen-GluHis-OH: 3N-Boc-cyclen – 55 mg (102 μM, 1.0 eq) in 200 μL, Fmoc-Glu(tBu)-® - 387 mg (204 μM, 2.0 eq), HOBT – 29.0 mg (214 μM, 2.1 eq) in 50 μL DMF, BB - 27 μL, DIC – 153 μL (306 μM, 3.0 eq) in 50 μL DMF. Yield: crude- 106 mg (83.1%), pure – 54 mg (42.3 %); MS (M + H^+) 498.

DOPA-HisHis-OH: Fmoc-His(trt)-® - 470 mg (250 μM, 1.0 eq), Fmoc-DOPA(ac)-OH – 150 mg (326 μM, 1.3 eq) in 450 μL DMF, HOBT – 51.0 mg (375 μM, 1.5 eq) in 300 μL DMF, BB - 32 μL, DIC – 288 μL (575 μM, 2.3 eq). Yield: crude- 117 mg (76.0%), pure –76.6 mg (49.7 %); MS (M + H^+) 472.

Cytotoxicity Assay:

Monitoring of cell growth with the RTCA DP Instrument

Experiments were carried out using the xCELLigence RTCA DP instrument (Roche Diagnostics GmbH, Mannheim, Germany)which was placed into a incubator (37 °C and 5% CO_2). Cell proliferation and cytotoxicity experiments were performed using modified 16-well plates (E-plate, Roche Diagnostics GmbH, Mannheim, Germany). Microelectrodes were attached at the bottom of the wells for impedance-based detection of attachment, spreading and proliferation of the cells. Initially, 100 μL of cell-free growth medium (10% FBS, 1% MEM) was added to the wells.

Cells were harvested from exponential phase cultures by a standardized detachment procedure using 0.25% Trypsin-EDTA and counted automatically using Roche's CASY Cell Counter and Analyzer. 100 μL of the cell suspension was seeded into the wells as 10^5 Hep G2 cells/ml and 10^6 HEK-293T cells/ml for cytotoxicity experiments. Twenty-four hours after cell seeding were added tested substances dissolved in water (concentration 50 μg/ml) and also during a period of 72 hours with. Water was added to control wells. CI (cell index) was monitored every 60 min during the experiment for 72 hours. This results into growth curves (dependence of the impedance expressed by the "cell index" value on time) of monitored cells in the presence of individual substances.

Hep G2 - ATCC® HB-8065™ cells are derived from human liver and HEK-293T - ATCC® CRL-11268™ are epithelial cells derived from kidney of human fetus. HEK-293T cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; with 4.5 g/L glucose and L-Glutamine), Hep G2 in RPMI 1640 (Sigma Aldrich) medium, bothe of them supplemented with 10% fetal bovine serum (FBS) and 1% of MEM (mix of vitamines – Gibco, GB.. Cells were cultured at 37 °C and 5% CO_2; cultivation medium was changed every 2 to 3 days. For experimental procedures, cells were seeded in 96-well plates at a concentration of 10^5 cells/ml per well.

The cytotoxicity assay showed (Fig.6-9) that cyclen-dipeptide hybrids are non-toxic for cell line Hep G2 - ATCC® HB-8065™ (cells are derived from human liver) and HEK-293T - ATCC® CRL-11268™ (epithelial cells derived from kidney of human fetus).

Cell line HEK 293T (concentration of cell 10^6 cell/ml)
Concentration of samples 50 μg/ml

Fig.6. Green – control, blue – sample cyclen-HisHis, red – medium

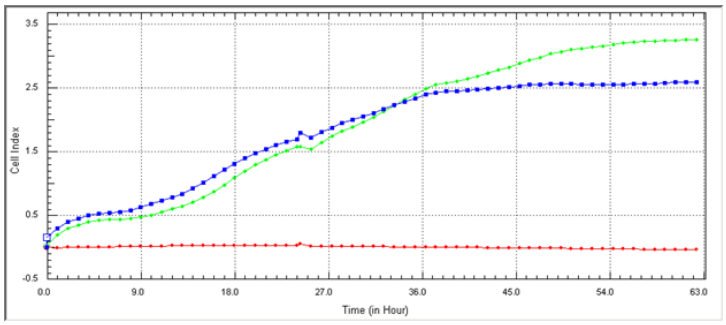


Fig.7. Green – control, blue – sample cyclen-GluHis, red – medium

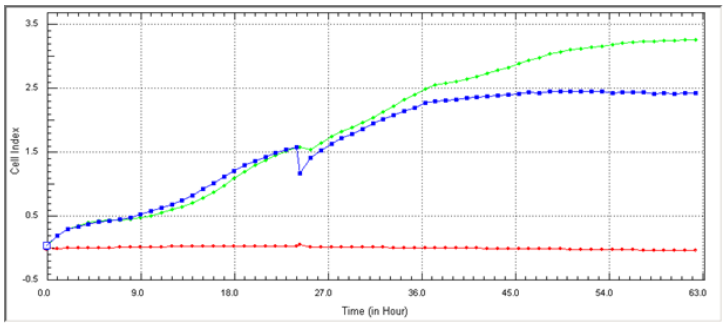


Fig.8. Green – control, blue – sample cyclen-AspHis, red – medium

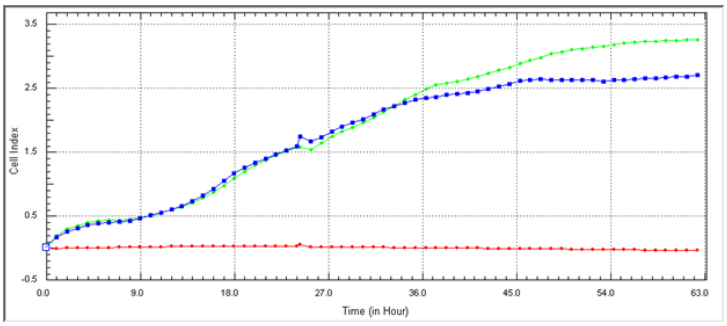
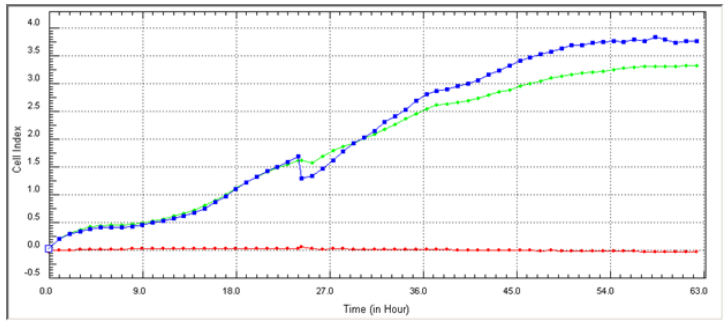


Fig.9. Green – control, blue – sample Zn(cyclen)-AspHis, red – medium



Conclusions:
The new small peptide functionalized cyclen and DOPA derivatives - cyclen-HisHis, cyclen-AspHis, cyclen-GluHis, DOPA-HisHis, as well as their Cu(II) and/or Zn (II) coordination compounds were prepared. The solid-phase synthesis strategy was used for preparation of new compounds. Synthesized cyclen- and DOPA-oligopeptide hybrid conjugations were purified by HPLC and analyzed using MS-ES spectrometer. The *in vitro* testing of cytotoxicity showed that cyclen-dipeptide and Dopa hybrids are non-toxic compounds for cell line Hep G2 - ATCC® HB-8065™ (cells are derived from human liver) and HEK-293T - ATCC® CRL-11268™ (epithelial cells derived from kidney of human fetus), and their antioxidant and anticancer activities will studied based on the obtained toxicity results.

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