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Click chemistry-based imaging to study tissue distribution of curcumin-protein complex in mice

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Abstract

Previous studies have shown that curcumin, a bioactive dietary compound with a thiol-reactive α,β-unsaturated carbonyl moiety, can covalently modify protein thiols. However, most of the previous studies were performed in cultured cells or cell-free enzyme systems, it remains unknown whether curcumin could covalently modify proteins after oral administration *in vivo*. Using click chemistry-based fluorescence imaging, here we show that oral administration of dialkyne-curcumin (Di-Cur), a "click" probe mimicking curcumin, results in covalent modifications of cellular proteins in colon and liver tissues, but not in other tissues, in mice. This result suggests that oral administration of curcumin leads to formation of curcumin-protein complex in a tissue-specific manner, which could contribute to the biological effects and/or pharmacokinetics of curcumin. Further studies to elucidate the identities of curcumin-binding proteins could greatly help us to better understand molecular mechanisms of curcumin, and develop novel strategies for disease prevention.

Keywords

Curcumin, click chemistry, in-gel fluorescence imaging, protein thiols

Introduction

The α,β-unsaturated carbonyl moiety (-C=C-C=O) is a common structural feature of many bioactive dietary compounds, such as curcumin and shagoal, and is widely regarded to contribute to the health-promoting effects of these dietary compounds [1-3]. Indeed, previous studies have shown that once these compounds enter the cells, the α,β-unsaturated carbonyl moiety can rapidly react with protein thiols via a Michael reaction, leading to post-translational modifications of cellular proteins, some of which play critical roles in cell signaling, resulting in activation or attenuation of cell signaling pathways. The thiols of some cellular proteins, such as Keap1 or NF-κB proteins, play critical roles in regulating antioxidant and inflammatory responses [4, 5]. These results support the critical roles of the α,β-unsaturated carbonyl moiety in the health-promoting effects of these dietary compounds, through covalent modification of protein thiols. In addition, the formation of intracellular protein-compound complex could result in reduced concentrations of free-form dietary compounds in tissues, and therefore could contribute to the metabolic stability and pharmacokinetics of these dietary compounds. However, most of the previous studies were performed in cell culture or cell-free systems, whether these compounds could covalently modify cellular proteins *in vivo*, and the detailed tissue distribution of the protein-compound complex, are largely unknown. Considering that these α,β-unsaturated carbonyl-bearing compounds could react with a large array of cellular proteins, it is challenge to analyze the presence and identities of the protein-compound complex, hampering our understanding of the molecular mechanisms of these compounds.

Alkyne and azide [3+2] cycloaddition “click” chemistry-based imaging is a powerful and innovative tool for analysis of the interactions of proteins with small compounds [6]. This strategy involves several steps: (1) design and synthesize a “click” probe of the bioactive compound, by functionalizing it with an alkyne- or azide- moiety, (2) treat the cells or animals with the “click” probe, during this period, the protein targets of the bioactive compound are labelled with the alkyne- or azide- moiety, and (3) the labelled protein targets are further labelled with a fluorescence dye and/or biotin moiety via the click reaction, which allows fluorescence imaging to visualize the target proteins and LC-MS/MS analysis to elucidate the identities of the target proteins (**Fig. 1**) [7-11]. In a previous study, we have designed and synthesized click probes of curcumin and found that 4 h after a single oral administration of the click probe, the presence of curcumin-protein complex was only observed in colon and liver, but not in other tissues [12]. However, it remains unknown how a long-time treatment with the curcumin probe modifies tissue proteins. In this study, we further evaluated the effects of the click chemistry probe of curcumin to label cellular proteins *in vitro* and *in vivo*, and used the probe to investigate the detailed tissue distribution of protein-curcumin complex in mice.

Material and Methods

Materials and Chemicals

Curcumin (>98% purity) was purchased from Thermal Fisher Scientific (Waltham, MA), and its click-chemistry probe dialkyne-curcumin (Di-Cur) was chemically synthesized as described in

our previous report [12]. The structure and purity of the synthesized Di-Cur were validated by HPLC, MS, and ^{1}H NMR, as we described [12].

Cell Culture of MC38 colon cells

Murine colonic adenocarcinoma cell MC38 cells (a kind gift from Prof. Ajit Varki at the University of California San Diego) were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza, Allendale, NJ) supplemented with 10% fetal bovine serum (FBS, Corning, NY) in a 37 °C incubator under an atmosphere with 5% CO_2.

Treatment with the curcumin click-chemistry probe Di-Cur in MC38 cells

MC38 cells were treated with curcumin, Di-Cur, or DMSO vehicle in either serum-free medium or complete medium. At the end of the experiment, the medium was discarded, the cells were washed with PBS, centrifuged at 1,000 rpm at 4 °C for 5 min, extracted using RIPA lysis buffer with phosphatase/protease inhibitors (Boston BioProducts, Ashland, MA) on ice for 1 hour, and centrifuged at 12,000 rpm for 15 minutes at 4 °C. The supernatant was collected for subjected to click chemistry-based SDS-PAGE in-gel fluorescence imaging.

Click Chemistry-based fluorescence imaging

The supernatant from MC38 cells (containing 80-100 μg protein, as assessed by the BCA assay) was incubated with $CuSO_4$ (50 μM, Thermo Fisher Scientific), ascorbic acid (50 μM, Thermo Fisher Scientific), Tris-benzyltriazolylmethylamine (THPTA) ligand (50 μM, Lumiprobe, Hunt Valley, MD), and CruzFluor sm™ 8 azide (50 μM, Santa cruz, Dallas, TX) for 1 h at room

temperature in dark. The reaction mixture was then 1:1 mixed with the SDS-PAGE loading buffer (Amresco Inc., Solon, OH) and heated at 95 °C for 10 min, and resolved using 12% SDS-PAGE gel. The gel was scanned with wavelength at 800 nm using an Odyssey imaging system (LI-COR Biosciences, Lincoln, NE). The total protein levels in the gels were measured by Coomassie blue staining.

Intraperitoneal injection of the curcumin click-chemistry probe Di-Cur in Mice

This study was performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (NIH Publication No. 85-23 Rev. 1985). All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts-Amherst. C57BL/6 male mice (age = 6 weeks) were purchased from Charles River (Wilmington, MA), fed with standard chow, and maintained in a standard Specific-Pathogen Free (SPF) animal facility. After animal adaption, the mice were treated with curcumin or Di-Cur (dose = 100 mg/kg), or DMSO vehicle, via intraperitoneal injection. After 1-2 hours, the mice were sacrificed, and the liver tissues were collected, ground up using liquid nitrogen, and extracted with RIPA lysis buffer with phosphatase/protease inhibitors (Boston BioProducts) on ice for 1 hour. The obtained tissue lysates were subjected to click chemistry-based SDS-PAGE in-gel fluorescence imaging, as described above.

Oral gavage of the curcumin click-chemistry probe Di-Cur in mice

Swiss Webster male mice (age = 6 weeks) were purchased from Charles River, fed with standard chow, and maintained in a standard SPF animal facility of the University of Massachusetts-

Amherst. After animal adaption, the mice were treated with curcumin or Di-Cur (dose = 70 mg/kg/day), or polyethylene glycerol 400 (PEG 400, Thermal Fisher Scientific, Waltham, MA) as vehicle, once per day for 7 days, via oral gavage. At the end of the experiment, the mice were sacrificed, and tissues (including brain, heart, lung, spleen, stomach, kidney, liver, small intestine, and colon) were collected, extracted, and lysed as described above. The obtained tissue lysates were subjected to click chemistry-based SDS-PAGE in-gel fluorescence imaging as described above.

Results

Curcumin click probe Di-Cur labels cellular proteins in colon cells *in vitro*

In our previous study, we used a click chemistry probe of curcumin, mono-curcumin (mono-Cur), to study the curcumin-protein interactions *in vitro* and *in vivo* [12]. To establish the broader range of validity of the observed curcumin-protein interactions, here we used a different probe, Di-Cur, that was designed and synthesized in our previous study [12]. To test whether Di-Cur can covalently label cellular proteins, we treated MC38 colon cancer cells with Di-Cur, then performed click chemistry-based in-gel fluorescence imaging (see scheme of experiment in **Fig. 2A**). We found that in both serum-free and complete medium, treatment with Di-Cur can efficiently label cellular proteins in MC38 cells, demonstrating the formation of curcumin-protein complex *in vitro* (**Fig. 2B-C**). Di-Cur labelled cellular proteins in MC38 cells in a time- and dose-dependent manner (**Fig. 2B-C**). Together, these results further support that it is feasible to use the Di-Cur probe to study the formation of curcumin-protein complex.

Intraperitoneal injection of curcumin click-chemistry probe Di-Cur labels liver proteins *in vivo*

After we found that Di-Cur can label the cellular protein *in vitro*, we further tested its effect on protein labelling *in vivo*. We treated the mice with vehicle control (DMSO), curcumin, or Di-Cur via intraperitoneal injection, then performed click chemistry-based in-gel fluorescence imaging in the liver tissue (see scheme of experiment in **Fig. 3A**). We found that after 2-hour treatment, Di-Cur efficiently labelled proteins in the liver tissue, suggesting that the probe can reach the liver tissue via systemic circulation and form covalent complex with the liver proteins. Such effect was not observed after 1-hour treatment, demonstrating a time-dependent effect. And as expected, treatment with vehicle control or curcumin showed little fluorescence signal (**Fig. 3B**). Together, these results further support that it is feasible to use the Di-Cur probe to study the formation of curcumin-protein complex.

Oral administration of curcumin click-chemistry probe Di-Cur selectively labels tissue proteins *in vivo*

We next use the curcumin click chemistry probe Di-Cur to study tissue distribution of curcumin-protein complex in mice. To this end, we treated mice with vehicle control, curcumin, or Di-Cur via oral gavage, once per day for 7 days, then collected tissues for click chemistry-based imaging (see scheme of experiment in **Fig. 4A**). We found that after 1-week treatment, Di-Cur efficiently labelled cellular proteins in the colon and liver tissues of the treated mice, suggesting the presence of curcumin-protein complex in these two tissues (**Fig. 4B**). Notably, the colon tissue showed stronger fluorescence signals compared with the liver tissue, supporting a higher

concentration of curcumin-protein complex in the colon tissue (**Fig. 4B**). In contrast, in other tissues such as lung (**Fig. 4B**), brain, heart, stomach, spleen, small intestine, and whole blood (**Fig. S1**), Di-Cur treatment resulted in weak or little fluorescence signal, suggesting the absence of curcumin-protein complex in these tissues. And as expected, treatment with vehicle control or curcumin showed little fluorescence signal (**Fig. 3B** and **Fig. S1**).

Discussion

Previous studies have shown that curcumin could covalently modify protein thiols, which could contribute to its health-promoting effects [13, 14]. In addition, the formation of intracellular curcumin-protein complex could also contribute to the poor pharmacokinetics of curcumin. However, most of the previous studies were performed in cultured cells or cell-free enzyme systems, it remains unknown whether curcumin could covalently modify proteins after oral administration *in vivo*. Here we used click chemistry-based SDS-PAGE in-gel fluorescence imaging to visualize the presence of covalently-linked curcumin-protein complex. Our central finding is that after repeated oral administration of the curcumin probe Di-Cur, it can covalently modify cellular proteins in the colon and liver tissues of the treated mice. These results support that oral administration of curcumin could form curcumin-protein complex in the colon and liver tissues *in vivo*, which could contribute to the biological effects and/or pharmacokinetics of curcumin. Besides curcumin, other α,β-unsaturated carbonyl-bearing dietary compounds could also be capable of modifying cellular proteins *in vivo*, and a better understanding of the

protein-compound interactions could help us to better understand their health-promoting mechanisms and design effective and safe strategies for disease prevention.

We found that the curcumin-protein complex was only observed in the liver and colon, but not in other tissues (including lung, brain, heart, stomach, spleen, small intestine, and whole blood) of the treated mice. This result is in agreement with our previous studies, which showed that a single-time oral administration of monoalkyne-curcumin (Mono-Cur), another curcumin probe, also only labelled cellular proteins in the liver and colon tissues in mice [12]. Our previous study showed that mono-Cur is a better probe compared with Di-Cur [12]. Here we used Di-Cur, mainly because of the difficulties to chemically synthesize mono-Cur. Furthermore, our data here showed that Di-Cur is an effective probe to study curcumin-protein interactions. Using two different curcumin probes, we got the same result that the formation of protein-curcumin complex was only observed in liver and colon tissues. This could be, at least in part, because the colon and liver tissues are the major organs involved in absorption and/or metabolism of curcumin [15, 16]. Notably, after oral intake of food (e.g. curcumin), the food stays in colon for 10 hours to several days [17], suggesting that there is substantial time for the ingested curcumin to interact with colon tissues for the Michael reaction to occur. Substantial studies have shown that after oral administration of curcumin, the circulating and tissue concentrations of curcumin are very low [18], which could contribute to the absence of curcumin-protein complex in other tissues such as lung and heart. Together, these results support that oral administration of curcumin could result in covalent modification of cellular proteins in a tissue-specific manner; and biological significance of the observed effects remains to be determined.

A limitation of our study is that we only performed SDS-PAGE in-gel imaging to show the presence of curcumin-protein complex in tissues, while the identities of the curcumin-binding proteins are unknown. Previous studies have shown that the click chemistry-based proteomics approach is a powerful method for identification of target proteins. Indeed, after treatment with the click probe of the bioactive compound (e.g. curcumin) in cells or in animals, the target proteins are labelled with alkyne- or azide- moiety, which can be enriched by click chemistry and affinity purification, and the identities of the enriched protein targets can be elucidated by LC-MS analysis [7-11]. Using this strategy, our previous study has shown it is feasible to use this method to identify curcumin-binding proteins in MC38 colon cancer cells [12]. It is important to use this approach to elucidate the identities of curcumin-binding proteins in animal models, and determine the biological significance of the identified proteins in the health-promoting effects of curcumin. This could greatly help our understanding for the molecular mechanism of curcumin. In addition, the identified protein(s) could serve as novel cellular targets for disease prevention.

In summary, here our results support that oral administration of curcumin could result in formation of protein-curcumin complex in a tissue-specific manner, which could contribute to the biological effects and/or pharmacokinetics of curcumin. Further studies using click chemistry-based proteomics to elucidate the identities of curcumin-binding proteins could greatly help us to better understand molecular mechanisms of curcumin.

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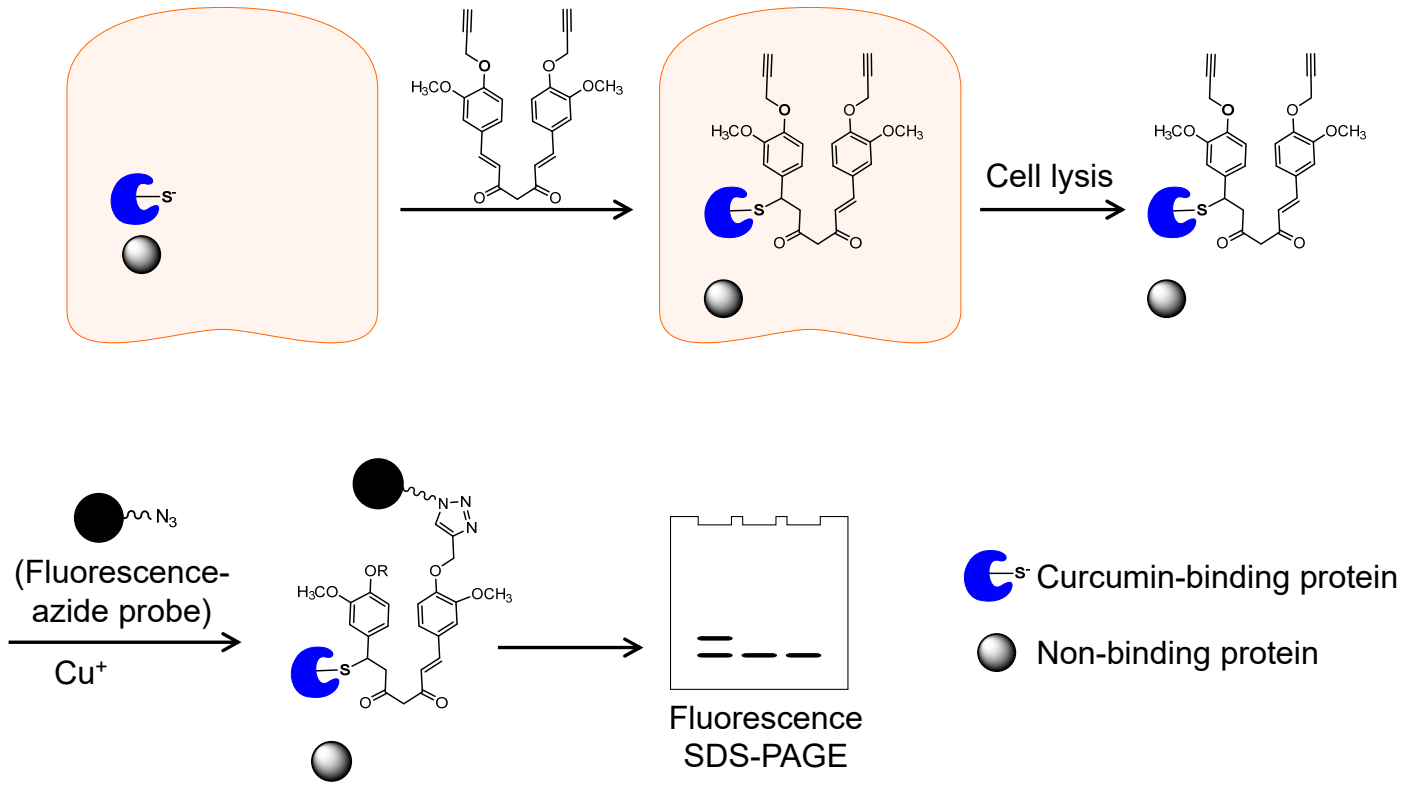

Figure 1. Click chemistry-based fluorescence imaging to detect formation of curcumin-protein complex *in vitro* and *in vivo*.

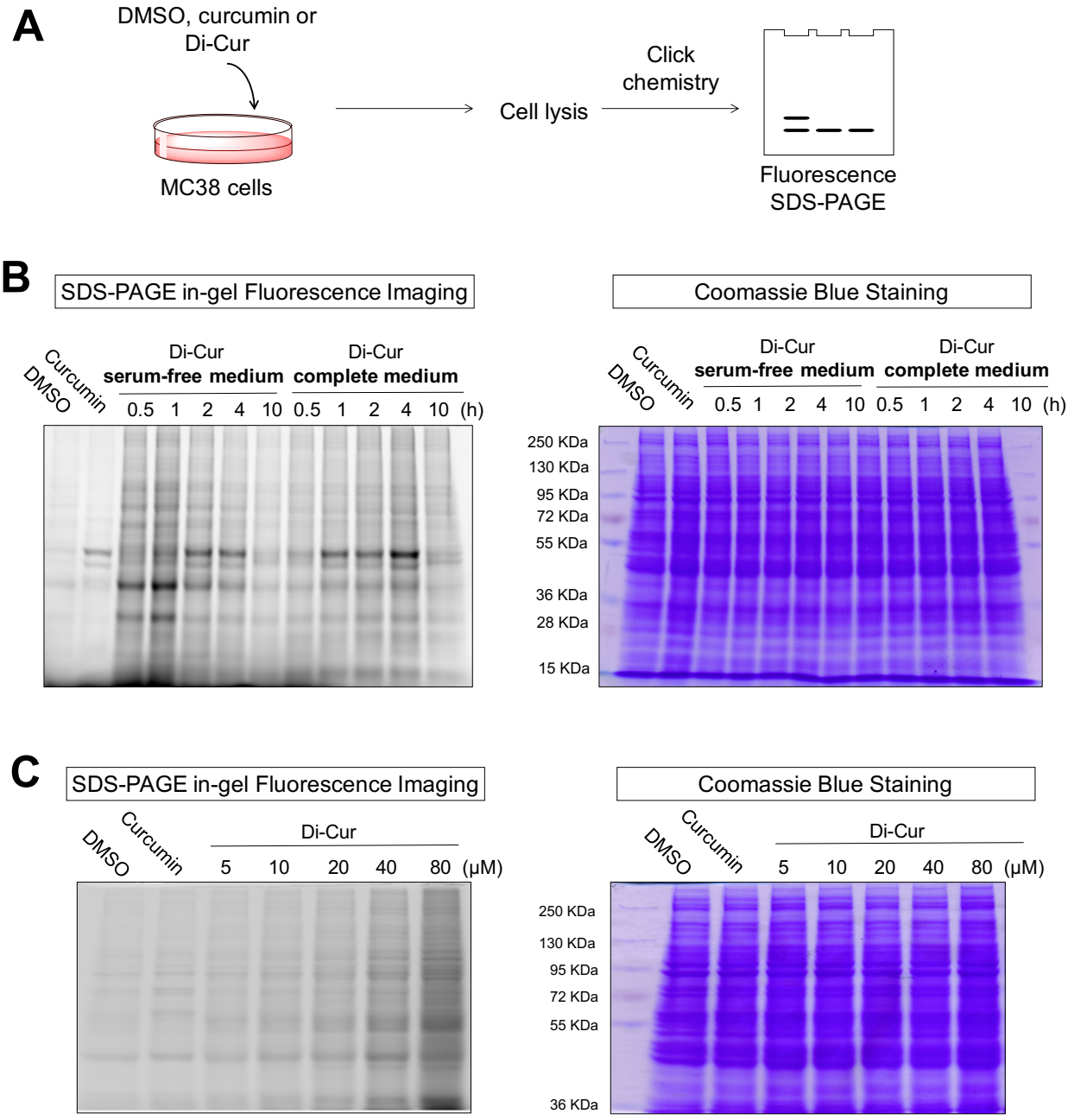

Figure 2. Effect of Di-Cur probe on protein labelling *in vitro*. (A) Scheme of experiment. MC38 cells were treated with curcumin, Di-Cur, or DMSO vehicle control in either serum-free or complete medium. After incubation, the cells were lysed and subjected to click chemistry-based SDS-PAGE in-gel fluorescence. (B) Di-Cur labelled cellular proteins in MC38 cells in a time-dependent manner (concentration of curcumin or Di-Cur = 30 μM). (C) Di-Cur labelled cellular proteins in MC38 cells in a dose-dependent manner (treatment time: 2 hours).

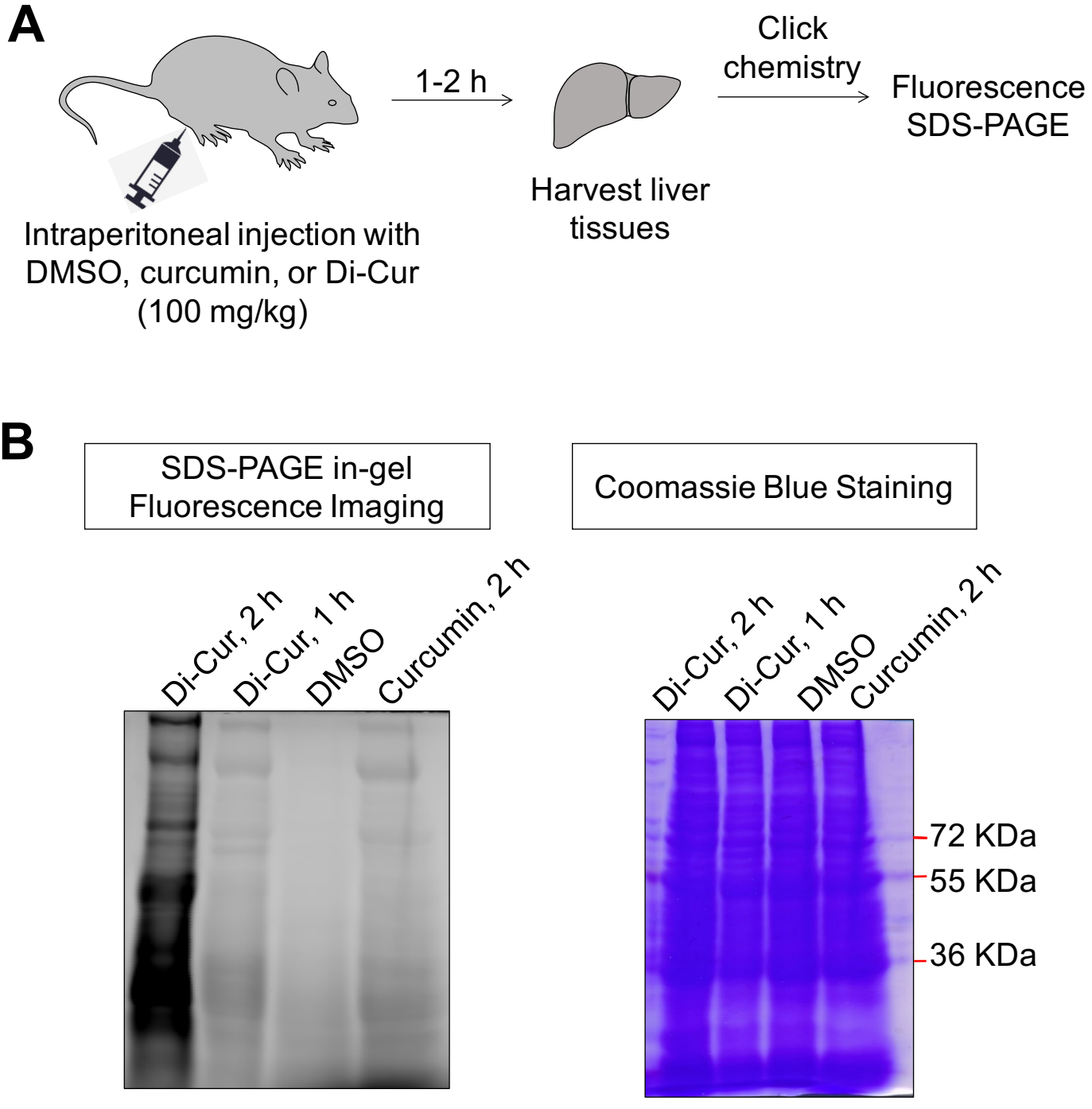

Figure 3. Intraperitoneal injection of curcumin click-chemistry probe Di-Cur labels liver proteins *in vivo*. (***A***) Scheme of experiment. (***B***) Effects of Di-Cur on labelling cellular proteins in the liver tissue. Left panel: SDS-PAGE in-gel fluorescence. Right panel: Coomassie blue staining.

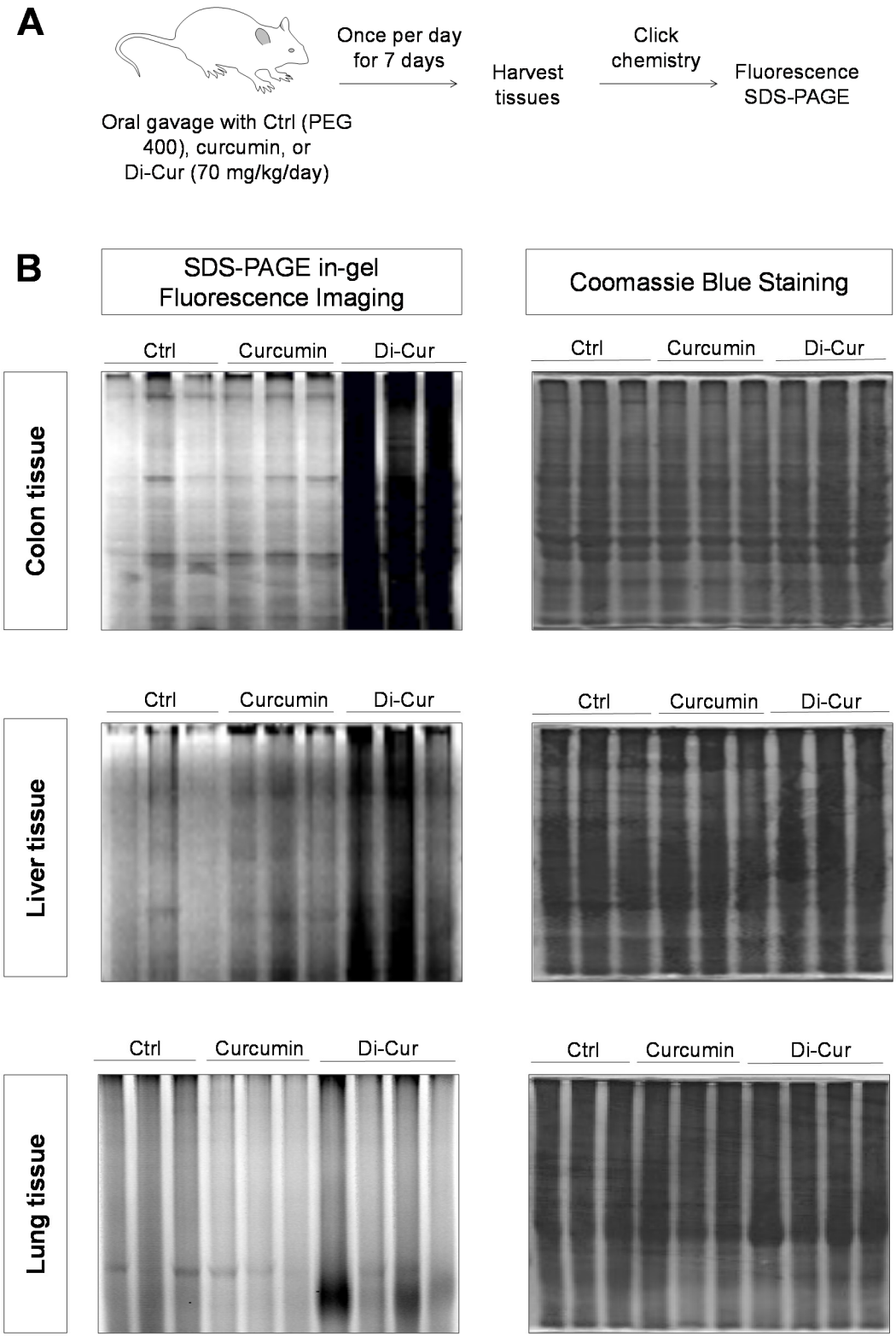

Figure 4. Oral administration of curcumin click-chemistry probe Di-Cur labels cellular proteins in a tissue-specific manner *in vivo.* (***A***) Scheme of experiment. (***B***) Effects of Di-Cur on labelling cellular proteins in tissues. Left panel: SDS-PAGE in-gel fluorescence. Right panel: Coomassie blue staining.