COMPLEXES OF ^{65}Zn and Phe-D-Trp-Lys-Thr TETRAPEPTIDE CONJUGATES WITH AZACROWN ETHERS: SYNTHESIS, *IN VITRO* AND *IN VIVO* STABILITY

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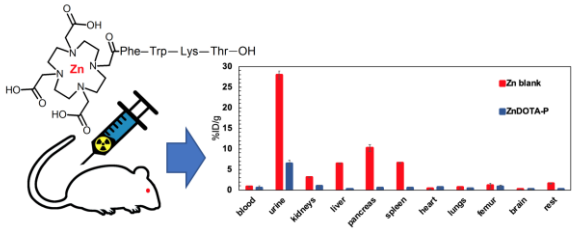
Abstract

This communication is devoted to the investigation of the complexes of ^{65}Zn and conjugates of azacrown ethers, bearing four and six heteroatoms in the macrocycle, with a tetrapeptide—a somatostatin analog. The conditions for binding of the radionuclide and stability of the resulting complexes in the presence of serum proteins are studied. The data on the distribution in a healthy mouse and *in vivo* stability of complex ZnDOTA-P are presented.



Key words: radiopharmaceutical, conjugate, tetrapeptide, azacrown ether, zinc.

Introduction

The main approach for the creation of radiopharmaceuticals is the combination of a radionuclide with an appropriate chelator and a biological vector [1]. One of the problems is the search for new ligands that would be able to form stable complexes with metal radionuclides. Of particular interest are macrocyclic ligands which can afford complexes featuring high stability and low dissociation rates [1–5]. Radiopharmaceuticals based on octreotide, a synthetic analog of somatostatin, are widely used for diagnosing neuroendocrine tumors [6]. It was shown that the specificity of octreotide to this type of tumors is provided by a Phe-D-Trp-Lys-Thr moiety [7]. Furthermore, the short peptides in which L-Trp was substituted for D-Trp were found to exhibit longer half-lives than their natural analogs [8]. In this respect, the conjugates of tetrapeptides—somatostatin analogs—and macrocyclic ligands hold great promise for the application in nuclear medicine.

Among a variety of metals which isotopes are of certain interest as components of radiopharmaceuticals, zinc is one of the most underexplored metals [9], although a series of its isotopes have huge potential for nuclear medicine owing to their physical characteristics. Thus, ^{63}Zn (β^+, $T_{1/2}$= 38.5 min) can be used in positron emission tomography (PET) [10, 11]. ^{62}Zn (β^+, $T_{1/2}$ = 9.26 h), besides its own application in PET, can be used as an *in vivo* generator for more short-lived nuclide ^{62}Cu (β^+, $T_{1/2}$ = 9.7 min) [12, 13]. Attempts were made to consider ^{69m}Zn (IT, β^-, $T_{1/2}$ = 13.76 h) for biomedical applications [14–17]. ^{65}Zn (EC, β^+, $T_{1/2}$ = 244.26 days) can find only limited use for medical purposes due to a relatively long half-life [18, 19]; however, it can be used as a convenient tool for evaluation of the stability of radiopharmaceuticals with zinc for various purposes.

Herein, we report on the synthesis of ^{65}Zn complexes with DOTA-P and L-P conjugates (Fig. 1), investigation of the conditions for complex formation and the stability of the resulting complexes in the presence of serum proteins. Furthermore, the data on *in vivo* distribution of [^{65}Zn]ZnDOTA-P is presented.

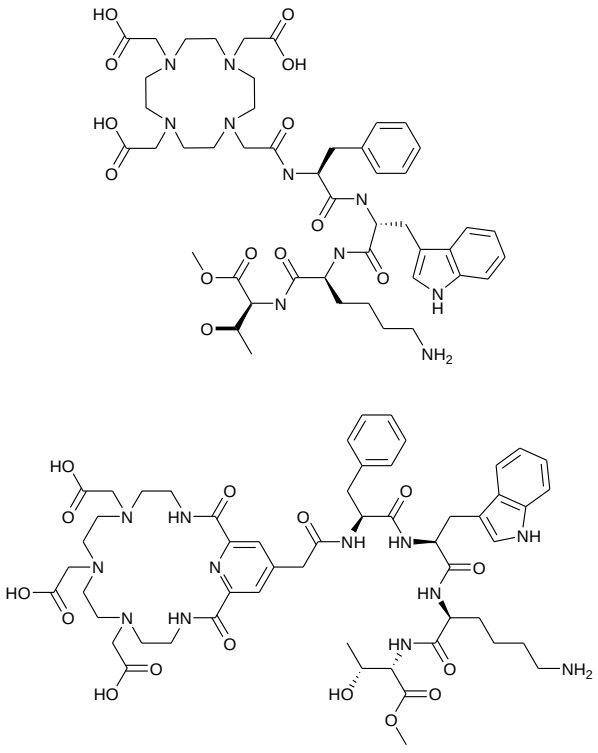


Figure 1. Structures of the DOTA-P (at the top) and L-P (at the bottom) conjugates.

Results and discussion

The L-P conjugate was obtained by the condensation of the ligand (HOOC-L-(*t*-Bu)$_3$) with the protected tetrapeptide (H-Phe-D-Trp-Lys(Boc)-Thr-OMe) using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylamine tetrafluoroborate (TBTU) as a coupling agent, *N*-hydroxybenzotriazole (HOBt), and diisopropylethylamine (DIPEA) (Fig. 2).

The protected conjugate was sequentially subjected to hydrolysis of the methyl ester and removal of the protecting groups (for the detailed description of the synthetic procedures, see Experimental). According to the HPLC data, the content of the target product through all the steps composed 28.4%; therefore, the conjugate was purified by preparative HPLC. This afforded 33 mg of the target product in the form of a trifluoroacetate salt.

The conditions for the production of ^{65}Zn complexes with the conjugates were chosen using TLC followed by separation of the plates according to the values of R_f for the unbound cation and the complexes and their gamma spectrometry. It was shown that the binding degree of Zn^{2+} with DOTA-P at the ligand concentration above $1\cdot10^{-4}$ M composes 93–97%. In the case of complex ^{65}ZnL-P, the stable binding with the ligand was observed also at the concentration of L-P above $1\cdot10^{-4}$ M, after which the yield of complexation dramatically declined with a reduction in the concentration (Fig. 3). In both cases, the binding with the radionuclide occurred for no more than 10 min; no additional time for the incubation of the complex solution was required. This evidences that there are no kinetic hindrances for the binding of ^{65}Zn with the ligand, which is crucial for further potential application of these compounds as radiopharmaceuticals. The stability constant logarithm for the complex of Zn^{2+} with DOTA is 21.0 [20], and that for L is 12.6 [21]. Therefore, at the same concentrations, the binding degree of ^{65}Zn for L is naturally, but only slightly lower than that for DOTA.

For preliminary evaluation of the stability of the resulting complexes in biological media, a series of experiments on the competitive binding with blood serum proteins were carried out. These experiments revealed that, for the first hours after mixing of the complex solution with serum, a major part of the radionuclide remains in the complex composition (Fig. 4). However, after incubation with serum for 1 day, about 30% of the radionuclide in the case of ZnL-P undergoes transchelation with blood proteins, whereas the analogous complex with DOTA-P remains stable under the same conditions. The lower stability of ZnL-P can be associated with the absence of a macrocyclic effect for the relatively small cation Zn^{2+} (d = 1.5 Å [22]) compared to the cavity of the macrocycle of L (2.6–3.2 Å [21]). This results in the relatively easy transchelation of the metal cation due to the absence of steric hindrances for external chelators. For the conjugate with DOTA, in contrast, the coordination environment is optimal for chelation of Zn^{2+}: the zinc cation is surrounded by all four nitrogen atoms of the macrocycle and two carboxy groups [23], which affords steric hindrances for transchelation and leads to stability in the presence of serum proteins.

Taking into account the *in vitro* stability of ZnDOTA-P, it seemed reasonable to study its behavior under *in vivo* conditions. The biodistribution of [^{65}Zn]ZnDOTA-P was explored in healthy mice (groups of three mice). A solution of [^{65}Zn]$ZnCl_2$ with pH = 7 was used as a reference (Zn blank). The organ distribution of accumulated radioactivity is presented in Figs. 5 and 6. As can be seen, zinc cations were accumulated predominantly in the pancreas, liver, and spleen, which is in

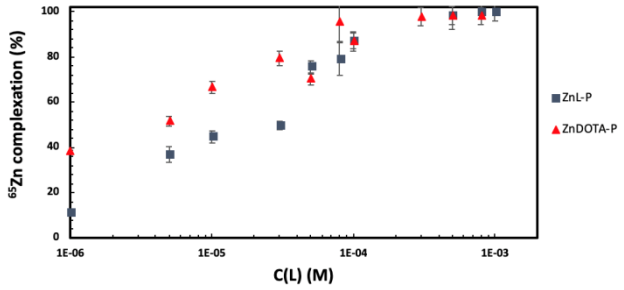


Figure 3. Complexation degree of the radionuclide depending on the conjugate concentration.

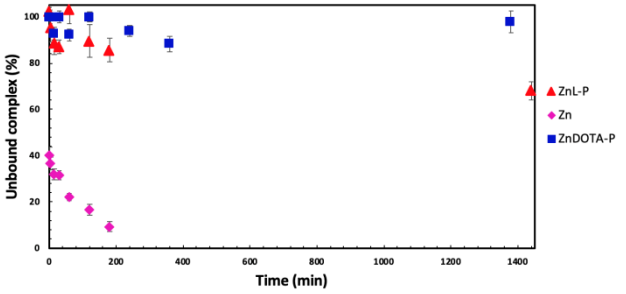


Figure 4. Binding degree of the zinc radionuclide with serum proteins depending on time.

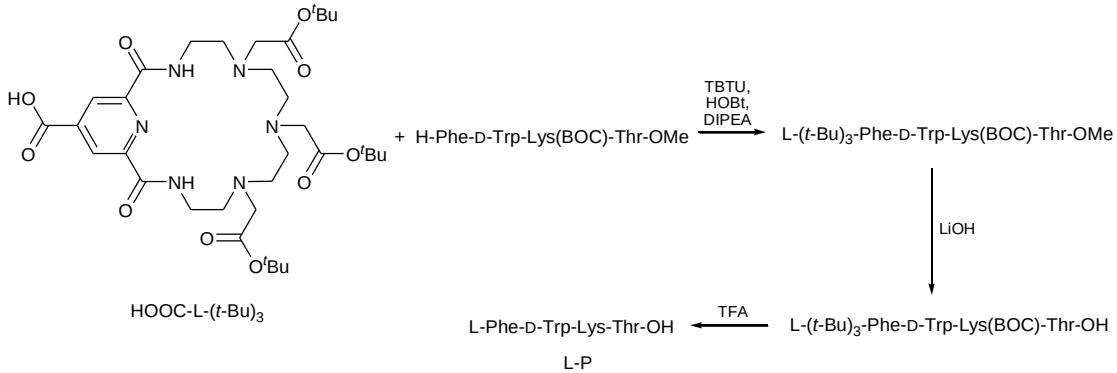


Figure 2. Synthesis of the L-P conjugate.

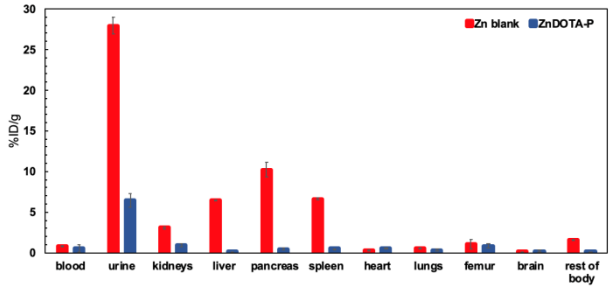


Figure 5. Distribution of complex ZnDOTA-P in organs of a laboratory mouse in 1 h.

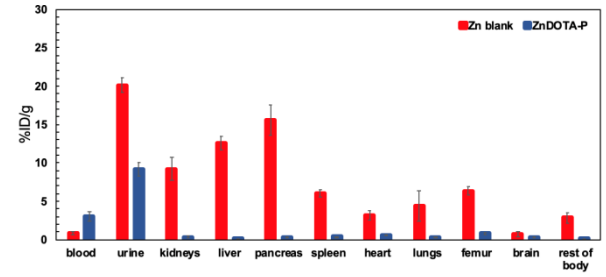


Figure 6. Distribution of complex ZnDOTA-P in organs of a laboratory mouse in 6 h.

good agreement with the metabolism of this element [24]. At the same time, already in 1 h, ZnDOTA-P almost did not undergo accumulation in any organ, and a major part of radioactivity was detected in urine. This fact testifies that the introduction of the peptide moiety does not affect the stability of a Zn complex with DOTA and does not lead to its accumulation in healthy organs, as in the case of unbound Zn^{2+} cations. Hence, further investigation of the conjugates of macrocyclic ligands with tetrapeptide P seems to be very interesting.

Experimental

Synthesis of the DOTA-P and L-P conjugates

The DOTA-P tetrapeptide was synthesized according to the procedure described by Zubenko *et al.* [25]. The synthesis of ligand L was carried out using the approach suggested by Fedorov *et al*. [21].

The L-P conjugate was obtained from L(*t*-Bu)$_3$-OH and tetrapeptide H-Phe-D-Trp-Lys(Boc)-Thr-OMe in DMF. L(*t*-Bu)$_3$-OH (150 mg, 0.21 mmol) and the mentioned tetrapeptide (160 mg, 0.23 mmol) were dissolved in DMF (8 mL). Then, a solution of TBTU (74 mg, 0.23 mmol), HOBt (43 mg, 0.25 mmol), and DIPEA (33 mg, 0.25 mmol) in DMF (5 mL) was added. The reaction mixture was stirred at room temperature for 12 h, then diluted with ethyl acetate (50 mL), sequentially washed with 5% aq. citric acid (2×10 mL), water (10 mL), 5% aq. sodium bicarbonate (2×10 mL), and a saturated aqueous solution of NaCl (10 mL). The organic phase was separated, dried over anhydrous sodium sulfate, and evaporated under vacuum to give the target product as a beige powder. Yield: 290 mg. The content of the main component according to HPLC was 86.5%. ESI-MS (m/z): 1384.1 $[M + H]^+$.

The resulting protected conjugate was used without purification for further hydrolysis of the methyl ester. A solution of LiOH (10.5 mg, 0.25 mmol) in water (1 mL) was added to a solution of the protected conjugate (290 mg, 0.21 mmol) in methanol (5 mL) at 5 °C. The resulting solution was stirred at room temperature for 1 h and, then, washed and dried analogously to the above-described procedure. The organic phase was dried over anhydrous sodium sulfate and evaporated under vacuum to give the target product as a beige powder. Yield: 290 mg. The content of the main compound according to HPLC was 80.7%. ESI-MS (m/z): 1370.2 $[M + H]^+$ (Figs. 7, 8).

The intermediate obtained was used without purification for further removal of the protecting groups of the conjugate. The process was carried out in trifluoroacetic acid (TFA). A mixture of trifluoroacetic acid (5 mL), triisobutylsilane (30 μL), anisole (30 μL), mercaptoethanol (30 μL), and water (25 μL) was added to L(*t*-Bu)$_3$-Phe-D-Trp-Lys(Boc)-Thr-OH. The reaction mixture was stirred at room temperature for 12 h and, then, diethyl ether (15 mL) cooled to 0 °C was added. The resulting precipitate was filtered off and rinsed with diethyl ether to give the target product as a trifluoroacetate salt of the conjugate as a beige powder. Yield: 260 mg. The content of the main compound according to HPLC was 28.4%. ESI-MS (m/z): 1101.9 $[M + H]^+$ (Figs. 9 and 10).

The resulting product was purified by preparative HPLC. To purify the conjugate, a chromatographic system was used that

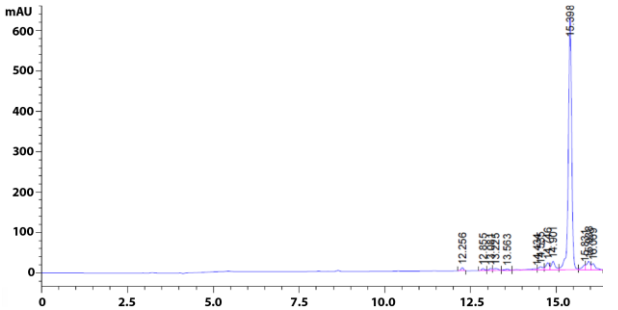


Figure 7. HPLC chromatogram of L(*t*-Bu)$_3$-Phe-D-Trp-Lys(Boc)-Thr-OH.

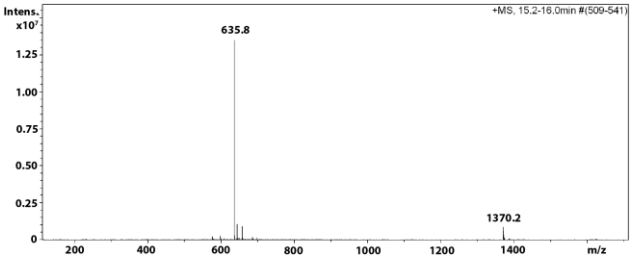


Figure 8. Mass spectrum of L(*t*-Bu)$_3$-Phe-D-Trp-Lys(Boc)-Thr-OH.

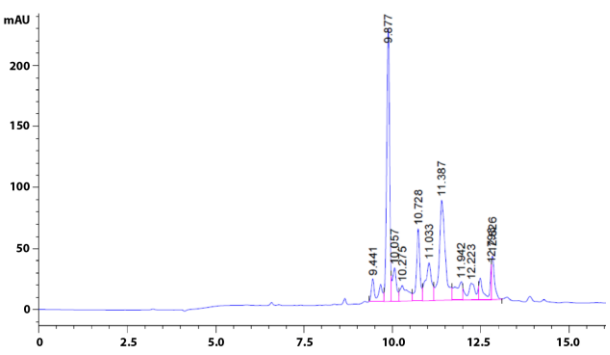


Figure 9. HPLC chromatogram of L-Phe-D-Trp-Lys-Thr-OH.

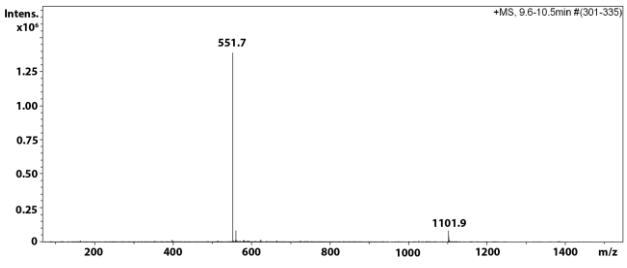


Figure 10. Mass spectrum of L-Phe-D-Trp-Lys-Thr-OH.

included two GILSON 306 pumps (USA) with the productivity up to 25 mL per minute, a GILSON 805 manometric module, and a UVV-105 spectrophotometric detector (JET Chrom production). The separation was carried out on a ReproSil-PurAQ C18 5 μm column (250×10 mm) with a ReproSil-PurAQ C18 10 μm precolumn (30×8 mm) using a system of two eluents: eluent A—an aqueous solution of ammonium acetate (0.04 M) with pH = 5, adjusted by the addition of acetic acid; eluent B—acetonitrile with pH = 5, adjusted by the addition of acetic acid. The separation was performed in a gradient mode from the initial concentration of eluent B at the zero minute to the final value of the concentration at the 40th minute. The limiting values of the concentrations of eluent B in a mobile phase were 40÷80%. The volume rate of the pumps during separation composed 6 mL per minute. The selected fractions with the pure product were combined. Acetonitrile was removed under reduced pressure. The resulting solution was lyophilized to give the target product as a trifluoroacetate salt (one trifluoroacetate anion per one molecule of L-P). Yield: 33 mg.

Preparation of labeled compounds

To obtain the complex compounds, an aqueous solution bearing the L-P or DOTA-P conjugate (upon selection of the conditions for complex formation, the ligand concentration was varied from $1{\cdot}10^{-6}$ to $1{\cdot}10^{-3}$ M) and 0.15 M $AcONH_4$ buffer were added to a solution of $[^{65}Zn]ZnCl_2$ at room temperature. Immediately after mixing of solutions, the mixture pH was adjusted to 7.0 using aqueous solutions of NaOH or HCl.

Thin-layer chromatography and autoradiography

Thin-layer chromatography was carried out on i-TLC plates (Agilent) using 10% $AcONH_4$:MeOH (1:1) as an eluent for ZnDOTA-P and on cellulose plates (Sigma-Aldrich) using 10 mM NaOH in 0.9% NaCl as an eluent for ZnL-P. The values of retention factors for free cations in these systems composed 0.05 and 0.15, respectively, for ZnDOTA-P 0.80, and for ZnL-P 0.75.

The visual analysis of the TLC plates was conducted by autoradiography on the plates from BaFBr doped with europium (Multisensitive phosphor screens) using a Cyclone Plus Storage Phosphor System (Perkin Elmer); the resulting images were analyzed using an Optiquant software.

The gamma-ray spectra were analyzed with a GC-3020 gamma spectrometer (Canberra Packard Ind., USA) with a semiconducting detector from ultrapure germanium. The efficiency of the detector registration for γ quants with different energies was determined based on the measurement of the activity of standard sample sources.

Isolation of ^{65}Zn

The ^{65}Zn radionuclide was isolated from a metallic copper target (target mass 1.5 g) irradiated with deuterons with the energy of 14.8 MeV on a cyclotron in 2 days after irradiation. The copper target was dissolved in 65% aq. nitric acid, then the solution was evaporated and the resulting residue was redissolved in 2M HCl. The solution obtained was purified on a column filled with a Dowex 1×8 sorbent, which was preliminarily kept in 2M HCl. After charging the solution, the column was washed with 2M HCl, and after the removal of copper, zinc was washed out with distilled water. The fractions bearing the target radionuclide were evaporated and redissolved in HCl with pH = 2. The radionuclide purity was controlled using gamma spectrometry.

Evaluation of *in vitro* stability

A solution of the corresponding complex (100 μL) with the ligand concentration of $1{\times}10^{-3}$ M was mixed with fetal bovine serum (FBS, HyClone) in the volume ratio V(ML solution):V(FBS) = 1:9. The resulting mixture was incubated at 37 °C. The aliquots (100 μL) of this mixture were taken at specific time intervals, mixed with ethanol (300 μl), and cooled down to 2–4 °C for precipitation of proteins. The resulting precipitate was separated by centrifugation at 4000 g over 5 min and decantation of the mother liquor (300 μL). The activity of the resulting mother liquor was measured using gamma spectrometry. A reference sample was obtained by the addition of serum (225 μL) to an aliquot of the initial solution (75 μL). The blank experiments were carried out analogously using the solutions of the radioactive label that did not contain the ligands.

Analysis of *in vivo* distribution of $[^{65}Zn]ZnDOTA$-P

$[^{65}Zn]ZnDOTA$-P was dissolved in 0.9% aq. NaCl in the presence of $AcONH_4$ buffer; pH was adjusted to 7.0. The resulting complex was introduced intraperitoneally in the amount of 100 μL. A reference sample was an analogous solution of $[^{65}Zn]ZnCl_2$ in an acetate buffer. The radioactivity of the introduced agent was 5kBq in one injection. The mouse was euthanized by cervical dislocation and decapitation (as a duplicate method of euthanasia) in 1 and 6 hours after the administration. The organs (liver, kidneys, spleen, lung, heart, pancreas, brain, and femur) were isolated post mortem, rinsed in a normal saline, and weighed. The radioactivity accumulated in the organs was measured using gamma spectrometry. Blood after decapitation was collected and mixed with a heparin solution (100 μL). A percentage of the introduced dose per gram of the organ was calculated by the following formula:

$$\%\frac{ID}{g} = \frac{A}{A_{\text{introd}} \cdot m_{\text{org}}} \cdot k_{\text{vol}} \cdot 100$$

where A is the measured activity accumulated in the organ, A_{introd} is the total activity of the introduced agent, m_{org} is the organ mass, k_{vol} is the calibration factor that takes into account the volume of the measured sample.

Conclusions

The conjugate of the azacrown ether and the L-P tetrapeptide was obtained and characterized. An optimal concentration of the ligands for the formation of zinc complexes with the DOTA-P and L-P conjugates was found to be equal to $1 \cdot 10^{-4}$ M. The binding degree of the radionuclide at this concentration composed over 90%. [^{65}Zn]ZnDOTA-P exhibited higher *in vitro* stability than its counterpart based on L-P. It was shown that [^{65}Zn]ZnDOTA-P can be removed from an organism of a live mouse already for the first hour, which makes it a promising radiopharmaceutical for further studies.

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