

Proteome-Wide Profiling of Targets of Cysteine reactive Small Molecules by Using Ethynyl Benziodoxolone Reagents

Daniel Abegg, Reto Frei, Luca Cerato, Durga Prasad Hari, Chao Wang, Jerome Waser, and Alexander Adibekian*

Abstract: In this study, we present a highly efficient method for proteomic profiling of cysteine residues in complex proteomes and in living cells. Our method is based on alkynylation of cysteines in complex proteomes using a “clickable” alkynyl benziodoxolone bearing an azide group. This reaction proceeds fast, under mild physiological conditions, and with a very high degree of chemoselectivity. The formed azide-capped alkynyl–cysteine adducts are readily detectable by LC-MS/MS, and can be further functionalized with TAMRA or biotin alkyne via CuAAC. We demonstrate the utility of alkynyl benziodoxolones for chemical proteomics applications by identifying the proteomic targets of curcumin, a diarylheptanoid natural product that was and still is part of multiple human clinical trials as anticancer agent. Our results demonstrate that curcumin covalently modifies several key players of cellular signaling and metabolism, most notably the enzyme casein kinase I gamma. We anticipate that this new method for cysteine profiling will find broad application in chemical proteomics and drug discovery.

Despite their relatively low abundance in proteins, cysteines are vital for cellular biochemistry. For example, cysteines form disulfide bridges, are actively involved in enzyme catalysis as nucleophiles and can be posttranslationally oxidized or modified through palmitoylation, prenylation or nitrosylation.^[1] Many of the so-called functional cysteines display pronounced hyperreactivity and these reactive hot spots can be precisely identified in complex proteomes with broad-range electrophilic probes.^[2] Such probes represent particularly useful tools for competitive activity-based protein profiling^[3] (ABPP) of targets of covalently binding cysteine-reactive drugs and metabolites, as impressively demonstrated in recent publications.^[4] Over the past few years, a broad variety of novel methods for broad-spectrum, yet chemoselective targeting of cysteine residues have been reported.^[5] However, while most of these transformations deliver excel-

lent results on peptides and recombinant proteins, only very few of them have been shown to be also effective in labeling cysteines in cells or cellular lysates and therefore suitable for proteomics applications. Iodoacetamide (IAA) is widely accepted as gold standard among MS-compatible cysteine-reactive chemical probes,^[6] but it displays rather low chemical stability in aqueous buffers and it only reacts with a fraction of functional cysteines.^[7] Moreover, iodoacetamide also modifies lysines and the formed artifacts lead to misassignment of lysine ubiquitination sites by mass spectrometry.^[8] Thus, there is a clear need for new electrophilic probes complementary to IAA that would enable labeling of so far “inaccessible” subsets of proteomic cysteines, which would then allow more comprehensive target profiling. Herein, we present discovery and an in-depth evaluation of a “clickable” alkynyl benziodoxolone as a highly efficient and chemoselective cysteine-reactive probe with an iodoacetamide-complementary proteomic profile.

Small-molecule thiols are readily alkynylated by alkynyl benziodoxolones (EBX reagents).^[9] This class of reagents is selective for thiols in presence of other nucleophilic functional groups such as aromatic rings, alcohols, amines or carboxylates. While these transformations were fast, high-yielding and air-tolerant, they were performed on dipeptides in water/organic solvent mixtures and required stoichiometric amounts of base. Thus, reaction of the alkynyl benziodoxolones with cysteines directly on proteins and under native physiological conditions would constitute a formidable challenge. We chose the azide-functionalized alkynyl benziodoxolone JW-RF-010 for our studies, as the azide group can be used for further functionalization via the copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC), whereas the obtained internal thioalkynes are usually inert in copper-catalyzed cycloadditions (Figure 1A). Compared to fluorescent dye- and biotin-conjugated ABPP probes that have poor cell membrane permeability and are too bulky to enable efficient binding to some of the proteins, such two-step labeling approach is advantageous for applications in cells^[3b] and animals^[10] and is now widely used.

Before starting with the labeling experiments, it was important to test the aqueous stability of JW-RF-010 by obtaining ¹H NMR spectra of this reagent in D₂O over prolonged period of time. Even after 14 days in D₂O, only traces (< 3 %) of decomposition were detectable (Figure S1 in the Supporting Information). Next, we compared the glutathione (GSH)-directed reactivity between the azide-functionalized alkynyl benziodoxolone JW-RF-010 and iodoacetamide through a colorimetric GSH-binding assay (Figure S2). While both probes showed rather moderate activity in this

[*] D. Abegg, L. Cerato, C. Wang, Prof. Dr. A. Adibekian
School of Chemistry and Biochemistry
NCCR Chemical Biology, University of Geneva
30 quai Ernest-Ansermet, Geneva (Switzerland)
E-mail: alexander.adibekian@unige.ch

Dr. R. Frei, Dr. D. Prasad Hari, Prof. Dr. J. Waser
Laboratory of Catalysis and Organic Synthesis
Ecole Polytechnique Fédérale de Lausanne
EPFL SB ISIC LCSO, BCH 4306, 1015 Lausanne (Switzerland)

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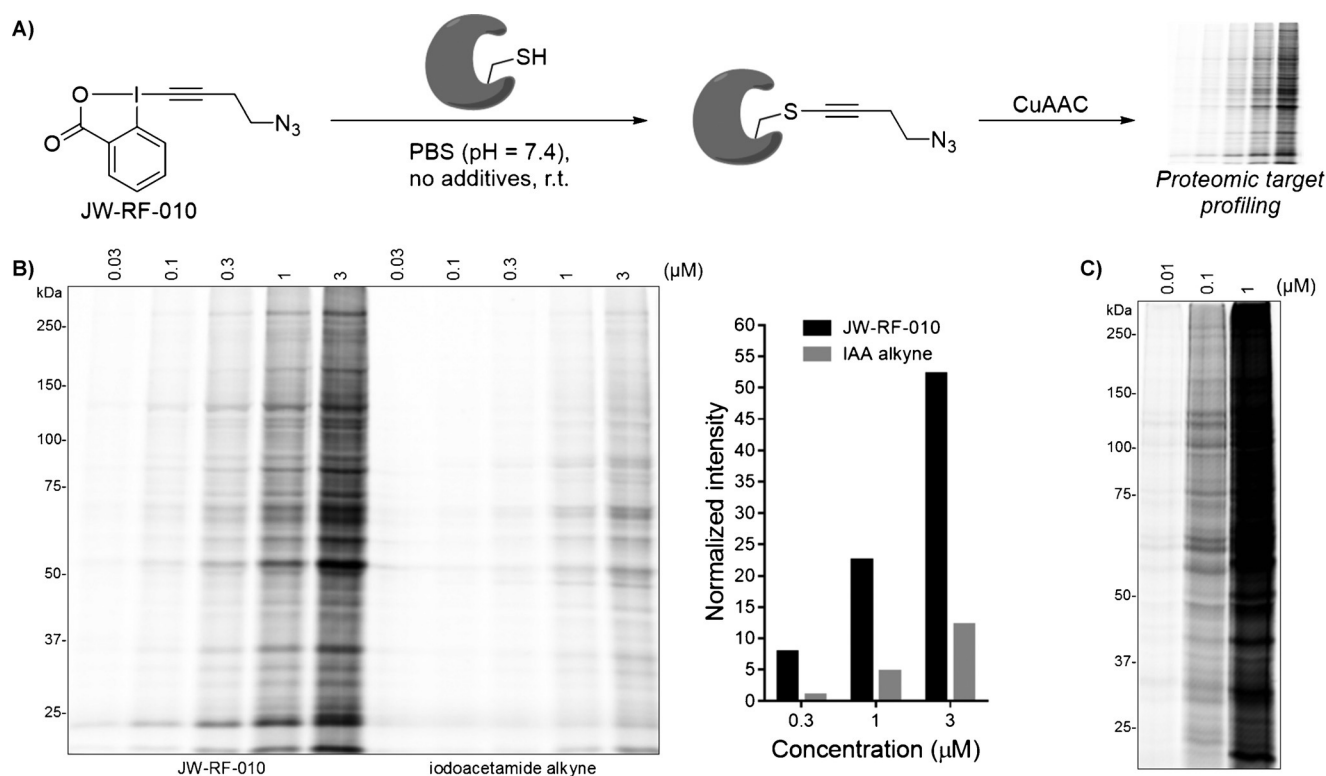


Figure 1. Evaluating the proteomic reactivity of JW-RF-010 by SDS-PAGE. A) A general reaction scheme showing alkylation of cysteine-containing proteins by JW-RF-010. B) Gel-based fluorescence image of HeLa cell lysates labeled with various concentrations of JW-RF-010 or iodoacetamide alkyne (left) and quantification of the fluorescence labeling (right). C) Gel-based fluorescence image after labeling of living HeLa cells with various concentrations of JW-RF-010.

experiment, we observed a twofold higher activity for JW-RF-010 ($RC_{50} = 75 \mu M$) compared to IAA ($RC_{50} = 145 \mu M$). Interestingly, when monitoring this transformation by mass spectrometry we did not detect increased disulfide bond formation or any other cysteine oxidation as possible side reactions (Figure S3). We then compared the efficiency of labeling of purified protein catalase by an EBX reagent versus iodoacetamide using a gel-based competitive assay (Figure S4). Here, catalase was labeled with $5 \mu M$ 2-iodo-*N*-(prop-2-yn-1-yl)acetamide (iodoacetamide alkyne), then reacted with carboxymethylrhodamine (TAMRA) azide through CuAAC, and visualized by in-gel fluorescence scanning after SDS-PAGE. In a competitive labeling with the methyl alkyne-substituted EBX reagent JW-RF-001 a ca. 3 to 5-fold higher efficiency compared to iodoacetamide was observed (Figure S4). To assure that the alkynyl cysteine adduct is stable under physiological conditions even after longer period of time, we labeled catalase with $1 \mu M$ of JW-RF-010, then removed the excess of JW-RF-010 by membrane filtration and incubated the reacted protein for various amounts of time prior to “clicking” with TAMRA alkyne and visualization by SDS-PAGE (Figure S5). Even after 18 h of incubation, no noticeable loss of fluorescence was detectable, implying that the formed alkynyl cysteine adduct is indeed stable under the tested conditions.

Having demonstrated the efficiency of the alkynyl benziodoxole probes on both peptides and proteins, the stage was now ready for the much more challenging labeling in total cell

lysates. We started by incubating HeLa cell lysates in phosphate-buffered saline with different concentrations of JW-RF-010 for 1 h. Multiple fluorescent bands were detected even at submicromolar concentrations of JW-RF-010 and clear concentration-dependent increase in labeling intensity was observed (Figure 1B, S6). With iodoacetamide alkyne as probe, an about fivefold lower overall fluorescence intensity compared to JW-RF-010-treated proteomes was observed. Moreover, the higher proteome reactivity of EBX reagents compared to iodoacetamide (IAA) was also clearly observable in cross-competition experiments (Figure S7). Here, pretreatment with high concentrations of JW-RF-001 completely abolished proteome labeling by iodoacetamide alkyne, but not vice versa. Interestingly, while the overall labeling pattern of both probes showed significant degree of similarity, several fluorescence bands appeared to be specifically present in JW-RF-010-labeled proteomes.

We next examined whether JW-RF-010 could also be used for labeling proteomes directly in living cells. Because broad range cysteine-reactive electrophilic probes are intrinsically cytotoxic,^[11] we compared the cytotoxicities of JW-RF-010 and IAA in HeLa cells using WST-1 assay (Figure S8). Whereas significant cytotoxicity was observed after 20 h in cells treated with $1 \mu M$ IAA, JW-RF-010 did not show any cellular toxicity at concentrations up to $1 \mu M$, while at $3 \mu M$ significant cell death was observed. We then tested whether JW-RF-010 could permeate the cellular membranes and react with the intracellular proteome. HeLa cells were treated with

1 μM JW-RF-010 for 4 h, fixed and TAMRA alkyne and CuAAC reagents were added. The obtained confocal microscopy images showed homogeneous intracellular TAMRA labeling in JW-RF-010 treated cells, while no significant fluorescence was detected in cells treated with DMSO as control (Figure S9). To visualize the proteomic targets by gel-based ABPP, HeLa cells were again treated with JW-RF-010 for 4 h, lysed, and the probe-labeled proteins were reacted with TAMRA alkyne and separated by SDS-PAGE. The gel scan documented strong fluorescence labeling at the concentration as low as 100 nM (Figure 1 C, S6), thus well below the concentration toxic to cells.

Having successfully established proteome labeling in vitro and in situ, we continued our studies by comparing the exact modification sites of JW-RF-010 and IAA alkyne in complex proteomes using a capture-and-release strategy coupled with LC-MS/MS analysis that allowed us specific enrichment and detection of probe-labeled peptides (Figure 2 A). In total, 2257 cysteine-containing peptides were identified as enriched in proteomes labeled by 10 μM JW-RF-010 and 2184 peptides were enriched by 10 μM IAA alkyne. While 1391 peptides were commonly found in both datasets, 866 peptides (38 %) were only enriched by JW-RF-010 (Figure 2 B and Table S1). Thus, the proteomic profile of JW-RF-010 shows significant complementarity to IAA alkyne to allow more comprehen-

sive profiling of proteomic cysteines. To exclude the possibility of labeling of amino acids other than cysteines as possible reason for the large amount of unique peptides observed, the resulting MS data were searched for mass adducts on all nucleophilic amino acids (Cys, Asp, Glu, His, Lys, Ser, Thr, Tyr) using the Sequest HT algorithm^[12] and the percentage of unique peptides carrying the covalent modification on each amino acid was determined (Figure 2 C and Tables S1, S2). Gratifyingly, formed alkynyl adducts were predominantly found on cysteines (97 %). For comparison, we observed significantly lower cysteine selectivity with 10 μM IAA alkyne as probe (91.4 %). This is an important advantage of the EBX probes, as they will limit the presence of “false positives” in proteomics experiments.^[8]

Finally, to demonstrate the utility of our method for drug target discovery applications, we used JW-RF-010 to identify the proteomic targets of curcumin (Figure 3 A). Curcumin, a diarylheptanoid natural product isolated from turmeric (*Curcumin longa*), has been extensively studied over the last few decades as an anticancer and an anti-inflammatory agent.^[13] Due to these intriguing biomedical properties and also the fact that even multigram high dosages of curcumin are well tolerated by humans, curcumin has been part of many human clinical trials including multiple myeloma, colon and pancreatic cancer.^[14] While countless studies have been

performed with the goal of identification of entire biochemical and signaling pathways modulated by curcumin,^[15] surprisingly little is known about the direct molecular targets of this natural product.^[16] For instance, several studies have demonstrated that curcumin treatment results in decrease of phosphorylation of S473 in Akt,^[17] a modification that is crucial for the full activation of this potent oncogene. Akt is a suppressor of apoptosis and a major target for anticancer drug discovery, but the molecular link between curcumin and Akt is not understood. Examining the chemical structure of curcumin made us speculate whether possible covalent bond formation between the α,β -unsaturated ketone group of this natural product and the proteomic cysteines could potentially explain the connection to Akt signaling and also many other intriguing bioactivities of curcumin.

We first tested and confirmed the cytotoxic effect of curcumin on HeLa cells using the WST-1 assay (Figure 3 B, S10). Using the caspase-directed fluorescent probe FITC-VAD-FMK and con-

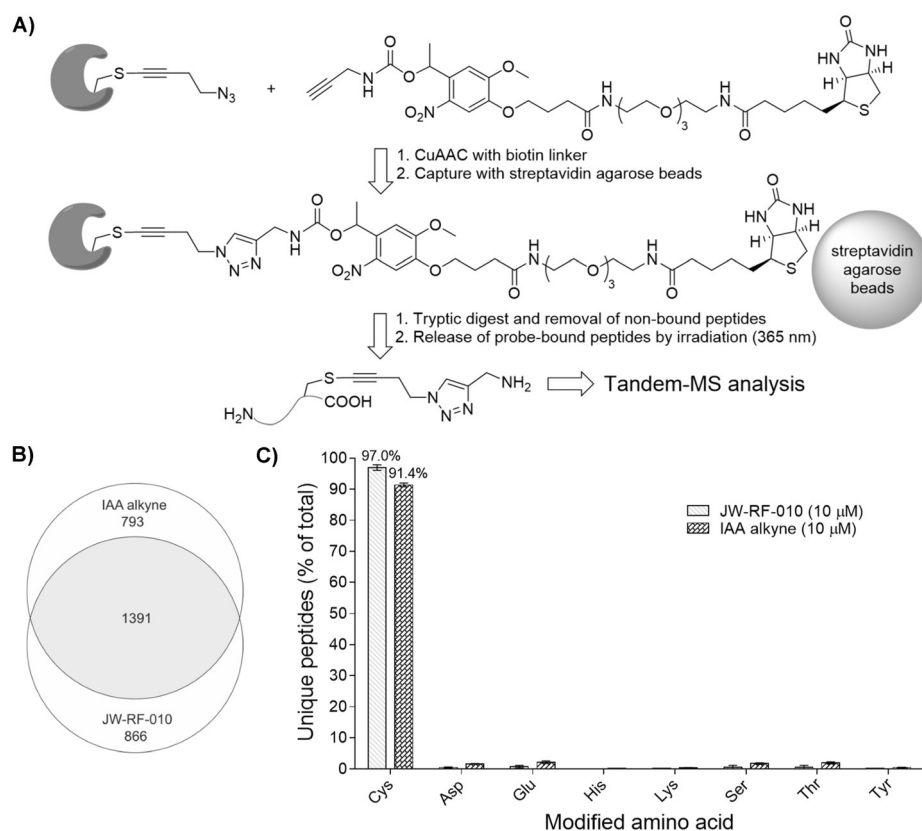


Figure 2. Comparative evaluation of JW-RF-010 versus iodoacetamide alkyne using chemical enrichment and LC-MS/MS analysis. A) A capture-and-release strategy using a photocleavable biotin alkyne linker for detection of exact probe modification sites in complex proteomes. B) A Venn diagram of cysteine-containing peptides enriched by 10 μM JW-RF-010 versus 10 μM iodoacetamide alkyne ($n=4$). C) Chemoselectivities observed in HeLa lysates using 10 μM JW-RF-001 versus 10 μM IAA alkyne ($n=4$). Error bars represent standard deviation (SD).

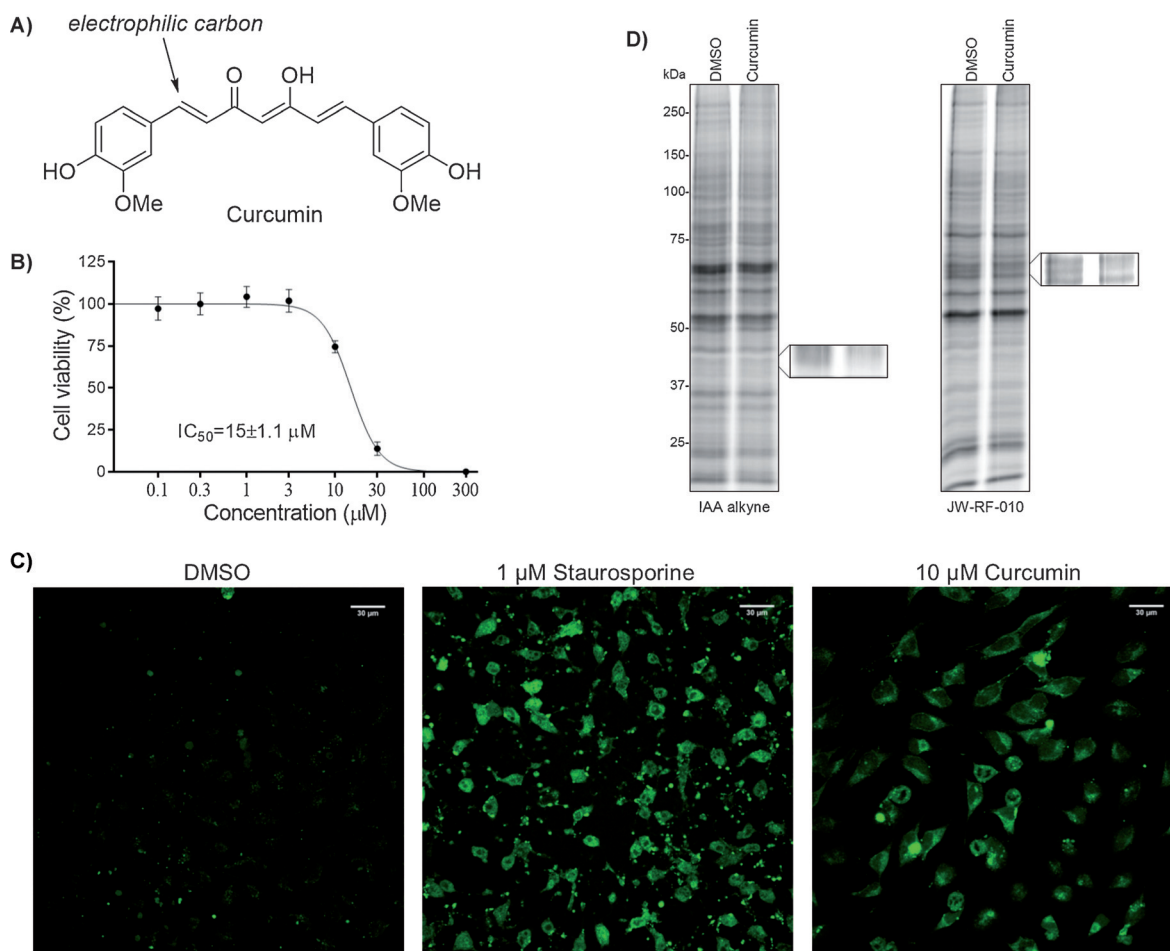


Figure 3. Effect of curcumin treatment on HeLa cells. A) Chemical structure of curcumin. B) HeLa cell viability curve after treatment with various concentrations of curcumin ($n=3$). C) Detection of caspase activation and apoptosis in HeLa cells after treatment with 1 μM staurosporine (positive control) or 10 μM curcumin using FITC-VAD-FMK fluorescent marker (confocal microscopy images are shown). D) Gel-based competitive profiling of curcumin targets after treatment of HeLa cell lysates with 30 μM curcumin and using 10 μM JW-RF-010 or 10 μM iodoacetamide alkyne as cysteine-reactive probes. The contrast of the IAA alkyne gel was adjusted for better comparison between both probes.

focal microscopy, we determined that the cell death is caused by caspase activation and subsequent apoptosis (Figure 3C). A gel-based competition experiment in HeLa cells employing 10 μM IAA alkyne or 10 μM JW-RF-010 as probes evidenced disappearance of few protein bands upon pretreatment with 30 μM curcumin, suggesting that curcumin may indeed have reacted with proteomic cysteines (Figure 3D).

Based on this observation, we then performed a competitive LC-MS/MS experiment using both cysteine-reactive probes at 10 μM concentration and employing the capture-and-release strategy described above coupled with the method of stable isotope labeling of amino acids in culture (SILAC).^[18] A detailed experimental workflow is shown in Figure 4A. In total, 2689 different cysteine-modified peptides were enriched from HeLa cells by both probes and quantified (Table S3), but labeling of only 57 of 2689 peptides, corresponding to 57 different proteins, was $\geq 75\%$ competed by 30 μM curcumin (SILAC ratio ≤ 0.25). To further confirm the identified targets we synthesized an alkyne-tagged derivative of curcumin and confirmed this probe's bioactivity in HeLa cells using the WST-1 assay (Figure S11). We then treated HeLa cells with 30 μM curcumin alkyne and identified

the “click”-enriched proteins by LC-MS/MS (Table S4). Although enrichment with curcumin alkyne allowed identifying and confirming target proteins, in contrast to our comparative profiling approach, it did not provide the exact modification sites due to complex fragmentation pattern of cysteine–curcumin adducts and some high abundance proteins, known as contaminants in proteomic pulldown experiments, were also enriched by this probe. In total, 42 of 57 (74%) initially identified candidate curcumin targets were also enriched by curcumin alkyne (Figure 4B and Table S5). To the best of our knowledge, only 1 of 42 identified proteins has been reported as direct biological target of curcumin before.^[16a] Remarkably, among the 42 targets, 19 proteins were only enriched by iodoacetamide alkyne and 16 proteins were exclusively enriched by JW-RF-010, further underscoring the importance of using multiple cysteine-reactive probes for more comprehensive proteome-wide target profiling. For instance, MAP2K4 and MAP2K6, both members of the same family of mitogen-activated protein kinase kinases, were enriched exclusively by IAA alkyne or JW-RF-010, respectively (Figure 4B).

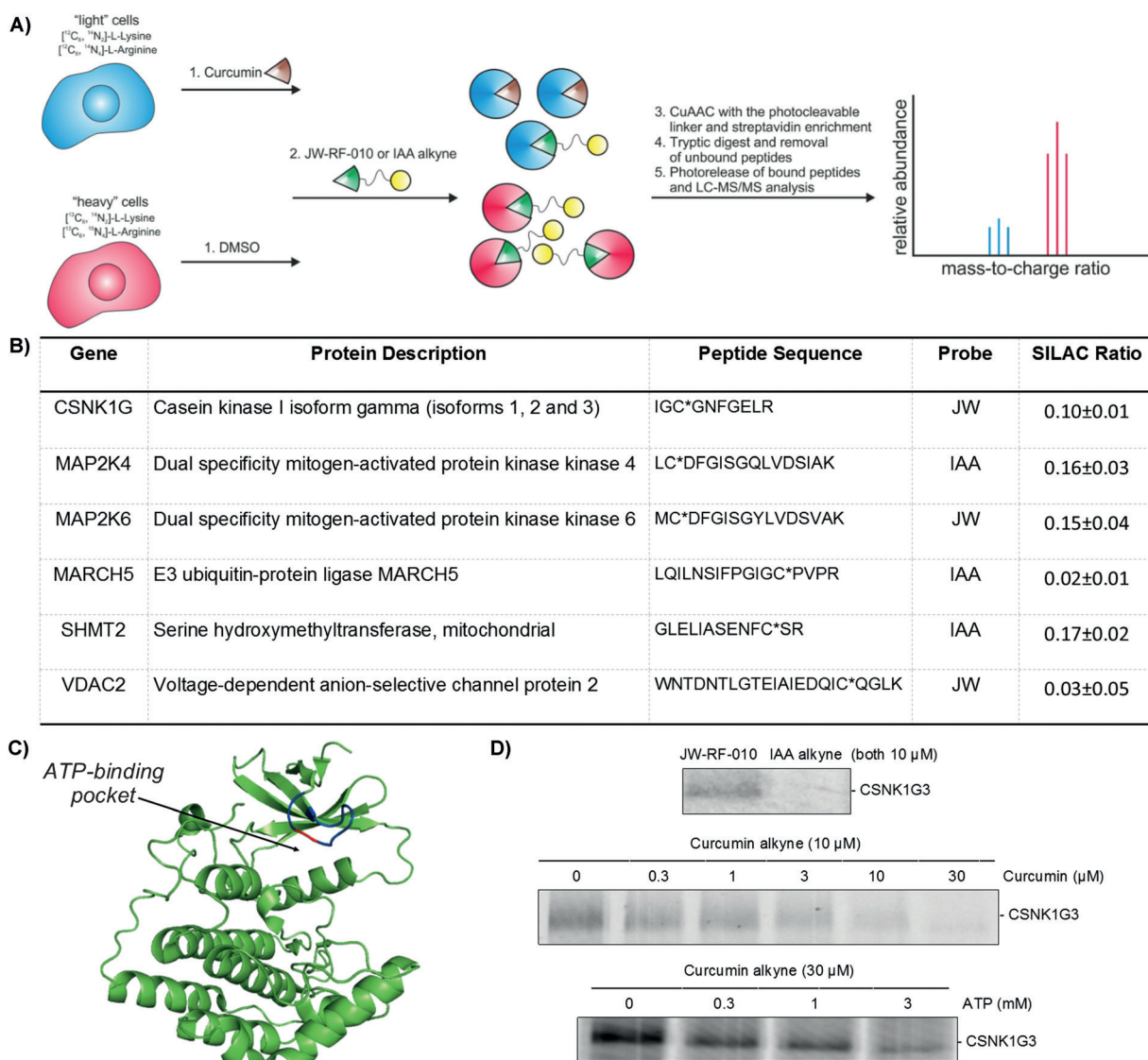


Figure 4. Identification of molecular targets of curcumin in HeLa cells using cysteine-reactive probes JW-RF-010 (10 μM) and IAA alkyne (10 μM) and SILAC proteomics. A) Experimental workflow for the competitive proteomic profiling of curcumin targets in SILAC-labeled HeLa cells. B) Selected proteomic targets of curcumin in HeLa cells. The corresponding enrichment probes and the "light"/"heavy" SILAC ratios ± SD are indicated ($n=3$). C) Protein structure of *H. sapiens* CSNK1G3 (PDB: 2IZS) with the P loop displayed in blue and Cys⁵¹ displayed in red. The image was created using PyMOL (V1.7.2.1). D) Recombinant human CSNK1G3 is labeled by JW-RF-010, but not by IAA alkyne (both 10 μM; top). CSNK1G3 is labeled by 10 μM curcumin alkyne and the labeling is competed by curcumin in concentration-dependent manner (middle). The labeling of CSNK1G3 by 30 μM curcumin alkyne is also competed by ATP in concentration-dependent manner (bottom).

Most intriguingly, one of the identified targets, casein kinase I gamma (CSNK1G, all three isoforms), may provide a direct mechanistic link to Akt phosphorylation. The protein kinase CSNK1G isoform 3 (CSNK1G3, EC:2.7.11.1) was shown to directly participate in Akt signaling and siRNA knockdown of CSNK1G3 reduced the phosphorylation on S473 of Akt.^[19] CSNK1G3 knockdown in combination with an Akt inhibitor treatment also resulted in improved killing of renal carcinoma cells.^[19] In our LC-MS/MS-based proteomic profiling experiments CSNK1G was labeled exclusively by JW-RF-010. The modification was identified on Cys⁵¹ that is located in the glycine-rich loop (P loop) in the immediate proximity to the ATP-binding pocket of this kinase (Figure 4C, S12, S13) and was ≥ 90% competed by curcumin

pretreatment. Cysteine residues in P loops of other protein kinases are well known as binding sites for some ATP competitive, covalent kinase inhibitors, for example, pan-FGFR inhibitors.^[20] As expected, purified human CSNK1G3 was labeled by 10 μM JW-RF-010, but not by 10 μM iodoacetamide alkyne (Figure 4D, top and S14). We confirmed CSNK1G3 as direct molecular target of curcumin by labeling the protein with curcumin alkyne and competing this labeling with curcumin (Figure 4D, middle and S14). Labeling by curcumin alkyne was also competed by ATP in dose-dependent manner (Figure 4D, bottom and S14), confirming that curcumin does indeed bind in the close proximity to the ATP-binding pocket of CSNK1G3. Hence, treatment with 30 μM curcumin also inhibited ATP hydrolysis by recombi-

nant CSNK1G3 (Figure S15), indicating that curcumin not only binds, but also inhibits the catalytic activity of CSNK1G3. More detailed biochemical follow-up studies are currently in progress in our laboratories and these results will be reported in due course.

In summary, we have presented the discovery and detailed proteomic evaluation of EBX reagents as biocompatible cysteine-reactive chemical probes. A range of advantageous properties render them desirable probes for many life science applications. EBX reagents are stable in water and cysteine alkylation can be performed under physiological conditions and with high chemoselectivity. Moreover, the described modification can be performed directly in living cells with up to micromolar probe concentrations. Finally, our methodology is especially well suited for chemical proteomics applications, because the formed thioalkyne adducts are readily detected in routine LC-MS/MS experiments and, when the probe contains an azide group, can be further functionalized via CuAAC. We demonstrated the utility of alkynyl benziodoxolones as useful, iodoacetamide-complementary probes for chemical proteomics by successfully identifying the biological targets of curcumin in HeLa cells. These results will hopefully provoke new drug discovery endeavors towards identification of pharmacologically improved analogs of curcumin as promising anticancer drugs.

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