Report on the works in frame of the Eduard H. Schuchman Fellowship to Dr. Gita Naseri and Christian Kappe, group of Prof. Christoph Arenz

Update July 2022, with special focus on the outlook given in January 2022

Introduction

Acid sphingomyelinase deficiency (ASMD) or Niemann-Pick Disease Types A and B

Types A and B of Niemann-Pick Disease (NPD) are rare lysosomal storage disorders resulting from an Acid Sphingomyelinase Deficiency (ASMD) and the subsequent accumulation of sphingomyelin, cholesterol and other membrane lipids in the cells of affected individuals.[1] NPD type A is characterized by neurodegeneration and death in early childhood. In contrast, NPD type B is more heterogenous with little or no neurodegeneration, but usually leads to severe maladies like growth retardation, frequent pulmonary infections, hepatosplenomegaly and hematological disorders. Types A/B of NPD originate from recessively inherited point mutations in the SMPD1 gene, encoding the ubiquitously expressed lysosomal enzyme, acid sphingomyelinase (ASM).

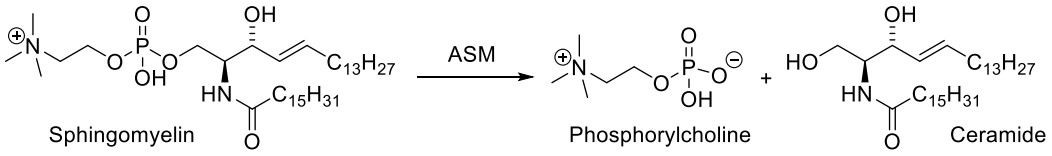

Figure 1: Reaction catalyzed by acid sphingomyelinase (ASM).

In a number of related lysosomal storage disease, it has been shown that the variant enzymes produced by the patients are less stable and often aggregated in ER/Golgi compartments or get degraded by the proteasome or other mechanisms. A study published by Schuchman et al shows for some of the most prevalent ASM variants that these are neither degraded nor aggregated inside the cells. Our own unpublished work shows that some but not all of the ASM variants show full activity after heterologous expression and purification in a liposomal assay or even after cellular delivery. These two observations suggest that the molecular reasons for reduced enzymatic activity could differ from other sphingolipidoses and even differ among variousNPD-ASM variants. The fact that several ASM variants are active after purification and that a small molecule, Arimoclomol, reduces lipid storage in ASMD cells, clearly suggest a potential for small molecules in ameliorating the ASMD pathology.

For other sphingolipidoses, small molecules that can stabilize disease-related enzyme variants have been described, termed “site-specific pharmacological chaperones” (SSPC). These compounds prevent degradation or aggregation of the variant enzymes. In the past, SSPC were almost exclusively developed from (competitive) inhibitors of the proteins of interest. This paradox however arises from the fact that there are known principles of rational inhibitor design, but enzyme activator development remains a widely unexplored field. Nonetheless, it is thought that allosteric binders may also be able to stabilize or even activate the protein of interest, but without exerting significant inhibition. Such activators however can barely be found by rational approaches, but rather by screening if an efficient and valid activity assay is known.

The Arenz group has developed a series of FRET probes that not only facilitate in vitro enzyme assays, but can also be used to specifically indicate ASM activity within the lysosomes of living cells.[7,9] Recently, a visible range sphingomyelinase FRET probe was established in combination with flow cytometry for quantitation of ASM activity in live cells.[8] This technical development allows for a more-rapid identification of cellular ASM activators independent of

their individual mode of action. However, it be stressed here again that different genetic mutations leading to ASMD might require different small molecules for treatment.

Scientific problem

For the Arenz group, the biggest obstacle in the research towards NPD was the non-availability of suitable NPD cells. Although various patient-derived cells have been obtained from various sources. Since these cells were primary cells, they showed strongly restricted vitality after a few passages. Furthermore, most cells were heterozygous with respect to the NPD mutation. Also, the often common overexpression of an NPD B ASM variants from lymphocytes of NPD A patients always was considered too artificial to justify the laborious characterization of potential ASM activators. Especially if a larger number of chemical compounds are to be tested for possible improvement of the NPD pathology at a later stage, the choice of test system is of utmost importance.

A deeper investigation of the flow cytometry ASM assay with our FRET probes showed that the probe is penetrating the cells only very slowly, while cleavage of the latter inside the cells is comparatively fast. We therefore reasoned that a faster delivery of the ASM FRET probe could make flow cytometry ASM assays much more sensitive, which is a prerequisite for efficient search for ASM activating molecules.

Goals of the project

The most important goal of the project funded by the NNPF was to establish genetically defined, immortalized NPD cell lines homo-allelic for the most common NPD mutations, using CRISPR/Cas technology. Moreover, we planed to adapt and optimize our FRET probe-based flow cytometry assay for maximum sensitivity. The ultimate goal was to test compounds in living cells for a reversion of lipid storage and other parameters of NPD pathology.

Results (Update July 2022)

1) Cellular NPD Models

We have already reported the CRISPR modification of HEK 293 cells into NPD mimicking cells expressing the ASM variants H421Y, R289H and DR608. The small difference in our FACS-based intracellular ASM assay prompted us to re-investigate the assay and to compare the results with other models of ASM deficiency. Possible reasons were the low responsiveness were that the cells were not fully modified, that the underlying cell type would be inappropriate as a model, or that our assay would not adequately represent the existing activity differences.

To further investigate the assay and the cell-based model we have

a) Performed the ASM assay in murine ASM knock out cells (SMPD1 -/- MEFs in comparison to WT MEFs).
b) Changed the loading protocol for the probe into the cells
c) Established a collaboration with Dr. Eugen Mengel from SphinCS GmbH, who will provide us with several homozygous NPD patient-derived lymphocytes to compare the results with the ones from CRISPR modified HEK cells
d) Established a collaboration with Dr. Soumya Pati (Shiv Nadar University Noida, India), who will establish further NPD mutations with CRISPR/Cas in embryonic stem cells, which then can be differentiated into different kinds of cells.

Results:

a) We have used SMPD1 -/- murine embryonic fibroblasts (MEF) to improve our ASM assay. As expected, the knock-out cell line compared to wild-type showed significantly less fluorescence (~20fold) in the green fluorescence channel at 527 nm, indicating that probe cleavage was low, while the fluorescence in the red channel at 613 nm showed only subtle reduction, compared to the wild-type cells, in accordance to previous results. As a consequence, the green/red fluorescence ratio was significantly reduced (Figure 2, left). Also similar to previous results, there was no obvious difference between knock out cells and inhibitor (Arc39) treated cells. However, the same data showed significant differences between knock-out and inhibitor treated wild-type cells, when the ratio was inverted to red/green, suggesting that Arc39-mediated inhibition was close but still different from 100%. We concluded, that a proper mathematical evaluation will be key to highlight even subtle differences between different cells with low levels of ASM, as needed for Niemann-Pick Disease related research.

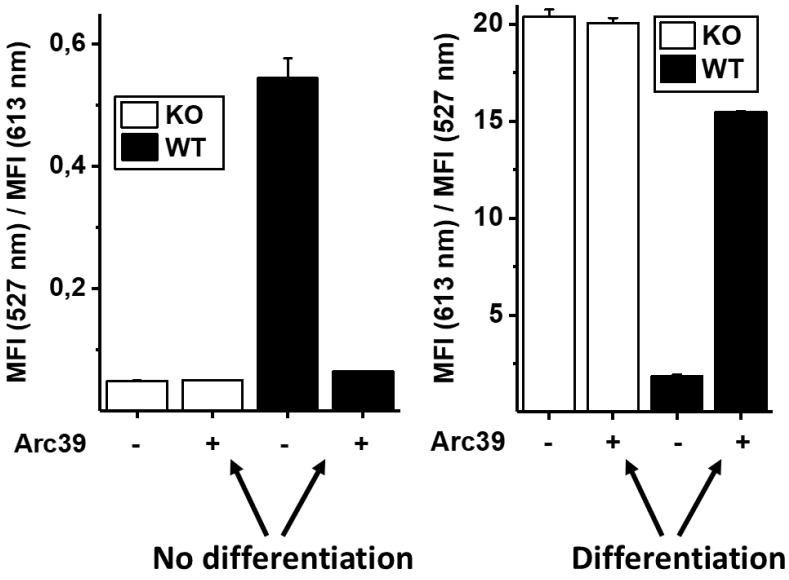

Figure 2: FACS assay using the ASM FRET probe in MEFs with SMPD1 -/- (KO) or wild-type (WT) genotype. Left: green/red ratio does not provide any differentiation between knock-out and inhibitor. Right: red/green ratio uncovers significant difference between knock-out and Arc39-treated cells.

b) One obvious problem is that the ASM FRET probe enters target cells only slowly and a continuous influx of probe into cells during experiments has to be taken into account. To solve this problem we have manufactured liposomes containing the FRET probe among other typical membrane lipids (PC, PE, Cholesterol). We expected a faster probe entry and thus lower probe concentrations needed for performing our FACS assay. Indeed, liposomes containing only 10% of the probe compared to DMSO solution yielded in a higher cell staining. Surprisingly, we observed that a two high loading of cells with FRET probe was associated with a loss in differentiation, even between wild-type and ASM knock-out MEFs (not shown), suggesting that an appropriate loading of cells with the FRET probe is also key to a successful high-throughput assay. In order to further increase the background fluorescence of cells having no or low ASM activity, we have synthesized two quencher lipids that should

minimize green fluorescence for intact FRET probe containing liposomes (Figure 3). The quencher lipids will in future be added to liposomes containing the FRET probe.

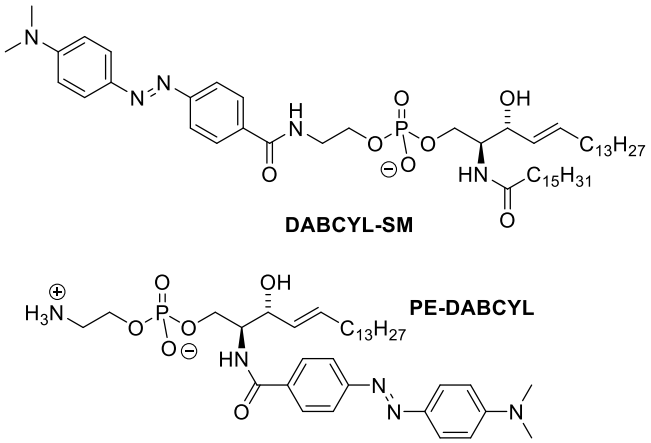

Figure 3: Two different quencher lipids to be added to liposomes containing the ASM FRET probe in order to minimize background fluorescence for cells with low ASM activity.

c) and d) These collaborations just have started.

2) Test of further potential ASM activators in vitro and in cellulo

We have tested another 40 activating compounds in dose-response setups. Unfortunately, most of these compounds did not show conclusive dose-response curves similar to the two compounds identified last year. Only another three compounds were chosen to be tested with regard to their potential to change the cellular lipidome. The probes have been submitted to the group of Dr. Burkard Kleuser for evaluation, but the results are still pending.

3) Synthesis of potential stabilizers of rec ASM

As the focus of our work has been changed from molecular biology to synthetic chemistry, we have investigated in another project that has been suggested by Dr. Ed Schuchman to Dr. Christoph Arenz during a conference in 2017. We have synthesized an acid-labile inhibitor/chaperone of ASM that might be useful to support Olipudase alfa treatment in patients. In oral conversations with Dr. Mengel, we have learned that the half-life time of Olipudase alfa in the blood of patients is high, which is obviously associated with a loss in enzymatic activity, before the enzyme can enter target cells and organs.

According to the suggestion of Dr. Schuchman we have thought of a compound that might bind to ASM at neutral pH thereby stabilizing it, but is decomposed at lower pH values. We have started the synthesis of various potential compounds that could have such properties.

Figure 4 shows the evaluation of a first compound incubated together with ASM in pure serum (FCS). At the shown concentration (5 µM in pre-incubation, 1 µM in final assay) the compound doubled the remaining ASM activity after 6 h of pre-incubation from 20% to 40%. The same experiment at other (lower or higher) concentrations showed similar but somewhat lower effects. A detailed investigation showed that the decomposition of the compound KP404 was only slowly to be active within the time frame of the assay used

(150 min). Therefore, further compounds with even more acid labile goups are currently synthesized.

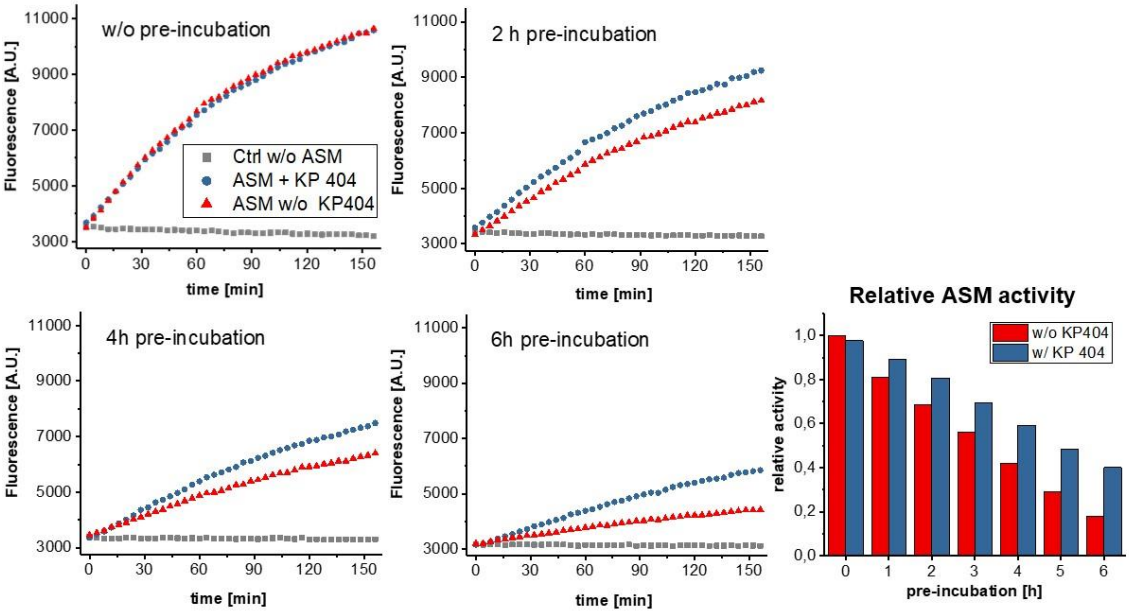

Figure 4: KP404 rescues FCS mediated decay of ASM acticvity. Activity of ASM w/o and after 2h, 4h or 6h pre-incubation in FCS in presence (blue) or absence (red) of KP404. After pre-incubation, the pH was changed to 4.5 and FRET probe was added, which resulted in a fivefold dilution of ASM and inhibitor. Right bottom: time-dependence of ASM decay in presence (blue) or absence (red) of KP404.

Our results indicate, that higher concentrations of KP404 are inhibiting ASM even at low pH. The reason is that within these 150 minutes, there is still enough KP404 present, which inhibits the activity of ASM that actually has been protected from FCS-mediated decay. We are therefore currently following another approach that will lead to faster pH mediated decomposition of our inhibitor at pH 4.5. This should enable us to use higher concentrations resulting in an even better protection.

With the results from the Eduard H Schuchman Fellowship we were able to produce preliminary results that have been used to apply for a full grant from the German DFG funding agency. The application is still pending, the result will be expected in September or October of this year.

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