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Enhancing cognate target elution efficiency in gel-free chemical proteomics

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ABSTRACT

Gel-free liquid chromatography mass spectrometry coupled to chemical proteomics is a powerful approach for characterizing cellular target profiles of small molecules. We have previously described a fast and efficient elution protocol; however, altered target profiles were observed. We hypothesised that elution conditions critically impact the effectiveness of disrupting drug-protein interactions. Thus, a number of elution conditions were systematically assessed with the aim of improving the recovery of all classes of proteins whilst maintaining compatibility with immunoblotting procedures. A double elution with formic acid combined with urea emerged as the most efficient and generically applicable elution method for chemical proteomics

1. Significance

The majority of drugs are surprisingly promiscuous, thus a powerful approach to characterize the cellular target profiles of small molecules is imperative. An acid-based chemical proteomic elution protocol compatible with gel-free liquid chromatography mass spectrometry (LCMS) is effective; however, altered target profiles were observed. We have optimised and implemented a new strategy that decidedly enhanced cognate target elution efficiency. This was evident for both chemical immobilization of compounds on an inert matrix and also for biotinylated compounds on avidin-functionalized resins.

2. Introduction

Understanding the molecular mechanisms of drugs is of utmost importance as this knowledge may guide target-based improvement of lead compounds; whilst revealing off-target effects that lead to toxicity [1–3]. If chemical entities could be matched to biological processes at the molecular level throughout the drug discovery and development process, the attrition rates for tool compounds and drugs could potentially decrease. Concomitantly, therapeutic efficacy should also improve. Thus, deciphering the target spectra of bioactive compounds can lead to exploitation of the full potential of drug candidates. Some examples where this is applicable are in aiding the identification of novel therapeutic applications or elucidating side effects [4–7]; and/or pharmacological tool compounds that are used to dissect complex biological processes [8]. There is a growing body of data that supports the notion that the majority of drugs are promiscuous and that the 'one drug, one target' paradigm seldom applies [9]. The more we understand drug properties, the more we realize that it is not so much the question of if a compound has off-targets, but how many there are and how these contribute to the biological effects. Several methods have been employed in the identification of small molecule-protein interactions, such as chemical proteomics or gene expression-based methods [3,5,10,11].

Abbreviations: MS, mass spectrometry; LC, liquid chromatography; LCMS, liquid chromatography mass spectrometry; iTRAQ, isobaric tags for relative and absolute quantitation; SDS, sodium dodecyl sulphate; CML, chronic myeloid leukemia; TEA, triethylamine; HPLC-MS, high-performance liquid chromatography mass spectrometry; TEAB, triethylammonium bicarbonate; FA, formic acid; ACN, acetonitrile; XX-NHS, biotin-biotinamidohexanoyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester; DTT, dithiothreitol; TLCK, *N*-alpha-tosyl-L-lysinyl-chloromethylketone; U/FA, 3 M urea and 50 mM FA; B, boiling; dNSAF, distributed normalized spectral abundance factor; dSAF, spectral abundance factor; uSpC, unique spectral count.

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Chemical proteomics is a post-genomic affinity chromatography-based approach that is enabled by modern mass spectrometry (MS) and bioinformatic capabilities [3,12–17]. There are various protocols in use, but the most widely-accepted procedure entails the elution of interacting proteins from a drug-affinity matrix with sodium dodecyl sulphate (SDS) followed by analysis of the eluate by one- or two-dimensional SDS-PAGE and *in situ* tryptic digestion of the proteins. In most cases, the resultant peptide mixture is analyzed by nano-liquid chromatography (LC) coupled to nano-electrospray (ESI) tandem MS[18]. Whereas this gel-based proteomic workflow (often referred to as GeLCMS) has been highly successful and has led to a number of landmark publications that describe several important novel drug-protein interactions [8,19–21], there are also a number of important limitations. These include high labor demand, high cost, and an increased risk of keratin contamination as a consequence of multiple sample handling steps. GeLCMS is also not directly compatible with quantitative proteomic approaches that utilize post-digestion chemical labeling with isobaric tags (*e.g.*, iTRAQ, TMT) [22]. Therefore, gel-free proteomic methods are receiving more widespread interest.

We have recently shown that adaptation of a gel-free approach resulted in a significant reduction in sample preparation and MS instrument time, and ultimately led to an increase in absolute numbers of identified proteins [23]. Target recovery was also improved such that a 5-fold decrease in the protein input was enabled without loss of data quality [23]. Furthermore, we have demonstrated the compatibility of our approach with subsequent relative quantitative proteomics using iTRAQ labeling [24,25]. Despite these advancements, the method has only been used in a few studies from our groups [26–30], as there were some questions raised concerning cognate target recovery (especially for receptor tyrosine kinases, RTKs) and decreased immunoblot efficiency. For example, the detection of the BCR-ABL fusion oncoprotein, which is the biochemical hallmark of chronic myeloid leukemia (CML) and a major drug target of several kinase inhibitors (*e.g.*, imatinib and dasatinib), was compromised. This observation was apparent with dot blots and western gel-based immunoblot assays. Therefore, we hypothesised that elution conditions critically impact the effectiveness of disrupting drug-protein interactions. Subsequently, the final drug-protein profile can be altered. Surprisingly, this aspect is rarely addressed and often overlooked in biochemical approaches linked to mass spectrometry-based proteomics. Thus, the ultimate aim of this current study was to systematically and thoroughly assess a number of different elution conditions to determine the best, yet generic, protocol that efficiently eluted a broad range of cognate targets encompassing several protein classes. Dasatinib was initially selected as the test compound. This drug is a multi-kinase inhibitor approved for the treatment of patients with imatinib-resistant CML and BCR-ABL-positive acute lymphoblastic leukemia (ALL). Dasatinib is not only a potent inhibitor of the large 210 kDa fusion protein BCR-ABL [31], but also of the cytosolic TEC family kinase BTK [6] and the membrane-bound receptor tyrosine kinase DDR1 [21]. In addition, we have previously generated a dasatinib analog suitable for chemical proteomics that we have validated and successfully employed in different studies [21,32,33].

A number of elution conditions were assessed with the objective to improve: (i) compatibility with immunoblot analysis, which is an important quality control; and (ii) the overall recovery of *bona fide* targets. The protocol optimised on the coupleable analog of dasatinib was further extended to a biotinylated derivative of the drug; and also to a second compound with a different target profile. Compared to dasatinib, sunitinib [34] inhibits a complementary fraction of kinases [28]. The drug is an oral, multi-targeted kinase inhibitor, which has been approved for treatment of imatinib-resistant gastrointestinal stromal tumor and renal cell carcinoma. Additionally, sunitinib is in clinical trials for CML and myelodysplastic syndromes. Sunitinib is a potent inhibitor of receptor tyrosine kinase c-KIT [35], and the serine/threonine protein kinase PRKAA1 (AMPK1α) and CAMK2 [36]. Overall, we could show that our optimised elution method brought a universal improvement in elution efficiency with two different drug-coupling strategies and two different kinase inhibitors.

3. Materials and methods

3.1. Chemicals

All chemicals used were of analytical grade, unless stated otherwise and obtained from commercial suppliers.

3.2. Biological material

K562 and HEL cells were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen). Antibodies used were rabbit polyclonal anti-DDR1, anti-BTK (E9) and anti-KIT (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-ABL (21–63) (generated in house).

3.3. Compounds and immobilization

Dasatinib and sunitinib were purchased from Selleck Biochem (Houston, Texas Area). c-dasatinib was synthesized by WuXi PharmaTech (Shanghai, China) [21], and c-sunitinib was obtained from Indus Biosciences Private Limited (Hyderabad, India) through Gateway Pharma (Freeland, UK). Compounds were immobilized on NHS-activated Sepharose 4 Fast low (Amersham Biosciences, Amersham, UK). Beads were washed with dimethyl sulfoxide (DMSO) and incubated overnight with 1 mM compound and 100 mM triethylamine (TEA) at room temperature (RT) with constant agitation. After incubation, the supernatant was analyzed by high-performance liquid chromatography mass spectrometry (HPLCMS) in order to determine whether the compound was completely immobilized. Unreacted functional groups were subsequently blocked with 0.8 M ethanolamine for at least 8 h at RT, washed with DMSO and either stored at 4 °C in isopropyl alcohol or used immediately for affinity chromatography. For the biotinylated drug experiments, c-dasatinib was incubated with biotin amidohexanoyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester (biotin-XX-NHS, Sigma–Aldrich, St. Louis, MO) in the presence of TEA overnight at RT with constant agitation. The supernatant was then analyzed by HPLC-MS for residual reagents and the reaction yield. Dasatinib coupled to biotin was incubated with UltraLink immobilized streptavidin plus beads (Pierce, Rockford, IL) on a roto-shaker for 30 min at 4 °C and used for affinity chromatography.

3.4. Affinity purification

The same affinity purification protocol was used for c-dasatinib, c-sunitinib and c-dasatinib-XX-biotin. K562 and HEL cell lysates were prepared using a lysis buffer comprised of 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 0.2% NP-40, 5% glycerol, 1.5 mM $MgCl_2$, 25 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 10 μg/mL *N*-alpha-tosyl-L-lysinyl-chloromethylketone (TLCK), 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 10 μg/mL soybean trypsin inhibitor. In order to minimise sample variability, cell lysates were prepared in large batches, aliquoted and stored at −80 °C until required. Before application to the pre-equilibrated affinity matrices, cell suspensions were clarified by centrifugation. Lysates (5 and 10 mg total protein for K562 and HEL, respectively) were incubated with drug-