Targeting the anion exchanger 2 with specific peptides as a new therapeutic approach in B lymphoid neoplasms

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SUPPLEMENTAL METHODS

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Peptide design, synthesis and characterization

Linear peptides were synthesized by the solid phase method of Merrifield using the Fmoc alternative, as previously described [1]. The purity of the peptides was analyzed by HPLC.

Design of cyclic peptides of p17AE2

The 3D structure of the p17AE2 peptide was predicted using the *de novo* prediction server PEP-FOLD [2,3]. The three sOPEP-based ranked models where minimized in MOE (MOE 2015.10. Chemical Computing Group, Inc. Molecular Operating Environment, Montreal, Quebec, Canada) using Amber12:EHT as force field and the Generalized Born model as implicit solvation energy. For each model, a molecular dynamic simulation of 50 ns each was performed with MOE after an equilibration stage of 5 ns with gradual increase of the temperature to the simulation temperature of 300 K (Nosé-Poincaré-Andersen equations of motion and time step of 0.002 ps). The distance between the N- and C-terminal extremes was monitored along the full trajectory (N of NH main chain of K1 and C of carboxy terminal group of F15). On average, this distance was < 5 Å, between 5 and 10 Å and > 10 Å for 62%, 10% and 28% of the snapshots, respectively. For each of these three states, the minimal energy conformation of p17AE2 was chosen for further examination. Analysis of the preferred conformation (< 5 Å), suggested that direct head-to-tail cyclation of the extremes (p17AE2-HT) would be well tolerated, without causing a great conformational change. Additionally, a series of linkers of different lengths bearing amide, aliphatic, piperidine and piperazine moieties were explored for the remaining two conformations of

p17AE2 with the aim of covering potentially optimal increased distances between both terminal extremes. Among them, peptide p17AE2-Amide was finally chosen (Figure SXB) as it showed a good compromise between conformational stability and synthetic accessibility.

Synthesis of cyclic peptides

P17AE2-Amide peptide was synthesized by solid phase synthesis using Fmoc-Lys(Boc)-CTC Resin (0.3 mmol, 0.5 mmol/g). The other amino acids (KKKFFWAFFILF and 4-(9H-fluoren-9-ylmethoxycarbonylamino)butanoic acid) were coupled with HBTU (2.85 eq) and DIEA (6.0 eq) stirring for 1 hour. Then, (9H-fluoren-9-yl)methyl (3-oxopropyl) carbamate (1.0 eq) and trimethoxymethane (6.5 eq) in THF (10 mL) were added and the mixture was stirred for 5 minutes. Then $NaBH_3CN$ (4.0 eq) was added and the solution was stirred overnight. Last two aminoacids (KK) were coupled with HBTU (2.85 eq) and DIEA (6.0 eq) stirring for 1 hour. The Fmoc protecting group was removed after each coupling cycle with 20% piperidine in DMF. Then, the resin was washed with MeOH (three times) and dried under vacuum for 2 hours. The linear peptide was cleavage from the resin with a solution containing 1% TFA in DCM (10 mL) stirring for 5 minutes. Then the product was filtered. The TFA-mixture was added into 300 mL DMF and cyclization was performed with DIC (2.0 eq) and HOBt (2.0 eq) stirring for 16 hours. Then, the solvent was evaporated to give the crude peptide. Side chain protecting groups were removed with a mixture of 95% TFA, 2.5% TIS and 2.5% H_2O stirring for 2 hours. The crude peptide was precipitated with cold methyl tert-butyl ether (100 mL) and centrifuged (5000 rpm, 2 minutes). The supernatant was decanted and the precipitate was washed one more time (50 mL). The crude peptide was dried under vacuum for 2 hours and then purified by preparative reverse phase HPLC (method 1) to obtain pure p17AE2-Amide peptide (10.0 mg, 1.56% yield). ESI-MS *m/z* 1065.9 $[M/2+H]^+$ calc. for $C_{114}H_{168}N_{24}O_{16}$. Purity: 94.677%.

P17AE2-HT peptide was synthesized by solid phase synthesis. To a mixture containing CTC resin (4.0 mmol, 1.0 mmol/g) and Fmoc-Lys(Boc)-OH (0.80 eq) was added DCM (25 mL) and DIEA (4.0 eq) and the mixture was stirred for 2 hours. Then, MeOH (4.0 mL) was added and the solution was stirred for 30 minutes. Then, Fmoc-Lys(Boc)-OH (3.0 eq) was coupled using HATU (2.85 eq) and DIEA (6 eq) in DMF (15 mL) for 1.5 hours. The other common amino acids were coupled with HBTU (2.85 eq) and DIEA (6 eq) in DMF (15 mL) for 1 hour. After each coupling the resin was washed with DMF (5 times) and Fmoc protecting group was removed with 20% piperidine in DMF. After last coupling the resin was washed with MeOH (3 times) and dried under vacuum for 2 hours. The linear peptide was cleavage from the resin with a solution containing 1% TFA in DCM (10 mL) stirring for 5 minutes (2 times). Then, cyclization was performed with DIC (10.0 eq) and HOBt (10.0 eq) in a mixture DMF/NMP (1:1, 1500 mL) stirring for 24 hours. Then, the solvent was evaporated to give the crude peptide. Side chain protecting groups were removed with a mixture of 95% TFA, 2.5% TIS and 2.5% H_2O stirring for 1 hour. The crude peptide was precipitated with cold methyl tert-butyl ether (300 mL) and centrifuged (3000 rpm, 3 minutes). The supernatant was decanted and the precipitate was washed two more times (300 mL). The crude peptide was dried under vacuum for 2 hours and then purified by preparative reverse phase HPLC (method 2) to obtain pure p17AE2-HT peptide (105.3 mg, 1.3% yield). ESI-MS *m/z* 994.7 $[M/2+H]^+$ calc. for $C_{107}H_{154}N_{22}O_{15}$. Purity: 95.861%.

As preparative reverse phase HPLC purification methods we have utilized:

Method 1: Reverse phase HPLC (Gilson 281) was carried out on Luna C18 (200 x 25 mm; 10 μm) and Gemini C18 (150 x 30 mm; 5 μm) in series. Solvent A: water with 0.075% trifluoroacetic acid; Solvent B: acetonitrile. Gradient: at room temperature, 20% of B to 50% of B within 60 minutes at 25 mL/min; then 90% B at 25 mL/min over 25 minutes, UV detection (wave length = 215 nm).

Method 2: Reverse phase HPLC (Gilson 281) was carried out on Luna C18 (200 x 25 mm; 10 μm) and Gemini C18 (150 x 30 mm; 5 μm) in series. Solvent A: water with 0.075% $HClO_4$; Solvent B: acetonitrile. Gradient: at room temperature, 40% of B to 70% of B within 60 minutes at 25 mL/min; then 90% B at 25 mL/min over 25 minutes, UV detection (wave length = 215 nm).

The HPLC-analysis was performed using an Agilent 1200 HPLC-BE (1-614) instrument with a Gemini-NX C18 column (150 x 4.6 mm, 5 μm) at room temperature and UV detection. Solvent A: water with 0.1% TFA; Solvent B: acetonitrile with 0.1% TFA. Gradient: 15% of B to 45% of B within 20 minutes at 1 mL/min.

Metabolic stability of synthesized peptides

Metabolic stability of the peptides was measured by Wuxi AppTech. Briefly, peptides (1 μM, 5% MeOH in potassium phosphate buffer) were incubated with human (BD Ultrapool) and mouse liver microsomas (Xenotech) at 37 °C for 10 min. The reaction was started by the addition of NADP cofactor solution and stopped by the addition of stop solution (acetonitrile at 4 °C, including tolbutamide and labetalol as an internal standard) after 20 min of incubation. The samples were shaken for 10 min and then centrifuged for 20 min at 4000 rpm. An aliquot of the supernatant was analysed by LC-MS/MS (Agilent 1200 Binary Pump API 4000). An injection volume of 10 μL was added to an ACE 5 Phenyl 2.1*50 mm ACE-125-0502 column eluting with formic acid in water or acetonitrile at a flow rate of 800 μL/min. The percent loss of parent compound was calculated from the peak area ratio of the analyte/internal standard. Compounds and positive controls were tested in duplicate. Equations of first order kinetics were used to calculate half-life time ($T_{1/2}$) and clearance (CL):

$$C_t = C_0 \bullet e^{-k_e \bullet t}$$

$$when\ C_t = \frac{1}{2} C_0\ ,$$

$$T_{1/2} = \frac{Ln2}{-k_e} = \frac{0.693}{-k_e}$$

$$CL = \frac{0.693}{T_{1/2}} \bullet \frac{1}{mg/mL\ microsomal\ protein\ in\ reaction\ system}$$

where final concentration of liver microsome solution preparation is 0.5 mg protein/mL.

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