Multiplexed TMT-Based Quantitative Proteomics Identified Essential Players Involved in the Mechanism of Action of SENS-401 Observed Under Normal or Ototoxic Conditions in Intact Cochlear Organ Cultures

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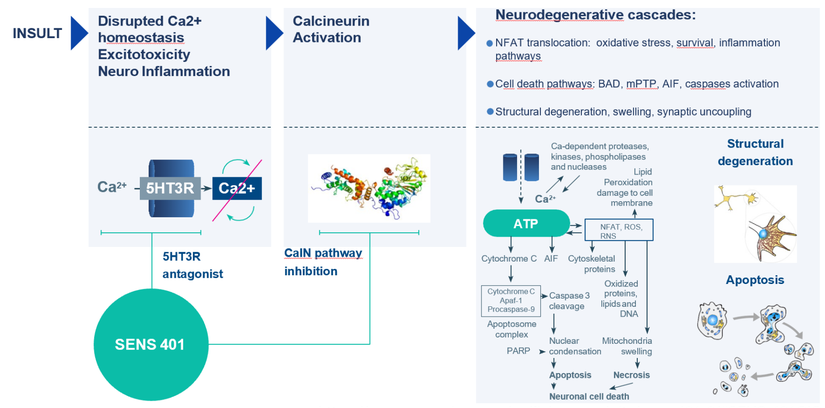
Background

SENS-401 (R-azasetron besylate) is a first-in-class drug candidate to treat Sudden Sensorineural Hearing Loss (SSNHL) and in clinical development for inner ear protection against Cisplatin-Induced Ototoxicity (CIO) and hearing preservation after cochlear implantation. In all three indications SENS-401 demonstrates a promising potential to improve hearing (https://www.sensorion.com/en/) via its actions on known targets, the 5-HT3 receptor and the calcineurin (see schematic on the right). The goal of this study is to further understand the protection granted by SENS-401 by conducting a systematic analysis of proteins and pathways involved its protective action. In this perspective, we aim to identify early players involved in CIO and SENS-401 protection using unbiased proteomics approach.

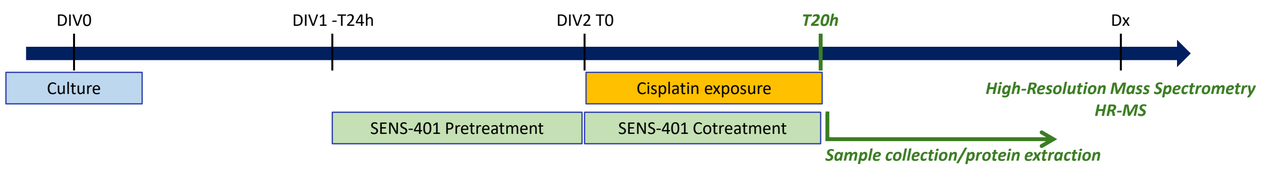


https://www.sensorion.com/en/fiches-produits/sens-401-2/#SENS-401CIO

Methods

- P3 to P5 Wistar rat organotypic explant cultures, including both spiral ganglion and organ of Corti intact tissue, were prepared in MatTek dishes. The concentration of cisplatin and time of lesion, at which earliest apoptotic effects were observed with early apoptosis marker cleaved caspase-3, was predetermined in previous time-course experiments and fixed at 20 µM for 20h. The concentration of SENS-401 was also predetermined in previous dose-response experiments and was fixed at 10 µM. Here, explants were therefore treated with 10 µM SENS-401 for a total of 44h and exposed to 20 µM cisplatin for the last 20h.

Organ culture for SENS-401 evaluation of action



- To identify all early players of both CIO and SENS-401, we used unbiased tandem mass tag (TMT) multiplexed quantitative proteomics coupled with high performance liquid chromatography and mass spectrometry (Exploris 480, FPP Montpellier). Bioinformatics data processing was done with MaxQuant & Perseus software. A total of 5040 proteins were identified with a False Discovery Rate (FDR) of 1 % at protein level, each protein having at least 1 unique peptide. Differentially expressed proteins (DEPs) obtained by comparing conditions were analysed using the Database for Annotation, Visualization and Integrated Discovery (DAVID), and KEGG database resources. Differential analysis with corrected t-test (250 permutations, FDR 5 %, and s0=1) was considered significant when the q-value was < 0.05 and allowed the identification of DEPs. Principal Component Analysis (PCA) was used to define clusters from collected tissue samples based on their similarity in the treated conditions. A heatmap presentation was chosen to demonstrate our quantitative proteomics data to represent the homogeneity of our samples in each condition and the potential changes in protein profile. STRING Core Data Resource was used for protein-protein interaction network analysis.

Results

Proteomics analysis delivered homogenous results

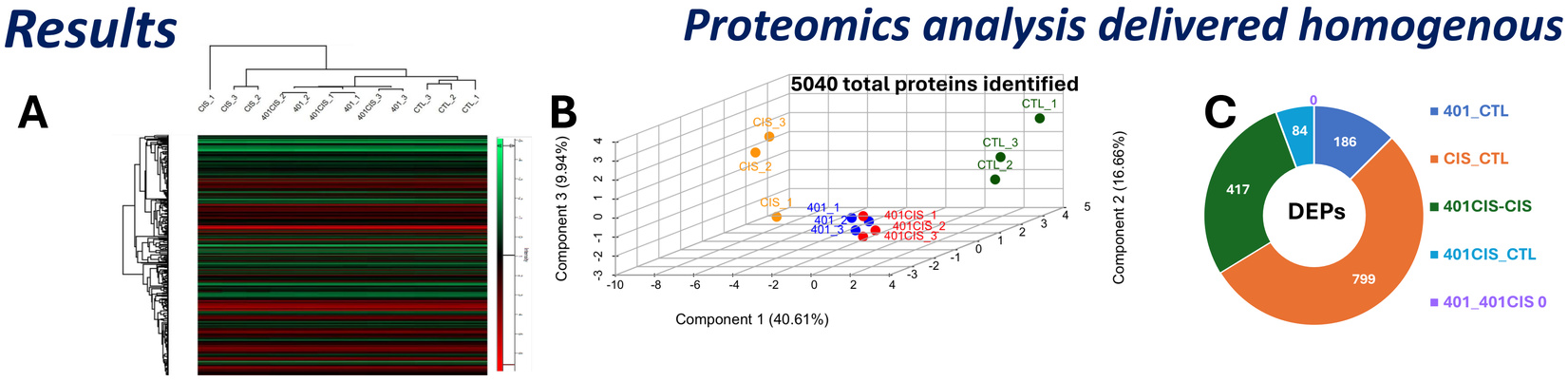
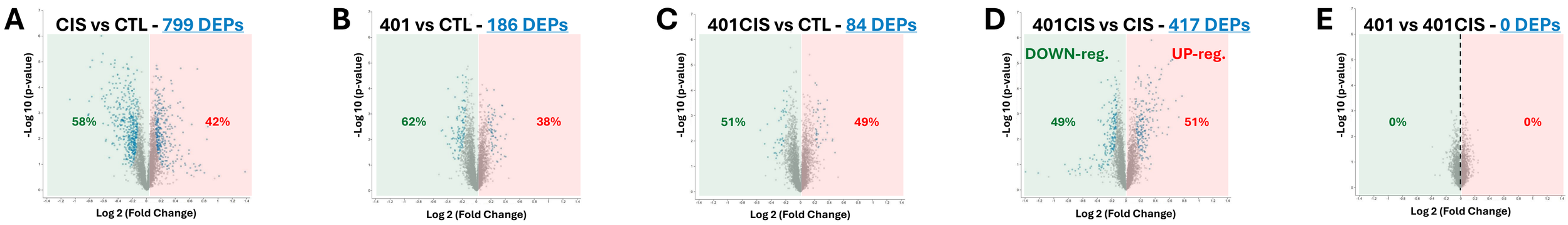


Figure 1: A. Proteomics heatmap of protein abundance differences showing a reliable grouping within conditions. Red color indicates high, green low abundance. **B.** PCA analysis of the 5040 proteins identified leads to homogenous clustering of triplicates of the same condition, indicating clear sample set differences between the control (CTL)- and the cisplatin (CIS)-treated tissues. SENS-401 (401)- and SENS-401+cisplatin (401CIS)-treated clusters are very close to each other, signifying a higher degree of protein profile similarity and thus a global impact of SENS-401 on CIO effect. SENS-401-treated cisplatin sample profiles show a clear trend towards the control sample profiles. **C.** Numbers of DEPs identified between the different conditions, clearly reflecting the broad effect of cisplatin with most DEPs identified (799), while demonstrating a strong attenuating effect of SENS-401, with less DEPs involved (417).

Proteomics identified DEPs involved in CIO & SENS-401 protective effect



- Comparisons of proteomics data are shown for a total of 5040 proteins identified in the different conditions
- The plots are representing magnitude of change (x axis) versus significance (p-value from a Student's t-test on y axis)
- Significance in protein expression (in blue) between the compared groups were analyzed applying an FDR of 5%, an assumed systematic error s0 of 1, and a correction of values by permutation (250)

Figure 2: Volcano plots of protein expression differences between conditions illustrating significantly changed proteins (blue dots). While only 186 proteins are significantly different in their expression profile in the comparison between the SENS-401 and the control conditions **(B)**, a large number of DEPs (799) are differentially affected in the comparison between the cisplatin and the control conditions **(A)**, reflecting a broad effect induced by cisplatin and a narrower effect instigated by SENS-401. Interestingly, the cotreated tissue samples (SENS-401+cisplatin) show fewer protein changes when compared to the control condition (84 DEPs) **(C)**, suggesting that SENS-401 treatment strongly restores protein profiles closer to normal. Also, comparison of protein expressions between SENS-401+cisplatin and cisplatin conditions identifies a large number of DEPs (417) **(D),** implying that SENS-401 has an important effect in the context of the cisplatin pathology. Finally, and strikingly, the comparison between SENS-401 and SENS-401+cisplatin conditions demonstrates no significant differences in protein profiles **(E)**, emphasizing that cisplatin does not induce significant protein changes in the context of SENS-401 treatment and thus has no more impact on our inner ear explant model. The percentages indicate the portion of DEPs, which were up- or downregulated in the respective comparisons.

Identification of key pathways and proteins underlying CIO and SENS-401 effects

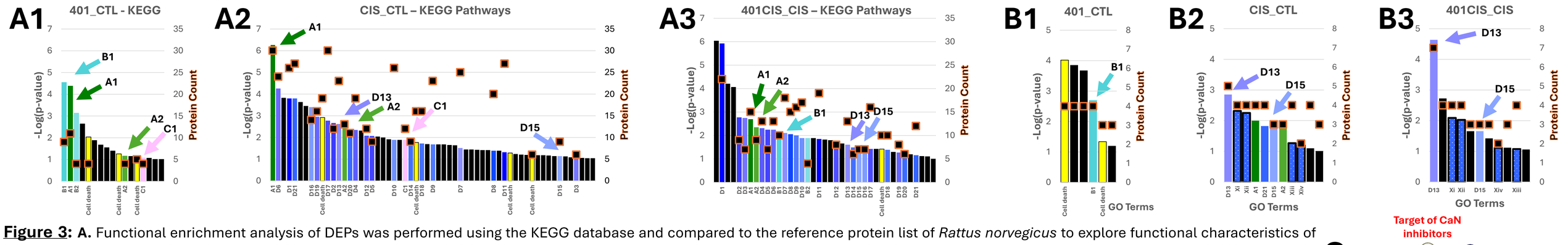
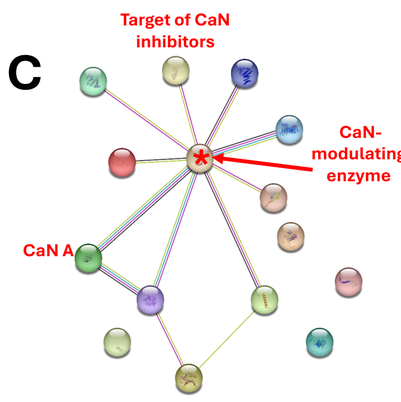


Figure 3: A. Functional enrichment analysis of DEPs was performed using the KEGG database and compared to the reference protein list of *Rattus norvegicus* to explore functional characteristics of identified DEPs. **A1.** The Gene Ontology (GO) analysis revealed 21 KEGG pathways for the 186 DEPs identified within the context of SENS-401, which are involved in the mechanism of SENS-401. **A2.** In contrast, 59 KEGG pathways were discovered for the 799 DEPs identified within the context of CIO. As observed in the volcano plots **(Figure 2 B & C)**, the effects of SENS-401 are narrower than the one of cisplatin. Within the cisplatin context, SENS-401 impacts a considerable number of pathways (39), which were covered by the 417 DEPs identified **(A3)**. To facilitate the comparability between graphs, identical pathways are indicated with the same colors across all graphs. Interestingly, a few of the pathway terms were recurrent in all three conditions (2 green tones with labels A1 & A2), indicating the involvement of similar functional processes under all conditions. These two pathways, largely impacted by cisplatin showing an important number of proteins **(A2)**, are also involved, but to a lesser extent, in the actions of SENS-401 **(A1)**. These two pathways identified in the context of the cisplatin pathology **(A2)** are of particular interest as players for the protective actions of SENS-401 **(A3)**, and the number of proteins involved decreased in the presence of SENS-401 (see green arrows). **B.** Bioinformatics analysis represents the pathways identified for the top 30 highly significant DEPs (q-value ranking) added up to the top 30 highly changed DEPs (fold change ranking). A few distinct pathways have been detected within the cisplatin but also the SENS-401+cisplatin treatment, indicating that SENS-401 has an effect on the pathways that were affected by cisplatin and could potentially reverse the CIO effect to prompt protection. **C.** Protein-protein interaction network analysis using STRING Core Data Resource was performed for 16 DEPs, which were identified as Calmodulin/Calcineurin/NFAT-associated key molecules in our HR-MS and were modified by either by SENS-401, or by cisplatin, or by the SENS-401+cisplatin treatment. The STRING network allowed to determined a key protein, which presents a core molecule within the Calmodulin/Calcineurin/NFAT-pathway (red asterisk). **These data confirm the involvement of the CaN pathway in the protective effect of SENS-401, in line with its pharmacology**.



Conclusions

- ✓ **Cisplatin effect is strongly minimized by SENS-401, in line with previous *in vivo* protection data** (Petremann *et al.*, 2017; Otology & Neurotology 38(9):p 1355-1361)**, with no direct impact on cisplatin antitumoral activity**
- ✓ **Identification of potential novel actionable therapeutic pathways and targets**
- ✓ **Identification of key proteins and pathways involved in**
  - ✓ **the early stages of CIO (relating to pathways leading to apoptosis)**
  - ✓ **the MoA of SENS-401 protection against CIO (involving calcineurin-related pathways)**

Acknowledgements

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