Label-Free Mass Spectrometry-Based Quantitative Proteomics to Evaluate the Effects of the Calcium-Sensing Receptor Agonist Cinacalcet on Protein Expression in Rat Brains and Livers

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Background: Cinacalcet is a calcium-sensing receptor agonist that is clinically approved for the treatment of secondary hyperparathyroidism in chronic kidney disease and hypercalcemia in patients with parathyroid carcinoma. This study aimed to use quantitative mass spectrometry-based label-free proteomics to evaluate the effects of cinacalcet on protein expression in rat brains and livers.

Material/Methods: We randomly assigned 18 Wistar rats to 2 groups: an untreated control group (n=6) and a group treated with cinacalcet at a dose corresponding to the maximum dose used in humans (2 mg/kg/body weight, 5 days/week) divided into 7-day (n=6) and 21-day (n=6) treatment subgroups. A mass-spectrometry-based label-free quantitative proteomics approach using peptides peak area calculation was used to evaluate the changes in protein expression in examined tissues. Bioinformatics analysis of quantitative proteomics data was done using MaxQuant and Perseus environment.

Results: No changes in protein expression were revealed in the 7-day treatment subgroup. We detected 10 upregulated and 3 downregulated proteins in the liver and 1 upregulated protein in the brain in the 21-day treatment subgroup compared to the control group. Based on Gene Ontology classification, all identified differentially expressed proteins were indicated as molecular functions involved in the enzyme regulator activity (36%), binding (31%), and catalytic activity (19%).

Conclusions: These findings indicate that long-term cinacalcet therapy can impair phase II of enzymatic detoxication and can cause disturbances in blood hemostasis, lipid metabolism, and inflammatory mediators or contribute to the acceleration of cognitive dysfunction; therefore, appropriate patient monitoring should be considered.

Keywords: Cinacalcet • Pharmacology, Clinical • Proteomics • Renal Insufficiency, Chronic

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Cinacalcet is a type II calcimimetic. It is recommended in secondary hyperparathyroidism or calciphylaxis, mainly in patients with end-stage chronic kidney disease (CKD), especially hemodialysis or primary hyperparathyroidism, when the patients are not subjected to surgical treatment [1].

Renal dysfunction is associated with metabolic disorders of calcium, phosphorus, and vitamin D, including abnormalities of the Ca-sensing receptor (CaSR), vitamin D receptor, and fibroblast growth factor 23. The physiological response to these abnormalities includes increased serum parathyroid hormone (PTH) and parathyroid hyperplasia. It is associated with cardiovascular disease and fracture risk factors, resulting in lower quality of life and higher mortality [2, 3]. Secondary hyperparathyroidism occurs in about 70% of patients with advanced CKD and about 55% of hemodialyzed patients [4, 5]. The global all-age prevalence of CKD was 9.1% in 2017, which equals 697.5 million cases, with a constantly increasing trend since 1990 [6]. CaSR detects minor changes in serum concentration of Ca²⁺ and maintains PTH accordingly. CaSR is located on the cell membrane of chief cells of the parathyroid gland and is coupled with 7-transmembrane domain G-proteins. Once activated, CaSR inhibits the accumulation of cAMP and simultaneously stimulates the phosphoinositide-phospholipase C pathway, ultimately leading to suppression of PTH secretion [7].

Cinacalcet acts as an allosteric activator of the CaSR, but it is not a true ligand. Cinacalcet changes the structural conformation of CaSR, thus increasing the sensitivity to eCa²⁺. It shifts the Ca-PTH concentration-response curve to the left and finally decreases PTH secretion in a dose-dependent manner [8, 9]. The recommended starting dose of cinacalcet for adults is 30 mg, and the maximum dose is 180 mg administered once daily. Cinacalcet has dose-proportional pharmacokinetics. After oral administration, its peak plasma concentration occurs after 2-6 hours, with absolute bioavailability of 20%-25%. The half-life elimination is 30-40 hours, and the steady-state concentration is achieved after about 7 days [10, 11]. Cinacalcet is principally eliminated by oxidative metabolism in the liver mediated by hepatic cytochrome P450 enzymes. The primary route of its metabolism is N-dealkylation leading to carboxylic acid derivatives and oxidation of the naphthalene ring to form dihydrodiols. Metabolites of cinacalcet in humans are excreted in urine and feces in the proportion of about 80% to 15%, in the form of glycine conjugates (urine), and as glucuronide conjugates (urine and feces) [11-14].

Cinacalcet has some adverse effects, for which the mechanism remains unclear. Such adverse effects include nausea, vomiting, dizziness, asthenia, anorexia, and hypertension [14]. Cinacalcet is widely used among patients with CKD. Those patients, especially in the last stage, require many other drugs; therefore, the possible interaction between cinacalcet and other drugs should always be taken into account. Additionally, the therapy of CKD patients is long-term [15]; therefore, possible interactions and adverse effects could accumulate. Taking into account the mode of metabolism and elimination of cinacalcet, and its direct or indirect possible influences on the central nervous system resulting in some of the adverse effects listed above, we aimed in this study to examine the influence of cinacalcet on protein expression in rat brain and liver tissues. Over the past few years, mass spectrometry (MS)-based proteomics has evolved significantly [16-20]. Sample preparation and the efficiency of separation techniques are still evolving and improving instrument performance. To evaluate changes in protein expression after cinacalcet therapy, we used a novel methodology with a mass spectrometry-based label-free quantitative proteomics approach using peptides peak area calculation.

Material and Methods

All experiments were performed following the relevant European Union guidelines and regulations for the care and use of laboratory animals. The appropriate Ethics Committee approved the experimental protocol (permission number: WAW2/055/2018, Warsaw, Poland). A mass-spectrometry-based label-free quantitative proteomics approach was used to evaluate the changes in protein expression in brain and liver tissue following treatment with cinacalcet. The method used was based on the measurement of peak areas, which covers calculating and comparing the mean intensity of peak areas for all peptides from each protein in the biological sample. The methodology includes the following steps: (1) sample preparation, including protein extraction, reduction, alkylation, and in-solution digestion; (2) chromatographic separation by liquid chromatography (nano-UHPLC) and analysis by a high-resolution mass spectrometer (Orbitrap); and (3) bioinformatics analysis of quantitative proteomics data to identify differentially expressed proteins. The experimental workflow of label-free quantitative proteomics is summarized in Figure 1.

Samples

Eighteen adult male Wistar rats (Rattus norvegicus) aged 10-16 weeks and with similar body weight were used as described previously [18, 19]. Rats were randomly assigned to the following groups: the control group (n=6) and the group treated with cinacalcet (7-day group and 21-day group, n=6 in each group, with dose of 2 mg/kg/body weight, 5 days/week). Fresh frozen brain and liver tissues were used during the experiments.

Figure 1
Mass spectrometry-based proteomics

Brain and liver tissue samples → In-solution digestion → Chromatographic separation → High-resolution mass spectrometry

Search for significantly regulated proteins

MS data → Proteome identification and quantification → Statistical & bioinformatics → Differentially expressed proteins

Figure 1. Experimental workflow of label-free differential quantitative proteomics.

Instrumentation and reagents

The instrumentation used for sample preparation were: vacuum centrifuge 5804/5804 R (Eppendorf, USA), mechanical homogenizer Ultra-Turrax (IKA, Germany), laboratory incubator CLN 240 (MultiSerw, Poland), vortex shaker (IKA, Germany), thermomixer Eppendorf Comfort (Eppendorf, USA), and vacuum concentrator SpeedVac Concentrator Plus (Eppendorf, USA). Separations of peptides were obtained using a nano-UHPLC by UltiMate 3000 nanosystem (Dionex Ultimate Series UHPLC, Thermo Scientific, USA) equipped with an in-house-packed fused silica capillary C-18 column (75 μm×500 mm, particle size 1.9 μm) coupled to a high-resolution tandem mass spectrometer (Orbitrap Fusion Tribrid™ Mass Spectrometer, Thermo Scientific, USA). Analytical-grade reagents for proteomics analysis were obtained from Thermo Scientific (USA), Promega (USA), Merck (Germany), Waters (USA), and EMD Millipore (Germany).

Sample Preparation, Digestion, and Peptide Extraction

Each tissue piece (approx. 50 mg wet weight) was homogenized with 1 mL of lysis buffer (1% SDS and cOmplete™ EDTA-free protease inhibitor) (Merck, Germany) in 100 mmol/L ammonium bicarbonate (Merck, Germany), at room temperature for 15 min supported by a mechanically operated homogenizer. Protein supernatants were obtained by centrifugation for 30 min at 20 000×g. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA) following the manufacturer’s instructions. The volumes of protein extracts derived equivalent to 100 μg of proteins were precipitated overnight with acetone (-20°C, 800 μL; Merck, Germany). The samples were then centrifuged (20 000×g) at 4°C, and the supernatants were discarded. Dried protein pellets were then re-suspended in 50 μL of 0.1% RapiGest (Waters, USA) in 50 mmol/L ammonium bicarbonate (Merck, Germany). Then, the proteins were reduced with 75 μL of 5 mmol/L 1,4-dithiothreitol (Merck, Germany) for 45 min at 56°C and alkylated with 75 μL of 30 mmol/L acrylamide (Merck, Germany) for 30 min at room temperature in the dark. Alkylation was quenched by adding an equal-molar amount of 1,4-dithiothreitol and incubation at room temperature for 15 min. The samples were enzymatically digested for 18 h at 37°C with 75 μL of 20 ng/L trypsin Promega (USA) and 100 μL of 50 mmol/L ammonium bicarbonate (protein: enzyme weight ratio ~ 100: 1). The reaction was quenched by adding 150 μL of 5% formic acid aqueous solution (Merck, Germany). Pierce™ Peptide Desalting Spin Columns (Thermo Scientific, USA) were used to purify peptides.

LC-MS/MS Analysis

Dried peptide fractions were re-suspended in 100 μL of 5% acetonitrile (Merck, Germany), 0.1% formic acid, and subjected to LC-MS/MS analysis. Peptide mixtures (1 μg) were separated on a reverse-phase 50 cm long in-house packed C18 fused silica column (75 μm inner diameter, ReproSil Gold 120 C18 1.9 μm beads, Dr. Maisch, Germany) using a UHPLC instrument (Ultimate 3000 nano-UHPLC, Thermo Fisher Scientific, USA) coupled on-line to an Orbitrap Fusion™ Tribrid™ mass spectrometer (Thermo Fisher Scientific, USA). Elution was performed over 90 min at a flow rate of 300 nL/min using gradient elution consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in 80% acetonitrile/20% water (solvent B). The optimal conditions for ion source were: positive ionization mode and capillary voltage of 1.9 kV. The other parameters of the mass spectrometer were: auxiliary gas flow: 15 L/min; ion transfer tube temperature: 300°C; 60 000 resolving power of Orbitrap for survey scan and 15 000 for MS2; type of fragmentation: higher-energy collisional dissociation and m/z range: 350-1600.

Data analysis and Statistical Analysis

Bioinformatics analysis of MS/MS raw data was performed using MaxQuant version 1.6.1.0 and searched using the Andromeda search engine [21,22]. The sequence database (Rattus norvegicus) used in the analysis was accessed from the UniProt database (n=29 928 protein isoforms, retrieved July 2021) [23]. Fixed modification was propionamidation on cysteine (C), which is the result of using acrylamide as an alkylating agent during sample preparation. Variable modifications were: fluorination of alanine (A), phenylalanine (F), tryptophan (W), and tyrosine (Y); oxidation of methionine (M); and...
acetylation of protein N-terminal were set as variable modifications. The trypsin enzyme (Trypsin Gold, Mass Spectrometry Grade, Promega) was adjusted as specific enzymatic digestion. Bioinformatic analysis was carried out in no-labeling mode and multiplicity was set to 1. During the main search, parent masses were allowed an initial mass deviation of 5 ppm and fragment ions were allowed a mass deviation of 10 ppm. The search for common contaminants was also considered during analysis. Peptides with a minimum of 7 amino acid lengths were considered. Identifications were filtered to a false discovery rate (FDR) of 0.01 at the PSM (peptide spectrum match) and protein level using the target-decoy approach to control for false discoveries. Quantitation of proteins was performed using LFQ (label-free quantification) with consideration of only razor and unique peptides. For protein quantification, the minimum ratio count was adjusted as 2. All other MaxQuant settings were arranged as default according to the protocol proposed by Tyanova et al [24]. Perseus software (version 1.6.14.0, Max Planck Institute of Biochemistry, Germany) was used to perform bioinformatics and statistical analysis using the output file from MaxQuant [25]. Using the obtained dataset, principal component analysis (PCA) was conducted using Perseus software. Perseus contains PCA analysis based on singular value decomposition [22]. Analysis of variance (ANOVA) was used to test for significant global differences between all 3 groups (control, after 7 days, and after 21 days of cinacalcet therapy). To compare the specific changes in protein expression between 2 groups (control vs after 21 days), the t test was used. A protein was considered to be differentially expressed when the difference between groups had an adjusted P value of ≤0.05. The fold change cut-off was set at 2 (s0=1).

**Results**

**Proteomics Analysis**

To investigate changes in the proteins’ expression after cinacalcet therapy in rat liver and brain tissue, we conducted bioinformatics analysis of the LC-MS/MS data using MaxQuant and Perseus software. These analyses identified about 2200 and 2500 proteins in brain and liver tissue samples, respectively.

To obtain an overview of the proteomic variability between the 3 groups (control, 7 days, and 21 days), principal component analysis was carried out (Figure 2). Data represent the results of PCA of log2 LFQ intensity values from each sample. Technical replicates are shown by matching colors – green: control group, blue: after 7 days, red: after 21 days. In case of differences in the histogram plots for individual sample replicates and no agreement in the Pearson correlation, replicates that were not well correlated were not considered in further analysis. The correlations between the replicates suggest a strong, positive association and higher than between samples from different groups. Principal component analysis of obtained data showed significant differences between the control and study groups (after 7 days and 21 days, respectively, of cinacalcet therapy).

Ten upregulated and 3 downregulated proteins in the liver and 1 upregulated protein in the brain showed altered expression levels in the group after 21 days of cinacalcet therapy in comparison with the control group. No downregulated proteins were detected for brain tissues after using very stringent statistical criteria (FDR=1%; s0=1; adjusted P<0.01) using the Perseus proteomics data environment. However, only 13 up/downregulated proteins after applying very stringent statistical criteria (FDR=1%; s0=1; adjusted P<0.01) during bioinformatic analysis were used for further analysis. The very stringent statistical criteria allow identifying only those changes in protein expression that are statistically most significant. The complete lists of all differentially expressed proteins are provided in Tables 1 and 2.

**Gene Ontology Analysis**

All differential proteins identified were assigned a gene symbol using the UniProt database [23]. Protein classification was performed based on functional annotations using gene ontology (GO) for molecular function [23]. Gene ontology classification of differentially expressed proteins under cinacalcet therapy was carried out (Figure 2). The correlations between the replicates suggest a...
therapy (Figure 3) is related to global changes in proteins expression identified in the liver and brain. All differentially expressed proteins were primarily involved in the enzyme regulator activity (36%), binding (31%), and catalytic activity (19%), the contribution of proteins in a given process (in parentheses) is indicated in relation to the number of all proteins involved in all processes.

### Discussion

The results of our study revealed a set of proteins in investigated tissues whose expression was increased or decreased after cinacalcet administration. It has been previously reported that long-term use of cinacalcet as the fluorinated drug induces fluorination of selected proteins in brain and liver tissue [18], but the possible relationship between these 2 phenomena is unclear. After 21 days of cinacalcet administration with a dose comparable to the mean dose used in humans, we identified only 1 protein with an increased concentration in brain tissue – Histone H1.0 N-terminally processed. According to UniProt [23], histones are usually found in cells that are in terminal stages of differentiation or that have low rates of cell division. In general, this protein is involved in DNA binding, which can negatively regulate chromatin silencing, DNA recombination, and transcription by RNA polymerase. It also participates in nucleosome assembly and positioning and regulation of transcription regulatory region DNA binding. According to Reactome [26], Histone H1 is present in 2 pathways: DNA damage and telomere stress-induced

| Student's t-test difference (protein fold) | Gene symbol | Protein description |
|------------------------------------------|-------------|---------------------|
| (a) Up-regulated protein expression (21 days vs control) in rats liver | TF | Serotransferrin |
| 3.86 | MGST2 | Microsomal glutathione S-transferase 2 |
| 2.93 | ERG28 | Ergosterol biosynthesis 28 homolog |
| 2.58 | LOC299282 | Serine protease inhibitor A3N |
| 2.47 | LRRP1 | Succinate dehydrogenase [ubiquinone] cytochrome b small subunit, mitochondrial |
| 2.43 | IGHG | Ig gamma-1 chain C region |
| 2.15 | KNG1L1 | T-kininogen 2 |
| 2.06 | EBP | 3-beta-hydroxysteroid-Delta(8) |
| 2.06 | FADS2 | Fatty acid desaturase 2 |
| 1.97 | KNG1 | T-kininogen 1 |

| Student's t-test difference (protein fold) | Gene symbol | Protein description |
|------------------------------------------|-------------|---------------------|
| (b) Down-regulated protein expression (21 days vs control) in rats liver | EIF4E | Eukaryotic translation initiation factor 4E |
| -1.81 | GSTA2 | Glutathione S-transferase |
| -3.10 | RPS28 | 40S ribosomal protein S28 |

Table 1. List of upregulated and downregulated protein expression after 21 days cinacalcet therapy in rat livers (FDR=1%; s0=1, adjusted P<0.01).

| Student's t-test difference (protein fold) | Gene symbol | Protein description |
|------------------------------------------|-------------|---------------------|
| (a) Up-regulated protein expression (21 days vs control) in rats brain | H1F0 | Histone H1.0 N-terminally processed |

Table 2. List of upregulated and downregulated protein expression after 21 days cinacalcet therapy in rat brains (FDR=1%; s0=1, adjusted P<0.01).
after cinacalcet administration, regulate the translation process. Those proteins, whose concentration in liver tissue decreased, regulate the translation process increased possess various and multiple functions. Some of them, such as Serotransferrin and Succinate dehydrogenase (ubiquinone) cytochrome b mitochondrial small subunit, are involved in metal ion transport, especially iron. Ergosterol biosynthesis 28 homolog and Fatty acid desaturase 2 are involved in lipid metabolism, while Succinate dehydrogenase (ubiquinone) cytochrome b mitochondrial small subunit is involved in the tricarboxylic acid cycle. Some of them, such as Serine protease inhibitor A3N and Ig gamma-1 chain C region, play a role in the inflammatory reaction, both innate and acquired, by, among others, positive regulation of cytokines production. Kininogens also play roles in immune reaction, such as in regulating hemostasis and acute-phase reaction. It was revealed that cinacalcet-treated HepG2 cells in the presence of oleic acid exhibited a 19% increased triglycerides content, and there was a 50-300% elevation in the expression of pro-inflammatory cytokines. These studies concluded that stimulation of CaSR in hepatic cells could elevate steatosis and increase proinflammatory cytokines.

Those proteins, whose concentration in liver tissue decreased after cinacalcet administration, regulate the translation process and mitotic cell cycle (Eukaryotic translation initiation factor 4E) or are the structural constituent of ribosome (40S ribosomal protein S28) [23].

Interestingly, the influence of cinacalcet administration on microsomal glutathione S-transferase 2 is reciprocal, in contrast to its influence on cytosolic glutathione S-transferase/glutathione S-transferase alpha-1. Glutathione transferases play an important role in phase II of enzymatic detoxication of xenobiotics. This detoxication is divided into 3 separate phases, but they are integrated with each other. Phase I is catalyzed mainly by cytochrome P450 (CYP). The cytochrome P450 superfamily is a family of enzymes, including CYP2D6. Standard nomenclature for this family includes prefix CYP, followed by the number for the family, then the number for the subfamily, and finally the number for the specific gene. It had previously been reported that cinacalcet is a strong inhibitor of CYP2D6 [11,30]. Glutathione S-transferases (GSTs) are the major set of phase II enzymes that catalase the reactions of conjugation of activated xenobiotics to reduced glutathione; however, they also play other roles. Phase III refers to the elimination of the metabolites from the cell [31]. GSTs can be found in the cytosol, they are associated with microsomal membranes, and they are encoded by separate genes. The microsomal class of GSTs, quite distinct from the cytosolic enzymes, is designated as “membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG)”. MAPEG consist of 6 proteins, including 3 types of microsomal glutathione S-transferase: 1, 2, and 3 (MGST1, 2, and 3, respectively). MGST2 affects glutathione S-transferase and glutathione-dependent peroxidase. It is involved in cellular defense mechanisms against harmful highly reactive lipophilic compounds of exogenous and endogenous origin, including metabolites produced during oxidative stress. It catalyzes the conjugation of LTA4 (Leukotriene A4) with glutathione, which further leads to the production of particles that are inflammatory mediators [32]. Cytosolic GSTs are classified into Alpha, Mu, and Pi classes; the Alpha and Mu classes include several enzymes, while the Pi class includes only 1.
Glutathione S-transferases catalyze the nucleophilic attack of the sulfur atom of glutathione on the electrophilic groups of a wide range of exogenous and endogenous compounds, thus being also involved in the defense against carcinogens and therapeutic drugs by modifying their serum concentration, and in consequence, the bioavailability and therapeutic action. It also has an endogenous non-detoxification function, including the formation of glutathione conjugates of prostaglandins, hormone biosynthesis, and defense against peroxidase-induced cell death and the role in the metabolism of oxidized fatty acids [23,31,33,34]. The change in GSTs concentration in liver tissue after cinacalcet administration can influence many functions and activities of the liver and the organism as a whole. Based on our results, we cannot speculate about possible outcomes. We also cannot predict whether the increase in MGST balances the decrease in cytosol GSTs; however, this effect of cinacalcet should be further explored because it could have, for instance, a crucial impact on the metabolism and the activity of concomitant drugs administered to the individual patient, especially since the influence of cinacalcet on phase I of detoxification is already well known [10].

Limitations

This experiment has some limitations. The sizes of the investigated groups were rather small. We conducted the experiment only among healthy individuals, and therefore we did not check the influence of cinacalcet on the concentration of proteins in selected tissues in health and disease and did not compare these results. We also did not check the outcomes of the change in the expression of investigated proteins.

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Conclusions

In conclusion, chronic administration of cinacalcet results in changes in the concentration of selected tissue proteins. Some of these are involved in the enzymatic detoxification process in the liver. It is well known that cinacalcet is a strong inhibitor of the CYP2D6 enzyme, engaged in phase I of enzymatic detoxication. Our results indicate that cinacalcet therapy could also impair phase II of enzymatic detoxication. Therefore, dose adjustment of concomitant drugs is required, not only in the case of drugs that are mainly metabolized in phase I but also in phase II. Other proteins may cause disturbances (for instance, in blood hemostasis, lipid metabolism, and inflammatory mediators) or contribute to the acceleration or intensification of dementia and cognitive dysfunction; therefore, close monitoring of patients subjected to long-term cinacalcet therapy may be required.

The results obtained in this experiment cannot be the basis for particular recommendations associated with cinacalcet therapy; however, they clearly indicate the urgent need for further research to explore the possible impact of cinacalcet on the comprehensive therapy of CKD patients and clinical outcomes.

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Declaration of Figures’ Authenticity

All figures submitted have been created by the authors, who confirm that the images are original with no duplication and have not been previously published in whole or in part.
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