Perspective

EU-OPENSCREEN: A Novel Collaborative Approach to Facilitate Chemical Biology

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Abstract

Compound screening in biological assays and subsequent optimization of hits is indispensable for the development of new molecular research tools and drug candidates. To facilitate such discoveries, the European Research Infrastructure EU-OPENSCREEN was founded recently with the support of its member countries and the European Commission. Its distributed character harnesses complementary knowledge, expertise, and instrumentation in the discipline of chemical biology from 20 European partners, and its open working model ensures that academia and industry can readily access EU-OPENSCREEN's compound collection, equipment, and generated data. To demonstrate the power of this collaborative approach, this perspective article highlights recent projects from EU-OPENSCREEN partner institutions. These studies yielded (1) 2-aminoquinazolin-4(3*H*)-ones as potential lead structures for new antimalarial drugs, (2) a novel lipodepsipeptide specifically inducing apoptosis in cells deficient for the pVHL tumor suppressor, (3) small-molecule-based ROCK inhibitors that induce definitive endoderm formation and can potentially be used for regenerative medicine, (4) potential pharmacological chaperones for inborn errors of metabolism and a familiar form of acute myeloid leukemia (AML), and (5) novel tankyrase inhibitors that entered a lead-to-candidate program. Collectively, these findings highlight the benefits of small-molecule screening, the plethora of assay designs, and the close connection between screening and medicinal chemistry within EU-OPENSCREEN.

Keywords

chemical biology, screening, medicinal chemistry, open access, compound library

Scientific Concept and Operational Model of EU-OPENSCREEN

Chemical biology is an interdisciplinary research field that has emerged in the past decade from classical pharmacology and cell biology, and it studies the effects of chemical compounds on biological systems. In parallel, postgenome biology, with its powerful technologies of genome sequencing, transcriptomics, proteomics, metabolomics, and genome editing by CRISPR/Cas9, as well as new model systems such as microphysiological systems, has provided a rapidly expanding range of information on new cellular targets for basic research and early drug discovery. However, the availability of selective "tools or probes" for systematic biochemical investigation of target function still remains a limiting factor in many investigations. Reviews on the use of chemical compounds that elicit a well-defined biological effect highlight the opportunities (and also the limitations) of these tool compounds for the modulation of functions of biological targets and for studying the underlying molecular mechanisms at the biochemical, cellular, tissue, and organismal levels.[1] Chemical biology as a discipline is well represented in, but not restricted to, academia, and increasingly, the drug discovery workflows in pharmaceutical companies

feature chemical biology studies. The discovery and optimization of probe compounds form important parts of industrial tractability and "go versus no-go" decision-making on novel targets, as well as the study of target translatability as part of the early design of subsequent clinical phases.[2]

EU-OPENSCREEN is a research infrastructure (RI) of screening and medicinal chemistry platforms that was established by seven European member states and one observer state in 2018, under the legal framework of a European Research Infrastructure Consortium (ERIC), in order to enable chemical biology in an open-access setting (www.eu-openscreen.eu). The RI will provide scientists access to a chemical library, assay development and screening facilities, medicinal chemistry and informatics platforms, and associated supporting facilities for protein production, cell line generation (e.g., 2D and 3D models and patient-derived cells), computational and structural biology, and structure-based drug design (**Fig. 1**). As it begins the operational phase of its work, EU-OPENSCREEN anticipates that a high demand exists from users to access its platforms. In the year before initiation of EU-OPENSCREEN, the partner sites that came together to form the EU-OPENSCREEN network participated in more than 135 screening and medicinal chemistry projects, involving academic, small and medium-sized enterprise (SME), and large industry users coming from 22 European and non-European countries. Historically, the partner sites of EU-OPENSCREEN have been involved in multiple projects, which reached clinical and/or preclinical stages for indications including leukemia, epilepsy, autism, myotonic dystrophy, Parkinson's disease, and colon cancer.

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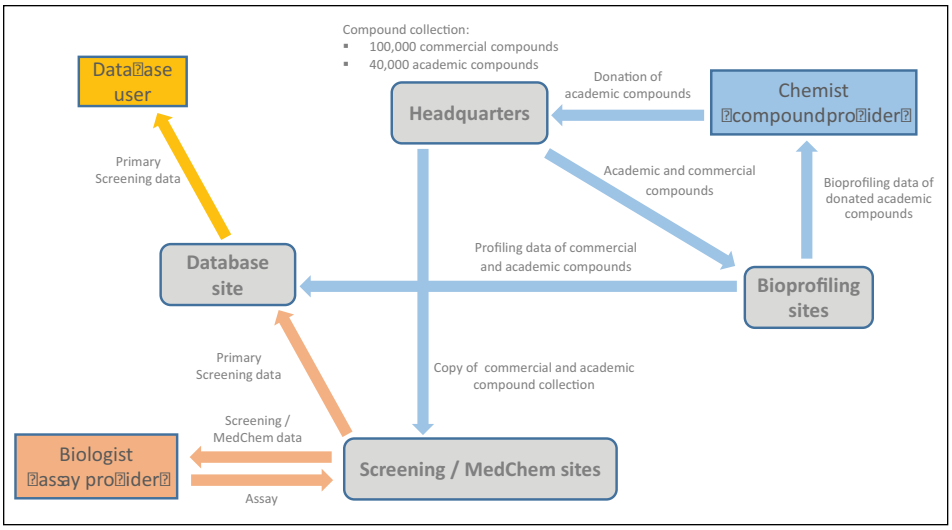

Figure 1. Working model of the EU-OPENSCREEN ERIC. Three categories of users are envisioned (assay provider, compound provider, and database user). The compound collection comprising academic and commercial compounds is stored centrally at the headquarters. Bioprofiling of academic and commercial compounds occurs at the bioprofiling sites. Compound screening and medicinal chemistry are carried out at the individual EU-OPENSCREEN partner sites. Data are stored at the database site and can be accessed via the EU-OPENSCREEN database.

The central office of the EU-OPENSCREEN ERIC acts as a single point of contact for users, assists in identifying the appropriate partner site, and helps to formulate the project design. Herein, the central office receives the project requests from users and contacts potential partners with appropriate technologies and expertise. Together with the partner sites, the technical feasibility, the scientific novelty of the proposed project, and the most suitable partner site are evaluated, and if deemed feasible and novel, the project will be initiated in collaboration with the most suitable EU-OPENSCREEN partner. Depending on the individual needs of the users, projects take place at 1 of 15 specialized and high-capacity screening partner sites or 6 medicinal chemistry sites. The assay development phase preceding the actual screening campaign emphasizes the quality, integrity, robustness, and pharmacological relevance of the readouts. Projects have access to the EU-OPENSCREEN compound collection[3] containing 100,000 commercially available as well as 40,000 academic compounds crowdsourced through a network of national chemical biology networks. A 5000-compound subset—carefully selected to represent the diversity of the entire collection and also containing 2500 known bioactive compounds—is available for the process of assay validation in pilot studies. Notably, during the selection process for commercial compounds pan-assay interference compounds (PAINS)[4] are partially selected against. Furthermore, access to a wider range of chemical diversity will be provided through the isolation and characterization of natural products by some of the EU-OPENSCREEN partner sites, and moreover, a fragment library for fragment-based screening will be acquired. These two additions are currently the only expansions envisioned for the EU-OPENSCREEN compound collection, and future expansions will depend on the funding situation. The compound collection is stored and managed at the headquarters located in Berlin, and copies of the screening collection, as well as library updates, are distributed to the EU-OPENSCREEN partner sites. Results from the primary screens will be made available globally in an open-access database. To allow for publication of data or securing intellectual property, a grace period of up to 3 years is provided between completion of the primary screen and data presentation in the EU-OPENSCREEN database. After the official start of EU-OPENSCREEN in 2018, the database is currently still under construction. A link will be provided on the EU-OPENSCREEN website as soon as the database development has been completed.

While EU-OPENSCREEN just started its operational phase, its 20 partner institutions are already operational as stand-alone institutes. Hence, in the following sections recent successful chemical biology projects at selected partner institutes are presented to give an overview of the capacities and expertise present within the EU-OPENSCREEN network, as well as an outlook as to how EU-OPENSCREEN can facilitate academic and applied science in the future.

Project A: 2-Aminoquinazolin-4(3*H*)-ones as Potential New Nonpeptidomimetic Lead Structures for Antimalarial Agents

Emerging drug resistance of *Plasmodium* parasites against approved antimalarial drugs represents a major threat for preventing and treating malaria, one of the most detrimental infectious diseases, in the future.[5] Hence, substantial efforts are currently being made to identify novel drug candidates that interrupt the life cycle of the parasite by yet unknown mechanisms of action. In this context, the Latvian Institute of Organic Synthesis (LIOS), a medicinal chemistry partner

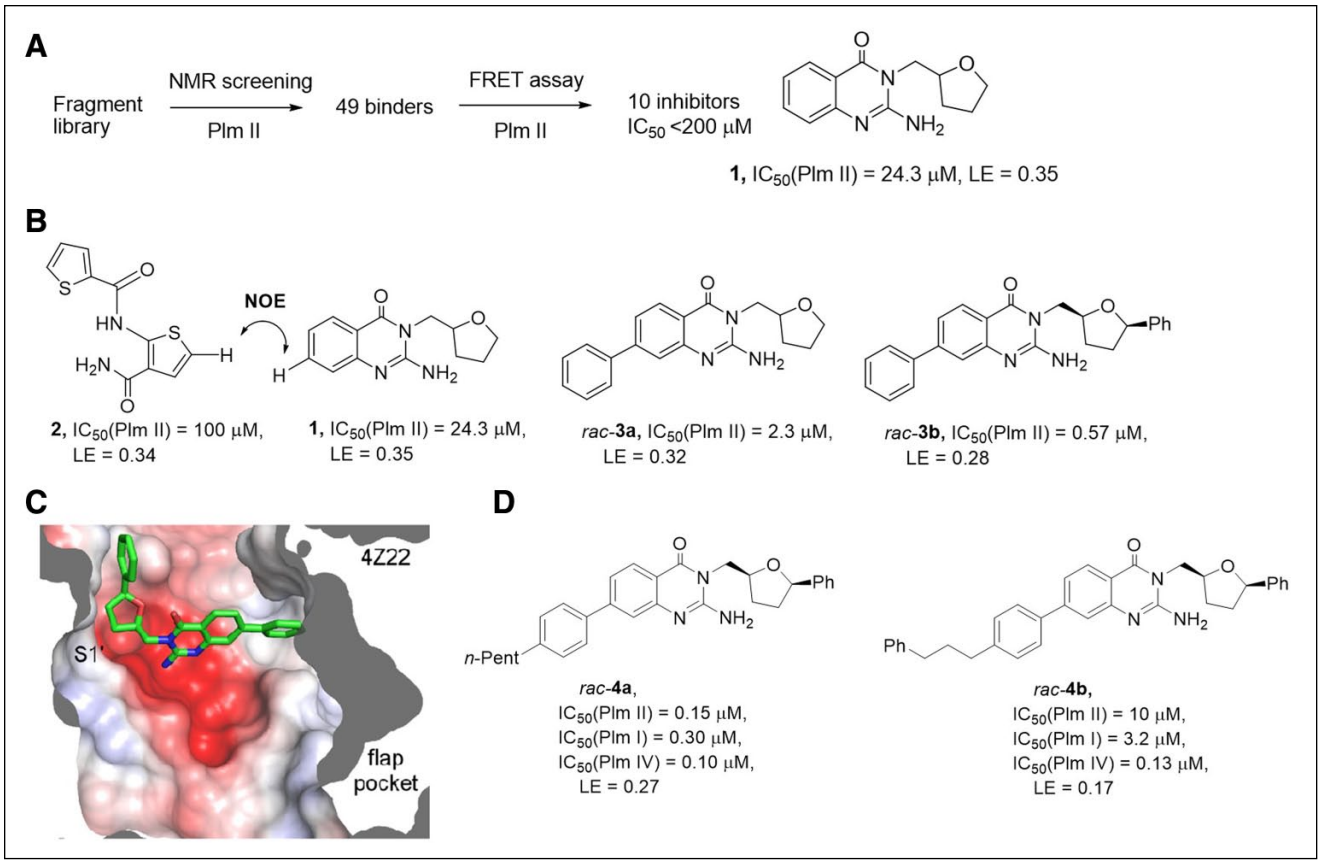

Figure 2. 2-Aminoquinazolin-4(3*H*)-ones as potential new nonpeptidomimetic lead structures for antimalarial agents. (**A**) Experimental pathway that led to the discovery of the 2-aminoquinazolin-4(3*H*)-one scaffold. (**B**) Optimized inhibitor hit *rac*-**3a** from NMR studies of fragment hits **1** and **2**, and inhibitor *rac*-**3b** from molecular modeling studies. (**C**) X-ray structure of *rac*-**3b** bound to Plm II (PDB ID 4Z22). (**D**) Chemical structures of the two Plm inhibitors *rac*-**4a** and *rac*-**4b**. Figure adapted with permission from Rasina et al. (*J. Med. Chem.* 59, 374–387).[6] Copyright 2016 American Chemical Society.

site of EU-OPENSCREEN, recently spearheaded a study that identified 2-aminoquinazolin-4(3*H*)-ones as potent inhibitors of three digestive aspartic proteases (i.e., digestive plasmepsins), namely, Plm I, II, and IV.[6] Digestive plasmepsins (i.e., Plm I, II, and IV and HAP), localized in the digestive vacuole of the *Plasmodium* parasite, contribute toward the metabolism of hemoglobin into amino acids, and represent a subgroup of the extensively studied family of aspartic plasmepsin proteases, which have been considered potential drug targets against malaria parasites for more than 10 years.[7–9] Due to their high potential as drug targets, numerous inhibitors have been developed against plasmepsins over the past years.[10–12] Nonpeptidomimetic compounds show better selectivity against human aspartic proteases and tend to bind to the open-flap form of *Plasmodium* plasmepsins, whereas peptidomimetic compounds preferably bind to the closed-flap form of the proteases. Herein, opening of the flap results in accessibility of a protein subpocket, which can subsequently be occupied by parts of the nonpeptidomimetic inhibitor. In close collaboration with the Biomedical Research and Study Centre in Riga and the Francis Crick Institute in London, a nuclear magnetic resonance (NMR)-based fragment screening approach[13,14] was used to identify 2-aminoquinazolin-4(3*H*)-ones as nonpeptidomimetic inhibitors of digestive plasmepsins (**Fig. 2A**). To this end, a diversity subset comprising 976 Astex "rule of 3"-compliant compounds of the ChemBridge fragment collection (ChemBridge, San Diego, CA) was screened against binding to the digestive plasmepsin Plm II. STD, T1*p*, and Water-LOGSY NMR spectra were taken in both the presence and absence of the Plm II target, and specificity was assessed by competition experiments with the potent aspartic protease inhibitor pepstatin A.[15] Binding to Plm II detected by at least two of the three NMR techniques, as well as competition with pepstatin A, was set as a hit criterion and yielded a total of 49 fragment hits. Subsequent fluorescence resonance energy transfer (FRET)-based enzymatic assays identified fragment **1**, which is based on the 2-aminoquinazolin-4(3*H*)-one scaffold (**Fig. 2A**), as the most potent hit with one of the highest ligand efficiencies (IC_{50}(Plm II) = 24.3 μM, LE =

0.35). Subsequent chemical optimization experiments were thus performed using this scaffold as a starting point. An initial hint for the medicinal chemistry optimization strategy came from NMR competition experiments using fragment **1** in combination with fragment **2**: the two fragments did not bind to the same binding site, and an observed interligand NOE between H-7 of fragment **1** and H-5 of fragment **2** indicated close proximity of the two fragments—thus implying an additional binding pocket around H-7 of fragment **1** (**Fig. 2B**). Following this hint, the addition of a phenyl group at position 7 of fragment **1** yielded the more potent inhibitor *rac*-**3a** (IC_{50}(Plm II) = 2.3 μM, LE = 0.32) (**Fig. 2B**). Subsequent docking experiments with *rac*-**3a** using Schrödinger Glide software[16] (Schrödinger, New York, NY) suggested high-quality binding to the open-flap conformation of Plm II with hydrogen bonding interactions between the N1 and 2-amino group of fragment **1** and catalytic Asp34-Asp214 dyad and indicated an extension of the added phenyl residue toward the deep flap pocket, while the tetrahydrofuran (THF) moiety of fragment **1** resided in the S1′ pocket of Plm II. Moreover, docking experiments suggested additional space in S1′, and indeed, adding a lipophilic *cis*-phenyl group to the 5-position of the THF group yielded *rac*-**3b** as the more potent inhibitor of Plm II (IC_{50}(Plm II) = 0.57 μM, LE = 0.28) with a more than 10-fold selectivity for Plm II when compared with human Cat D (**Fig. 2B**). A co-crystal of *rac*-**3b** and Plm II was obtained, and the x-ray structure (2.7 Å) confirmed binding of the inhibitor to the open-flap conformation previously suggested by the docking experiments and thus provided a better understanding of the binding of the inhibitor to Plm II (**Fig. 2C**). Importantly, the x-ray structure also showed that the flap pocket is for the most part unoccupied by *rac*-**3b**, and thus suggested that adding hydrophobic groups to the 7-phenyl substituent could help target the flap pocket; indeed, literature-informed[10,11,17] addition of the *n*-pentyl group to the para position of the phenyl group yielded the even more potent inhibitor *rac*-**4a** (IC_{50}(Plm II) = 0.15 μM, LE = 0.27) (**Fig. 2D**). Notably, adding a 3-phenylpropyl group to the *para* position of the phenyl group yielded the Plm IV specific inhibitor *rac*-**4b** (IC_{50}(Plm IV) = 0.13 μM, LE = 0.17) (**Fig. 2D**), whereas *rac*-**4a** inhibited the three digestive plasmepsins Plm I, II, and IV to similar extents. Selectivity over human Cat D was comparable to previous reports on nonpeptidomimetic Plm inhibitors for both *rac*-**4a** and *rac*-**4b**.[10] Interestingly, homology modeling of Plm IV using Plm II as a template suggested that the flap pocket of Plm IV is slightly more spacious. Both structure models could accommodate the smaller *n*-pentyl group of *rac*-**4a** well, while the bulkier 3-phenylpropyl group of *rac*-**4b** could only fit into the more spacious flap pocket of Plm IV. This suggests that specifically targeting the flap pockets of digestive plasmepsins could yield additional subtype-specific inhibitors. Both *rac*-**4a** and *rac*-**4b** were tested in an in vitro growth assay using the *Plasmodium falciparum* clone 3D7 and showed growth inhibition at levels close to 1 μM (*rac*-**4a**: 1.1 ± 0.2 μM; *rac*-**4b**: 1.2 ± 0.2 μM), which is considered as a criterion for promising leads for potential new antimalarial drugs.[10]

This study nicely demonstrates several aspects that are also relevant in the context of the EU-OPENSCREEN network. First, the EU-OPENSCREEN compound collection will include a fragment-based subset of compounds, and the LIOS will be involved in the design and setup of this library subset. Second, the expertise in fragment-based drug discovery present at LIOS represents an important addition to the EU-OPENSCREEN capabilities, and future EU-OPENSCREEN users designing similar experimental setups will find LIOS to be a competent collaboration partner for running their experiments. Lastly, the study shows that EU-OPENSCREEN covers expertise regarding assay systems, screening as well as subsequent chemical optimization of hit compounds. This will allow prospective users to identify promising hits and develop them into lead structures for further drug discovery. Here, the complementary expertise of currently 20 partner institutions ensures that prospective users will be directed to the collaborator who best matches their individual experimental needs.

Project B: Discovery of the Novel Lipodepsipeptide MDN-0066, a Natural Product That Specifically Induces Apoptosis in Cells That Do Not Express the Functional pVHL Tumor Suppressor

Inactivation of the von Hippel–Lindau tumor suppressor gene (pVHL) occurs in many renal cell carcinomas (RCCs) as well as in other cancers. In clear cell renal cell carcinomas (CC-RCCs), for instance, either genetic or epigenetic inactivation of pVHL function is observed in 70%–80% of reported cases.[18,19] pVHL is ubiquitously expressed throughout human tissues, and its loss of expression is thus restricted to the tumor tissue. Inactivation is usually accompanied by high levels of tumor vascularization and poor prognosis for the patient. Moreover, it has been shown that reintroduction of functional pVHL into RCC cells restores tumor suppression.[20] Collectively, its high rate of inactivation in RCCs, the local restriction of pVHL inactivation to the tumor tissue, and its important role for malignancy make pVHL an ideal target for novel drug candidates, which will possibly have only few side effects in unaffected tissues due to the differential expression of functional pVHL in healthy and diseased cells. To identify novel chemical agents that exhibit cell toxicity only in the context of pVHL inactivation, the EU-OPENSCREEN partner site Fundación MEDINA used a quantitative whole-cell assay—previously developed[21–23]—on site[24] to screen pVHL-deficient human

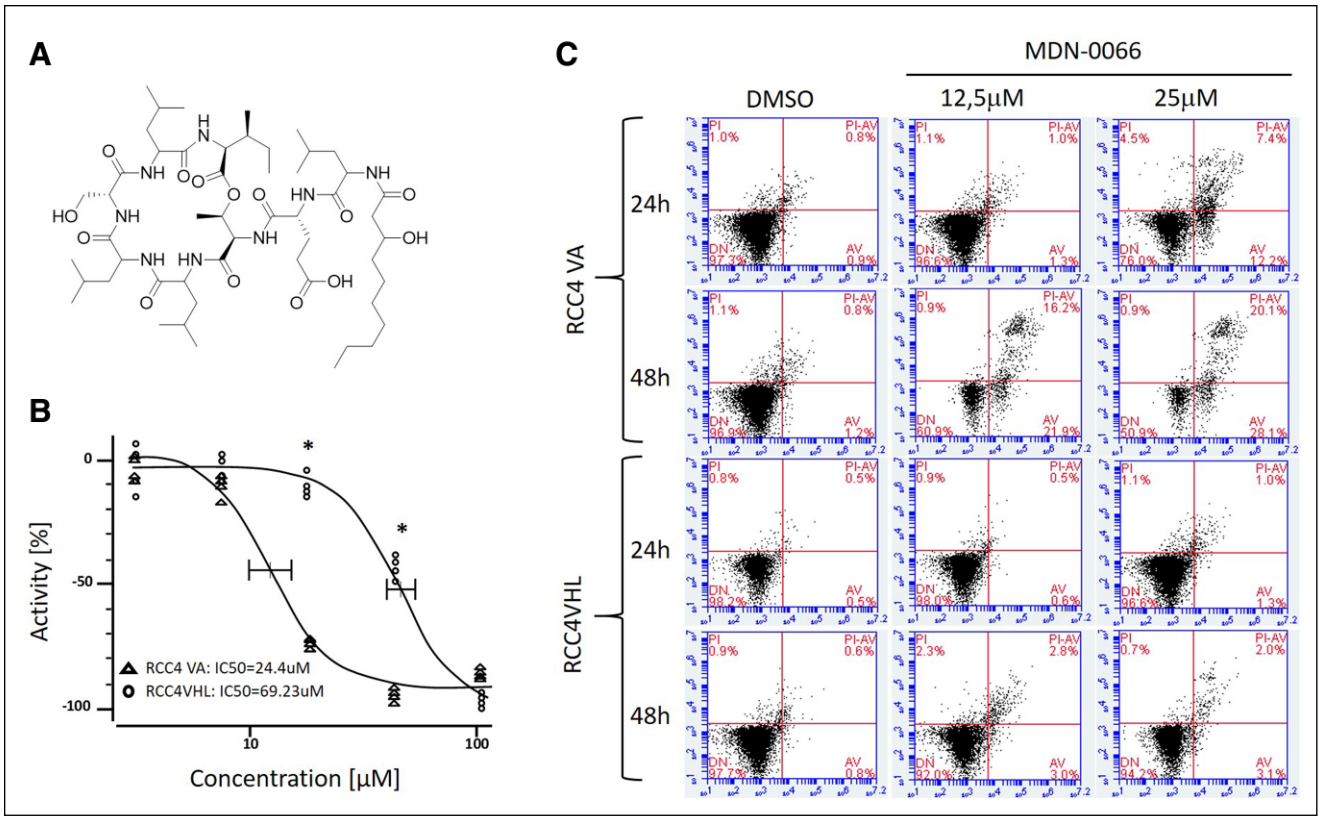

Figure 3. MDN-0066 as a potential new lead structure inducing apoptosis specifically in cells deficient for the tumor suppressor pVHL. (**A**) Structure of the newly discovered lipodepsipeptide MDN-0066. (**B**) Concentration–response curve of MDN-0066 showing increased sensitivity to MDN-0066 in pVHL-deficient cells compared with the pVHL rescue cell line. (**C**) Annexin V/PI staining confirming induction of apoptosis specifically in RCC4-VA cells in response to MDN-0066 exposure. Lower left quadrant = live cells; lower right quadrant = early apoptotic cells; upper right quadrant = apoptotic cells; upper left quadrant = necrotic cells. Figure adapted from Cautain et al. (*PLoS One*, 2015, 10, e0125221).[25]

renal carcinoma cells (RCC4 cell line) transformed either with an empty vector (RCC4-VA) or with a vector expressing a functional rescue copy of pVHL (RCC4-VHL) against a collection of microbial extracts.[25] Herein, the decision to use natural products for the screen was informed by the fact that microorganisms are capable of producing a vast variety of diverse chemical structures, which makes them highly interesting for drug discovery, and explains why natural products keep showing desirable bioactivities in various therapeutic areas.[26] A total of 1040 microbial acetone extracts were isolated from 117 unicellular microbial species grown in a set of different growth conditions. Microbial extracts that induced cell death in RCC4-VA but not in RCC4-VHL cells were subsequently fractionated, fractions were rescreened, and the ones recapitulating the RCC4-VA-specific induction of cell death were further analyzed by liquid chromatography–high-resolution mass spectrometry (LC/HRMS) and NMR to identify the active natural product and assess its novelty by chemical dereplication. These dereplication experiments allowed rapid focusing on active fractions of the promising strain F 278,770^{T}, which eventually turned out to be a previously not described *Pseudomonas* species, now named *P. granadensis* sp. nov.[27] Subsequent large-scale fermentation, assay-guided compound isolation, and structural elucidation eventually yielded the natural product MDN-0066, a lipodepsipeptide, which was not described previously in the literature and, due to its unique structure, established a novel class of lipodepsipeptides (**Fig. 3A**). Dose response analysis showed that the pVHL-deficient cell line RCC4-VA was more sensitive to purified MDN-0066 (IC_{50} = 24.4 µM) than the pVHL rescue cell line RCC4-VHL (IC_{50} = 69.23 µM), and demonstrated that MDN-0066 already induced cell death in RCC4-VA cells at concentrations that did not show an effect in the rescue cell line (**Fig. 3B**). Importantly, it could be shown that MDN-0066 is capable of inducing apoptosis specifically in pVHL-deficient cells as judged by PARP cleavage detection. This was confirmed by annexin V/propidium iodide (PI) staining, which demonstrated specific induction of apoptosis in pVHL-deficient cells upon MDN-0066 treatment (**Fig. 3C**). This finding is promising with respect to drug development programs, as pVHL-deficient

cells have previously been described as being resistant to apoptosis.[20] Moreover, flow cytometric DNA content analysis of pVHL-deficient RCC4-VA and RCC4-VHS cells upon 48 h exposure to MDN-0066 revealed an S-phase arrest unique to RCC4-VA cells (6.0 ± 2.1 to 18.5% ± 5.2; $p = 0.005$), which was correlated with a G0/G1 decrease (75.5 ± 4.2 to 37.5% ± 4.6; $p = 0.001$) and a G2/M increase (10 ± 0.5 to 26.2% ± 3.1; $p < 0.0005$) (see **Suppl. Fig. S1**). These results suggest that MDN-0066 is critical for the S-phase arrest in RCC4-VA cells, but can also be involved in G2/M arrest in the absence of pVHL. Loss of hypoxia-inducible factor 1α (HIF-1α) causes an increased progression into the S phase, rather than a growth arrest, and pVHL is associated with cell cycle arrest upon serum withdrawal.[28] Collectively, these results suggest that MDN-0066 has an effect on the VHL/HIF pathway, which is involved in cell cycle regulation. Ongoing research tries to establish the link between the cell cycle arrest and the induction of apoptosis.

In sum, this report represents the first potential lead compound from a natural source affecting cellular growth specifically in the absence of the pVHL tumor suppressor, and thus could have an important impact on the future treatment of renal cancers.

One-third of top-selling drugs are based on natural products, and they are used successfully in the treatment of numerous diseases.[26] To adequately cover this highly diverse class of potential drug candidates, EU-OPENSCREEN will include a set of natural products in its compound collection. Hence, having the expertise to isolate and characterize natural products represented in the network is of high importance to ensure that EU-OPENSCREEN satisfactorily serves the users' demand for structural diversity. Moreover, this study demonstrates the competency of the EU-OPENSCREEN network to develop novel assays that can be easily adapted for use in high-throughput screening campaigns. The screening of 1040 natural products represented just a starting point, and future screening campaigns using larger collections of natural products, including both pure compounds and extract/fraction libraries, are envisioned. The initial assay design and setup, which involves the screening of small library subsets, is of utmost importance for screening larger compound collections successfully later on, and EU-OPENSCREEN users can rely on competent collaborators, assistance, and advice during this crucial first step of the experimental workflow.

Project C: High-Content Screening to Enable Regenerative Medicine Strategies in Diabetes Mellitus

Human embryonic stem cells (hESCs) are pluripotent and hence are able to generate cell and tissue types of the three germ layers endoderm, mesoderm, and ectoderm. In this project, the EU-OPENSCREEN high-capacity screening site Fraunhofer IME Screening Port Hamburg worked closely with colleagues in HMGU Munich to identify small molecules able to direct efficient generation of definitive endoderm and serve as a platform to investigate the regulatory mechanisms of endodermal differentiation.[29] Access to such endodermal cell lineages in turn gives hope to treat diseases such as diabetes mellitus by means of transplantation therapies. As the main challenge in pancreatic transplantation therapy is the shortage of donor islets, the generation and expansion of functional β-cells from hESCs and human induced pluripotent stem cells (iPSCs) could represent a promising approach.[30] Current differentiation protocols act by mimicking the developmental steps in vitro and have facilitated generation and upscaling of pancreatic β-cells.[31] However, a major drawback of these protocols is the generation of immature β-cells compared with human β-cells found in islets.[32] One tactic to increase the efficiency of β-cell generation is to target specific pathways that are directly linked to insulin production in cultured cells.

Previous data indicate that the transcription factor FoxA2 is highly expressed in mesendoderm and definitive endoderm at the early stages of differentiation and is also expressed in all the endoderm-derived organs.[33] To facilitate high-throughput screening of novel compounds that might induce definitive endoderm, a mouse embryonic stem cell (mESC) line was used for primary screening. The mESC cell line was selected because it was relatively straightforward to perform large-scale cell culture needed to support screening and the line was previously well characterized with respect to signaling pathways involved in controlling endodermal differentiation.[33] The endoderm differentiation protocol used in the screen was based on previously published protocols with some modifications to the basal medium to achieve higher levels of FoxA2 expression in the mESCs in order to meet the needs of high-throughput assays. Under the influence of TGF-β pathway factors such as activin A, the cells differentiate successfully into FoxA2-positive cells while the undifferentiated stem cells show strong expression of the pluripotency marker Oct4.[29]

The initial high-throughput screen was performed in 384-well format with 23,406 small molecules (Enamine, Monmouth Junction, NJ), which were selected on the basis of chemical diversity and which excluded structural features associated with well-described interference effects.[4] The compounds were lead-like with a cLogP of <4.2 and a molecular weight between 180 and 460 Da. In addition, an FDA-approved drug set from Enzo (Lörrach, Germany) was also screened both with the aim to identify possible targets for active compounds and, in the longer term, for the possibility of drug repurposing.

For the primary assay, PerkinElmer Cell Carrier assay plates (Waltham, MA) were coated with autoclaved 0.1% gelatin, and compounds as well as controls were spotted by

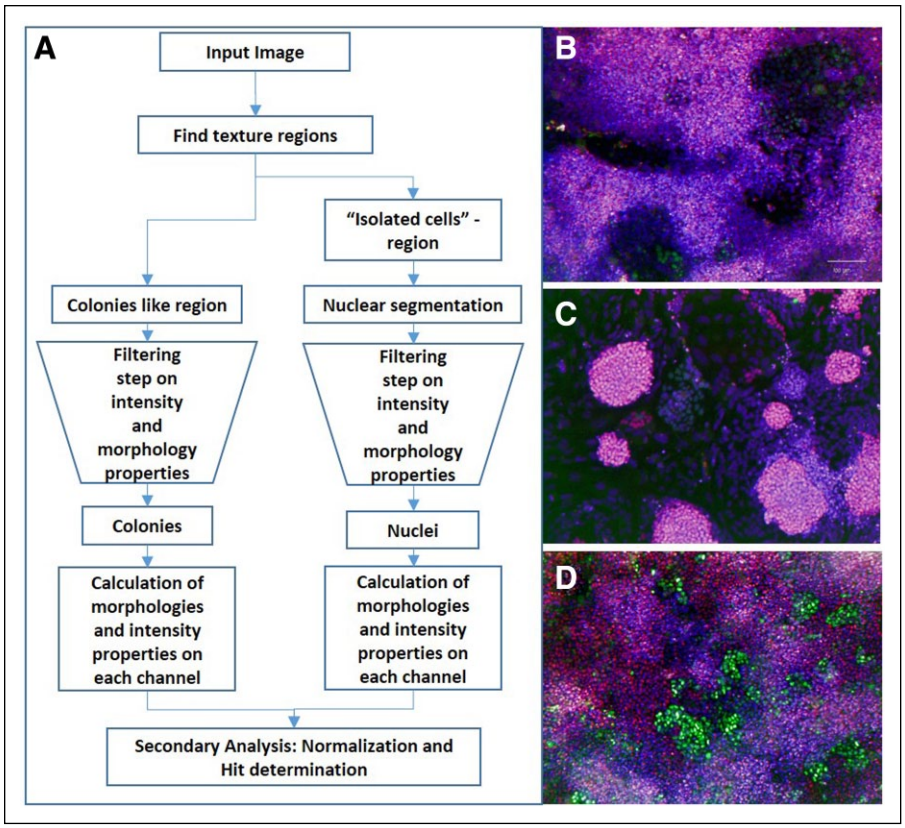

Figure 4. ROCK inhibitors as inducers of definitive endoderm formation. (**A**) General analysis workflow for primary hit selection. (**B**) DMSO solvent control. (**C**) Pluripotency control. (**D**) Differentiation control (blue: DAPI = nuclei stain; red: Alexa-Fluor 555 = Oct-3/4 detection; green: Alexa-Fluor 488 = FoxA2 detection). Figure adapted from Korostylev et al. (2017).[29]

an Echo liquid handling system (Labcyte Echo 550, Labcyte, San Jose, CA) into coated 384-well plates (source compounds at 2 mM in 100% DMSO). Wells with the differentiation control contained activin A and added Wnt3a (final concentrations of 12.5 ng/mL [activin A, R&D Systems, Minneapolis, MN] and 2 ng/mL [Wnt3a, R&D Systems]). For the pluripotency control, a GSK3β inhibitor (CT 99021, Axon Medchem, Reston, VA) was added to a final concentration of 3 µM. Negative controls were generated by adding only DMSO. Seeding of mESCs involved the addition of 50 µL of mESC suspension to give between 11,000 and 14,000 cells per well. Medium change with readdition of fresh compound and control solutions occurred on day 2 postseeding, and cells were then fixed and prepared for imaging at day 5 after seeding. Cells were permeabilized and blocked and primary (anti-FoxA2 goat polyclonal antibody [sc-6554, Santa Cruz Biotechnology, Dallas, TX] and anti-Oct-3/4 mouse monoclonal antibody [sc-5279, Santa Cruz Biotechnology)] and secondary (Alexa-Fluor 555 anti-mouse IgG and Alexa-Fluor 488 donkey anti-goat) antibodies (Thermo Fisher Scientific, Waltham, MA) were added under automated screening conditions. The image acquisition procedure used a PerkinElmer Opera Imaging System with DNA-DAPI staining. Image analysis used the features of the Columbus suite three Columbus Image Data Storage and Analysis System (PerkinElmer), which is a tool to access, store, and explore images. A multiple-step image analysis sequence was developed (**Fig. 4A**), and the DMSO control (**Fig. 4B**), pluripotency control (**Fig. 4C**), and differentiation control (**Fig. 4D**) were analyzed automatically to determine the relative response to compounds in terms of capacity to induce both proliferation and FoxA2 expression in mESCs.

For each phenotype, compounds that gave a minimum of 20% elevation in mean FoxA2 signal intensity relative to solvent control-treated cells were selected as "differentiation-inducing hits." A total of 400 primary differentiation-inducing hits from the primary screen were then tested in triplicates, and from these 67 compounds were identified for follow-up in dose response experiments. In addition, a further hit expansion was performed by selecting analogs of the 67 compounds from a set of 200,000 Enamine compounds, which were available as liquid stocks. Around three members of each cluster were selected to give 351 compounds for analysis in a seven-point dose response with serial compound dilutions and cell viability assessment to account for cytotoxicity using the CellTiter-Glo Luminescent Assay (Promega, Madison, WI). Based on the differentiation controls as well as cell viability, the ROCK inhibitor fasudil was selected for further studies to induce definitive endoderm in vitro in an analog-by-catalog approach. Out of the 16 analogs of fasudil tested, two analogs gave rise to endoderm differentiation (with 27% and 31% of FoxA2+ cells, respectively), which were comparable to the induction of FoxA2 by fasudil (25.5%) and Wnt3a/activin A (23%). In further profiling studies using the human ESC line

H9, the capacity of fasudil to induce definitive endoderm expression was confirmed by fluorescence-activated cell sorting (FACS) and qPCR analyses.[29]

This project shows the potential of chemical biology approaches for elucidating signaling pathways associated with cellular differentiation.[34–36] Small-molecule-based ROCK inhibition can play an important role in early lineage formation of stem cells. Together with low batch-to-batch variation compared with the recombinant proteins normally used for definitive endoderm induction, the ROCK inhibitor fasudil could prove to be a very useful small-molecule tool to promote differentiation of ESCs toward definitive endoderm, and therefore in the longer term act as a contributor to regenerative medicine approaches based on pancreatic transplantation therapy. Interestingly, at the CIPF EU-OPENSCREEN partner site in Spain, it was recently also demonstrated that ROCK inhibitors could have an impact not only on pancreatic cell transplantation therapies but also on transplantation of ependymal stem/progenitor cells (epSPCs) of the spinal cord in acute as well as chronic in vivo models of spinal cord injury (SCI).[37] In the context of EU-OPENSCREEN, the expertise in stem cell-based assays in conjunction with high-throughput and high-content screening present at the Fraunhofer IME Screening Port, as well as the CIPF partner sites, represents an important asset of EU-OPENSCREEN to provide new tool compounds to support the rapidly growing field of regenerative medicine.

Project D: Pharmacological Chaperones to Correct the Instability of Mutants Associated with Inborn Errors of Metabolism and Rare Forms of Cancer

Many inherited disorders are associated with defective anabolic or catabolic pathways in cell metabolism, affecting the synthesis, degradation, and storage of biomolecules. These disorders, collectively known as inborn errors of metabolism (IEMs), are typically caused by mutations in enzyme-coding genes, and although each of these disorders is rare, the combined incidence of IEM is approximately 1 in 2000 births worldwide, and new diseases continue to be recognized.[38] Nevertheless, only few IEMs can be treated effectively, and developing new therapies has proven to be challenging. Increasing knowledge on the pathogenic mechanisms has revealed that many IEM-associated mutations cause destabilization and misfolding of the coded variants. This link between mutation-associated destabilization and loss of function has been largely demonstrated for phenylketonuria (PKU),[39] which is the most prevalent IEM, caused by mutations in phenylalanine hydroxylase (PAH) that lead to neurotoxic levels of phenylalanine. Despite complex quality control systems, the folding of unstable mutant proteins is not properly achieved, and the consequent misfolding may cause increased protein degradation, aggregation, and/or mislocalization in the cell. This understanding points to pharmacological chaperones (PCs) as promising therapeutic strategies for IEM correction, earlier shown as a proof of concept for PKU.[40] PCs are low-molecular-weight compounds that stabilize and rescue variant proteins by stimulating their renaturation and preventing their misfolding in vitro and in vivo. The EU-OPENSCREEN partner site at the University of Bergen, Norway, is specialized in target-based biophysical screens and has developed concepts and a protocol for the discovery and development of PCs.

This protocol includes a primary screening of chemical libraries, searching for stabilizing binders of target proteins by differential scanning fluorimetry (DSF).[41] Customarily, DSF is performed in a real-time PCR instrument with 384-well microplates and a total assay volume of 25 μL in each well consisting of the protein sample at concentrations in the range 0.05–0.15 mg/mL in an optimal buffer, and the extrinsic dye SYPRO Orange that emits fluorescence when interacting with hydrophobic areas of denatured proteins (**Fig. 5A**). The compounds are added to the assay solution to a final concentration of 80 μg/mL and 4% DMSO. Negative controls with only 4% DMSO are routinely included on each plate. The unfolding curves are then registered from 20 to 95 °C at a 2 °C/min scan rate, and the midpoint melting temperature (T_m) and the corresponding shift relative to the reference (ΔT_m) are calculated for each compound (**Fig. 5A**). Thus, compounds with significant increases in ΔT_m are selected using in-house software and those showing adequate follow-up concentration-dependent DSF curves are considered primary stabilizing hits. Further selection requires secondary assays to corroborate binding and protein-target stabilization by several methods, preferentially surface plasmon resonance (SPR), which also provides the dissociation constant (K_D) for the primary hits. Additional secondary assays include enzymatic activity measurements to eliminate inhibitory compounds. Finally, the efficacy of the best compounds is validated in tests measuring the increased activity and steady-state levels of the target protein in eukaryotic cells and animal models (**Fig. 5B**).[42] The partner site at the University of Bergen has used this protocol in the early-stage discovery and validation of compounds for conformational stabilization of specific enzymes associated with IEMs.[43–46] Methylmalonic aciduria cblB type (MMA cblB) is caused by the impairment of ATP:cob(I)alamin adenosyltransferase (ATR), the enzyme that catalyzes the synthesis of adenosylcobalamin. ATR was screened for the first time for small-molecular-weight stabilizing ligands, and six potential PCs were discovered from the MyriaScreen Diversity Collection from Sigma Aldrich (St. Louis, MO), of which compound V (*N*-{[(4-chlorophenyl)carbamothioyl]amino}-2-phenylacetamide)

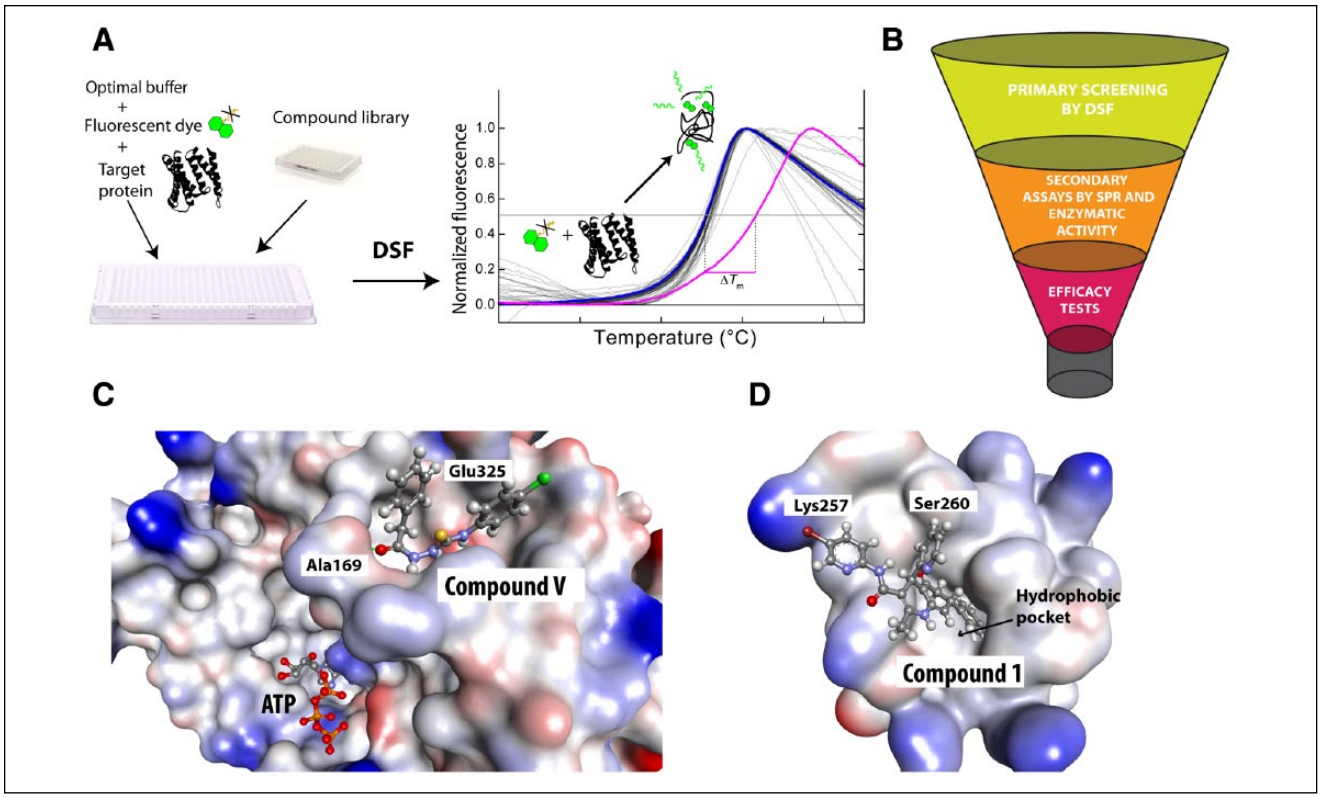

Figure 5. DSF-based screening and discovery of PCs. (**A**) In DSF screening the protein is mixed with SYPRO Orange and the compounds transferred from the chemical libraries. Fluorescence is recorded at increasing temperatures and stabilizing compounds (i.e., significant positive ΔT_m) with respect to the controls are selected. (**B**) Schematic funnel for the discovery of PCs, including the primary DSF screening, secondary assays by SPR, and enzymatic activity and validation by efficacy tests in cells and in vivo. (**C**) The binding site of hit compound V in one subunit of tetrameric ATR (PDB ID 2IDX), predicted by molecular docking, showing interacting residues and the location of the substrate ATP. See also Jorge-Finnigan et al. (2013).[43] (**D**) The binding mode of hit compound **1** to the C-terminal domain of NPM (PDB ID 2LLH), predicted by docking, wherein its phenyl ring is placed in a hydrophobic pocket establishing polar interactions with solvent-accessible residues. See also Urbaneja et al. (2017).[46] The proteins in **C** and **D** are shown as solvent-accessible surfaces with projected interpolated positive (blue) and negative (red) charges.

was very effective in increasing the stability and activity of common MMA cblB-associated ATR variants overexpressed in a cellular model, as well as in mice.[44,45] The K_D value for binding of compound V to ATR was 7.4 ± 0.4 μM, showing a relative high affinity for a primary hit. Molecular docking for compound V identified a probable binding site surrounded by residues 228–240 and 165–175 at each subunit in the ATR tetramer (**Fig. 5C**). Recently, a similar DSF-based screening has been applied to discover compounds that improve the conformational defect of mutant nucleophosmin (NPM). This is a multifunctional nucleolar protein where deletion mutations at Trp288 and Trp290, located in the C-terminal domain, lead to cytoplasmic localization and aggregation, features that are associated with acute myeloid leukemia (AML).[47] NPM is an established target in cancer, and as such has been the subject of several screening and medicinal chemistry campaigns; most of this activity is reviewed in Di Matteo et al.[48] However, previous studies did not consider NPM a target for pharmacological chaperoning. By using the DSF-based screening, the partner site in Bergen identified stabilizing hit compounds that bind to a hydrophobic pocket in NPM and show chaperoning potential. The phenyl ring of the best and novel NPM hit *N*-(5-bromopyridin-2-yl)-2-methyl-5-oxo-7-phenyl-4-pyridin-2- yl-4,6,7,8-tetrahydro-1*H*-quinoline-3-carboxamide (compound **1**) binds into the pocket, where it is surrounded by a number of hydrophobic residues adjacent to the AML mutation sites Trp288 and Trp290. Compound **1** additionally establishes favorable polar interactions with Lys257 and Ser260 (**Fig. 5D**). Furthermore, in a cellular system, this hit compound reduced the aggregation and increased nuclear localization of NMP mutants. The identified hits appear promising for PC-based therapies for AML.[47]

DSF constitutes a sensitive assay of ligand binding and an efficient biophysical readout for target-based screening, contributing to the EU-OPENSCREEN portfolio. As exemplified above, this primary screen is particularly appropriate

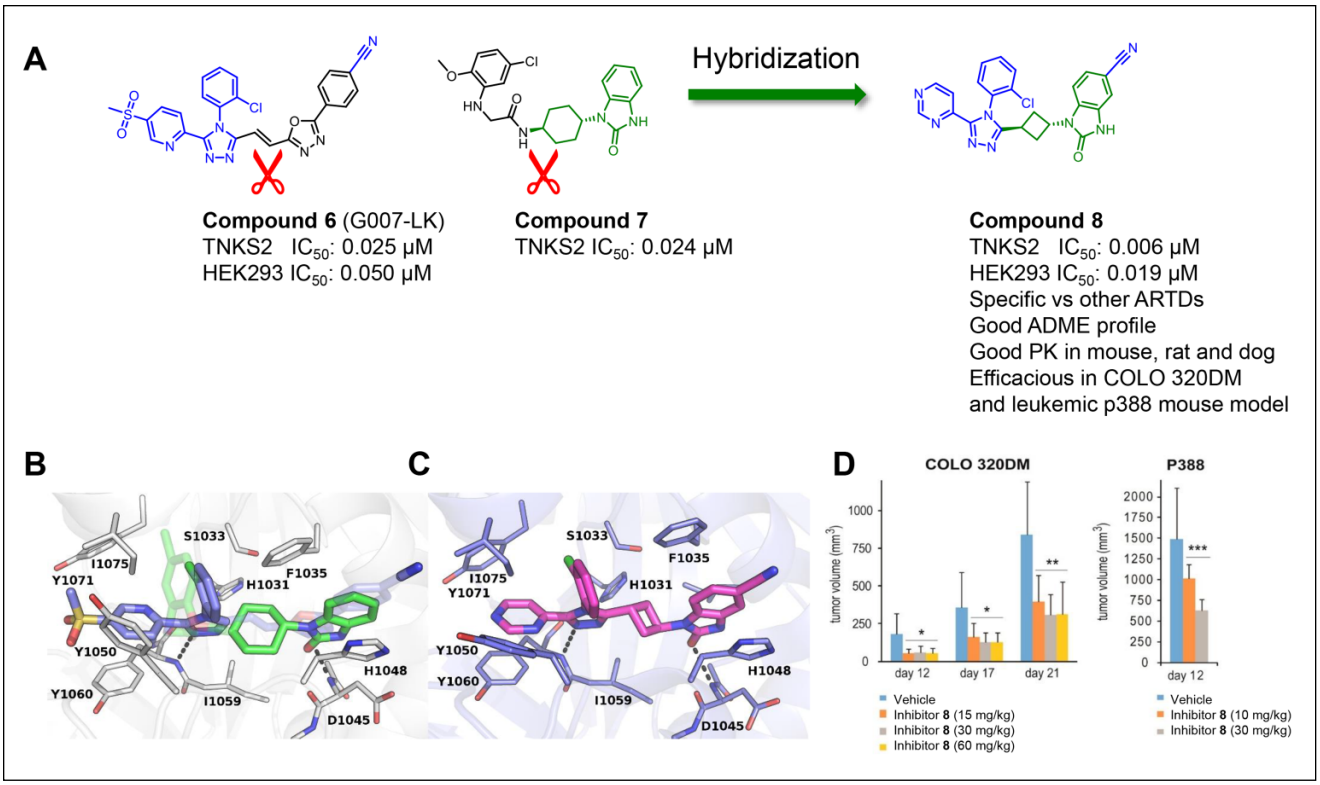

Figure 6. Structure-guided chemical hybridization approach toward a new specific tankyrase inhibitor as a high-quality chemical tool. (**A**) Structures of **6–8**. Hybridization logic to yield **8** from the deconstructed parent inhibitors **6** and **7**. (**B**) Superposition of **6** (blue) and **7** (green) co-crystal structures (PDB: 4HYF and 4K4E). Only TNKS2 protein is shown for clarity. (**C**) Co-crystal structure of **8** (magenta) with TNKS2 (PDB: 5NOB). (**D**) Antitumor activity of **8** in xenograft models. Left panel: COLO320DM colon cancer xenograft; right panel: isogenic p388 leukemia mouse model. Reduction of tumor volume (mm^3) versus vehicle-treated controls (blue) after once daily oral dosing of **8** at various depicted doses. Statistical significance is indicated: ANOVA on Ranks/Dunn's method, *$p < 0.05$; one-way ANOVA/Holm–Sidak method, **$p < 0.001$; and one-tailed ***$p < 0.05$. Figure compiled and modified with permission based on the original publication: Anumala et al. (*J. Med. Chem.* 60, 10013–10025).[53] Copyright 2017 American Chemical Society.

for the early-stage discovery of stabilizing ligands that can be developed into chaperoning treatments. Nevertheless, DSF-based screening has also been successfully applied for the discovery of effective inhibitors.[49,50] Most screening campaigns performed so far at the Bergen partner site have used diversity libraries of up to 18,000 compounds. However, recent updates in robotics allow the screening of libraries with a throughput of 50,000 compounds in about 2 weeks, paving the way for screening subsets of the EU-OPENSCREEN compound collection for selected targets in high-throughput screening times.

Project E: High-Quality Chemical Tools for the Perturbation of Aberrant Wnt/β-Catenin Signaling—Construction of Tankyrase Inhibitors by a Hybridization Approach

WNT/β-catenin signaling is altered in a variety of tumors, including tumors emerging from colorectal tissue, uterus, pancreas, skin, liver, thyroid, prostate, ovary, stomach, lung, lymphoid, bladder, brain, breast, and kidney. Increased β-catenin levels have been identified as a central factor in T-cell infiltration in melanoma specimens,[51] and a correlation between WNT/β-catenin pathway activation and immune exclusion has been observed across numerous human cancers.[52] The key effector in the hippo pathway, YAP, has also been identified as an oncoprotein whose expression is elevated in various human cancers.[53]

Tankyrases 1 and 2 (TNKS1/PARP-5a/ARTD5 and TNKS2/PARP-5b/ARTD6) are members of the poly-ADP-ribose polymerase (PARP) subfamily of the human ADP-ribosyltransfrase family of enzymes with homology to diphtheria toxin. Tankyrases have been identified as regulators of the WNT/β-catenin signaling pathway via interactions with AXIN protein and a regulator of the hippo-signaling pathway via interactions with members of the AMOT family of proteins.[53] The inhibition of tankyrases produces elevated AXIN protein levels and reduced levels of cellular β-catenin even in the absence of a dysfunctional and truncated form of APC protein. The inhibition of tankyrases

also stabilizes the AMOT family proteins, thereby suppressing YAP oncogenic functions.

The work presented here is a close collaboration between the FMP, which is an EU-OPENSCREEN medicinal chemistry partner site; the Department of Immunology and Transfusion Medicine, Oslo University Hospital; and the Faculty of Biochemistry and Molecular Medicine, Biocenter Oulu, University of Oulu.[54] The project started off with an initial screening campaign at the FMP Screening Unit—an EU-OPENSCREEN screening partner site—and yielded a 1,2,4-triazole-based selective tankyrase inhibitor JW74 with reasonable biochemical affinity (TNKS2: IC_{50} = 0.46 μM) but only moderate cellular activity (HEK293: IC_{50} = 1.01 μM).[55] While other reports on screening approaches in the context of tankyrase inhibitors have been published previously as well,[56–59] a subsequent optimization by a classical analoging strategy led to a first lead structure **6** (G007-LK) (HEK293: IC_{50} = 0.05 μM; TNKS2: IC_{50} = 0.025 μM[60]), which was highly potent and showed outstanding selectivity toward the other members of the ARTD family.[61] Crystal structures of tankyrase had revealed that the early inhibitors compete with the substrate NAD+ through binding to the conserved nicotinamide[62] or to the adenosine subpocket.[63] Analysis of the co-crystal structure of **6** showed that it binds to the adenosine subpocket, explaining the high selectivity toward tankyrases over other ARTDs. However, albeit showing an excellent oral bioavailability in mice, further development of **6** was hampered by poor pharmacokinetics in rats and concerns regarding solubility and photostability. Structurally, this was attributed to the extended, highly conjugated, aromatic system incorporating a vinylic bond in combination with the intrinsically high lipophilicity, low Fsp^3 content, and a critically high molecular weight for some derivatives. Collectively, this suggested that incremental structural changes by further classical optimization may not solve these issues. It was therefore reasoned that a structure-guided hybridization approach aiming to partially deconstruct the structure of **6** to a virtual fragment and merge/join it with a new privileged tankyrase binding motif would provide the necessary significant structural change while still preserving the binding mode and distinct interaction pattern for affinity and selectivity.[54] After analyzing several available tankyrase inhibitor co-crystal structures, it was hypothesized based on the co-crystal structures of TNKS2-**6** (PDB: 4HYF) and TNKS1-**7** (PDB: 4K4E)[64] that joining the diaryl substituted 1,2,4-triazole of **6** and the benzimidazolone of **7** with an appropriate linker would yield a suitable hybrid inhibitor (**Fig. 6A,B**). Three different linker types, phenyl, cyclohexyl, and cyclobutyl, were chosen as replacements of the vinylic bond to provide the appropriate distance and conformational adaptability within this new class of tankyrase inhibitors. Docking was used to check compatibility with the binding pocket, and synthesis of the three designed inhibitors was accomplished in a nine-step synthesis. Initial testing for TNKS1 and TNKS2 affinity in a biochemical assay and activity in human embryonic kidney HEK293 as well as in the human colon SW480 cell lines as functional assays of the WNT/β-catenin signaling pathway revealed compound **8** as the most potent hybrid tankyrase inhibitor for which activity was observed (HEK293: IC_{50} = 19 nM; SW480: IC_{50} = 70 nM), accompanied by a favorable biochemical IC_{50} (TNKS1: IC_{50} = 29 nM; TNKS2: IC_{50} = 6.3 nM). Inspection of the co-crystal structure confirmed that the hybrid inhibitor recapitulated the binding mode of both parent inhibitors (PDB: 5NOB; **Fig. 6C**). All essential contacts of the used fragments were preserved and addressed in the protein ligand complex TNKS-**8**. Selectivity profiling showed an exceptional selectivity over the other ARTD enzymes, and none of the other tested ARTD enzymes were inhibited at 100 or 10 μM, respectively. This confirmed the hypothesis that the selectivity characteristics of the parent inhibitors would also be inherited by the newly designed hybrid compound **8**. In addition, compound **8** showed no relevant inhibition in an extended kinase selectivity profiling (320 kinases with >50% inhibition at 10 μM: 4/320; CLK2: 73%; MELK: 70%; PRKG1: 66%; and TSF1: 52%). In line with the favorable calculated physicochemical properties and Lipinski rule-of-5 compliance, **8** showed good in vitro ADME properties, proved to be metabolically stable in human liver fractions across species and in human hepatocytes, and also showed good predicted absorption properties acceptable for in vivo studies. An overall good bioavailability in mouse (F = 47%), rat (F = 35%), and dog (F = 91%) upon oral administration, including a surprisingly low compound excretion in urine and feces in rat, underscored the suitability of **8** as a chemical tool for a subsequent pharmacological in vivo evaluation.

Next, **8** was evaluated in mouse xenograft models using the human colorectal cancer cell line COLO 320DM cells in male Balb/c nude mice and a *syngeneic* leukemic p388 mouse model. Chronic treatment with **8** was well tolerated and resulted in 53%, 63%, and 63% statistically significant tumor size reductions after 21 days at 15, 30, and 60 mg/kg, respectively, once daily oral administration in the COLO 320DM xenograft model. Similarly, in the leukemic p388 mouse model statistically significant tumor size reductions of 32% and 57% for 15 and 30 mg/kg, respectively, were observed after 10 days (**Fig. 6D**).

These results highlight the suitability of compound **8** as a new high-quality chemical tool to investigate the role of tankyrases in cellular but also in in vivo pharmacological models. This novel lead structure has been further developed in an extensive medicinal chemistry lead-to-candidate program in collaboration with Mercachem, Nijmegen, Netherlands.[65]

This example shows how essential subsequent medicinal chemistry approaches are for optimizing initial screening hits to obtain high-quality probes with cellular and biochemical IC_{50} values in the nanomolar range. Moreover, it illustrates how nonclassical medicinal chemistry approaches can capitalize from available structural biology information and how a highly interdisciplinary project environment will favorably impact future chemical tool compound development within the EU-OPENSCREEN network.

Summary and Outlook

The case studies described above represent only a small subset of the EU-OPENSCREEN network capabilities. The infrastructure will provide the enabling framework and resources for fostering discoveries and multinational, multidisciplinary collaborations in research and development in the field of chemical biology. For example, a chemical compound discovered or synthesized in Spain will be included in the EU-OPENSCREEN compound collection, will subsequently meet a biological target from Finland in a screening experiment conducted at a facility in Germany, and as a consequence of these collaborative efforts, will trigger a new research and development program. Moreover, the integration of diverse scientific disciplines is also demonstrated by the inclusion of the entire natural product drug discovery pipeline in EU-OPENSCREEN, starting with the isolation of microorganisms from extreme environments, through fermentation, extraction, and screening, to isolation and chemical characterization of the bioactive natural products. In a further extension of EU-OPENSCREEN's capabilities, a fragment collection of 1000 compounds will be established in cooperation with the structural biology infrastructure, INSTRUCT (https://www.instruct-eric.eu), and used to support structure-based discovery efforts on isolated targets. EU-OPENSCREEN will provide users access to the fragment collection for their screening campaigns and also to medicinal chemistry expertise to further optimize the identified fragment hits into leads and tool compounds. Other additions to the library, such as DNA-encoded libraries or large-molecular-weight compounds other than natural products, are currently not envisioned, and further library expansions will depend on the funding situation.

Furthermore, EU-OPENSCREEN is building synergies as it will cooperate with a number of other initiatives in Europe (European Strategy Forum on Research Infrastructures [ESFRI], Joint Programming Initiatives [JPIs], European Research Area Networks [ERA-NETs], and Innovative Medicines Initiatives [IMIs]), which promote innovation in the health and environment sectors. In this way, chemical biology research in Europe can reach a scale competitive to that of other similar large communities in the United States, Asia, and elsewhere. Common quality and operating standards, which have been defined in the frame of EU-OPENSCREEN by all participating research groups, will for the first time allow screening results of different European platforms to be collected and comparatively analyzed in a joint database. Such data (e.g., screening results, assay protocols, and chemical information about the substances and their biological activities), which are generated in expensive research projects, are currently published only in a limited and restricted manner (e.g., only selected positive data supporting a hypothesis). The EU-OPENSCREEN working model will link its database to other life sciences databases and informatics resources, and actively promote interoperability of produced data.

In the example projects, the wide range of methodological approaches used illustrates the broad expertise available within the EU-OPENSCREEN partner site network and the ability to meet the future demands from users. In the malarial target-based drug discovery project from the Latvian Institute for Organic Synthesis, the original fragment screen, NMR analyses, and medicinal chemistry design and synthesis allowed for a structure-informed optimization taking hits with potencies in the range of 25 μM to selective compounds with nanomolar potency and a well-defined structure–activity relationship (SAR) for the target protein. In the RCC project from Fundación MEDINA, a natural product workflow was described that identified a novel lipodepsipeptide, not previously reported in the literature. The compound MDN-0066 was shown to have an effect on cell cycle regulation via the VHL/HIF pathway, inducing apoptosis specifically in pVHL-deficient cells. In the ESC differentiation project from Fraunhofer IME, a high-content phenotypic screening approach was implemented with assays extending up to 5 days with complex multistep media replacement, fixation, staining, and imaging protocols. These analyses revealed pathway-specific inhibitors, which were further elucidated using a cost-effective analog-by-catalog method, which confirmed the role of ROCK inhibition in the initiation of ESC differentiation into definitive endoderm. The elaborate DSF-based biophysical assay system developed at the University of Bergen yielded PCs with the potential to correct IEMs and rare forms of cancer, based on conformational stabilization of target proteins in a therapeutic setting. The nonclassical medicinal chemistry approaches based on available structural biology information, in conjunction with iterative screening at the FMP medicinal chemistry and screening partner sites of EU-OPENSCREEN, led to the discovery of novel tankyrase inhibitors, which recently entered a lead-to-candidate program.

EU-OPENSCREEN will ensure that it also meets the users' demand for exciting new technologies and innovative assay designs in the future by regularly updating existing and/or adding new partner institutions and member countries. Central to this will be the establishment of a new category of EU-OPENSCREEN partner sites focused on offering access to chemical proteomic technologies,

including affinity-based and biophysical readouts such as cellular thermal shift assay platforms. The newly planned sites will allow for the identification of the underlying targets of compounds identified in phenotypic screens, as well as off-target and liability-associated activities. These new platforms will be piloted in the next 3 years as part of the recently established Horizon2020 EU-OPENSCREEN DRIVE project. Altogether, the result of the work of the EU-OPENSCREEN infrastructure and its associated community of screening, medicinal chemistry, and disease biology expert teams will be high-quality chemical probes and bioactivity data sets for use by the wider life science as well as drug discovery communities.[66,67]

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Declaration of Conflicting Interests

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: M.N., S.K., and L.L. have filed a patent application on tankyrase inhibitors (WO2018118868). A.M. is co-inventor of a granted patent on pharmacological chaperones for MMA type cbIB (ES2485540B1 21/10-2015).

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