

Advanced functional protein kinase enrichment using the Dasatinib caproKit™

Capture Compound Mass Spectrometry (CCMS) is an innovative technology to reduce the complexity of biological samples through selective isolation of targeted protein or enzyme families. Small synthetic molecules (Capture Compounds™) interrogate native proteins, even lipophilic membrane proteins. CCMS technology enables an efficient complexity reduction of the proteome and allows discovering, isolating and profiling members of functional protein families within a variety of biological samples, e.g tyrosine kinases.

Introduction

The profiling of the kinomes of biological systems is a major focus of current functional proteomics investigations. Kinases are among the key players in virtually all signal transduction cascades in mammals and many other organisms. Dysregulation of kinases occurs within numerous pathophysiological events, such as cancer. The profiling of kinases may yield important information on cellular events. Capture Compound Mass Spectrometry (CCMS) is a technology designed to enable the in-depth profiling of protein families on the basis of their functional interaction with a small molecule that is the selectivity function of a trifunctional Capture Compound™ (CC). The recently introduced Stauro Capture Compound™ can address a wide range of kinases within the mammalian kinome, using the broadband kinase inhibitor staurosporine as the selectivity function (1). However, on the basis of current knowledge of the affinities of different kinase inhibitors (2), additional Capture Compounds™ are desirable that address complementary sub-kinomes. One of these

inhibitors is dasatinib, a protein kinase inhibitor and anti-cancer drug that primarily addresses a range of receptor and non-receptor tyrosine kinases (3). This application note presents data obtained from capture experiments with the Dasatinib Capture Compound™ (Figure 1) which is part of the Dasatinib caproKit™. The Dasatinib-CC displays a distinct capture profile that is complementary to the Stauro-CC. The Dasatinib-CC is capable of isolating a range of tyrosine kinases from HepG2 cell lysate and can also be used to study potential non-kinase off-targets of dasatinib.

Materials

The two caproKits™ used for the experiments were: Dasatinib caproKit™, containing the Dasatinib Capture Compound™ solution, the Dasatinib competitor, streptavidin magnetic beads, 5x concentrated wash buffer 1 and 5x concentrated capture buffer, as well as the non-receptor tyrosine kinase Src as a GST fusion protein as the positive control. The Stauro caproKit™ contains the Stauro

Capture Compound™, the Staurosporine competitor, and positive control and the other components as described for the Dasatinib caproKit™. A UV filter frame was applied during irradiation of the samples for the capture experiments. The chemical structures of the Dasatinib-CC and the Stauro-CC are shown in Figure 1.

Capture experiment

Cell lysate was prepared as follows: HepG2 cells were lysed by French Press in lysis buffer (6.7 mM MES, 6.7 mM NaOAc, 6.7 mM HEPES, pH 7.5, supplemented to 200 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA, and protease

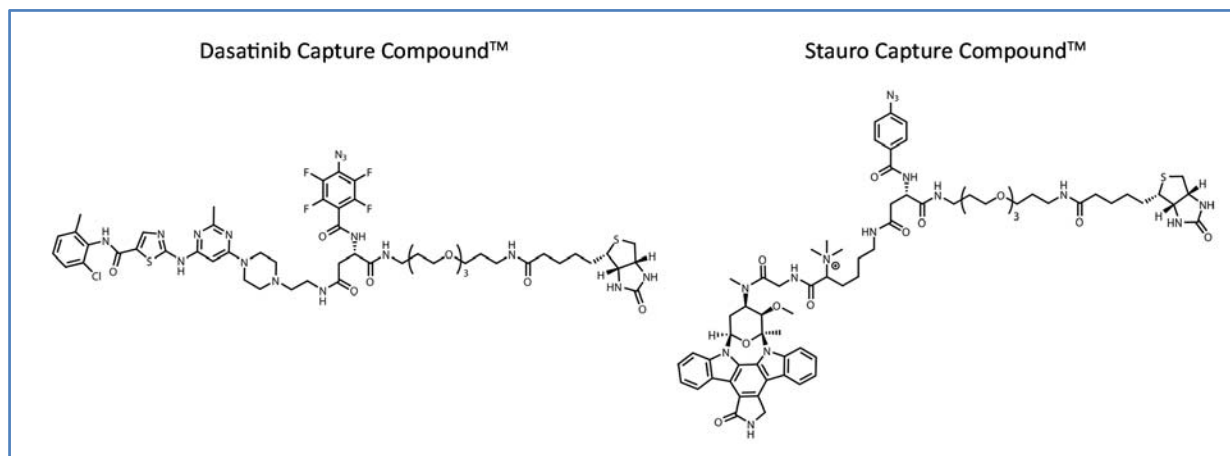


Figure 1 Structure of the Stauro and Dasatinib Capture Compound™.

The induction of the photo cross-link requires the caproBox™, a dedicated device for the controlled, reproducible irradiation and cooling of samples. The UV lamps in the caproBox™ have a broad spectrum with a maximum emission at $\lambda=310$ nm. The UV filter cuts off wavelength below $\lambda=330$ nm. This protects the CC selectivity functions dasatinib and staurosporin from irradiation damage, but still permits the photoactivation of the Capture Compounds™. The caproMag™ is recommended for the handling of the sample tubes for washing and isolation of the streptavidin magnetic beads. For sample preparation, 12 tube PCR tube strips (AB-1114, Thermo Scientific, component of the caproKits™) are used in the capture experiment. A tabletop centrifuge equipped with a butterfly rotor is required for optimal handling. HepG2 cell lysate was used as the complex biological sample (see Methods section).

inhibitor cocktail). Debris was removed by centrifugation at 30,000 x g for 60 min at 4 °C. The supernatant filtrated through a 0.2 μm filter and then dialysed against lysis buffer for the removal of small molecules. After dialysis, centrifugation at 4000 x g was carried out to remove protein precipitated during dialysis. For capture experiments using the Dasatinib caproKit™ or the Stauro caproKit™, 500 μg of total HepG2 lysate protein were used per assay. The experiments were conducted in the “OffBead” configuration, i.e. the Capture Compounds are allowed to interact with their target proteins in solution, and only afterwards streptavidin magnetic beads are added to isolate Capture Compound-protein conjugates from the complex protein mixtures. For both Dasatinib-CC and Stauro-CC it is important to keep samples in the dark at all times prior and after irradiation in the caproBox™, until washing of the beads with the captured proteins has been accomplished. The assay and competition control samples, that latter of which serve to distinguish non-specifically interacting proteins from specifically interacting proteins by subtraction of the proteins identified in the competition from those identified in the Capture Compound assay, were assembled as follows: first, the required amount of water was pipetted into the tubes, followed by 20 μl of capture buffer. Then, the

required volume of lysate was added. To the competition samples, 20 µl of Dasatinib or Stauro competitor were added and allowed to incubate for 30 minutes. Corresponding volumes of water were added to the assay samples. For sample calculation, use the Reaction volume calculator at <http://www.caprotec.com/support/downloads>. Then, Capture Compounds™ (10 µl) were added to assays and competitions and incubated for 2 hours in the dark at 4 °C on a rotating wheel. The final volume of assay and competition samples was 100 µl. Then, samples were placed in the caproBox™, tube lids opened, the UV filter frame applied, and samples irradiated for 10 minutes. Then 25 µl of 5x concentrations wash buffer 1 were applied to each tube, streptavidin magnetic beads added, and samples rotated on a rotation wheel for 1 hour at 4 °C. Note that samples at this stage still need to be kept in the dark. Samples were washed six times with 1x concentrated wash buffer 1 (see guideline for caproMag at <http://www.caprotec.com/support/downloads>). For processing of the samples for LC-MS/MS analysis, samples were washed another three times with 80 % acetonitrile, once with water, and then subjected to OnBead tryptic digest as described in SDS-Page of Captured Proteins and LC-MS Analysis at <http://www.caprotec.com/support/downloads>. For comparison with “shotgun samples” that contained no capture reaction, 10 µg of HepG2 cell lysate were digested with trypsin and desalted via stage tips (Proxeon).

Mass spectrometry

LC-MS/MS experiments were performed using a nanoflow HPLC system (Proxeon) coupled on-line to an ESI-LTQ-Orbitrap system. From the LC column, peptides were eluted during an 90-min linear gradient from 5 % ACN/0.1 % FA to 40 % ACN/0.1 % FA followed by additional 2 min to 100 % ACN/0.1 % FA and remaining at 100 % for another 8 min with a controlled flow rate of 300 nl/min. MS/MS fragmentation was performed in a data-dependent mode using one survey MS scan followed by four MS/MS scans per second. Proteins were identified by automated database searching against the

human UniProtKB/Swiss-Prot database using SEQUEST and Tandem X! as implemented in the software scaffold 2.0. Specific search parameters were 5 ppm precursor tolerance, 1 amu fragment ions tolerance, and full trypsin specificity allowing for up to two missed cleavages. Phosphorylation at serine, threonine, and tyrosine, oxidation of methionines, deamination at asparagines and glutamine, acetylation at lysine and serine, formylation at lysine, and methylation at arginine, lysine, serine, threonine and asparagine were allowed as variable modifications. No fixed modifications were used in database search.

Results

Samples analysed in triplicate by the more sensitive direct LC-MS/MS analysis revealed that kinases are specifically and robustly identified from a typical HepG2 lysate sample size of 500 µg. Comparison of the Stauro-CC, the Dasatinib-CC and the shotgun approach in HepG2 cell lysate showed distinct profiles for the different approaches. Some kinases are detected only in the shotgun samples. These are highly abundant kinases involved in metabolic processes and are not specific targets for neither dasatinib nor staurosporine. The two caproKits™ exhibited complementary profiles and enriched kinases that were not detectable by the shotgun approach. While the broad band kinase inhibitor staurosporine enriched 46 kinases, dasatinib showed higher selectivity and enriched 18 kinases. The competition experiments demonstrated the specificity of the Capture Compound™. 11 kinases were only detected with the Dasatinib-CC. The different capture profiles and, for comparison, shotgun kinases are shown in an overview in Figure 2. The most prominent dasatinib target in this HepG2 preparation was the tyrosine kinase CSK. In contrast, ribosomal S6 kinase KS6A3 was the most robust hit in the staurosporine experiments. Both kinases were not detected in the shotgun samples, demonstrating the massive enrichment and functional reduction of proteome complexity by the CCMS assay. In addition, the Stauro- and the Dasatinib-CC can be used complementarily to

comprehensively profile kinases in biological samples.

Table 1 shows which kinases were captured from HepG2 cell lysate with the Dasatinib-CC or with the Stauro-CC. The specificity of the capturing is demonstrated by the substantial reduction in or absence of the captured kinases from the competition samples, on the basis of normalized spectrum count for each kinase from LC-MS/MS data. For comparison, the data of the same kinases in the shotgun samples are provided in the table. Most of the captured kinases were not identified in the shotgun runs, indicating that these kinases are too low abundant for shotgun analysis and are only detected when CCMS is applied.

compared. All reported identifications were from capture experiments with low amounts of complex protein mixture of HepG2 cell lysate with only 500 µg starting material. The Stauro caproKit™ and the Dasatinib caproKit™ can be effectively used for the isolation of kinases from crude protein mixtures, with significantly distinct selectivity profiles for the different Capture Compounds™. The Dasatinib caproKit™ is an effective tool for the isolation of dasatinib binding proteins from complex protein mixtures. The selectivity profile is complementary to the Stauro caproKit. The selectivity of the analysis is demonstrated by the competition experiments. CCMS is an outstandingly sensitive method to discover, isolate and profile members of functional

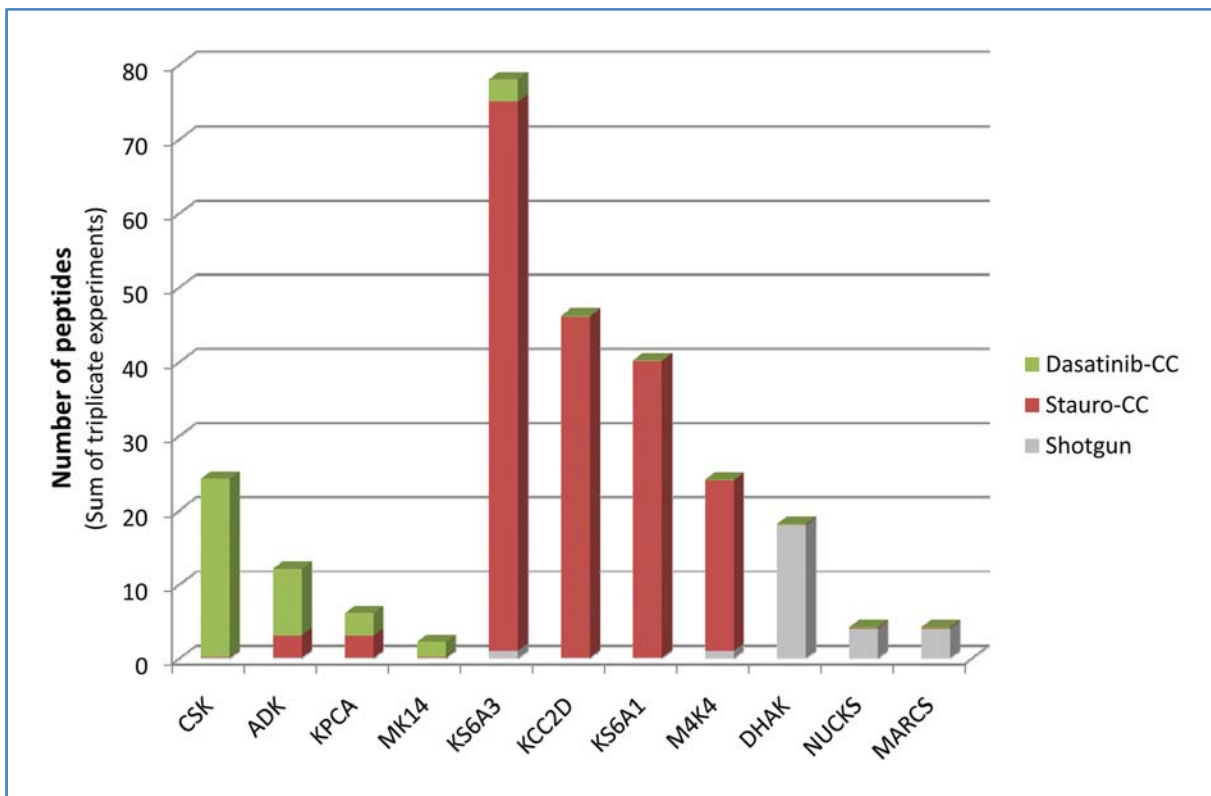


Figure 2 Kinases identified from capture experiments by using Dasatinib and Stauro caproKit™ in comparison to shotgun experiments. Number of unique peptide is given.

protein families within a variety of biological samples.

Conclusions

The capture profiles of the Dasatinib caproKit™ and the Stauro caproKit™ with respect to kinases were

Table 1 Kinases identified from capture experiments by using Dasatinib and Stauro caproKit™ in comparison to shotgun experiments (continued next page).

Protein	AN	MW	DA	DC	SA	SC	SG
Tyrosine-protein kinase CSK	CSK	51 kDa	24	0	0	0	0
Adenosine kinase	ADK	41 kDa	9	0	3	0	0
Protein kinase C alpha type	KPCA	77 kDa	3	0	3	0	0
Galactokinase	GALK1	42 kDa	2	0	4	7	0
Mitogen-activated protein kinase 14	MK14	41 kDa	2	0	0	0	0
Bone morphogenetic protein receptor type-1B	BMR1B	57 kDa	1	0	0	0	0
Casein kinase I isoform alpha-like	KC1AL	39 kDa	1	0	0	0	0
Serine/threonine-protein kinase PFTAIR-1	PFTK1	53 kDa	1	0	0	0	0
Diacylglycerol kinase zeta	DGKZ	124 kDa	1	0	0	0	0
Dual specificity mitogen-activated protein kinase kinase 1	MP2K1	43 kDa	1	0	0	0	0
Serine/threonine-protein kinase MRCK beta	MRCKB	194 kDa	1	0	0	0	0
Nucleoside diphosphate kinase, mitochondrial	NDKM	21 kDa	1	0	0	0	0
Extracellular signal-related kinase 1b	Q8NHX1	40 kDa	1	0	0	0	0
Serine/threonine-protein kinase PAK 4	PAK4	64 kDa	1	0	0	0	0
Ribosomal protein S6 kinase alpha-3	KS6A3	84 kDa	0	0	74	3	1
Calcium/calmodulin-dependent protein kinase type II delta chain	KCC2D	56 kDa	0	0	46	0	0
Ribosomal protein S6 kinase alpha-1	KS6A1	83 kDa	0	0	40	0	0
Mitogen-activated protein kinase kinase kinase 4	M4K4	142 kDa	0	0	23	0	1
Proto-oncogene tyrosine-protein kinase Src	SRC	60 kDa	0	0	15	0	0
Calcium/calmodulin-dependent protein kinase type II alpha chain	KCC2A	54 kDa	0	0	11	0	0
Proto-oncogene tyrosine-protein kinase FER	FER	95 kDa	0	0	10	0	0
Ribosomal protein S6 kinase alpha-2	KS6A2	83 kDa	0	0	9	0	0
5'-AMP-activated protein kinase catalytic subunit alpha-1	AAPK1	64 kDa	0	0	7	0	0
Putative nucleoside diphosphate kinase	NDK8	16 kDa	0	0	5	0	3
Calcium/calmodulin-dependent protein kinase type 1	KCC1A	41 kDa	0	0	5	0	0
Calcium/calmodulin-dependent protein kinase type II beta chain	KCC2B	73 kDa	0	0	5	0	0
Calcium/calmodulin-dependent protein kinase type II gamma chain	KCC2G	63 kDa	0	0	5	0	0
Proto-oncogene tyrosine-protein kinase Fyn	FYN	61 kDa	0	0	5	0	0
Pyruvate kinase isozymes M1/M2	KPYM	58 kDa	0	0	4	3	63
Nucleoside diphosphate kinase A	NDKA	17 kDa	0	0	4	0	2
Focal adhesion kinase 1	FAK1	119 kDa	0	0	4	0	0
Rho-associated protein kinase 2	ROCK2	161 kDa	0	0	4	0	0
Proto-oncogene tyrosine-protein kinase Yes	YES	61 kDa	0	0	4	0	0
Pyridoxal kinase	PDXK	35 kDa	0	0	3	0	1
Phosphoglycerate kinase 1	PGK1	45 kDa	0	0	2	0	31
5'-AMP-activated protein kinase subunit gamma-1	AAKG1	38 kDa	0	0	2	0	0
Serine/threonine-protein kinase 6	STK6	46 kDa	0	0	2	0	0
Serine/threonine-protein kinase TBK1	TBK1	84 kDa	0	0	2	0	0
Calcium/calmodulin-dependent protein kinase kinase 2	KKCC2	65 kDa	0	0	2	0	0
3-phosphoinositide-dependent protein kinase 1	PDPK1	63 kDa	0	0	2	0	0
Tyrosine-protein kinase receptor TYRO3	TYRO3	97 kDa	0	0	1	0	0
Dual specificity tyrosine-phosphorylation-regulated kinase 3	DYRK3	66 kDa	0	0	1	0	0
Cell division cycle 2-related protein kinase 7	CD2L7	164 kDa	0	0	1	0	0
Protein kinase C delta type	KPCD	78 kDa	0	0	1	0	0

Protein	AN	MW	DA	DC	SA	SC	SG
Phosphatidylinositol-4-phosphate 3-kinase C2 domain-containing alpha polypeptide	P3C2A	191 kDa	0	0	1	0	0
Serine/threonine-protein kinase N2	PKN2	112 kDa	0	0	1	0	0
Dual specificity mitogen-activated protein kinase kinase 6	MP2K6	37 kDa	0	0	1	0	0
Serine/threonine-protein kinase ICK	ICK	71 kDa	0	0	1	0	0
Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)	DHAK	59 kDa	0	0	0	0	18
UMP-CMP kinase	KCY	22 kDa	0	0	0	0	6
Nuclear ubiquitous casein and cyclin-dependent kinases substrate	NUCKS	27 kDa	0	0	0	0	4
Myristoylated alanine-rich C-kinase substrate	MARCS	32 kDa	0	0	0	0	4
Nucleoside diphosphate kinase B	NDKB	17 kDa	0	0	0	0	2
Ribosomal protein S6 kinase alpha-6	KS6A6	84 kDa	0	0	0	0	1
Myosin light chain kinase, smooth muscle	MYLK	211 kDa	0	0	0	0	1
Pyruvate kinase isozymes R/L	KPYR	62 kDa	0	0	0	0	1
MAP kinase-activating death domain protein	MADD	183 kDa	0	0	0	0	1
Adenylate kinase 2, mitochondrial	KAD2	26 kDa	0	0	0	0	1

MS/MS View: Identified Proteins (1570); AN = Annotation; MW = Molecular Weight; SG = Shotgun; SA = Stauro Capture Compound™ assay; SC = Stauro competition control; DA = Dasatinib Capture Compound™ assay; DC = Dasatinib competition control. For full Stauro-CC kinase list, please see Stauro application note at www.caprotec.com and ref. 1

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- 1) Fischer et al. (2010). Comprehensive identification of staurosporine-binding kinases in the hepatocyte cell line HepG2 using Capture Compound Mass Spectrometry (CCMS). *J Proteome Res* **9**, 806-817
- 2) Karaman, M.W. et al. (2008) A quantitative analysis of kinase inhibitor selectivity. *Nat Biotechnol* **26**, 127-132
- 3) Fischer et al. (2011) Dasatinib, imatinib and staurosporine capture compounds. *J Proteomics* **75** (10), 160-168

Downloads:

www.caprotec.com/support/downloads

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Ordering information:

Item Nr.	Description
1-1080-050	Dasatinib caproKit™ 50 reactions
1-1080-010	Dasatinib caproKit™ 10 reactions

The caproKits include the respective Capture Compound, all buffers, protein controls, competitor, and Streptavidin magnetic beads.

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