A rapid 3D *in vitro* screening-based discovery approach for selecting and prioritizing NASH drug candidates

Simon Ströbel[1], Radina Kostadinova[1], Jana Rupp[1], Katia Fiaschetti[1], Agnieszka Pajak[1], Katarzyna Sanchez[1], Mathieu Petitjean[2], Li Chen[2], Manuela Bieri[1], Armin Wolf[1], Eva Thoma[1]
[1]InSphero AG, Schlieren, Switzerland; [2]PharmaNest, 100 Overlook, Princeton, NJ 08540, USA

Introduction

Non-alcoholic steatohepatitis (NASH) is a severe, progressive disease characterized by fat accumulation, inflammation, and fibrosis of the liver. Despite the severity and increasing prevalence of this disease, no approved treatments are as yet available. Ongoing drug discovery and development has proven challenging due to the lack of suitable *in vivo* and *in vitro* preclinical models that recapitulate all aspects of NASH.
The aim of this study was to develop a scalable, high-throughput-screening platform for drug efficacy testing, based on a human-cell-based 3D *in vitro* NASH model, to enable predictive and efficient screening of NASH compounds and combination therapies.
We produced scaffold-free 3D microtissue co-cultures of primary human hepatocytes, Kupffer cells, liver endothelial cells and hepatic stellate cells, then induced NASH using a cocktail of lipotoxic and inflammatory stimuli (free fatty acids and LPS) in media containing high levels of sugar and insulin. Compared to untreated controls, disease-induced models displayed key pathophysiological features of NASH after 10 days of treatment: 1) increase of intracellular triglyceride content as an indicator of fat accumulation; 2) secretion of inflammatory cytokines/chemokines, such as IL-6, MIP-1α, TNF-α, IL-10, MCP-1 and IL-8; and 3) increased fibril collagens deposition and secretion of procollagen type I and III peptides. Whole transcriptome analysis of NASH-treated models versus control revealed activation of pathways and differential regulation of genes associated with key processes in NASH, such as lipid metabolism, inflammation, and fibrosis induction. Treatment with anti-TGF-β antibody and ALK5i (TGFβRI inhibitor) concentration dependently decreased secretion of pro-collagen type I/III. Decreased deposition of fibrosis based on phenotypic quantification of Sirius-Red staining (PharmaNest) was observed in microtissues treated with anti-TGF-β antibody and ALK5i. Importantly, treatment with Firsocostat affected tissue lipid accumulation indicative of disease progression, and results were to a large extent in line with clinical observations.
In summary, this high-throughput screening platform is a promising research tool for rapid evaluation and selection of most effective novel NASH drug candidates to advance in the NASH development pipeline.

Methods & Technology

Using proprietary Akura™ 96 plate technology for 3D cell culture (Figure 1), we produced 3D human liver microtissues using human primary cell types relevant for NASH disease induction and progression: PHH, KC, EC and HSC (Figure 2 A). To recapitulate NASH pathogenesis *in vitro*, microtissues were treated for 10 days with media containing high sugars, FFA, and LPS (Figure 2B). Lipid accumulation was assessed using Nile Red staining and high content imaging. To measure tissue triglyceride levels, Glycerol-Triglyceride-Glo™ (Promega) was used. Human Magnetic Luminex Assay (R&D Systems) was used for cytokine/chemokine secretion measurments. Procollagen type I and III secretion was measured using Procollagen type I-propeptide (HTRF) and Procollagen-III-propeptide (PIIINP) ELISAs (CisBio). TIMP-1 was measured by Enanta Pharmaceuticals Inc. using Meso Scale technology. Collagen fibril deposition was visualized by Sirius Red staining and phenotypically quantified by highly sensitive FibroNest software (PharmaNest). Gene expression signatures were determined by TempO-seq® targeted mRNA sequencing (BioSpyder). Bioinformatical analysis and subsequent visualisation was done using an analysis pipeline developed internally using R. Differential expression analysis (DEA) and pathway analysis (PA) were executed as implemented in DESeq2 R library and clusterProfiler R library, respectively.

Akura™ 96 Plate Technology

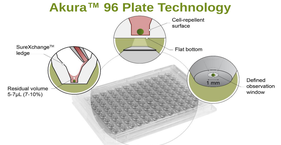
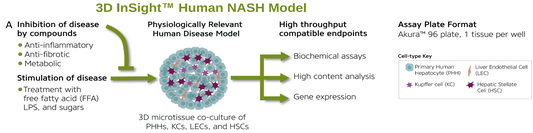


Figure 1. Akura™ technology for optimal 3D cell culture. Akura™ 96 format facilitates formation of uniform 3D human liver spheroid microtissues with robust functionality for high-throughput drug efficacy and safety testing.

3D InSight™ Human NASH Model



NASH Disease Induction and Treatment Schedule

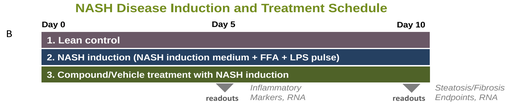


Figure 2. Assessment of anti-NASH compound efficacy using 3D InSight™ Human NASH Model. A) Induction of NASH in medium with high levels of sugars, FFA and LPS. B) Description of three NASH model treatment conditions (Lean control, NASH induction, NASH induction with treatment) and schedule for endpoint measurement. Compound efficacy studies were performed using NASH induction medium and stimuli to investigate the preventive role of compounds in NASH disease progression.

Results

Recapitulation of Pathophysiological Hallmarks of NASH Using Quantitative Endpoints

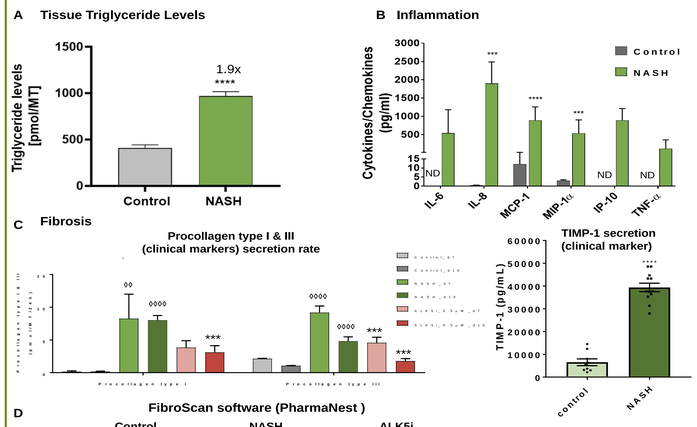
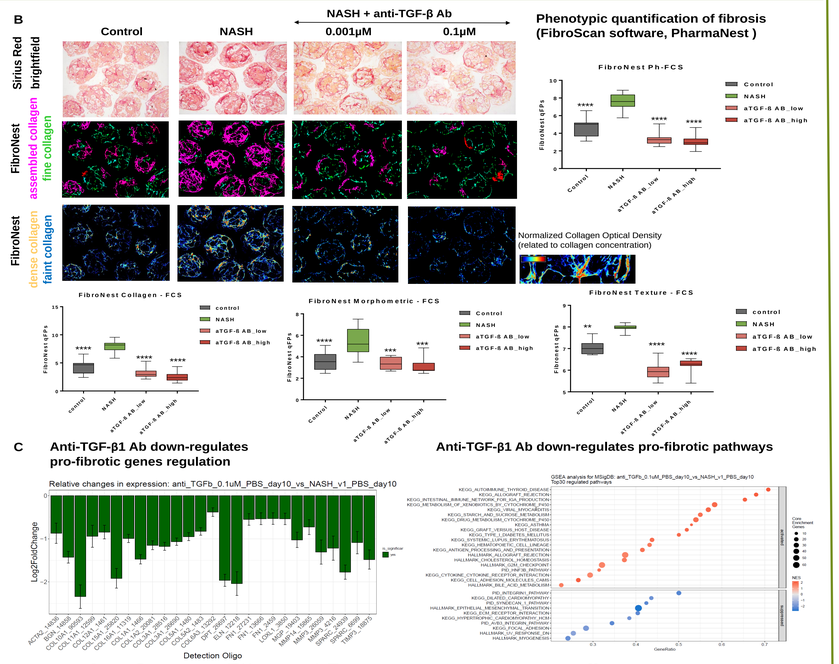


Figure 3: Recapitulation of hallmarks of NASH *in vitro*. A. Increased intracellular levels of triglycerides are detected within NASH-treated as compared to control-treated tissues at day 10 of treatment. Mean +/-SD, n=4 models, **** p≤0.0001 (t-test), NASH vs control. **B.** NASH stimuli induced the release of cytokines and chemokines in NASH-treated models at day 5 of treatment. Mean +/-SD, n=4 MTs, *** p≤0.001, **** p≤0.0001 (t-test), NASH vs control, 1 of 3 exp.. ND = below detection limit. **C.** Increased procollagen type I and III secretion (day 5-7 and 7-10 of treatment) as well as TIMP-1 secretion (day 5 of treatment, measured by Enanta Pharmaceuticals, Inc) indicates presence of fibrosis in NASH-treated models. Mean +/-SD, n=4-12 models, **** p ≤ 0.0001, ◊◊◊◊p ≤ 0.0001, ◊◊p ≤ 0.01 NASH vs lean control, *** p≤0.001, NASH vs NASH+ALK5i; 1 of 3 exp. (t-test). **D)** Sirius Red staining and phenotypic quantification of fibrosis (FibroScan, PharmaNest) indicate an increase collagen fibrils deposition in NASH conditions vs control. ALK5i decreases the fibrils deposition vs NASH. Ph-FCS (phenotypic fibrotic composite score). Mean +/-SD, n=7-9, *p≤0.05, **p≤0.01 ***p≤0.001, ****p≤0.0001(t-test), NASH vs control or NASH+ALK5i. **E)** DEA followed by PA revealed up-regulation of inflammation-, fibrosis-, and apoptosis-related genes and pathways, and downregulation of lipid metabolism-related genes and pathways in NASH as compared to control models. Adjusted p-value ≤0.05 was set as cut-off for significance level in both DEA and PA. NES: Normalized Enrichment Score.



Anti-Steatotic Effects of Firsocostat (now in phase II clinical trials)

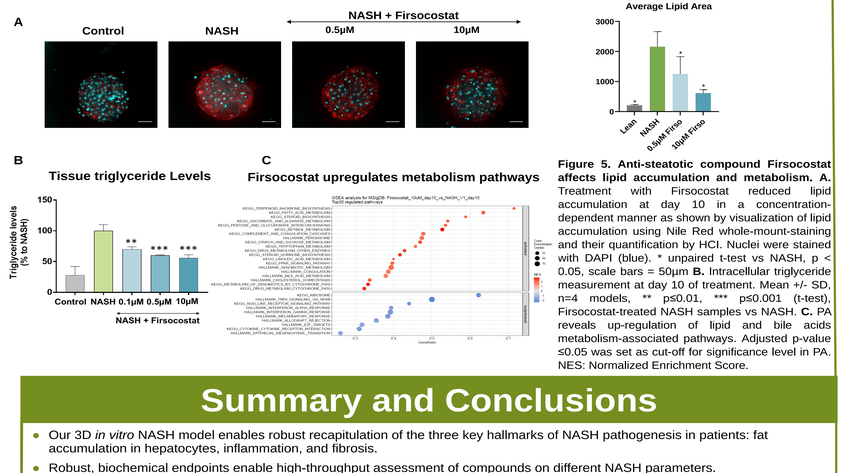
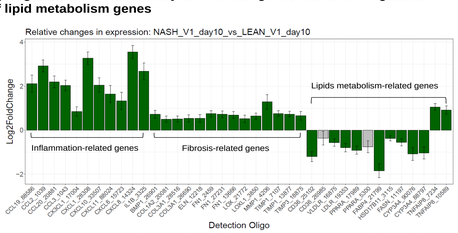
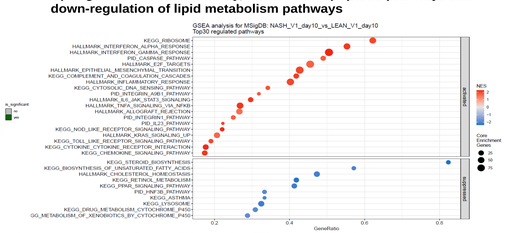


Figure 5. Anti-steatotic compound Firsocostat affects lipid accumulation and metabolism. A. Treatment with Firsocostat reduced lipid accumulation at day 10 in a concentration-dependent manner as shown by visualization of lipid accumulation using Nile Red whole-mount-staining and their quantification by HCI. Nuclei were stained with DAPI (blue). * unpaired t-test vs NASH, p < 0.05, scale bars = 50µm **B.** Intracellular triglyceride measurement at day 10 of treatment. Mean +/- SD, n=4 models, ** p≤0.01, *** p≤0.001 (t-test). Firsocostat-treated NASH samples vs NASH. **C.** PA reveals up-regulation of lipid and bile acids metabolism-associated pathways. Adjusted p-value ≤0.05 was set as cut-off for significance level in PA. NES: Normalized Enrichment Score.

Up-regulation of inflammatory and fibrotic genes and down-regulation of lipid metabolism genes



Up-regulation of inflammatory, fibrotic and apoptotic pathways and down-regulation of lipid metabolism pathways



Proof-of-Concept: Anti-TGF-β Antibody as an Anti-fibrotic Therapeutic

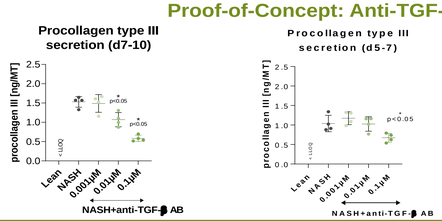


Figure 4. Proof-of-concept: Assessment of anti-fibrotic effects of anti-TGF-β1 Antibody. A) Anti-TGF-β1 antibody (Ab) treatment led to concentration-dependent decrease of procollagen type III secretion. Mean +/-SD, n=4 models, * p ≤ 0.05 (t-test), NASH vs NASH+anti-TGF-β1 Ab. **B)** Sirius Red staining brightfield (BF) and phenotypic quantification of fibrosis (FibroScan, PharmaNest) indicate increase of collagen fibrils deposition in NASH conditions vs control. Anti-TGF-b AB decreased the fibrosis vs NASH-treated samples. Mean +/-SD, n=7-10 models, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 (t-test), NASH vs control or NASH+anti-TGF-β AB. **C)** DEA followed by PA revealed down-regulation of fibrosis-associated genes and pathways in Anti-TGF-β1 antibody treatment group. Adjusted p-value ≤0.05 was set as cut-off for significance level in both DEA and PA. NES: Normalized Enrichment Score.

Summary and Conclusions

- Our 3D *in vitro* NASH model enables robust recapitulation of the three key hallmarks of NASH pathogenesis in patients: fat accumulation in hepatocytes, inflammation, and fibrosis.
- Robust, biochemical endpoints enable high-throughput assessment of compounds on different NASH parameters.
- Sirus Red staining combined with phenotypic quantification of collagen fibers using many quantitative fibrotic parameter can be used as a powerful tool for efficacy assesment of anti-fibrotic NASH compounds.
- Whole transcriptome analysis (RNASeq) confirms induction of NASH phenotype and expected effects of compounds
- Proof-of-concept studies demonstrate power of 3D NASH model for efficacy assessment of compound treatment for fat accumulation in hepatocytes (steatosis, Firsocostat) and collagen fibrils deposition (fibrosis, anti-TGFβ AB and Alk5i).

InSphero AG | Wagistrasse 27a, CH-8952 Schlieren, Switzerland | Phone: +41 44 515 04 90 | info@inspheo.com | www.insphero.com