D5.2 Mode of action or compound distribution report for compounds from selected projects from phase 2

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1 Executive summary

In EU-OPENSCREEN-DRIVE, the work package (WP) 5 team has continued to provide transnational infrastructure access to support basic and applied scientists seeking new chemistry-driven mechanistic insights into disease biology. WP5 has contributed to elucidating novel mechanisms, targets, and related drug discovery parameters using sensitive chemoproteomic and spatially resolved mass spectrometry imaging (MSI) methods. The studies performed have been highly interdisciplinary in nature, involving scientists from across the spectrum of biological, chemical, bioinformatic, and biophysical sciences, both as providers of platform access and as users. Previously in WP5, we focused on establishing chemoproteomics as a new service category which was previously not part of the EU-OPENSCREEN service portfolio (www.eu-openscreen.eu/). Furthermore, through two open calls in September 2019 and April 2021, five chemoproteomic projects and one MSI project were selected and implemented at seven EU-OPENSCREEN-DRIVE partners facilities. Deliverable 5.2 "Mode of action or compound distribution report for compounds from selected projects from phase 2" covers results from three projects selected during Phase 2 of the open calls that were reported in D5.1, as well as further work from two Phase 1 projects (PID9068, PID9452). Building upon the experience acquired during Phase 1, infrastructure access to second-stage projects was provided in an integrated "multi-site" model to account for the complexity of the service offered (see Annex 4.1 for a list of partners). This model was described in detail in Deliverable 1.2, "Handbook and defined user workflows for new categories of partner sites" (https://drive.eu-openscreen.eu/fileadmin/user_upload/DRIVE_D1.2_Handbook-UserWorkflowsForNewCategoriesOfPartnerSites.pdf).

2 Report on the deliverable

2.1 Overview

In recent years, the number of approaches available for target deconvolution has increased significantly. However, access to the technologies necessary for this research has been fragmented and incoherent, and these technologies are often unavailable to scientists seeking to translate disease understanding and drug pharmacology at the tissue or cellular level into a comprehensive molecular-level description. The EU-OPENSCREEN-DRIVE WP5 community has provided users access to excellent academic and biotechnology organisations dedicated to chemoproteomic and MSI technologies as part of six user access projects across phases 1 and 2.

By offering users access to chemoproteomics and MSI infrastructures, the aim of WP5 was to provide users the opportunity to exploit technologies that enable the following:

- Elucidation of new therapeutic targets and identification of novel mechanism(s) of action (MoA) linked to compounds identified in phenotypic drug discovery using disease-relevant *in vitro* models with high intrinsic predictive value for clinical translation.
- Better understanding of the MoA of chemical biology-derived tool compounds in the context of their interactions with cells. This will lead to the development of next generation chemical biology tools and an increased understanding of fundamental biological processes.
- Increased understanding of off-target properties of lead compounds and drug molecules and the underlying liability and toxicity-associated mechanisms.

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- Quantification of the distribution of lead compounds and drugs and their pharmacodynamic (PD) responses at the multicellular and tissue level using MSI. This quantification will enable a better understanding of the engagement of compounds with their targets and the concentration dependence of associated PD responses.

The chemoproteomic and MSI fields offer tremendous opportunities for progress in European drug discovery, especially due to technological advances in mass spectrometry instrumentation and new experimental paradigms based on biophysical concepts. However, its efforts have been generally dispersed and have not aligned with the needs of users in both industry and academia. In WP5 we have uniquely combined existing and novel tools provided by the starting community to cater to the needs of the industrial and academic drug discovery community; provide enhanced and better structured access to the research infrastructures for chemoproteomics and MSI fields; and develop a skilled multi-stakeholder user community. Through these actions, we have thereby supported the pathway for applying these technologies towards the elucidation of drug action, distribution, and safety.

The Phase 2 projects were selected after a rigorous external scientific review process, as described in Deliverable 5.1 (https://drive.eu-openscreen.eu/fileadmin/user_upload/DRIVE_DeliverableD5.1-ModeOfAction-CpdDistributionReport.pdf). Project selection criteria also included the potential to cover broad aspects of chemoproteomics and MSI disciplines and therefore allow the EU-OPENSCREEN-DRIVE teams to evaluate infrastructure performance and capacity to deliver diverse services relevant to the needs of the wider user community.

The indication areas of the selected Phase 2 stage projects were neurodegenerative diseases (PID16073) as well two cancer related topics, including molecular mechanism elucidation (PID16252) and compound disposition analysis in organ and colorectal cancer models (PID18898). The continued Phase 1 project PID9068 covered target identification for compound-induced centrosome clustering phenotypes in cancer, while PID9452 developed and characterized new tools for the specific visualization of CB1 and CB2 receptors.

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2.2 Detailed report on the work carried out in WP5 trans-national access user projects

As described above, a group of seven WP5 members provided experimental support to three access projects (PID16073, 16252, 18898) from the second phase of applications and two projects from the first phase. A summary of services provided is reported in Table 1 and the detailed work is described in sections 2.2.1 through 2.2.3. Due to the public nature of this deliverable, sensitive and project-specific information (e.g., compound structures, targets, etc.) are not disclosed.

Table 1 Summary of Phase 1 & 2 transnational access projects to SINTEF, USC, TUM, FVB-FMP, IBCH-PAS, HZI, and MUAS

Entry stage (phase)	Project	Research area	Service	Partner	Project status
1	PID8335	Mood disorders (e.g., depression, obesity, etc.)	Advanced mass spectrometry-based proteomics	SINTEF, USC	Reported in D5.1, work finalized
1	PID9452	Inflammatory diseases	Synthesis, purification and spectroscopic characterization of probes	FVB-FMP	Initial work reported in D5.1, extended here
			Advanced mass spectrometry-based proteomics	TUM, USC	Initial work reported in D5.1, extended here
1	PID9068	Cancer	Synthesis, purification, and spectroscopic characterization of probes	IBCH-PAS	Initial work reported in D5.1, extended here and finalized
			Advanced mass spectrometry-based proteomics	USC	Initial work reported in D5.1, extended here
2	PID16073	Neurodegenerative diseases	Advanced mass spectrometry-based proteomics	TUM	Work finalized
2	PID16252	Anticancer target identification	Synthesis, purification, and spectroscopic characterization of probes	HZI	Work finalized
			Advanced mass spectrometry-based proteomics	TUM	Work finalized
2	PID18898	Cancer	Advanced mass spectrometry imaging of drug and metabolites	CeMOS (MUAS)	Work finalized
			Mass spectrometry analysis of drug and metabolites	SINTEF	Work finalized

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2.2.1 PID9452: "Traceless labelling of cannabinoid receptors"

Abstract: The aim of the project, in close collaboration with several EU-OPENSCREEN-DRIVE partners (FVB-FMP, TUM, USC), is to develop new tools for the specific visualization of Cannabinoid receptor type 1 (CB1) and Cannabinoid receptor type 2 (CB2). A central requirement for the advancement of novel clinical and therapeutic indications is a deeper understanding of the CB receptors' molecular pharmacology. However, sufficiently reliable and specific tools for this purpose are currently not available. By developing small molecule-based ligand-directed labeling strategies, the team at FVB-FMP successfully tagged the CB1 and CB2 receptors with a fluorescent cargo. Thorough chemical synthetic strategies were developed for this purpose. We found that, using a particular fluorescent label, we are able to sense conformational changes of SNAP-Lumi4Tb-tagged CB receptors, making this construct a biosensor.

Synthesis, purification, and spectroscopic characterization of probes: As described in Deliverable 5.1, chemical probe synthesis was based on a common structural motif (diethyl glycine), and variations on linker attachment site and length (PEG1-4, thioalkyl PEG3-4), pharmacophores (three drug derived scaffolds, CB1/CB2), and labeling moieties (O-NBD, tosylalkyl, acyl-imidazole, N-acyl-N-alkyl sulfonamide) were explored. Most promising synthetic routes were consolidated, and an effective amide bond-based probe design was established. A convergent synthesis route for ligand-directed labeling of N-acyl-N-alkyl sulfonamide electrophile was optimized. Different fluorophores (Alexa488, sulfoCy5, SiR, TAMRA), together with biotin, were introduced by copper-catalyzed click chemistry to achieve ligand-directed labeling probes for CB1 and CB2 receptor. Chemical synthesis steps included preparation, purification (silica gel, HPLC, precipitation), characterization (1H-NMR, 13C-NMR, HMBC-NMR, HSQC-NMR, COSY-NMR), and photophysical characterization (UV-Vis absorbance, fluorescence emission, quantum yield). Specifically, synthesis steps were extensively routed and optimized, together with purification procedures.

Chemoproteomics: The project partner at the University of Nottingham subjected the most promising compounds to purified membrane fractions derived from CB1/CB2-overexpressing HEK293 cells. Probe-labelled membrane fractions were distributed to project partners (TUM, USC) for further testing (proteomics) to i) confirm the successful labeling and ii) identify the labeling site. Protocols for optimizing conditions by in-gel analysis, pull-down proteomics, LC-MS/MS analysis, data-dependent acquisition (DDA), qualitative analysis, and SWATH mass spectrometry analysis were already developed and could be adapted to test these samples (USC). At TUM, samples were in-gel digested using different proteases to increase sequence coverage of labelled CB1 and CB2 and thus increase the chance of identifying the labeling site. The expected chemical modification was added to the database search in MaxQuant, and an additional unbiased search was performed using MSFragger. Data analysis is still ongoing.

Conclusions: Chemical probes for CB1R and CB2R were developed based on a common building block (>30). Different chemical variations had to be explored and judged by chemical accessibility and labeling functionality. A convergent synthetic route for ligand-directed labeling N-acyl-N-alkyl sulfonamide electrophile was selected as most promising. We achieved not only the design, synthesis, and functionality of novel ligand-directed labeling probes (>10) for both CB receptors, but also explored a facile chemical construction. The chosen design of the probes allows fast chemical access and rapid installation of various tags and reporter units. The obtained tools are useful for the investigation of novel aspects of CB receptor pharmacology. Among them are the

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specific visualization and biosensor construction. Membrane-bound receptor material labeled with probes of the series are currently under investigation at TUM. If the currently employed experimental design does not successfully identify the labelling site, another chemoproteomic strategy will be employed. Briefly, the click handle at the labelled protein will be covalently attached to a cleavable linker (clinker approach) immobilized on a solid support. After washing the matrix, the enriched peptides carrying the labeling tag will be released and identified using mass spectrometry.

2.2.2 PID16073: Identification of the pharmacological target of compounds modulating the activity of the cellular prion protein

Abstract: Researchers from the University of Trento have identified small molecules capable of abrogating prion protein toxicity, which is the cause for neurodegenerative diseases such as prion diseases and Alzheimer's. However, the actual cellular effector protein of these molecules was still unknown. Together with the EU-OPENSCREEN-DRIVE partner TUM, project-specific chemoproteomic affinity matrices were generated and used for competition pulldown experiments. Several molecular targets of the compounds of interest were successfully identified and will now undergo dedicated experimental follow-up to evaluate which target is responsible for the observed biological effect.

Chemoproteomics: After the initial screening hit and several rounds of compound optimization, the researchers found two potent compounds which were subjected to chemoproteomic target deconvolution experiments performed at TUM. One of the two molecular structures was converted into a linkable version by the addition of a primary amine to the molecular scaffold and coupled to NHS-activated Sepharose beads. Chemoproteomic pulldown experiments were performed in complex native HEK293 lysate using standard conditions. A quantitative label-free proteomics readout revealed three protein targets bound by both compounds of interest with nanomolar affinity.

Conclusions: The chemoproteomic experiments successfully identified hitherto unknown binding partners of the two compounds of interest. The obtained dose–response curves demonstrate binding affinities between 60 nM and 165 nM for these compounds, which aligns with the previously observed mid-to-low-nanomolar activity in various cellular, *ex vivo*, and *in vivo* models for prion disorders and Alzheimer's disease. The identified protein targets require further validation to determine i) their (patho)biological role in these neurodegenerative diseases and ii) whether they are the molecular effector for the compounds' mode of action. If this is the case, these molecules may well form a pharmacological starting point for the development of future therapies.

2.2.3 PID16252: Identification of targets responsible for anti-cancer activity of (5-hydroxymethyl) isophthalate derivatives

Abstract: Researchers from the Helmholtz-Zentrum für Infektionsforschung (HZI) collaborated with EU-OPENSCREEN-DRIVE partner TUM to investigate the biological targets of novel compounds that were identified by a user from University of Helsinki as potential anticancer drugs. These compounds were designed as protein kinase C (PKC) activators but were found to exhibit PKC-independent anticancer activity. The molecular mechanism for the anticancer activity of these compounds is not known but believed to be the result of interactions with unidentified cellular binding partners. We aimed to identify these cellular binding partners using competitive chemoproteomic pulldown experiments. For this approach, a linkable analogue of the drug

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candidates was synthesized and used to generate a chemoproteomic affinity matrix. Competitive pulldown experiments were performed in HEK293 cell lysates, followed by quantitative proteomic analysis. Unfortunately, no binding could be observed to the main target, PKC alpha, nor to any other protein.

Synthesis, purification, and spectroscopic characterization of probes: For the selected chemoproteomic approach, an affinity ligand is required – in this case, an analogue of the drug candidates bearing a linker with a handle for immobilization. The biological activity of the parent compound must be retained in the probe for it to be a suitable affinity probe**;** thus, a non-immobilizable analogue bearing the linker was also needed as a control. Expertise sharing between partner sites enabled the design of a linker functionalization strategy which does not reduce the binding of the known binding partner PKC by the probes. The structural variation from the parent molecules was significant enough to prevent the transfer of methods for preparation of the parent molecules to the probes. As such, the synthesis of these novel targets required investigation and adaptation of different methods to incorporate the necessary structural features. To reduce the time needed for preparation of both probes, a late-stage divergence strategy was employed. Ultimately, the synthesis of the chemoproteomic probes was achieved on a milligram scale over six steps (eight synthetic steps in total for the two probes). The probes were successfully prepared in the quantities required by HZI and were sent to both the user and TUM for further testing.

Chemoproteomics: Chemoproteomic target deconvolution was planned for three compounds of interest: 1) PKCa activator comprising both PKCa activity and PKCa-independent anticancer activity, 2) a negative control which does not bind to PKC and does not have anticancer activity, and 3) a PKCa control which binds to PKCa but has no anticancer activity. Protein targets that are hit by compound 1 but not by compounds 2 and 3 might be the cellular effector protein for the observed PKCa-independent anti-cancer activity. At TUM, the linkable probe was immobilized on NHS-activated sepharose beads, yielding a project-specific affinity matrix. Competitive pulldown experiments were performed in complex native HEK293 lysates produced by the user and in a full dose response. Unfortunately, PKCa could not be enriched by the immobilized probe. Furthermore, no convincing dose response was observed for any other protein.

Conclusions: Binding assays performed by the user confirmed that the structural variations incorporated do not impact binding of the known binding partner PKC by the probes, but cell viability assays showed that the probes were less potent than the parent compound. Unfortunately, the competitive pulldown experiments did not deliver the expected results. This negative outcome may have been due to low membrane permeability. However, according to the user the more likely explanation is that the binding with the potential anticancer target also happens within the plasma membrane akin to the primary target PKC. In this case, the lysate-based affinity matrix approach may not be suitable to identify this interaction. However, despite the negative results, this information was rated very useful by the user and leads them to pursue more focused experiments towards membrane proteins now. It was discussed with the user whether a photoaffinity labeling (PAL) approach may be beneficial to continue the project. Here, transient and low-affinity interactions are stabilized by a photoactivatable probe, which establishes a covalent bond to the target protein upon UV irradiation. Given that the probes most likely interact with their target proteins within the plasma membrane, this experimental strategy may also not lead to the desired outcome, and also the timeframe of the project was too short to pursue this option.

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2.2.4 PID18898: MSI as a powerful tool to study metabolites and tissue distribution of OATD-02 –a dual arginase inhibitor

Abstract: Molecure, a clinical-stage biotechnology company that discovers and develops small molecule drug candidates, collaborated with EU-OPENSCREEN-DRIVE partner sites CeMOS (MUAS) and SINTEF to assess the systemic and tumor responses against OATD-02, which was developed by the user as a potent dual ARG1/ARG2 inhibitor with immunomodulatory and antitumor functions. To this end, we aimed to detect possible PD response markers and assess the distribution of these markers and OATD-02 in tested organs and the colorectal cancer CT26 model using matrix-assisted laser desorption/ionization (MALDI)-MSI. An LC-MS/MS method for OATD-02 and metabolites was also developed.

Tumor, control-dosed, and OATD-02-dosed samples were received from the user. Initial ionization experiments were performed to optimize the experimental workflow. After experimental workflow optimization, MALDI-MSI was performed on samples to evaluate drug distribution and PD markers.

Description of the service offered: MALDI-MSI analyses (i.e., sample preparation, data acquiring, and MSI data analyses) were offered by CeMOS (MUAS), and some initial experiments were also performed at SINTEF. Initially, target plate analyses were performed to test the ionization of the compound, OATD-02. To do this, different MALDI matrices, matrix compositions, and concentrations were tested to obtain optimal signal intensities for the compound. Following these initial tests, ionization data from different concentrations of the compound were evaluated both on the target plate and control tissue by spotting the compound dilution series. The initial experiments helped to understand compound ionization and elucidated the compound's limit of detection (LoD).

Based on the workflow optimizations, MALDI-MSI was performed on the selected organs (liver, kidney, brain, spleen) and tumor samples from OATD-02-dosed CT26 tumor-bearing mice. To assess the time course differences on the potential PD markers and OATD-02 localization, samples collected in different time courses were compared with the control samples dosed with vehicle.

As a systemic response to OATD-02, MALDI-MSI analyses revealed that urea cycle metabolites were detected as PD response markers in liver and kidney tissues. Tumor samples showed a homogenous distribution of the drug metabolite with a corresponding increase in arginine levels. Finally, differences in polyamine levels upon drug administration were shown to indicate potential implications of drug effects in different time courses as well as a cytostatic effect of OATD-02.

The present data provided by CeMOS to the user supported the in-depth understanding of the drug metabolite localizations and drug metabolism (e.g., PD markers, changes in metabolites).

Conclusions: Based on the ionization results, the compound ionized well on the target plate and when spotted on the slide. The detection of the OATD-02 in dosed organs was limited; however, the major metabolite of the drug and its PD effects are clearly demonstrated in dosed organs.

Successful detection of drug metabolite and PD response markers using MSI supported the user to better understand the metabolic pathways altered by OATD-02. As a result of changes in polyamines, which are necessary for tumor progression, important preliminary findings have been presented to the user on how OATD-02 can inhibit cancer cell proliferation and progression.

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2.2.5 PID9068: Target ID of centrosomal clustering inhibitors

Abstract: The project aims to identify target candidates for optimized analogues of the antifungal drug griseofulvin with a low micromolar cellular potency for inhibiting centrosomal clustering in cancer cells.

Unlike normal cells, malignant cells possess supernumerary centrosomes which require clustering in two spindle poles for effective proliferation. Therefore, targeting this mechanism holds great potential for the development of cancer cell-specific antiproliferative agents.

The GF15 analogue of griseofulvin (GF) has been shown to suppress tumor cell growth both *in vitro* and *in vivo* through inhibition of this centrosomal reorganization, possibly by altering microtubules. However, pulldown experiments with biotinylated and photo-affinity probes did not deliver proteins which could be confirmed as real targets. Also, identification of 35 gene candidates in clones with acquired GF resistance by whole genome sequencing failed to deliver novel regulators of centrosome reorganization.

Synthesis, purification, and spectroscopic characterization of probes: A GF analogue with confirmed intracellular activity was selected as an affinity ligand and furnished with the uniquely designed linker 1 at the attachment point (ketoxime ether linkage). This linder-containing analogue has been previously shown to retain some of the biological activity. The novelty of the probe designed in this work compared to previously used probes lies in the introduction of a bifunctional covalent anchor moiety that reacts with nucleophiles on the surface of the protein when reversibly bound to the protein of interest by the ligand (GF analogue). This results in a covalent attachment of the pulldown handle (in our case, a click handle) to the protein of interest, leaving the active site unchanged and allowing for pulldown in denaturing conditions, leaving only direct binders in the proteomic fraction.

Several synthetic pathways, reactive motifs, linker characters (e.g. PEG vs. alkyl), and lengths were explored. In conclusion, alkyl-type linker 1 elicits far more stable intermediates than PEG-type linkers. First-generation probes (ProbeONE-1 α *and* β) were obtained as enriched mixtures (80:20 and 20:80) of regioisomers Z- and E- across ketoxime ether linkage. The immediate precursor not bearing the ligand (Boc-protected *O*-alkyl hydroxylamine ProbeONE-2) was also prepared as negative control to track non-specific labeling events. Both compounds were used in the pulldown experiments. Synthesis of second generation probes with alternative reactive groups / pull-down tags has been explored.

Proteomics analyses: A protocol for optimization of in-gel analysis and pulldown experiment was established, using a resin with a click chemistry handle and a chemically cleavable linker. Briefly, cell lysate samples with induction and without induction of centrosomal clustering were exposed to two different probe concentrations, followed by the pulldown experiments. The LC-MS/MS analysis of the resulting enriched protein fractions were analysed using DDA mass spectrommtry and SWATH-MS analysis. These experiments initially revealed 12 upregulated proteins and 11 downregulated proteins in induced cell lysate samples vs. non-induced samples.

Conclusions: The complex nature of the ligand/inhibitor (i.e., bearing alkylation-susceptible moieties), together with challenges associated with i) the formation of unsaturated system-conjugated ketoxime ether linkage and ii) intrinsic reactivity and hydrolytic susceptibility of the bifunctional covalent anchor (reactive group) at the linker, limited the number of stable probes that could be synthesized. Nevertheless, two probes (differing by linker) and compound variants as

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negative and positive controls have been prepared and tested in live cells (for activity) and cell lysates (for protein identification by MS).

A chemoproteomic strategy to identify target protein candidates for GF was developed by integrating chemical synthesis of functionalized click chemistry probes and protocols for probe enrichment by pulldown proteomics. Preliminary analyses of chemoproteomic data, based on first generation probes, yield a list of differentially abundant target proteins in sample sets related to the phenotype of interest (centrosome amplification) and probe enrichment. Synthesis of second generation probes have been explored to provide the user with the alternative for future testing in live cells and pulldown MS proteomics to be performed beyond the scope of EU-OPENSCREEN-DRIVE project.

3 Next steps

In collaboration with the users, the WP5 project teams will prepare scientific publications over the next period after securing any potential intellectual property associated with the work performed. Where possible, FAIR data will also be added to community repositories such as PRIDE and the European Chemical Biology Database in order to support eventual reuse by the wider community.

For project PID9068, further functional validation experiments will be performed by users to identify candidates involved in GF-induced centrosome amplification. The results of these experiments will be used to generate hypotheses about the mode of action for GF as well as the protein interactions relevant in centrosomal amplification. For project PID16073, further studies will be conducted on the identified candidate protein targets to elucidate their specific roles in neurodegenerative processes and to confirm their putative mechanisms of action and the routes by which they act as molecular effectors. For project PID9452, chemoproteomic experiments are still ongoing and will be finalized within the next 1–2 months.

One achievement of WP5 was its establishment of “chemoproteomics sites” as a new EU-OPENSCREEN partner site category. This category was formally adopted at the Assembly of Members (AoM) Meeting 10 on 22.09.2022. As a practical outcome, an open call for the nomination of new partner sites is planned for 2024, and this call will include opportunities to nominate sites under the new category. Preliminary discussions indicate that several sites across the EU-OPENSCREEN member country network will apply for the category, indicating the underlying need to provide these services to the wider user community. The inclusion of this new category will ultimately elevate the utility of EU-OPENSCREEN services and increase the overall impact of the research infrastructure.

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4 Annexes

4.1 Annex 1

EU-OPENSCREEN-DRIVE institute	Description of expertise offered
Institute of Bioorganic Chemistry Polish Academy of Sciences, Department of Molecular Probes and Prodrugs & Center for High Throughput Screening Studies (IBCH PAS)	IBCH PAS offers support for the synthesis of 2–4 probes to study target engagement and/or fluorescent labeling for one validated bioactive compound with an identified activity-benign point of attachment on its scaffold. Probes are composed of linkers with variable length connecting a ligand (derived by the user) with a specific tag (e.g., biotin for proteomics or fluorophore for imaging). Synthesis is performed in a convergent manner, and several linker–tag adducts are synthesized in parallel. The resulting probes are then attached through a stable moiety to the identified hit scaffold. IBCH PAS also provides advanced proteomic identification of the targets.
Helmholtz Centre for Infection Research, Department of Chemical Biology (HZI)	The Department of Chemical Biology at HZI aims to discover new antibacterial and antiviral drugs, characterize their functionality, and optimize their properties. HZI focuses on infection research and studies small molecules that can function as antimicrobial or antiviral agents, interfere with pathogenicity factors, or stimulate the immune system. Within this call, HZI offers expertise in the area of drug conjugates to synthesize functionalized chemical probes. HZI has ample experience in the synthesis of bacteria-targeting conjugates that combine a small molecule and an effector moiety (an antibiotic, a fluorophore, etc.) through cleavable and non-cleavable linkers. Using these probes, HZI will support the affinity-based target identification using chemoproteomics.
Leibniz Research Institute for Molecular Pharmacology (FVB-FMP)	The medicinal chemistry group at FMP offers chemical probe optimization (activity, selectivity, linker attachment exploration) and follow-up modification of labeled chemical probes for target deconvolution, fluorescent labeling, and crosslinking. Within this call, FMP designed and synthesized a novel series of CB2R and CB1R selective probes for ligand-directed traceless proximity labeling of these receptors. This enables the collaborators now to covalently label these receptors with a variety of reporters without the need of any genetic manipulation.

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SINTEF Trondheim, Department of Biotechnology and Nanomedicine (SIN)	SINTEF provides access to advanced mass spectrometry-based proteomics facilities for drug–target interaction studies. This includes facilities for quantitative proteomics to support non-targeted and targeted screening of drug–protein interactions. Using robotic handling, SINTEF offers high-throughput sample preparation (extraction, lysis, in solution digestion, FASP, SPE clean-up) for both targeted and shotgun proteomics analysis. For shotgun proteomics to support non-targeted screening of drug–protein interaction, a QExactive-HF-X mass spectrometer is employed (at a core facility). For targeted screening of identified interactions, LC-MS/MS (Agilent QQQ) analysis is offered to enable highly sensitive analysis of multiple peptides. Targeted, high-throughput screening of identified interactions is also offered using Rapidfire-MS/MS (Agilent). In targeted analysis, isotope-labelled peptide standards are employed. Data processing and bioinformatics data interpretation pipelines are also offered including statistical analysis. Moreover, within this call, SINTEF offers mass spectrometry imaging (MSI) for compound disposition studies. MSI facilities at SINTEF include sample preparation setups (cryostat sectioning, ImagePrep for matrix and reagent deposition) and sample analysis using ultra-high-resolution mass spectrometry on a Bruker Solarix XR 12T FTICR. Analysis of the resulting imaging data is provided though by multivariate statistical analysis (SCiLS software). The MSI-based facilities offered by SINTEF support the determination of discrete tissue distribution of the parent compound, its metabolites, synthetic derivatives, and endogenous molecules linked to pharmacological and toxicological mechanisms of the compound. Thus, the facilities enable the investigation of i) the relative compound disposition of close structural analogues and ii) the target activation and possibly modes of action for compounds at the site of action.
Technical University of Munich, Chair of Proteomics and Bioanalytics (TUM)	TUM Proteomics offers a broad range of advanced mass spectrometry-based proteomic methods and expertise on specialized chemical proteomics technologies for drug target deconvolution. Established chemical proteomics workflows include the generation and application of affinity matrices from small molecules and natural compound derivatives. Specialized affinity matrices enriching subproteomes (protein kinases, HDACs) are readily available at TUM and allow for selectivity profiling of small molecule kinases and HDAC inhibitors. In addition, TUM provides facilities for cellular thermal shift assays (CETSA) and isothermal dose–response (ITDR) experiments. They also provide a bioinformatics infrastructure (400 TB of online data storage, about 20 server computers totaling 300 CPUs, and about 1 TB memory, 6

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	GPUs) and operate ProteomicsDB, a powerful in-memory database for the real-time analysis of proteomic data.
Mannheim University of Applied Sciences, Center for Mass Spectrometry and Optical Spectroscopy (MUAS-CeMOS)	CeMOS, a research center for MUAS, contributes expertise in mass spectrometry and optical spectroscopy, with substantial expertise in application development for the chemical, pharmaceutical, MedTech and Biotech industries. Within this call, CeMOS provided access to Mass Spectrometry Imaging (MSI) for (quantitative) compound disposition studies, discovery and imaging of response markers and evaluation of toxicology findings. Focus areas included lipid/metabolite as well as infrared microscopy-guided MSI in fresh-frozen tissue sections, mainly in the therapy areas of oncology, neurodegeneration and metabolic diseases. CeMOS offers state-of-the-art instrumentation for MSI sample preparation (HTX M5 and M3 sprayers) and analysis using high-resolution mass spectrometry on a Bruker solariX XR 7T FTICR, ion mobility spectrometry MS on Bruker timsTOF PRO2 and timsTOF flex and high-speed mass spectrometry on a Bruker rapifleX. Suitable data analysis tools are available (SCiLSlab and in-house bioinformatics). CeMOS also offers high-speed mid-infrared imaging of tissues on Bruker Hyperion II and Perkin Elmer Spotlight 400. Moreover, CeMOS offers access to MALDI MS-based assays for drug discovery. Assay facilities include Analytik Jena CyBio Felix pipetting robots for sample preparation and Bruker mass spectrometers for measurements. Assays are currently run in 384-well format and can be either biochemical assays with enzymes causing mass changes or cell-based assays where enzyme inhibition causes an accumulation of the substrate.
	BioFarma at USC aims at discovering new drugs by identifying chemical matter with pharmacological activity. USC focuses on drug discovery research and small molecules interfering with relevant mechanisms of disease. USC offers expertise in chemoproteomics of functionalized chemical probes. USC has experience in the assay development, screening and target identification approaches. USC may support comprehensive biological characterization of probes for affinity-based target identification using chemoproteomics.

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5. Glossary

AoM: Assembly of Members

ARG1: Arginase 1

ARG2: Arginase 2

Alexa488: 3,6-diamino-9-(2,5-dicarboxyphenyl)-4,5-disulfo-Xanthylium

CB1: Cannabinoid receptor type 1

CB2: Cannabinoid receptor type 2

CETSA: Cellular Thermal Shift Assays

COVID-19: Coronavirus disease 2019

DDA: Data-dependent acquisition

ERIC: European Research Infrastructure Consortium

EU-OS: EU-OPENSCREEN ERIC

GF: Griseofulvin

HPLC: High performance liquid chromatography

ITDR: Isothermal dose response

1H-NMR: Proton nuclear magnetic resonance

13C-NMR: Carbon-13 nuclear magnetic resonance

HMBC: Heteronuclear multiple bond correlation

HSQC: Heteronuclear single quantum coherence

COSY: Homonuclear correlation spectroscopy

HEK cells: Human embryonic kidney cells

HRMS: High-resolution mass spectrometry

LCMS: Liquid chromatography–mass spectrometry

LC-MS/MS: Liquid chromatography–mass spectrometry/mass spectrometry

LDNASA: N-acyl-N-alkyl sulfonamide

LDSP: N-sulfonyl pyridine

LoD: Limit of detection

MoA: Mechanism of action

MALDI-MSI: Matrix-assisted laser desorption/ionization mass spectrometry imaging

MSI: Mass spectrometry imaging

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NBD: Nitrobenzoxadiazole

NMR: Nuclear magnetic resonance

PKC: Protein kinase C

PAL: Photoaffinity labeling

PD: Pharmacodynamics

PEG: Polyethylene glycol

PID: Project identification number

RI: Research infrastructure

SDS-PAGE: Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

SiR: Silicon rhodamine

SNAP-Lumi4Tb: O6-Benzylguanine-Lumi4-Tb commercialized by Cisbio

TAMRA: 5-Carboxytetramethylrhodamine

TR-FRET: Time-resolved fluorescence resonance energy transfer

SWATH-MS: Sequential window acquisition of all theoretical mass spectra

WP: Work package

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