

CCMS: A New Era in Toxicoproteomics

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Capture Compound Mass Spectrometry (CCMS) represents a powerful platform technology for the generic identification of a drug's mode of action, as well as the prediction of potential off-targets responsible for adverse side effects.

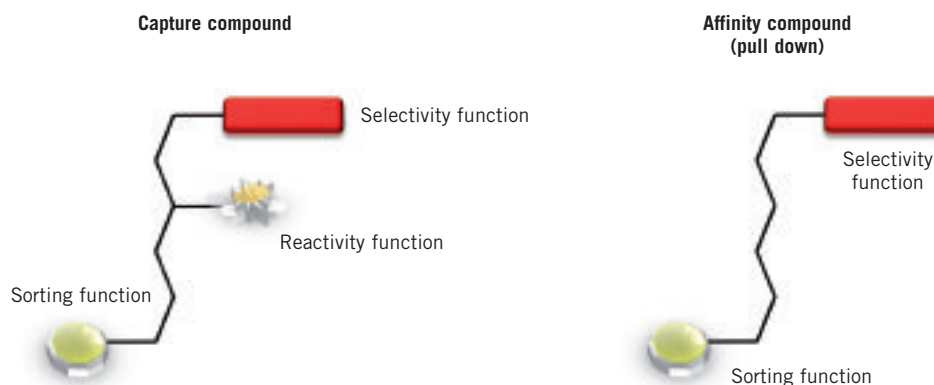
Over the last two decades, the process of drug discovery and development has changed rapidly from an *in vivo*-driven science to an *in vitro*-driven streamlined process (1). Many useful *in vitro* assays were developed to predict *in vivo* absorption, distribution, metabolism and excretion (ADME) and pharmacokinetic (PK) properties (2). In the 1990s it was declared that quantity beats quality, and the higher the number of compounds screened, the greater the success that could be anticipated. But this proved to be wrong, and along with more and more stringent regulations for drug filing and development, fewer and fewer drugs were approved (3). On the other hand, the compound libraries that had been produced prompted the pharma industry and its service providers to develop even faster *in vitro* methods to assess the properties of their compounds. It was possible to evaluate the compounds one thousand-fold in a statistically significant manner and compare them with older medications.

The combinatorial compound libraries produced during this period of time were more lipophilic than the old compound collections, and were causing huge problems in terms of solubility, permeability and selectivity, and consequently failed more often during drug development. As a consequence, Christopher Lipinski, along with many other scientists, developed rules to

counteract the development towards new chemical entities (NCEs) that were too lipophilic and undruggable (4). As a result, and in contrast to the growing molecular weight of compounds in screening libraries, other technologies such as fragment-based drug discovery (5) made their way into the drug discovery process. Fragment-based drug discovery produced smaller, water-soluble hits at the beginning of the drug discovery process. The concept of ligand efficiency was introduced and further developed in order to have a better understanding of drug-likeness (5).

But the biggest achievement of *in vitro*-driven drug discovery was the acknowledgment that it is indeed possible to assess and change certain drug properties such as permeability, solubility and stability early in the discovery process; this was the main reason why drugs had failed during the 1980s (6). The attrition rate due to pure pharmacokinetics was reduced dramatically; by contrast, after the introduction of *in vitro* ADME/PK screening campaigns, toxicity, lack of efficacy and commercial factors were the main reasons for attrition (7). Toxicity is still a big caveat and *in vitro* assessment of human toxicity outcome is not reliable (8); in particular, hepatotoxicity does not translate well into human toxic events. Although there have been attempts to predict accurately *in vivo* toxicities, the big success achieved with

Figure 1: Structural comparison of a Capture Compound™ and an affinity compound. The Capture Compound is a trifunctional molecule, which consists of a sorting function, reactivity function and a variable selectivity function. Traditional affinity compounds lack the reactivity function and consequently lose weak interacting partners during washing





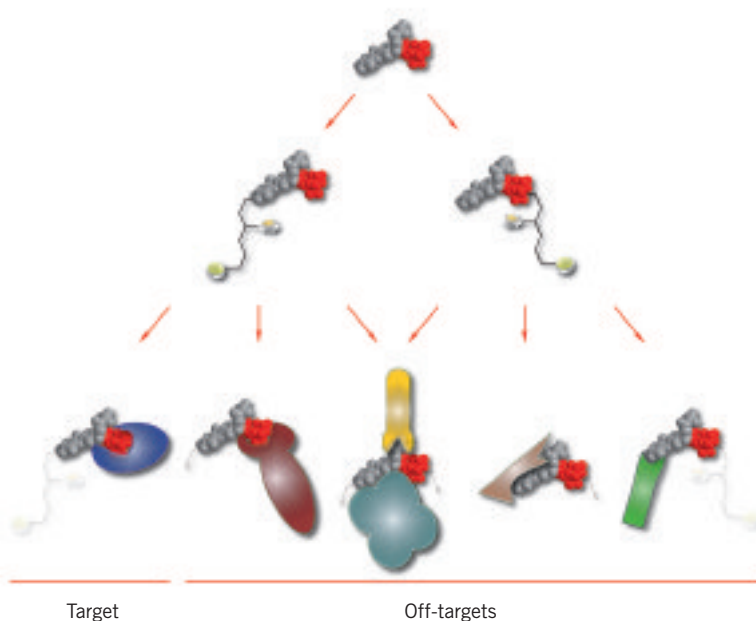
ADME *in vitro* screening models has not been repeated up to now. Drug developers are therefore in need of a method of predicting and identifying critical drug-protein interactions reliably – not just in the liver but in any tissue of the human body.

Despite the merits of pre-clinical toxicological studies in which a large number of potential human toxicities can be identified, there is still a significant percentage of new pharmaceuticals that have not exhibited toxicity during animal testing experiments, but have gone on to cause adverse side effects in humans. Instead of conducting cost-intensive clinical studies that – due to unforeseen human toxicities – bear a high risk of failure, a reliable up-front method for predicting potential interactions with off-target proteins would seem absolutely essential.

CCMS TECHNOLOGY

Capture Compound Mass Spectrometry (CCMS) is a versatile and straightforward in-solution affinity and photo cross-linking-based technology (9), that can be expected to have a significant impact on the landscape of drug R&D and safety. At the core of CCMS technology are small trifunctional synthetic molecules, Capture Compounds™ (CCs), that can selectively capture native target proteins – even lipophilic membrane proteins – from complex protein mixtures such as human lysates on the basis of small molecule-protein interactions (9). The schematic structure of CCs is illustrated in Figure 1. CCs integrate a small molecule selectivity function (the drug molecule to be investigated), a photo-activatable reactivity function (covalent cross-link of interacting proteins) and a sorting function for the direct isolation of CC-protein conjugates into a small molecule scaffold.

As pointed out above, finding the right drug candidates that ideally possess high efficacy and low toxicity is one of the major challenges in drug discovery and development. The CCMS approach enables the robust profiling of the mode of action of drugs under investigation in a fast and cost-effective manner. During drug development, CCMS can be used as a tool for the elucidation of drug-target interactions and the discovery of off-target molecules responsible for unwanted side effects. As shown in Figure 2, by changing the attachment site of the selectivity group, it is possible to specifically search for the actual target and/or potential off-targets. CCMS therefore helps to identify the toxicity of drug candidates in humans, and enables the exact prediction of the interaction profile of any small organic molecules with human proteomes of any source, thereby



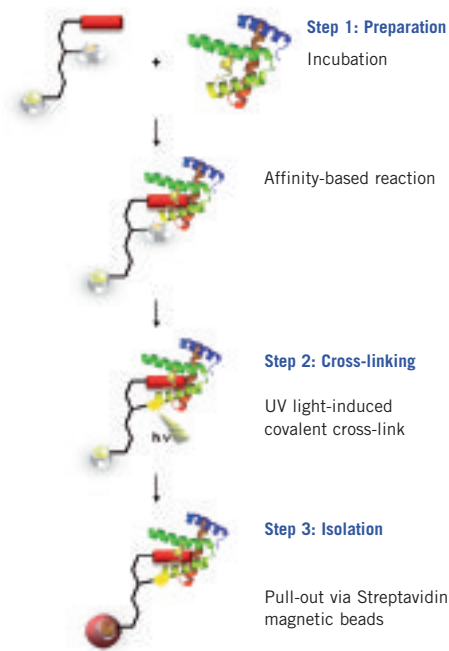
allowing the mode of action, selectivity and toxicity of the compounds to be foreseen.

The CCs are particularly suited to reflecting true small molecule interactions, since it is conducted in solution directly on proteins endogenous to complex biological samples; the proteins, including multi-spanning transmembrane receptors, are in their native state with intact protein-protein associations. No over-expression of recombinant proteins is required, or even desirable, for the discovery of proteins that interact with a given drug. The specificity of the capture experiment becomes obvious through comparison of the proteins identified in the capture samples with samples to which an excess of the selectivity group has been added prior to irradiation to compete for the specific capture of target proteins.

Compared with traditional affinity chromatography and affinity beads, CCMS offers major innovative advantages. As an in-solution approach, the technology exhibits no limitation by a solid support in terms of restricted ligand accessibility due to pore size distribution. The unique key feature of CCMS – a distinct photo-inducible reactivity moiety that can covalently attach the interacting partners to the CC – makes for stringent washing conditions and, therefore, the isolation and identification of weakly bound proteins (see Figure 1). This provides a significant difference compared with small molecule affinity bead approaches that are prone to lose specifically bound proteins, even during mild washing procedures. In terms of the number and quality of specific protein identifications, a dramatic benefit of the cross-link is observed in a direct comparison with pull-down experiments where no UV irradiation is performed and thus no immobilisation of protein targets occurs (see Figure 1).

Figure 2: caprotec's drug molecule profiling process: a pharmacologically active substance is linked to a CC scaffold at two spatially different locations. Different attachment points allow the identification of the original target and many off-target proteins. R: Capture Compound Scaffold attached to the small molecule

Figure 3: The CCMS approach comprises three easy steps. In Step 1, Capture Compounds bind proteins through reversible affinity interaction. In Step 2, a covalent bond between the Capture Compound and target protein is generated by photo cross-linking. In Step 3, Streptavidin-Biotin interactions are used to isolate captured proteins for Western blot or MS analysis, respectively



Although antibody-based immune precipitation is also carried out in solution, it is based on non-covalent affinity interaction and limited to known targets.

In contrast to the activity-based probe (ABP) approach, which is restricted to those small molecules as selectivity groups that have the intrinsic property to covalently bind to the active centre of enzymes such as serine proteases (10), CCMS can be applied to small molecules as selectivity groups that interact with their target proteins in a non-covalent fashion. This probably applies to the vast majority of existing drug molecules.

THREE SIMPLE STEPS

A typical capture experiment is accomplished in three simple steps (see Figure 3). In the first step, 50-500µg of total protein mixture input is used per assay; the assays are prepared in volumes of 100µl in PCR tube strips. After adding the CC to the protein sample, binding to the target protein via its selectivity group under

equilibrium conditions is allowed (Step 1, Figure 3). In the second step, the assays are placed in the caproBox™, a dedicated device for well-controlled reproducible sample irradiation under cooled conditions, and irradiated to induce a photo cross-link between the CCs reactivity group and the proteins that interact with the selectivity group (Step 2, Figure 3). Through this photo cross-link, the interaction is irreversibly fixed, and will withstand the harsh washing conditions that are applied in the third step to remove unbound proteins. For the washing procedure, the CC-protein conjugates are collected from the assay by streptavidin magnetic beads that bind to the biotin sorting function of the CC (Step 3, Figure 3).

Two principle configurations of the assay are possible: in the first ('OffBead'), the CCs are allowed to first react with the target proteins in solution, and, only after the irradiation, the streptavidin magnetic beads are added to collect the CC-protein conjugates. In the second configuration, the CCs are first loaded

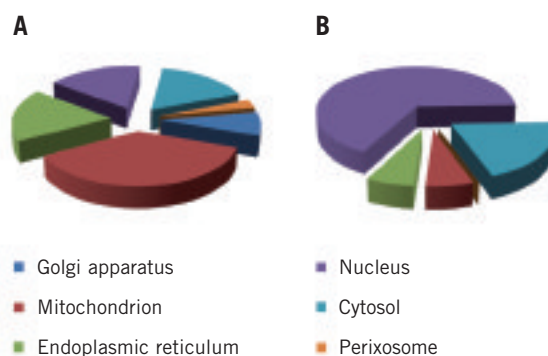
onto the streptavidin magnetic beads to yield caproBeads™ which are then added to the protein sample for target binding and photo cross-linking ('OnBead'). The magnetic beads are handled using the caproMag™, a device for the handling of magnetic beads in an assay of up to 12 tubes. In this configuration, the samples are washed in six cycles of collecting the beads, discarding the supernatant and re-suspending the beads in fresh washing buffer, all under high-salt conditions. Ultimately, for protein identification, the captured proteins are proteolytically cleaved by trypsin, and the peptides are subjected to high-resolution and high-accuracy nanoflow LC-electrospray-MS/MS

In a case study performed with a human hepatocyte cell line, CCMS was used for the first time to unravel the molecular basis of the hepatotoxicity of tolcapone, the catechol-O-methyltransferase inhibitor (COMT) used in the treatment of Parkinson's disease (Fischer *et al*, submitted). In this study, the protein capture profiles of CCs with tolcapone and the structurally related drug entacapone clearly showed that the former binds additionally to crucial proteins involved in β-oxidation and oxidative phosphorylation. It is likely that a malfunction of the respiratory chain or fatty acid β-oxidation leads to hepatotoxicity. This finding provides an explanation for the previously reported clinical and cell physiological observations (11). The cellular distribution of identified proteins captured by tolcapone-CCs (124 proteins in total) and entacapone-CCs (20 proteins) is depicted in Figure 4.

CONCLUSION

Taken together, these results indicate that CCMS represents a new era in toxicoproteomics. It is a powerful

Figure 4: Cellular localisation of proteins specifically interacting with (A) Tolcapone-CCs (124 proteins) and (B) Entacapone-CCs (20 proteins). (Assignment according to Gene Ontology's Cellular Component AmiGO)



platform technology for the generic identification of a drug's mode of action, as well as the prediction of potential off-targets responsible for adverse side effects. In combination with the minute amounts of starting material needed, CCMS can be customised for any drug target by basically exchanging the selectivity function – providing a highly versatile drug profiling tool with an unrivalled level of sensitivity. Besides shedding light on biochemical pathways that were previously not understood, CCMS will help in re-designing drugs already on the market, as well as clinical drug candidates, by structure optimisation. Thus, CCMS can be seen as filling a vital technological gap in the assessment of a drug's mode of action and safety – making it a uniquely versatile technology in the drug discovery and development toolbox.

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Dr Friedrich Kroll is Head of Medicinal Chemistry at caprotec GmbH (Berlin, Germany). He has 14 years' experience in industrial drug discovery and R&D, and has worked at Pharmacia, Lundbeck and Galapagos in managerial and project team roles of increasing responsibility. Friedrich has experience in the therapeutic fields of inflammation, CNS indications (depression, Parkinson's disease, Alzheimer's disease, pain) and in cardiovascular and metabolic disease. Prior to joining the pharmaceutical industry he spent three years as a Senior Medicinal Chemist at the University of St Andrews (Scotland). Email: friedrich.kroll@caprotec.com



Dr Mirko Glinski joined caprotec GmbH in June 2008 as Head of Analytical Chemistry. Prior to this, he was responsible for the R&D Analytics Department at the pharmaceutical company, IDEA (Munich, Germany). Mirko obtained a PhD in Chemistry from the University of Berlin in 1999; he was then appointed a Postdoctoral Fellow at the University of Berlin working on the biosynthesis of peptide antibiotics, before undertaking a Postdoctoral appointment at the Max Planck Institute of Molecular Plant Physiology (Potsdam, Germany) where he worked on protein phosphorylation dynamics in plant metabolism.



Dr Mathias Dreger joined caprotec in 2008 and heads the Biochemistry Department at caprotec bioanalytics, where he is responsible for the design and conduct of capture experiments in biological samples. Prior to his affiliation with caprotec, he worked as a Biochemist in the fields of Neurochemistry, Subcellular Biochemistry and Proteomics at the Free University, Berlin. For several years he ran a proteomics core facility lab at the Department of Physiology, Anatomy and Genetics at the University of Oxford (UK), with a focus on novel analytical strategies in proteomics.



Dr Erik Dülsner joined caprotec in 2008 and is an Application Scientist with caprotec bioanalytics. He studied Biochemistry at the Free University, Berlin, and completed his PhD in Biochemistry at the German Cancer Research Center (Heidelberg) working on COX-2 dependent signal transduction in the urinary bladder of K5.COX-2 transgenic mice. He then held a postdoctoral position at the Ludwig-Maximilians University Gene Center (Munich), working on the identification of biomarkers for pancreatic cancer.



Prof Dr Hubert Köster is Chief Executive Officer of caprotec bioanalytics. He founded and led several successful biotechnology companies, including Biosynthech, Biosearch, Milligen/Biosearch, Sequenom and caprotec. Hubert studied Chemistry at the University of Hamburg (Germany), obtained his doctorate in Chemistry at the Max Planck Institute for Experimental Chemistry (Göttingen) and undertook post-doctoral research at the Max Planck Institute for Virus Research (Tübingen). He has also been a Visiting Professor at Boston University (USA) and a Professor of Chemistry at the University of Hamburg.