S61 Early Players Involved in Both Cisplatin-Induced Ototoxicity and SENS-401 Protection in Intact Organ Cultures

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Introduction

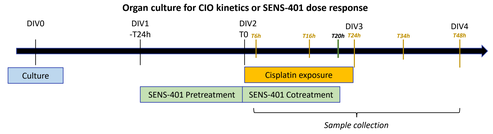
Hearing loss is affecting around 36% of adult and 40-60% of pediatric cancer patients treated with cisplatin, an impairment described as cisplatin-induced ototoxicity (CIO). Most of the deleterious effects described are cellular loss of essential auditory hair cells (HCs) within the organ of Corti (OoC) and spiral ganglion neurons (SGNs), representing mainly later stages of cisplatin impact. SENS-401, R-azasetron besylate, is aiming at preventing and protecting from CIO by acting on early-stage pathways. Our objective is to identify early players involved in CIO and SENS-401 protection. We aim to :

1. Characterize and optimize the intact model of organotypic explants containing both the OoC and the SGNs by i) confirming the presence of well-known SENS-401 targets, 5HT3a receptor and calcineurin A (CaN A), ii) determining kinetics of cisplatin exposure to assess the early phase of CIO, before apoptosis induction, and iii) defining the efficient dose of SENS-401 in this model.

2. Identify early players involved in CIO and SENS-401 protection by proteomic approach. First, we assess the quality of the proteomic experiment, then we identify global protein profiles and early-stage player categories for both CIO and SENS-401 effects.

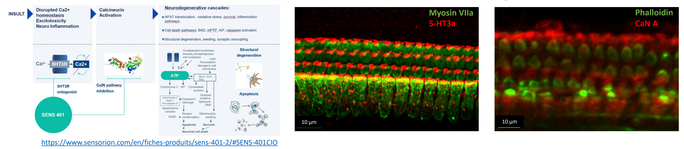
Methods

- Cochleae were dissected from P3 to P5 Wistar rat pups and either put in culture in MatTek dishes or fixed for immunofluorescence (IF) labeling. Organotypic explant cultures (whole explants), including both SG and OoC intact tissue, were prepared for CIO kinetics of early signaling (20 µM cisplatin at T6h, T16h, T24h, T34h, T48h) and SENS-401 dose response experiments (0.1, 1, 10, 30 µM).
- IF staining was performed using antibodies against cleaved caspase-3, an early apoptosis marker, myosin VIIa and phalloidin to label HCs, NF-200 to stain SGNs. and against the 5HT3a receptor (5-HT3aR)/calcineurin A (CaN A). Image analysis was performed using a confocal microscopy (Andor Dragonfly Spinning Disk).



- To identify the full early mechanisms of action of both CIO and SENS-401, we performed unbiased proteomics approaches using a mass spectrometer (Exploris 480) and quantification approach with Tandem Mass Tag. Explants were exposed to 20 µM cisplatin for 20h and treated with 10 µM SENS-401 for 44h. 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 proteomic data to represent the homogeneity of our samples in each condition and the potential changes in protein profile. Proteomics data was analyzed using Perseus and Panther overexpression system.

Well-known targets of SENS-401 (5-HT3aR & CaN A) observed in an intact organotypic explant model



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

Figure 1: A. Known mechanism of action of SENS-401 as 5-HT3 antagonist and CaN A inhibitor. **B-C**. Myo VIIa immunostaining **(B)** or phalloidin counterstaining **(C)** of HCs identifying three rows of outer HCs and one row of inner HCs. The 5-HT3a receptors and CaN A are present in the OoC of this intact organotypic explant model.

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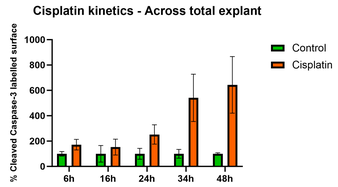
- Montpellier Ressources Imagerie (MRI, CNRS)

Results

Early apoptosis detected from 24h after cisplatin exposure



Protective effect of SENS-401 observed across doses 1-30 µM

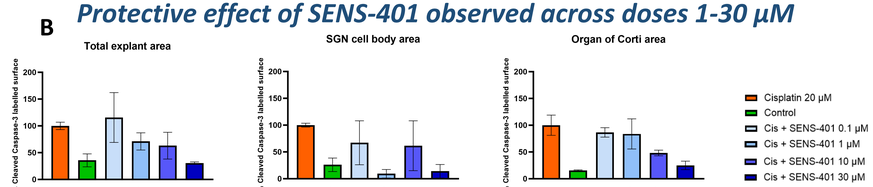


Figure 2: A. IF analysis of whole explants shows preliminary results indicating a tendency for the earliest apoptotic effects of cisplatin to occur from T24h after lesion onset. In order to identify early mechanisms, for proteomic experiment we choose to treat explants with cisplatin for 20h. **B.** Depending on the inner ear tissue, SENS-401 protects from CIO already at lower doses (≥ 1 µM) in SG, while an effect with SENS-401 is observed in the OoC tissue at ≥ 10 µM. To assure an overall protection across all inner ear tissues the more clinically relevant dose of 10 µM SENS-401 was chosen for mass spectrometry analysis. For the timeline experiments **(A)**, % were calculated by normalizing each timepoint of cisplatin treatment to its corresponding control condition, while in the SENS-401 dose response plots **(B)**, data was normalized to the cisplatin condition. Data is presented as mean ± SEM.

Proteomics analysis was homogenous and delivered congruent results

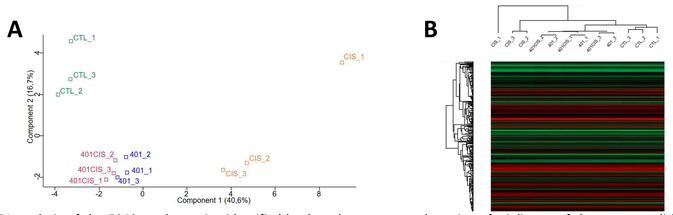


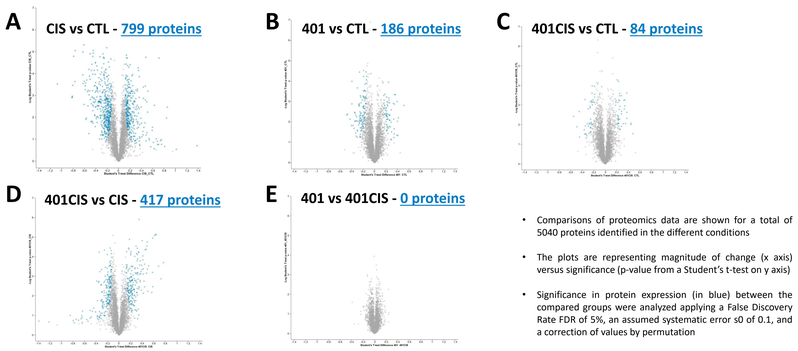
Figure 3: A. PCA analysis of the 5040 total proteins identified leads to homogenous clustering of triplicates of the same condition, indicating clear sample set differences between the control (CTL)- and the cisplatin (CIS)-treated tissues. SENS-401 (401)- and SENS-401+cisplatin (401CIS)-treated sample clusters are very close to each other, signifying a higher similarity level in proteins and therefore a global impact of SENS-401 on the cisplatin effect. SENS-401-treated cisplatin samples show a clear trend to bring back sample profiles closer to control condition. **B.** Heatmap showing a reliable grouping within conditions.

Conclusions

- ✓ **Implementation of an intact explant model to assess early mechanisms involved in CIO and SENS-401 protection by 20h exposure to cisplatin and a dose of 10 µM SENS-401**
- ✓ **Impact of SENS-401 on the cisplatin pathology protein profile. No more cisplatin effect is observed in the context of SENS-401 treatment. Results support our previous *in vivo* protection data** (Petremann *et al.*, 2017; Otology & Neurotology 38(9):p 1355-1361)
- ✓ **Identification of key proteins and pathways involved in the early stages of CIO and in the mechanism of action of SENS-401 protection against CIO**
- ✓ **Identification of potential novel actionable therapeutic pathways and targets**

Proteomics identified key players and pathways involved in CIO and the protective effect of SENS-401

Differences in protein expressions between conditions (with significantly changed proteins)



- 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 a False Discovery Rate FDR of 5%, an assumed systematic error s0 of 0.1, and a correction of values by permutation

Figure 4: Volcano plots of protein expression differences between treatment groups. While in the comparison between the SENS-401 and the control condition only 186 proteins are significantly different in their expression profile **(B)**, in the comparison between the cisplatin and the control condition a high number of proteins (799) are differentially affected **(A)**, reflecting a broad effect induced by cisplatin exposure, 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 proteins) **(C)**, suggesting that SENS-401 treatment strongly brings protein profiles back to normal condition. Also, comparison of protein expressions between SENS-401+cisplatin and cisplatin conditions identifies a high number of significantly different proteins (417) **(D)**, meaning that in the context of the cisplatin pathology SENS-401 has an important effect. Finally, and strikingly, the comparison between SENS-401 and SENS-401+cisplatin conditions demonstrates no significant differences in the protein profiles **(E)**, emphasizing that in a context of SENS-401 treatment cisplatin does not induce any significant protein changes, thus does not have any effect in our inner ear explant model. Overall, the proteomics results are consistent with the PCA analysis. It should be noted that SENS-401 does not impact the antitumoral activity of cisplatin *in vitro* (Petremann *et al.* 2017) and *in vivo* (unpublished data), suggesting different toxicity mechanisms at play in cancer cells and post-mitotic cells.

Identification of key molecular function classes and key pathways involved in CIO and SENS-401 treatment

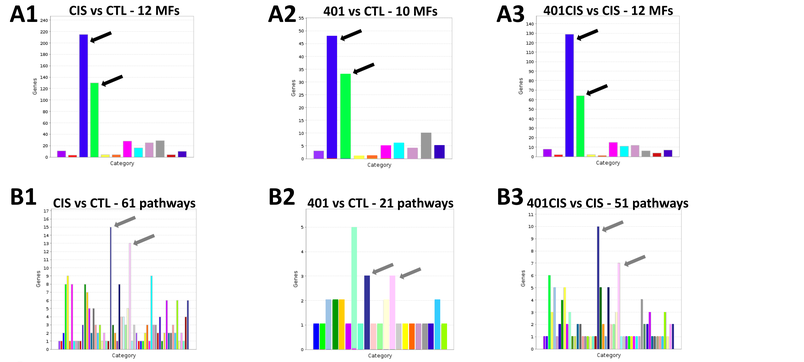


Figure 5: Global analysis of proteins with significant changes between groups was performed using the Panther Classification System and compared to the reference protein list of *Rattus norvegicus*. The molecular function (MF) **(A)** and Panther pathway (PP) **(B)** classes, in which these differential proteins were involved, were assessed. To facilitate the comparability between graphs, identical MFs and PPs are indicated with the same color across all graphs **A.** Interestingly, a few of the MF classes were recurrent in all three group comparisons (e.g. the blue and green bars indicated by black arrows). These two MF classes, showing an important number of genes potentially implicated in CIO **(A1)**, are also involved in the actions of SENS-401 **(A2)**. These two identified MFs in the context of the cisplatin pathology **(A3)** are of particular interest as players of the protective actions of SENS-401. **B.** 61 pathways were identified when the significant protein pool was compared between the cisplatin and the control condition **(B1)**, while only 21 pathways were significantly different between the SENS-401 and the control condition **(B2)**. As already observed in the volcano plot **(Figure 4B)**, this suggests that the effects of SENS-401 are narrower than the one of cisplatin. Moreover, in the cisplatin context, SENS-401 impacts a high number of pathways (51) **(B3)**. As for the MF, we can identify a few pathways, which were recurrent in all three group comparisons (e.g. the blue and pink bars indicated by grey arrows). Theses two PPs were both largely impacted by cisplatin **(B1)** and SENS-401 treatment **(B2)** and when comparing SENS-401+cisplatin with cisplatin **(B3)**. These pathways are of interest as players in the protective effect of SENS-401.