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In vivo biodistribution study of TAT-L-Sco2 fusion protein, developed as protein therapeutic for mitochondrial disorders attributed to *SCO2* mutations

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A B S T R A C T

The rapid progress achieved in the development of many biopharmaceuticals had a tremendous impact on the therapy of many metabolic/genetic disorders. This type of fruitful approach, called protein replacement therapy (PRT), aimed to either replace the deficient or malfunctional protein in human tissues that act either in plasma membrane or *via* a specific cell surface receptor. However, there are also many metabolic/genetic disorders attributed to either deficient or malfunctional proteins acting intracellularly. The recent developments of Protein Transduction Domain (PTD) technology offer new opportunities by allowing the intracellular delivery of recombinant proteins of a given therapeutic interest into different subcellular sites and organelles, such as mitochondria and other entities. Towards this pathway, we applied successfully PTD Technology as a protein therapeutic approach, *in vitro,* in *SCO2* deficient primary fibroblasts, derived from patient with mutations in human *SCO2* gene, responsible for fatal, infantile cardioencephalomyopathy and cytochrome *c* oxidase deficiency.

In this work, we radiolabeled the recombinant TAT-L-Sco2 fusion protein with technetium-99 m to assess its *in vivo* biodistribution and fate, by increasing the sensitivity of detection of even low levels of the transduced recombinant protein. The biodistribution pattern of [^{99m}Tc]Tc-TAT-L-Sco2 in mice demonstrated fast blood clearance, significant hepatobiliary and renal clearance. In addition, western blot analysis detected the recombinant TAT-L-Sco2 protein in the isolated mitochondria of several mouse tissues, including heart, muscle and brain. These results pave the way to further consider this PTD-mediated Protein Therapy Approach as a potentially alternative treatment of genetic/metabolic disorders.

1. Introduction

The rapid progress achieved in the development of many biopharmaceuticals, produced in genetically engineered bacteria, mammalian cells, plants or other systems, had a tremendous impact on the therapy of many metabolic/genetic disorders. This type of fruitful approach, called protein replacement therapy (PRT) [1], aimed to either replace the deficient or malfunctional protein in human tissues. Notably, in all these cases, the recombinant proteins (*e.g.* growth factors, monoclonal antibodies) act either in plasma membrane or *via* a specific cell surface receptor, by stimulating defective/inactive signaling pathways to deliver their pharmacological action. However, there are also many metabolic/genetic disorders attributed to either deficient or malfunctional proteins acting intracellularly, such as enzymes, metal binding carrier proteins, *etc.* In all these cases, although the corresponding recombinant proteins can be produced, they fail to cross the plasma membrane barrier and enter intracellularly into their target tissues.

The recent developments of Protein Transduction Domain (PTD) technology [2,3], by using small peptides that facilitate intracellular delivery of proteins, nucleic acids and other molecules, offer new opportunities, also permitting their subcellular localization in organelles, such as mitochondria.

Abbreviations: TAT-L-Sco2, 10xHis-Xa$_{SITE}$-TAT-L-Sco2-HA;; BSA, Bovine Serum Albumin;; COX, Cytochrome c oxidase;; FBS, Fetal bovine serum;; IBs, Inclusion bodies;; ID, Injected dose;; *i.p.*, Intraperitoneal;; *i.v.*, Intravenous;; L-Arg, L-Arginine;; *p.i.*, Post-injection;; PBS, Phosphate buffered saline;; PRT, Protein Replacement Therapy;; PTD, Protein Transduction Domain;; RA, Radioactivity;; RT, Room Temperature;; SD, Standard Deviation;; SEC, Size Exclusion Chromatography;; ^{99m}Tc, Technetium-99 m..

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Within this frame, our group has already exploited the PTD technology to produce and successfully deliver the human recombinant full length mitochondrial protein Sco2, fused to TAT, into the mitochondria of *SCO2*/COX deficient cell culture model [4,5], as a therapeutic approach to mitochondrial disorders due *SCO2* mutations and COX deficiency, since there are no cures for mitochondrial disorders and treatment is usually limited to supportive agents [6].

Mutations in human *SCO2* gene were found to be responsible for fatal infantile cardioencephalomyopathy and cytochrome *c* oxidase (COX) deficiency, by Papadopoulou, et al. (1999) [4], as well for other rare disorders, like Leigh disease [7]. COX refers to the complex IV of the Oxidative Phosphorylation System, a process that couples cellular respiration and production of ATP, playing crucial role in cell bioenergetics [4,5]. Human mitochondrial inner membrane Sco2 protein, a COX assembly protein, serves as a copper chaperone (along with Sco1) in mitochondria, as a mitochondrial redox signaling molecule [4,8,9], participating also in cell bioenergetics [10].

In the first study Papadopoulou, et al. (1999), the cloning of the full length wild-type DNA of human SCO2 gene, derived from a healthy individual, was conducted. During the following years, our team proceeded to the cloning of the recombinant bacterial expression pET-16b-TAT-L-Sco2-HA vector, in order to produce the full length TAT-L-Sco2 fusion protein in bacteria, which was further solubilized in 1 M L-Arg. Our team, Foltopoulou, et al. (2010), showed the successful intracellular delivery of the recombinant TAT-L-Sco2 fusion protein into the mitochondria of different types of mammalian cells (U-87 MG, T24, and K-562). In addition, Foltopoulou, et al. also achieved the transduction of TAT-L-Sco2 fusion protein in transiently SCO2 mRNA depleted and thus COX deficient human K-562 cells. Furthermore, [^{35}S]methionine-labeled TAT-L-Sco2 fusion protein, incubated with isolated intact mitochondria, was processed by the MPP, the matrix-located mitochondrial processing peptidase. Finally, our team also provided experimental evidence that TAT-L-Sco2 fusion protein successfully transduced in primary fibroblasts derived from SCO2/COX deficient patient, contributed to the recovery of suppressed COX activity through enzymatic assessment and histochemical staining for COX activity.

In the present study, we attempted to take a step forward and study the biodistribution pattern of recombinant TAT-L-Sco2 fusion protein by assessing the fate of the injected TAT-L-Sco2 fusion protein *in vivo*. Produced TAT-L-Sco2 fusion protein was radiolabeled by using Technetium-99 m (^{99m}Tc) to assess its *in vivo* biodistribution in mice, since radiolabeling will increase the detection sensitivity of even low levels of the transduced recombinant protein. The detection of TAT-L-Sco2, as a biopharmaceutical, in the mitochondria of peripheral tissues was further examined.

2. Materials and methods

2.1. Cell cultures

Human chronic myelogenous leukemia K-562 cells [11] were seeded in suspension culture at 2–3 × 10^5 cells/mL in RPMI-1640, supplemented with 10% fetal bovine serum (FBS) and an antibiotic-antimycotic cocktail [penicillin(100 U/mL)/streptomycin (0.1 mg/mL)/amphotericin B(0.25 μg/mL) (Gibco-Invitrogen)] and maintained in exponential growth at 37 °C, in 5% CO_2 humidified atmosphere. Cell number was assessed, as previously described [12].

2.2. Animal studies

All experiments were approved by the Aristotle University Committee for Animal Experimentation (License No 350880/3468), in accord with the European guidelines 2010/63/EU and PD 56 for animal experimentation. Balb/c mice, 31 animals in total (Online Resource Table S1), 10–12 weeks old, with a median weight of 20-25 g, were housed in proper animal facilities (Laboratory of Physiology, Pharmacology, Biochemistry-Toxicology, School of Veterinary Medicine, Aristotle University, EU License No EL 54 BIO 10) with food and water *ad libitum* in constant conditions of temperature and humidity and regular light cycles of 12/12 h light/dark. All animal experiments were conducted in light of 3 R' s (Replacement, Refinement, Reduction) and all mice used for the experiments were not subjected to pain or discomfort.

2.3. Expression, solubilization and analysis of the recombinant human TAT-L-Sco2

The expression/production/purification of recombinant 10xHis-Xa_{SITE}-TAT-L-Sco2-HA (TAT-L-Sco2) fusion protein was carried out in *E. coli* cultures, as previously described [5]. Briefly, a selected colony of transformed by pET-16b-TAT-L-Sco2-HA bacterial *E. coli* BL21 (DE3) cells was cultured in LB (Luria-Bertani) medium, containing ampicillin (50 μg/mL). Induction was performed with 0.3 mM isopropyl-LD-thiogalactoside (IPTG) (PanReac, AppliChem), at 37 °C for 2 h, and bacterial pellet was harvested in order to collect inclusion bodies (IBs), which were further purified and dissolved either in L-Arginine (L-Arg) [5,13] (Sigma-Aldrich) in 20 mM Tris–HCl, pH 7.5 [1–1.6 mg/mL protein] or in a salt buffer (300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) [0.3–1.2 mg/mL protein]. L-Arg solution has been found to facilitate folding of proteins [14]. Solubilized IBs were then centrifuged at 5000 x*g* and the supernatant, containing the recombinant TAT-L-Sco2-HA fusion protein, was collected and kept at RT (in case of dissolving in L-Arg) or at 4 °C (in case of dissolving in salt solution).

Bradford method, SDS − PAGE and western blot analysis were carried out, as previously described [5] and isolated mitochondrial proteins were blotted with either the purified polyclonal antiserum raised against recombinant human Sco2 mature protein [15] (anti-Sco2.IgG) (1:700) or the polyclonal antibody anti-HA.IgG (Santa Cruz Biotechnology) (1:3000) or the mouse monoclonal anti-His.IgG (1:2600) (Sigma-Aldrich). Finally, membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse.IgG-AP (1:1000) (Santa Cruz Biotechnology) or goat anti-rabbit IgG-AP (Sigma-Aldrich) (1,2500), as secondary antibodies. Proteins were visualized by using NBT/BCIP (Biotium) substrates for alkaline phosphatase.

2.4. Radiolabeling of TAT-L-Sco2 with ^{99m}Tc and stability

CAUTION! ^{99m}Tc is a gamma (γ)-emitting radionuclide ($t_{1/2}$ = 6 h, γ-energy,140 keV), and handling was performed following Greek legislation and the EU regulations (2013/59/Euratom). Various ^{99m}Tc-labeling protocols have been developed for the preparation of ^{99m}Tc-proteins [16].

^{99m}Tc was obtained as sodium pertechnetate in sterile saline from a commercial ^{99}Mo/^{99m}Tc generator (AHEPA Hospital, Thessaloniki, Greece).

Size Exclusion Chromatography (SEC), used for the analysis of the radiolabeled recombinant protein, was comprised of a HP/Agilent 1100 series HPLC system connected with a BIOSEP-SEC-S3000 column (300 × 7.8 mm, 5 μm), a Gabi, Raytest NaI(Tl) scintillator and UV detection set at 280 nm. Phosphate buffer 50 mM, pH 6.8, containing 0.3M NaCl and flow 0.5 mL/min were applied. Radioactivity (RA) measurements of ^{99m}Tc samples were done in a dose calibrator (ATOMLAB^{TM}100, Biodex Medical System) and in NaI(Tl) scintillator (Caprac®, Capintec). For the photometry measurements with the Ellman reagent (Thermo Scientific), a Perkin Elmer *lamda20* was used, set at 412 nm.

Samples of purified TAT-L-Sco2 (0.5–1.8 mg) were labeled with ^{99m}Tc, as previously described [17]. Since it was the first time for the radiolabeling of the recombinant TAT-L-Sco2 fusion protein, the preparation of the [^{99m}Tc]Tc-TAT-L-Sco2 samples was performed by two methods. Method I: The [^{99m}Tc]Tc-TAT-L-Sco2 was centrifuged (5 min, 5000 rpm), the supernatant was discarded and 3 mL of 1 M L-Arg were added to the solid. The suspension was then re-centrifuged and the

supernatant was collected. Method II: The [^{99m}Tc]Tc-TAT-L-Sco2 was centrifuged (5 min, 5000 rpm) in 10 K-MWCO centrifuge tubes (Vivaspin 0.5 mL, Sartorius Stedim Biotech GmbH) and the retentate was isolated. The protein concentration was also determined, *via* Bradford method.

The radiochemical yield of [^{99m}Tc]Tc-TAT-L-Sco2 was determined by radiochromatography, using the following systems: a) paper chromatography (P.Chr 1) and acetone as mobile phase; b) ITLC-SG (Gelman Sciences) and saline as mobile phase; and c) ITLC-SG and Bovine Serum Albumin (BSA) 5 mg/mL in EtOH:NH_3:H_2O, 2:1:1.5, as mobile phase. Same methods were used for the labeling and radiochromatography of [^{99m}Tc]Tc-mock. The labeling mixture of [^{99m}Tc]Tc-TAT-L-Sco2 was analyzed by SEC and its stability in the reaction solution was tested over 24 h.

According to the three radiochromatography separation systems applied: i) System I in acetone was used to determine the percentage of free pertechnetate, which migrates at the solvent front, and it was found to be <5%; ii) System II in saline was used to determine the percentage of the small-molecule impurities (*e.g.* ^{99m}Tc-gluconate) that migrate to the front of the solvent, while the labeled protein remains at the origin of the TLC plate; and iii) System III in BSA solution was used to calculate the formation of ^{99m}Tc-colloid, which remains at the origin of the TLC, while the labeled protein migrates to the solvent front ($R_f = 0.1–1$).

2.5. Assessment of stability in plasma and blood

IBs, enriched in full-length human recombinant fusion TAT-L-Sco2 (15 μg in 0.25 mL), were incubated with human plasma derived from a healthy donor at 37 °C, for various time intervals (5,15,30 min and 1,2,4,24,48 h), in a ratio volume 1:1 (final vol. 0.5 mL). At each timed point, samples (30 μL) were removed, analyzed by SDS-PAGE and western blot, using anti-Sco2.IgG. This experiment permitted evaluation of structural integrity (hence of the stability) of the TAT-L-Sco2 protein, based on changes occurring in its molecular mass, in a time-dependent assay.

For the stability studies of the [^{99m}Tc]Tc-TAT-L-Sco2, heparinized whole blood collected from healthy human donor (2 mL) was incubated with 500 μL of [^{99m}Tc]Tc-TAT-L-Sco2 (16KBq) at 37 °C, over 21 h. Portions of 250 μL were analyzed at 1 and 4 h, and a 500 μL portion at 21 h. The samples were centrifuged at 2000 *g*, for 10 min, the supernatant plasma was collected, and the blood cells were washed once more with RPMI-1640 medium (200 μL). Both the cells and the collected plasma were measured for RA in a γ-counter (Caprac®-Capintec). Furthermore, 400 μL acetonitrile (CH_3CN) were added in the plasma, the proteins were precipitated and then centrifuged at 5000 rpm, for 5 min. The RA of the protein pellet and the supernatant solution were measured in a γ-counter. For the 21 h time-point, a 500 μL-portion was analyzed and 1 mL CH_3CN was added for protein precipitation.

2.6. Assessment of intracellular delivery of [^{99m}Tc]Tc-TAT-L-Sco2 in cultured cells and subcellular distribution

Exponentially grown K-562 cells (3×10^7-1×10^8) were incubated with 250 μg of [^{99m}Tc]Tc-TAT-L-Sco2 (1–2.5 MBq), under serum-free conditions [Opti-MEM I Reduced Serum Medium (Gibco-Invitrogen)], for 3 h, at 37 °C, 5%CO_2. After treatment, cells were collected, washed twice with PBS(1×), and the RA of the cells was counted in a γ-counter to evaluate the total cellular uptake [18].

To assess the level of [^{99m}Tc]Tc-TAT-L-Sco2 fusion protein delivered into the mitochondrial fraction, K-562 cells were harvested with 0.6-1 mL ice-cold Mito-buffer solution (0.2 mM EDTA, 0.25 mM sucrose, and 10 mM Tris.HCl, pH 8.0), supplemented with protease inhibitors cocktail (Roche). The cell suspension was sonicated (up to two times) in an ultrasonic water bath (Bandelin, Sonorex) and the subcellular fractionation was conducted [18]. RA in both cytosolic fraction and mitochondrial pellet was counted in a γ-counter to determine total intracellular level of [^{99m}Tc]Tc-TAT-L-Sco2 and the level distributed in the mitochondrial fraction. The experiment was conducted in triplicate.

Furthermore, samples of mitochondrial extracts prepared, were heated at 65 °C, for 10 min, and analyzed in a 12% SDS-PAGE electrophoresis, without the denaturing agent β-mercaptoethanol (β-ME). The gel was fixed, dried and then autoradiographed with an intensifying film on Kodak XAR-5 film at −70 °C overnight (12-16 h), depending on the amount of sample RA.

2.7. Animal tissue subcellular distribution of [^{99m}Tc]Tc-TAT-L-Sco2

As a first step for the biodistribution studies, tissue subcellular distribution of [^{99m}Tc]Tc-TAT-L-Sco2 was assessed. For this, tissues (brain, heart, liver) from a healthy untreated Balb/c female mouse were removed to prepare sliced tissues as well as cultures of primary cells, derived from each tissue, by using collagenase treatment and form cell suspension. Tissues were placed into ice-cold RPMI-1640 medium (18 mL) and sliced in small pieces (sliced tissues). Part of the tissues were incubated with 2 mL of collagenase diluted in Hanks' Balanced Salt solution (Sigma-Aldrich) (4500-6000 units/mL), twice, at 37 °C, under vigorous shaking, followed by vortexing, until single-cells were released. The process was repeated three times and the three supernatants were centrifuged at 1100xrpm, for 5 min. The resulting pellet, corresponding to separated cells, was cultured in RPMI-1640 and maintained in exponential growth at 37 °C, 5% CO_2. The following day, cell number of each suspension culture was assessed (1.1×10^6–9.0×10^7).

Mouse sliced tissues and primary cell suspension cultures were treated with 3 μg of the [^{99m}Tc]Tc-TAT-L-Sco2 (1 MBq), in Opti-MEM, at 37 °C, for 1 h. Cells were harvested, washed twice with PBS(1×) and processed for subcellular fractionation. Briefly, cells were re-suspended in 1.4 mL ice-cold Mito-Buffer solution (0.2 mM EDTA, 0.25 mM sucrose, 10 mM Tris–HCl, pH 7.8), supplemented with protease inhibitors cocktail (Roche) and deep-freezed for 30 min, at −70 °C. Defrosted samples were disrupted by repeatedly passages through a 21-gauge needle and centrifuged at 1000 ×*g*, at 4 °C, for 10 min, to pellet nuclei and cellular debris. Post nuclear supernatant obtained was then centrifuged at 10,000 ×*g* for 15 min, at 4 °C, to collect intact mitochondria and stored at −70 °C. RA measurements were also conducted for each subcellular fraction.

2.8. Subcellular distribution of non-labeled TAT-L-Sco2 in mouse tissue's mitochondria

IBs, enriched in TAT-L-Sco2, were administered in healthy male BALB/c mice to study the subcellular distribution of TAT-L-Sco2 in the mitochondria of peripheral tissues, like brain, heart and liver. We used both *i.v.* and *i.p.* administration, to figure out if there is a significant difference in the biodistribution of protein. Three (3) mice were injected intravenously (*i.v.*) with 0.034 mg recombinant protein per 0.1 mL and other three (3) mice were injected intraperitoneally (*i.p.*) with 0.068 mg per 0.2 mL. Mice were euthanized, 30 min *p.i.,* by cervical dislocation. Organs and tissues of interest were excised rapidly and freezed at −70 °C. The tissue biodistribution of the TAT-L-Sco2 was evaluated in isolated mitochondrial protein fraction, followed by SDS-PAGE and western blot analysis, with anti-Sco2.IgG as well as anti-His.IgG. Anti-α-tubulin.IgG was used as constitutive control in the mitochondrial fraction, since tubulin is an inherent component of mitochondrial membranes [19].

2.9. Biodistribution of [^{99m}Tc]Tc-TAT-L-Sco2 in mice

For this experiment, twenty-four (24) male BALB/c mice, in total, were used. Firstly, nine (9) mice were *i.v.* injected *via* the tail vein with one dose of [^{99m}Tc]Tc-TAT-L-Sco2 (481 kBq/0.14 mg/0.1 mL) and divided in three (3) groups of three (3) mice, depending on the time point of sacrifice: at 1, 4 and 21 h, *p.i.* (post-injection). Furthermore,

another group of three (3) mice was injected *i.v.,* with one dose of [^{99m}Tc]Tc-TAT-L-Sco2 (370 kBq/0.12 mg/0.1 mL), and euthanized at 1 h, *p.i.*; this group of injected mice will be further referred as 1 h*.

Similarly, twelve (12) mice were *i.v.* injected with one dose of [^{99m}Tc]Tc-mock (370 kBq/0.18 mg/0.1 mL) and divided in three (3) groups of four (4) to be sacrificed, by cervical dislocation, 1, 4 and 21 h, *p.i.,* served as negative controls. The organs and tissues of interest were harvested, weighed and measured in a γ-counter.

The activity of the tissue samples was decay-corrected and calibrated by comparing the counts in the tissue with the counts of a standard solution, corresponding to 1% of the injected dose. Counts of the sample and calibration aliquots were measured in the γ-counter, at the same time. The amount of activity in the selected tissues and organs is expressed as a percent of the injected dose (ID) per organ/tissue (%ID/organ) or per gram tissue (%ID/g) [18]. Values are quoted as the mean ID% ± SD of the three or four mice per group. Blood, muscle and bone mass were estimated at 7, 43 and 10% of body weight, respectively. Tissues of interest were also evaluated by mitochondrial protein enrichment, followed by SDS-PAGE and western blot analysis, with the anti-Sco2.IgG and the anti-His.IgG. Anti-α-tubulin.IgG was also used as constitutive control for normalization purposes [19].

3. Results

3.1. Radiolabeling of TAT-L-Sco2 with ^{99m}Tc

In our previous studies, we have produced the fused TAT-L-Sco2 as a recombinant therapeutic protein (biopharmaceutical) to be delivered into the mitochondria of human fibroblasts, deficient in COX, due to mutations in the mitochondrial Sco2 protein.

Produced IBs, enriched in TAT-L-Sco2 protein, were proceeded in labeling with ^{99m}Tc, according to literature methods [17], as shown under material and methods, to perform biodistribution studies in animals. The protein was thiolated by 2-iminothiolane treatment prior to ^{99m}Tc-labeling (Online Resource Fig. S1). The final thiol concentration after the treatment, according to the Ellman's method, was approximately 1×10^{-3} M (the thiol concentration of the untreated TAT-L-Sco2 was 0.7×10^{-3} M). The radiochemical yield of the labeling mixture was determined by radiochromatography and found to be 73 ± 9%.

The stability of [^{99m}Tc]Tc-TAT-L-Sco2 was found to be quite high, even up to 24 h following the labeling reaction, as shown in the SE-chromatogram (Fig. 1, A, B). Further, the analysis of the intracellular uptake [^{99m}Tc]Tc-TAT-L-Sco2 after 1, 4 and 21 h (Table 1) indicated that the protein-bound RA (% of the total incubated RA) in the plasma, found to be unchanged for the different time-intervals, strongly supporting that [^{99m}Tc]Tc-TAT-L-Sco2 remains intact in plasma under *in vitro* conditions.

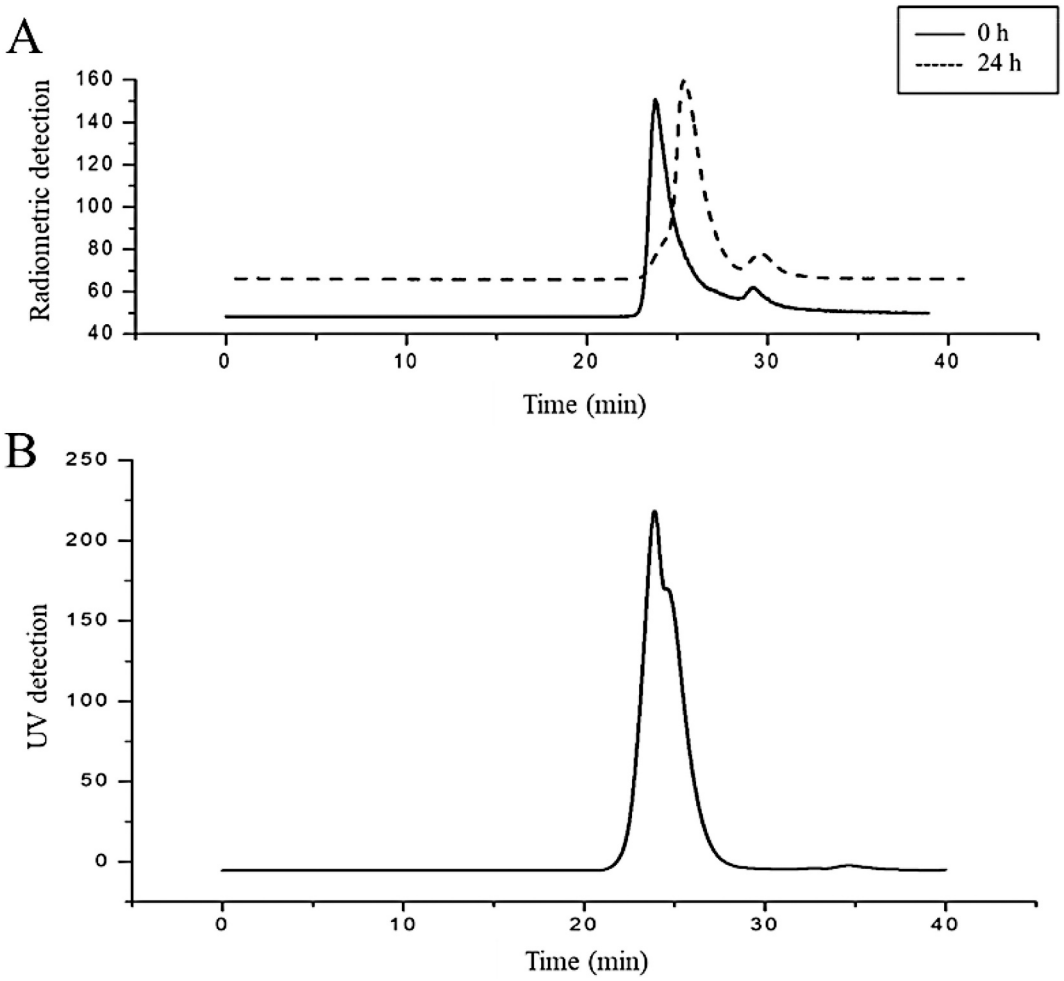

Fig. 1. Characterization of the labeling mixture of [^{99m}Tc]Tc-TAT-L-Sco2.
A. Size-exclusion chromatography of [^{99m}Tc]Tc-TAT-L-Sco2] at 0 h (solid line) and at 24 h (dashed line), R_f = 23.8 min; B. UV detection of the TAT-L-Sco2 protein, R_f = 24.3 min.
(^{99m}Tc,Technetium-99 m)

Table 1
Assessment of [^{99m}Tc]Tc-TAT-L-Sco2 cell uptake and stability in human plasma.

Duration of incubation	Cell uptake by blood cells	Protein-bound RA in plasma
1 h	28%	86%
4 h	20%	81%
21 h	15%	72%

(^{99m}Tc:Technetium-99 m)

3.2. Intracellular distribution of [^{99m}Tc]Tc-TAT-L-Sco2 into the mitochondria of K-562 cells

Cultured K-562 cells, incubated with [^{99m}Tc]Tc-TAT-L-Sco2, exhibited 18.5±12.9%(SD) of total RA accumulated intracellularly. Following K-562 cells lysis and isolation of the mitochondrial fraction, indicated that the 27±10.4%(SD) of the total intracellular RA was pertained to the isolated mitochondria, as shown by three biological experiments. Incubation of K-562 cells with only pertechnetate and ^{99m}Tc-gluconate conducted as control experiments, showed <1% of RA in the mitochondrial fraction.

Analysis of the mitochondrial protein extracts, prepared from K-562 cells incubated with [^{99m}Tc]Tc-TAT-L-Sco2 by SDS-PAGE and autoradiography confirmed the delivery of the [^{99m}Tc]Tc-TAT-L-Sco2 in the mitochondrial fraction, as expected [5] (Fig. 2, A). Similarly, analysis of the same mitochondrial extracts of K-562 cells by SDS-PAGE, followed by western blot analysis, with the use of anti-HA.IgG (Fig. 2, B), revealed the exogenously administered TAT-L-Sco2, thus confirming data derived from the autoradiography. This finding, overall, confirmed intracellular distribution of radiolabeled TAT-L-Sco2 fusion protein into mitochondria.

3.3. Subcellular distribution of [^{99m}Tc]Tc-TAT-L-Sco2 in sliced tissues and suspension cell cultures, derived from animal tissues

Sliced tissues as well as suspension cell cultures, derived from untreated mouse tissues (brain, heart and liver) were incubated with [^{99m}Tc]Tc-TAT-L-Sco2, for 1 h, at 37 °C, the corresponding mitochondrial fraction was isolated, and RA was counted. As shown in Table 2, [^{99m}Tc]Tc-TAT-L-Sco2 protein was taken up by both sliced tissues and cell suspension cultures and transduced into the mitochondria, as expected, based on our previous work [5].

3.4. Biodistribution of injected TAT-L-Sco2 in animals

A time-dependent stability studies of TAT-L-Sco2 protein in human plasma (derived from a healthy donor) was firstly conducted for various time intervals. As shown in Fig. 3, remarkable structural stability of TAT-L-Sco2 was observed from 5 min up to 48 h incubation in plasma.

IBs, enriched in TAT-L-Sco2 were *i.v.* and *i.p.* injected in two (2) groups of three (3) mice and 30 min, *p.i.*, organs were removed. Mitochondrial fractions, derived from each tissue, were prepared and analyzed by SDS-PAGE, complemented by western blot analysis and staining, with anti-Sco2.IgG (Online Resource Fig. S2). Due to the high structural homology of the human Sco2 to that of the mouse, it was considered critical to exclude possible cross reactivity with the anti-Sco2.IgG to recognize the naturally present endogenous murine Sco2. Thus, by immunostaining the same samples with the anti-His.IgG that selectively detects the histidine tag of the TAT-L-Sco2 (10xHis-Xa$_{SITE}$-TAT-L-Sco2-HA, 36 kDa) (Fig. 4), only the exogenously administered TAT-L-Sco2 was detected, while by immunostaining with the anti-Sco2. IgG both the transduced recombinant TAT-L-Sco2 and the endogenous murine Sco2 were detected. An increase in protein levels of TAT-L-Sco2 was observed over the control samples, in heart, liver and brain.

3.5. Biodistribution of [^{99m}Tc]Tc-TAT-L-Sco2 in mice

Intravenous administration of the [^{99m}Tc]Tc-TAT-L-Sco2 was performed in healthy mice and the biodistribution results, shown in Fig. 5 and Online Resource Tables S2, S3, indicate fast blood clearance and hepatobiliary excretion.

In particular, in blood 3.34 ± 0.47, 2.86 ± 1.5, 0.31 ± 0.07% ID/g at 1, 4 and 21 h (respectively), *p.i.,* was detected, while the liver received the majority of the injected dose, with % ID/g 28.26 ± 6.07, 22.08 ± 1.47 and 8.07 ± 1.05 at 1, 4 and 21 h, *p.i.* Furthermore, due to the TAT peptide, the protein can be intracellularly delivered also in the kidney cells, with % ID/g in the kidneys of 5.15 ± 1.65, 5.20 ± 0.20 and 3.48 ± 2.15 at 1, 4 and 21 h, *p.i.,* respectively. Significant urinary excretion was observed, with % ID 23.49 ± 12.00 and 26.56 ± 2.07 at 1 and 4 h, *p.i.* Even though proteins exhibited mainly hepatobiliary excretion, lower molecular weight fragments and/or metabolites could be excreted *via* the urinary tract. The spleen also exhibited notable accumulation, with % ID/g of 24.05 ± 3.15, 17.87 ± 3.99 and 8.77 ± 1.18 at 1, 4 and 21 h, *p.i.* The brain, the heart and the muscle exhibited low uptake of the

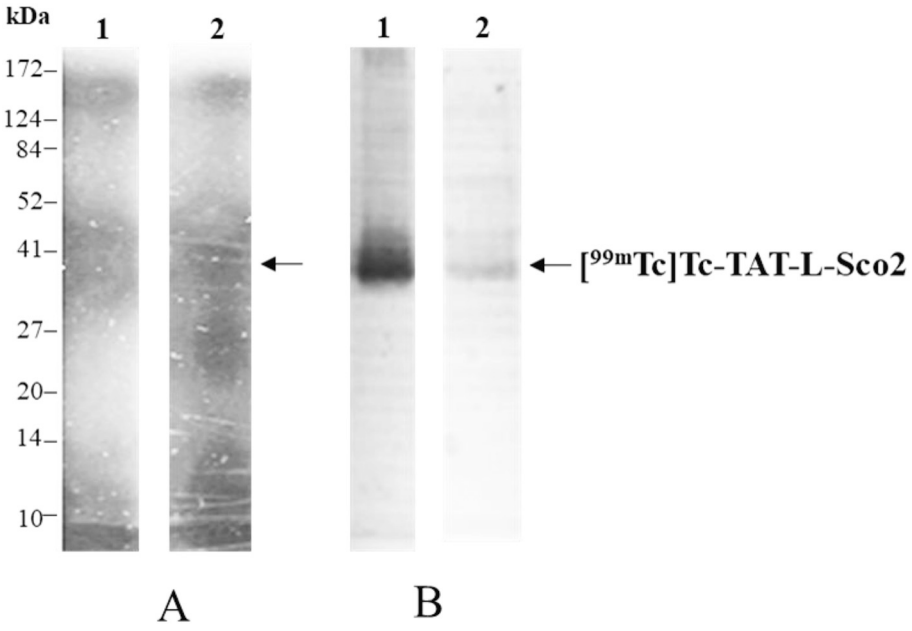

Fig. 2. Mitochondrial uptake of [^{99m}Tc]Tc-TAT-L-Sco2 in K-562 cells.
A. Autoradiogram; B. SDS-PAGE - western blot analysis, by using the anti-HA.IgG.
Lane 1: [^{99m}Tc]Tc-TAT-L-Sco2 (36 kDa); Lane 2: Mitochondrial protein extracts, derived from K-562 cells, incubated with [^{99m}Tc]Tc-TAT-L-Sco2.
(^{99m}Tc,Technetium-99 m)

Table 2
Subcellular distribution of [^{99m}Tc]Tc-TAT-L-Sco2, in sliced tissues and suspension cultured cells, derived from three tissues (brain, heart and liver).

	Mitochondria					Cytoplasm				
	Sliced tissues			Suspension cells		Sliced tissues			Suspension cells	
Tissue	% Mean	SD	n[a]	% Mean	n[a]	% Mean	SD	n[a]	% Mean	n[a]
Brain	56.6%	–	2	48%	2	43.4%	–	2	52%	2
Heart	41.7%	–	2	69.1%	1	58.3%	–	2	30.9%	1
Liver	60.3%	9.2%	4	51.1%	1	39.7%	9.2%	4	48.9%	1

(^{99m}Tc:Technetium-99 m; SD:Standard Deviation)
[a] n = number of independent incubation experiments.

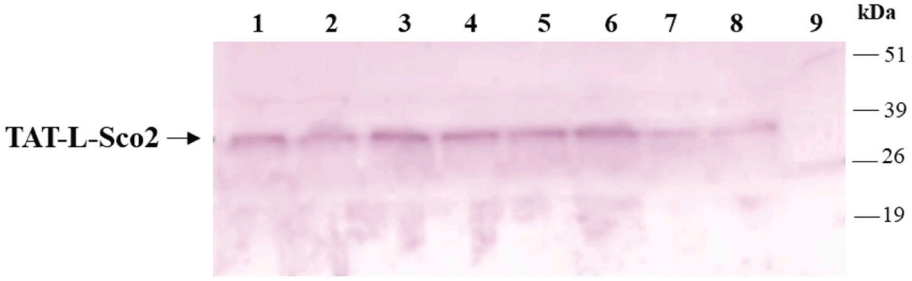

Fig. 3. Assessment of TAT-L-Sco2 stability in human plasma.
SDS-PAGE, 12%, followed by western blot analysis of 1 μg of TAT-L-Sco2 samples incubated in human plasma, using anti-Sco2.IgG, revealing structural stability as a function of time of incubation, from 5 min to 48 h.
Lane 1: 5 min; Lane 2: 15 min; Lane 3: 30 min; Lane 4: 1 h; Lane 5: 2 h; Lane 6: 4 h of incubation, Lane 7: 24 h of incubation; Lane 8: 48 h of incubation; Lane 9: protein molecular mass marker.

radiolabeled TAT-Sco2 fusion protein, with the lowest levels recorded in the brain.

In addition, biodistribution studies of [^{99m}Tc]Tc-TAT-L-Sco2 were repeated once more for 1 h, *p.i.*, and indicated as 1 h* (Fig. 5 and Online Resource Tables S2, S3). Considering the biodistribution after *i.v.* administration of [^{99m}Tc]Tc-TAT-L-Sco2 (481 kBq/0.14 mg/0.1 mL) for 1 h, in comparison with 1 h* treatment of [^{99m}Tc]Tc-TAT-L-Sco2 (0.12 mg/0.1 mL, 370 kBq), similar profile was obtain in almost all organs. The biodistribution profile of [^{99m}Tc]Tc-mock differs from [^{99m}Tc]Tc-TAT-L-Sco2' profile (Online Resource Tables S4, S5) mainly in a much higher stomach uptake, indicating more [^{99m}Tc]-pertechnetate formation.

Following the decay of radioactivity, mitochondrial fractions prepared from heart, muscle, liver, spleen and brain tissues from the biodistribution studies, as for 1 h*, were analyzed (Fig. 6). Western blot analysis confirmed the ability of the exogenously administered TAT-L-Sco2 to transduce the plasma membrane and localize in the mitochondria, by both anti-His.IgG staining (Fig. 6) and anti-Sco2.IgG (Online Resource Fig. S3).

4. Discussion

PTD technology permits intracellular delivery of therapeutic molecules, by facilitating penetration of almost all biological membranes, without interfering with the endogenous genetic information [2,20]. Our team has applied an *in vitro* PRT approach *via* the PTD technology, in the context of: a) β-thalassemia, with TAT-fused β-globin into K-562 cells to replace missing endogenous β-globin [13] and b) a mitochondrial disorder, due to *SCO2* mutations [5].

To pave the way to eventual clinical application of a TAT-mediated PRT for mitochondrial disorders attributed to *SCO2* mutations, we moved on to investigate the fate of TAT-L-Sco2 in peripheral tissues in mice, being able to cross the plasma membranes, due to TAT peptide, and reach mitochondria.

Radiolabeling with γ-emitting radionuclide enables efficient detection of protein in biological systems [16], permitting the assessment of its *in vivo* biodistribution, by increasing the sensitivity of the detection of even very low levels. ^{99m}Tc has excellent nuclear properties for *in vivo* diagnostic and imaging applications and was chosen for our biodistribution preclinical study. Radiolabeling of TAT-L-Sco2 with ^{99m}Tc was initially performed by direct labeling (without pretreatment with 2-iminothiolane) with stannous chloride and sodium dithionite as reducing agents, but low yields were obtained. The most efficient method proved to be the one involving thiolation with 2-IT and labeling with [^{99m}Tc]-Na[Tc-gluconate] with yields reaching up to 73 ± 9% of total protein being obtained. Small molecule impurities were removed by centrifugation, while [^{99m}Tc]Tc-TAT-L-Sco2 remained stable up to 24 h in solution. [^{99m}Tc]Tc-TAT-L-Sco2 protein was not only transduced in cultured K-562 cells, and individual cell suspension cultures prepared from animal tissues, but also detected in the mitochondrial fraction of animal tissue cell cultures, as expected, based on our previous results [5].

As a first step for the biodistribution studies, the stability of the radiolabeled TAT-L-Sco2 was assessed in human plasma and found to be quite high, while human whole blood stability studies of [^{99m}Tc]Tc-TAT-L-Sco2 indicated no decomposition of the radiolabeled protein to occur up to 21 h incubation. Upon both *i.v.* and *i.p.* administration of IBs, enriched in TAT-L-Sco2, in healthy mice, analysis of the mitochondrial fraction isolated from each peripheral tissue including heart, brain and liver removed from TAT-L-Sco2-treated mice, revealed that our recombinant protein, not only entered the cells, even in the brain, but it was also delivered directly into the mitochondria. Although in both cases, *i.v* and *i.p.,* the same concentration of recombinant protein is used, however, upon *i.p.* injection the total amount of protein was 2-fold higher, resulted also in the larger incorporation of injected recombinant protein in the brain mitochondrial fraction. The discrepancy in distribution between brain and other peripheral tissues is not easy to be explained; The fact that this difference in accumulation was not seen in the other tissues examined, could be attributed to: a) the physicochemical properties of the recombinant protein, affecting the rate of absorption, distribution (and finally transfer) of the recombinant protein from the site of injection (peritoneum) to the penetration of Blood Brain Barrier (BBB) and accumulation to the brain; b) the peculiar cellular, physiological and molecular structure of BBB *vs* the cellular components in the other tissues; or even c) the selective tissue-specific processing of the transduced TAT-L-Sco2 fusion protein administered.

As a next step, we proceeded in *in vivo* administration of [^{99m}Tc]Tc-TAT-L-Sco2 into healthy Balb/c mice. The biodistribution results revealed fast blood clearance, significant renal excretion and high liver and spleen uptake. These results are quite reasonable, as these latter organs are also the main pathway for catabolism and removal of large molecule proteins [21]. Furthermore, since this work serves also as a

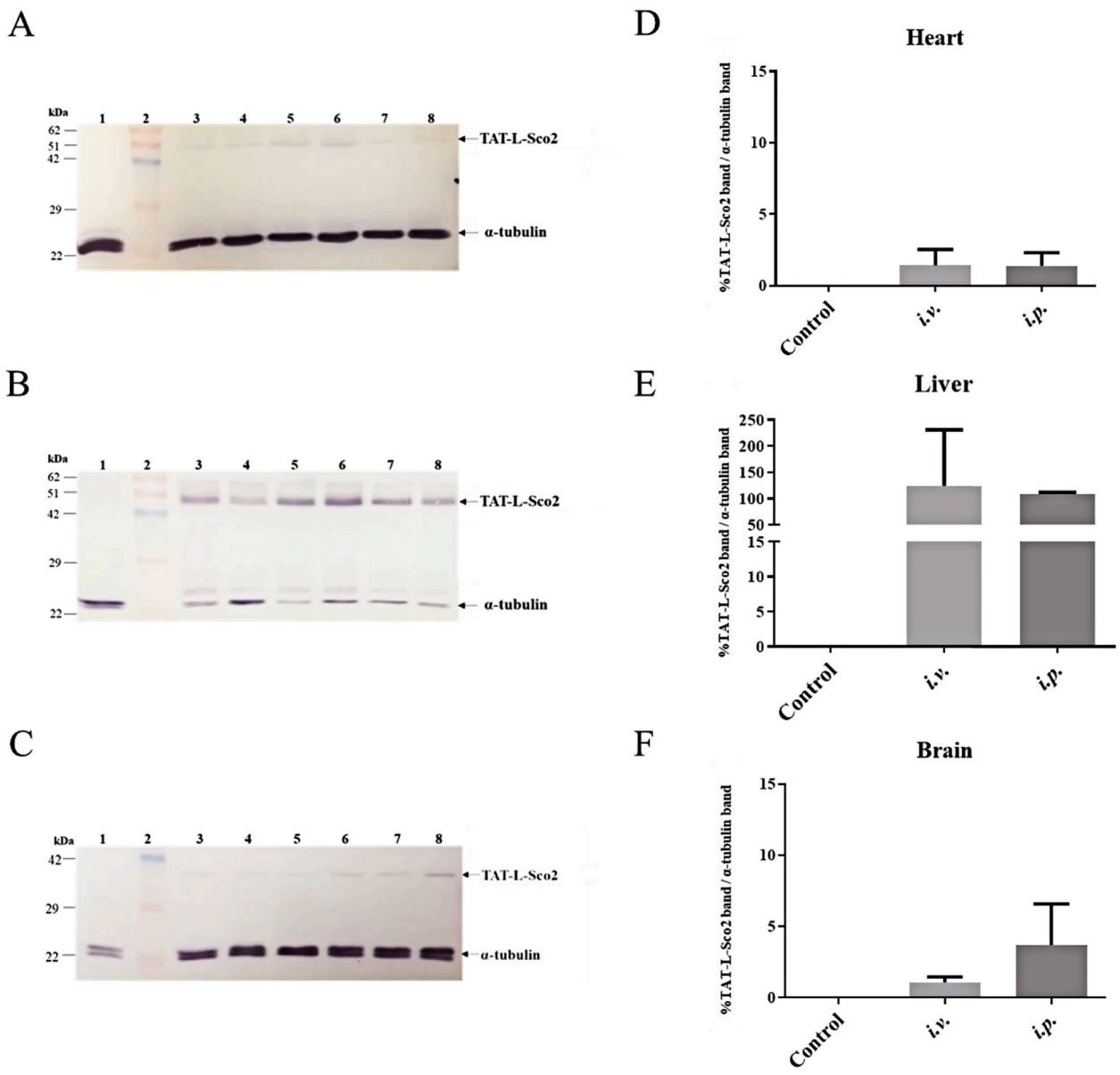

Fig. 4. Transduction of TAT-L-Sco2 in mitochondria derived from heart, liver and brain tissues, 30 min after administration (*i.v.* and *i.p.*) of IBs, enriched in TAT-L-Sco2, in mice.

Western blot analysis of electrophoresed mitochondrial protein extracts (70 μg): A. heart tissues, B. liver tissues and C. brain tissues, analyzed and blotted with anti-His.IgG/anti-α-tubulin.IgG.

Lane 1: Control (untreated); Lane 2: protein molecular mass marker; Lanes 3–5: three *i.v.* injected mice, with TAT-L-Sco2; Lanes 6–8: three *i.p.* injected mice, with TAT-L-Sco2.

Diagrams showing band intensity by comparison of TAT-L-Sco2/α-tubulin, after immunostaining with anti-His.IgG/anti-α-tubulin.IgG, based on Image Studio Lite, in control mouse and *i.v./i.p.* injected mice with the IBs, enriched in TAT-L-Sco2, in heart tissues (D), in liver tissues (E) and in brain tissues (F).

(IBs:Inclusion bodies; *i.p.*:Intraperitoneal; *i.v.*:Intravenous)

model system for protein therapy of mitochondrial disorders, it is worth mentioning that mutations in another *SCO*-like gene, *SCO1* [22], lead to neonatal-onset hepatic failure and encephalopathy. In a similar protein therapeutic approach, high liver uptake of a corresponding recombinant TAT-Sco1 would be in the right direction therapeutically.

Concerning the high energy demanding tissues, like heart, muscle and brain, low RA levels (% ID/g) were observed. However, steady-state levels of human *SCO2* transcripts from various tissues were much lower than that of *SCO1* and *β-actin*, based on exposure time during autoradiography [4]. Thus, even low levels of delivered TAT-L-Sco2 could be enough to complement the COX deficiency in tissues, like heart, muscle and brain.

Pertechnetate is a substrate of sodium-iodide symporter and therefore it is accumulated in tissues expressing it, like the gastric mucosa [23]. Elevated levels of RA in stomach could be due to breakdown of the radiolabeled protein [24]. Following the time, needed for the decay of ^{99m}Tc, isolation of mitochondrial fraction from several organs and measuring RA indicated the ability of the exogenously administered protein not only to reach organs, but also reach and accumulate into their mitochondria, as expected. Interestingly, western blot analysis of the mitochondrial samples with the anti-His.IgG (which selectively targets the histidine-tag of [10xHis-Xa$_{SITE}$-]TAT-L-Sco2-HA), revealed a clear band at ~51 kDa in heart, muscle, spleen and liver, instead of the expected band at 36 kDa for the TAT-L-Sco2, that is however the band

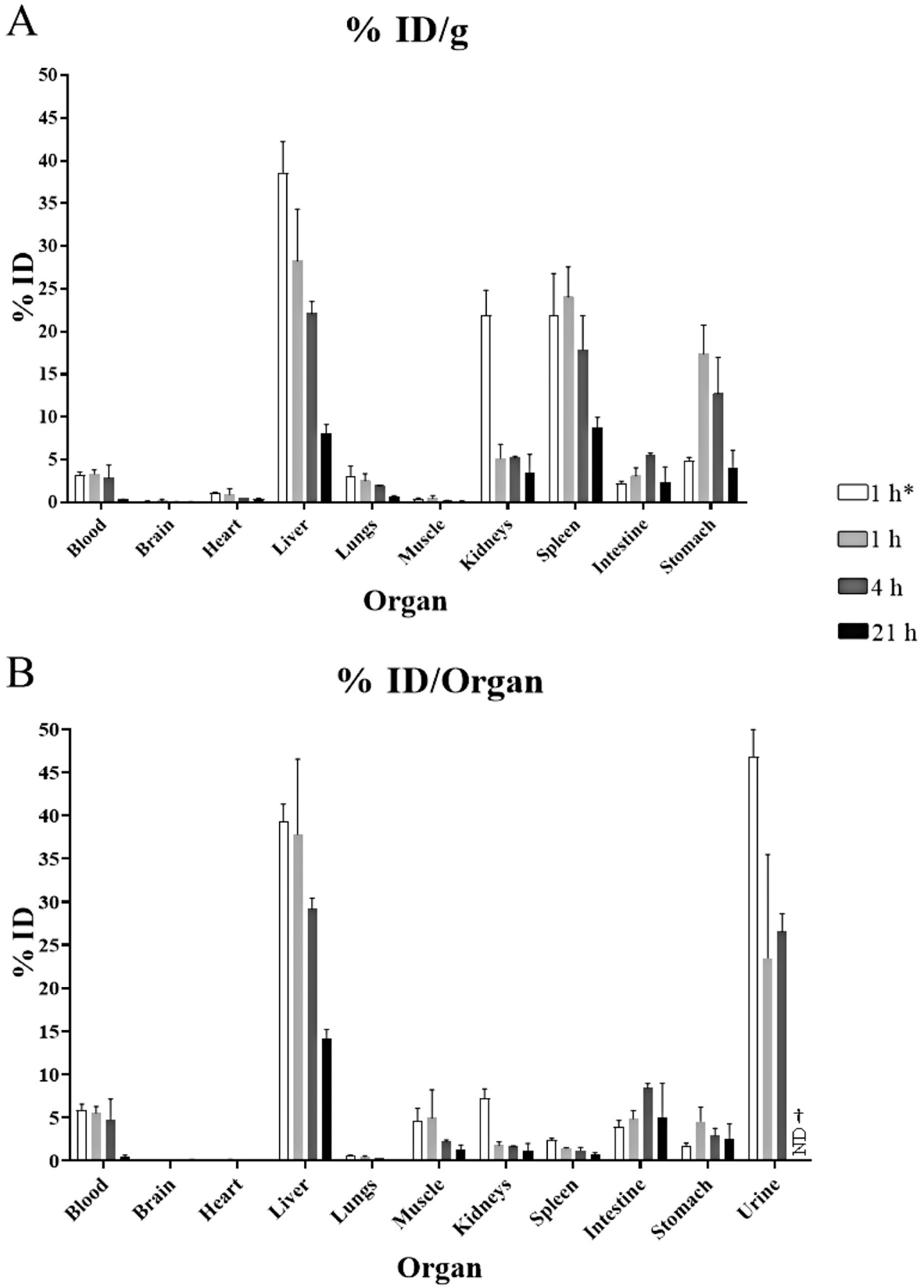

Fig. 5. Tissue biodistribution of [^{99m}Tc]Tc-TAT-L-Sco2 (0.12 mg/ 0.1 mL, 370 kBq) after 1 h (indicated as 1 h*) and [^{99m}Tc]Tc-TAT-L-Sco2 (0.138 mg/0.1 mL, 481 kBq) preparations in healthy Balb/c mice 1, 4, 21 h, following *i.v.* administration: A. Percentage of injected dose per gram (ID/g); B. percentage of injected dose per organ (ID/organ). (ND †: Not determined – Non-collected sample).
(^{99m}Tc,Technetium-99 m; ID:Injected dose; *i.v.*:Intravenous)

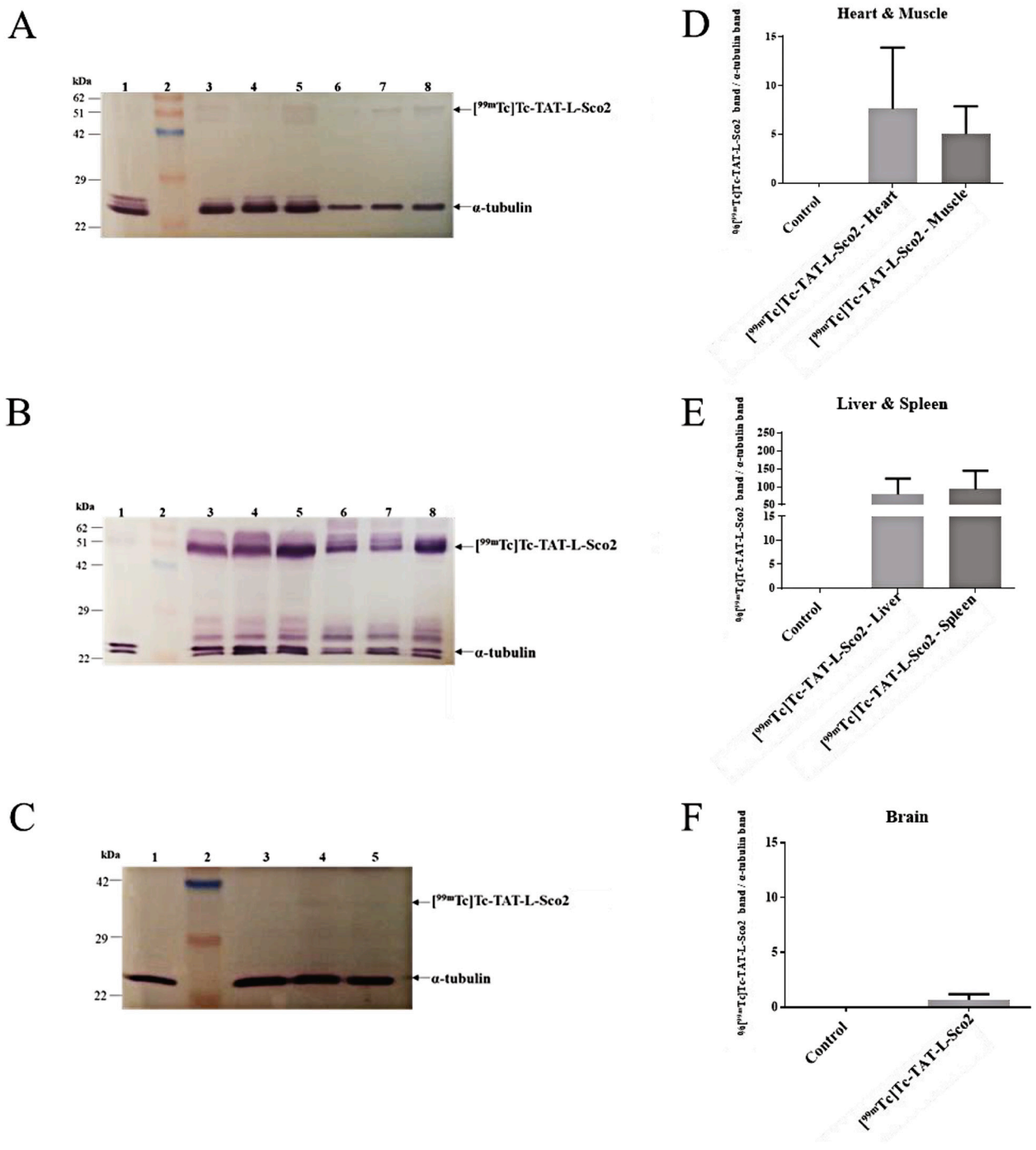

Fig. 6. Transduction of [^{99m}Tc]Tc-TAT-L-Sco2, in mitochondria derived from heart and muscle tissues, liver and spleen tissues and brain tissues, 1 h after *i.v.* administration of [^{99m}Tc]Tc-TAT-L-Sco2, in mice.

Western blot analysis, of electrophoresed mitochondrial protein extracts (70 μg): A. heart and muscle tissues, B. liver and spleen tissues and C. brain tissues, analyzed and blotted with anti-His.IgG/anti-α-tubulin.IgG.

A. Heart and muscle tissues. Lane 1: Control (untreated) heart tissue; Lane 2: protein molecular mass marker; Lanes 3–5: heart tissues of three *i.v.* injected mice, with [^{99m}Tc]Tc-TAT-L-Sco2; Lanes 6–8: muscle tissues of three *i.v.* injected mice, with [^{99m}Tc]Tc-TAT-L-Sco2.

B. Liver and spleen tissues. Lane 1: Control (untreated) liver tissue; Lane 2: protein molecular mass marker; Lanes 3–5: liver tissues of three *i.v.* injected mice, with [^{99m}Tc]Tc-TAT-L-Sco2; Lanes 6–8: spleen tissues of three *i.v.* injected mice, with [^{99m}Tc]Tc-TAT-L-Sco2.

C. Brain tissues: Lane 1: Control (untreated) brain tissue; Lane 2: protein molecular mass marker; Lanes 3–5: brain tissues of three *i.v.* injected mice, with [^{99m}Tc]Tc-TAT-L-Sco2.

Diagrams showing band intensity by comparison of [^{99m}Tc]Tc-TAT-L-Sco2/α-tubulin, after immunostaining with anti-His.IgG/anti-α-tubulin.IgG, based on Image Studio Lite, in control mouse and *i.v.*. injected mice with [^{99m}Tc]Tc-TAT-L-Sco2, in heart and muscle tissues (D), in liver and spleen tissues (E) and in brain tissues (F). (^{99m}Tc:Technetium-99 m; *i.v.*:Intravenous)

found in the brain samples. Since high levels of reducing agent was used in this experiment (710 mM of β-mercaptoethanol), the band of ~51 kDa could be attributed to either: a) different processing of the transduced recombinant protein or the reduction state of the proteins' thiol groups inside mitochondria in heart, muscle, spleen and liver than in brain; b) different conformation (chemical modifications, like glycosylation, ubiquitination, sumoylation) of this protein in these tissues, which could result in drastically retarded gel mobility shift; or c) different electrophoretic profile of the mitochondrial protein fractions, as observed with other proteins (*e.g.* p53) [25–27]. Alternatively, a dimer form of the recombinant protein could also be formed into these tissues, as we have previously shown [15], while the reduction state of the thiol groups in TAT-L-Sco2 fusion protein inside the mitochondria from the different tissues could also play a role.

5. Conclusions

In conclusion, we prepared the radiolabeled [^{99m}Tc]Tc-TAT-L-Sco2 fusion protein for the first time, which after administration into mice was shown to be biodistributed in the peripheral tissues, exhibits fast elimination from the circulation, and successfully delivered-transduced into mitochondria, *in vivo*.

This work aimed to potentially treatment of mitochondrial disorders, attributed to *SCO2* mutations, but also has provided a model system for the biodistribution of future novel biopharmaceutical proteins, *via* the PTD technology, in the frame of protein therapy of metabolic/genetic disorders.

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Availability of data and material

Data are available on request.

Author contributions

GCK performed all the experimental work, data analysis and contributed in writing. DP performed all the radiolabeling experiments and contributed in consulting and writing. AMN participated in the collagenase experiment and contributed in data analysis and preparation of manuscript. AnST participated in preliminary *in vivo* experiments with non-radiolabeled TAT-L-Sco2. PCC participated in preliminary *in vivo* experiments with non-radiolabeled TAT-L-Sco2. ACT participated in preliminary expression-purification experiments of TAT-L-Sco2. GAS consulted and participated in preliminary expression-purification experiments of TAT-L-Sco2. AST contributed to the writing of the paper and consulted on the overall organization of the manuscript. LCP designed, consulted and supervised experiments, analyzed and interpreted data and drafted the manuscript. She also provided the final approval of manuscript.

Declaration of Competing Interest

No conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgmr.2020.100683.

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