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In Situ Proteome Profiling of C75, a Covalent Bioactive Compound with Potential Anticancer Activities

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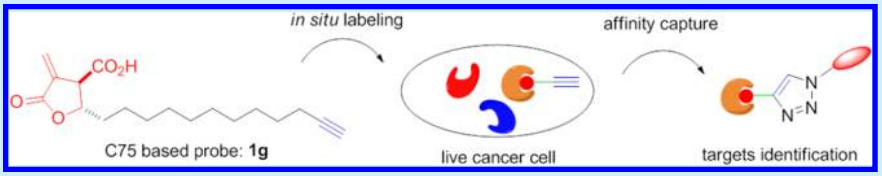
Supporting Information



ABSTRACT: A library of cell-permeable, minimally tagged C75 analogues was synthesized and used to uncover biological targets in human liver cancer cells. Known targets of C75, namely FASN and CPT1A, together with other unknown targets, including PDIA3, TFRC, and GAPDH, were thus identified.

Despite recent success with the use of covalent drugs, for example, orlistat (weight loss), clopidogrel (antiplatelet), and esomeprazole (peptic ulcer), there remain significant safety concerns over the large-scale development of covalent-based therapeutics.[1] Such drugs could not only modify proteins but also trigger an idiosyncratic immune response. Such concerns are in part attributed to the difficulty in establishing the spectrum of "off-target" binding events which can cause many undesirable side effects.[2]

To address this problem, we have recently developed a convenient chemical proteomic method to unravel the global target spectrum for any given covalent drug, using a click-based in situ profiling approach.[3,4] This method enables the repertoire of drug-target interactions to be probed within living systems. By introducing a very conservative modification onto the lead molecule (namely, an alkyne tag) at solvent-accessible sites (to retain pharmacodynamic properties), observations can be reliably made to determine molecular behavior and interactions within complex and dynamic cellular environments (Figure 1a).[2] We had previously applied this strategy to successfully profile the on- and off-targets of orlistat[3] as well as those of various kinase inhibitors.[5] Here we extend the strategy by building a library of a covalent bioactive compound, C75, which has been particularly promising as a weight-loss and anticancer agent through its ability to modulate fatty acid oxidation.[6,7] C75 is a more stable synthetic mimic of cerulenin (a fungal metabolite) (see the Supporting Information) and belongs to a class of α-methylene-γ-butyrolactones.[6] Various pathways have been found to be modulated by C75, including inhibition of fatty acid synthase (FASN) and activation of carnitine *O*-palmitoyltransferase-1 (CPT1).[7] The compound is also known to alter the level of neuropeptide Y and AMP-activated protein kinase (AMPK) activity in the hypothalamus, reducing food intake.[7b] Many common human cancers, including breast, ovarian, prostate, and colon, express high levels of FASN. Thus, decreased fatty acid production through FASN inhibition is seen as a viable anticancer strategy.[6] Prior work using isotopic labeling and immuno precipitation validated FASN as the cellular target of C75.[6b] In a separate study, a very similar butyrolactone scaffold was shown to bind histone acetyltransferase GCN5, with a weak IC_{50} of approximately 100 μM, highlighting that other proteins may have affinity to C75 as well.[8]

In the current study, we set out to identify unknown cellular targets (on and off) of C75 at its early stages of development as a potential therapeutic agent. A small library of eight activity-based probes (ABPs) was first designed from the C75 scaffold. The 5-membered-ring (4-methylene-2-octyl-5-oxotetrahydrofuran-3-carboxylic acid) nucleophilic acceptor was retained as the pharmacophore for binding to its respective targets. The linker lengths were varied between 2 and 10 carbon atoms, with both the *cis* and *trans* versions synthesized for probe **1f**. The terminal alkyne was introduced to the linker side chain. Several azide-containing reporters were also synthesized, with a fluorescent group (rhodamine B) and/or biotin (Figure 1b).

Briefly, by using **1g** as an example, bromocarboxylic acid **6g** was treated with lithium (trimethylsilyl)acetylide, which was made from trimethylsilylacetylene and butyllithium, to give compound **5g** (56% yield). Following reduction with lithium aluminum hydride and deprotection of the trimethylsilyl (TMS) group, **4g** was obtained. After oxidation with PCC and sodium acetate,[9] the aldehyde **3g** was eluted by diethyl ether through a pad of silica gel and carefully concentrated. By treatment with freshly prepared LDA and 6 N H_2SO_4, the desired aldehyde **3g** then was cyclized with intermediate **7** to give compound **2g**, which was deprotected under mild conditions to give final product **1g**.[8] Compound **2g** was not

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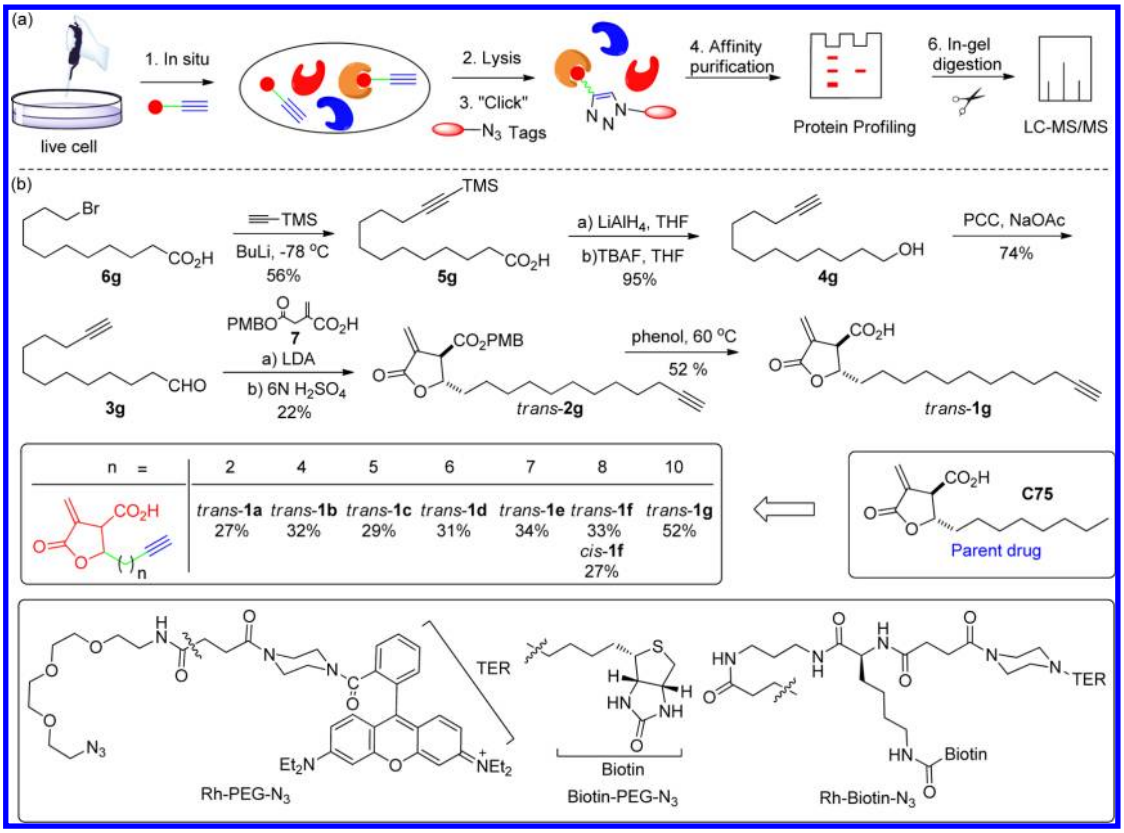

Figure 1. (a) Overall strategy for in situ profiling of potential C75 cellular targets. C75 analogues with an alkyne handle were applied to living cells, followed by a click reaction with the probe segment that contained the azide group. The labeled proteins were separated and identified by in-gel fluorescence scanning and LC−MS/MS analysis. (b) Representative synthesis of **1g**, structures of C75 and its analogues (**1a**−**g**), as well as clickable reporters used in this study.

stable at room temperature under open air, and it should be stored at low temperature and used in the next step under mild conditions.

To benchmark the activities of the modified C75 analogues, antiproliferation XTT assays were performed with HepG2 cancer cells. Compared to the original synthetic compound C75, **1g** produced the best antiproliferation activity among the various C75 analogues (Figure 2a). The longer hydrophobic side chain in **1g** might have afforded better cell permeability of this compound, giving rise to its improved cellular activities. The stereoisomers of *trans*-**1f** and *cis*-**1f** did not show significant activity differences. Subsequently, **1g** was used for further biological studies. For example, **1g** could be used as a small molecule imaging probe to assess the cellular uptake of C75 and detect its subcellular localizations (Figure 2b);[3,5] results showed **1g** was mostly localizing within the cytosol, indicating this is where most of C75's cellular targets reside. Next, in vitro and in situ labeling experiments were performed to test the proteome reactivity profiles of all probes (**1a**−**g**) by using mammalian cell lysates and live cells, respectively (Figure 2c, left). The probes were first incubated with the lysates of HepG2 cells and clicked with the fluorescent reporter Rh-PEG-N_3. Results from the in-gel fluorescence scanning showed that even 5 μM of the probes were able to produce numerous significant fluorescent bands, in a dose-dependent manner. The labeled bands could be competed away by pretreatment of C75, indicating that the corresponding labeled proteins were likely true cellular targets of C75 (see the Supporting Information). Further, the library was tested in situ at a higher 20 μM concentration with each of the **1a**−**g** analogues. This provided a compartmentalized living system in which to test the binding activities of these scaffolds (Figure 2c, left); probe **1g** remained the compound with the most intense proteome labeling profiles. Finally, to positively identify some of the putative protein targets visualized on the gels, a pull-down experiment was performed with **1g**. Instead of using Rh-PEG-N_3, a trifunctional reporter Rh-Biotin-$N_3$was used in the click reaction following proteome labeling by **1g**. A pull-down experiment was performed as previously described,[3,5] with avidin-immobilized agarose beads. The pulled-down samples were further separated by SDS-PAGE and analyzed by Western blotting to unambiguously confirm the presence of FASN and CPT1A (Figure 2d), two previously identified C75 targets.[6,7] These results indicate that our newly developed "clickable" C75 analogues, e.g., **1g**, were indeed suitable small molecule probes for large-scale, cell-based proteome profiling of potential targets of C75 in the cancer cells.

Reassured with these results, we proceeded with the pull-down/LCMS experiments, with 20 μM of **1g**, under the same conditions as in situ proteome profiling to identify putative cellular targets of C75.[3,5] A list of potential C75 targets was obtained after deduction of those nonspecific binding proteins that also appeared in negative control (DMSO) and subsequent filtering (summarized in Table S1 in the Supporting Information). We focused our attention on those candidates which possess known nucleophilic residues and play important roles in cancer cells (Figure 3a). FASN and CPT1A again emerged as candidates on this list, as should be expected. The vast majority of these hits also reappeared in list of the parallel pull-down/LC-MS experiment using more (100 μM) of **1g**

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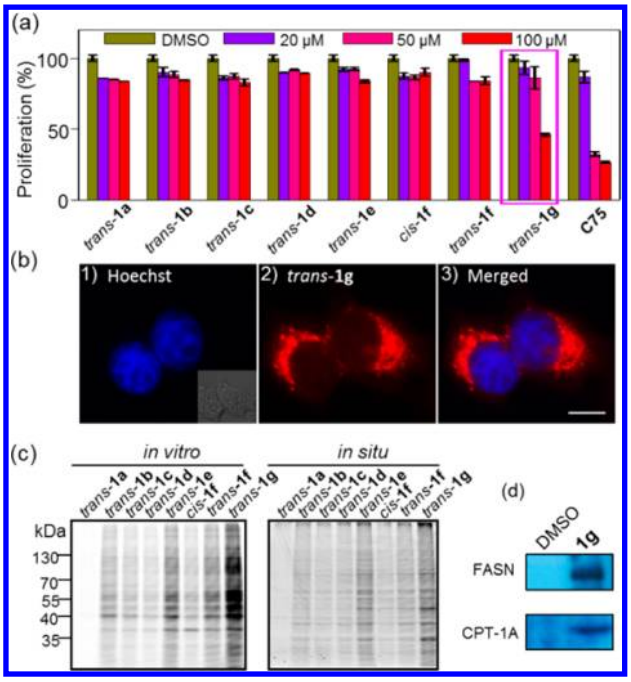

Figure 2. (a) Dose-dependent inhibition of HepG2 proliferation by C75 and its analogues **1a**–**g** (0, 20, 50, 100 μM) by XTT assays. (b) Fluorescence imaging of HepG2 cells with (1) Hoechst (blue) and (2) **1g** (20 μM, red) clicked with Rh-PEG-N_3; (3) merged imaging; DIC inset. Scale bar = 20 μm. (c) In vitro (5 μM, left) and in situ (20 μM, right) labeling of HepG2 proteomes with C75 probes **1a**–**g**. (d) Validation of known targets of C75 by pull-down and Western blotting experiments.

(Table S2 in the Supporting Information). A number of lipid- and/or fatty-acid-metabolism-related proteins were also identified, including acyl-CoA dehydrogenase (ACADVL) and sterol *O*-acyltransferase (SOAT1). Proteins possessing a cysteine active-site residue, including protein disulfide-isomerase A3 (PDIA3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transferrin receptor protein 1 (TFRC), asparagine synthetase (ASNS), cytoskeleton-associated protein 4 (CKAP4), protein disulfide isomerase (P4HB), thioredoxin reductase 1 (TXNRD1), and double-stranded RNA-specific adenosine deaminase (ADAR), were identified as potential C75 targets. Other proteins, including fructose-bisphosphate aldolase A (ALDOA), heat shock protein 90 βb (HSP90AB2P), l-lactate dehydrogenase A chain (LDHA) and methyl crotonoyl-CoA carboxylase (MCCC1), were also identified as potential targets and they all contained an active-site lysine residue. Based on antibody availability, we validated several of these hits, including PDIA3, TFRC, and GAPDH, by **1g** labeling/pull-down/Western blotting (Figure 3b); all three proteins were positively labeled by **1g** and successfully pulled-down, indicating they are likely true cellular targets of C75 which had not been identified previously. Further biological validation experiments are underway, and the results will be reported in due course.

In conclusion, we have successfully designed and synthesized a library of C75 analogues. The known targets, including FASN and CPT-1A, were validated, and other potential C75 targets were identified by this strategy. We have thus far carried out preliminary experiments to validate some of these newly identified C75 cellular targets. Further studies will be focused on elucidating the mechanism of C75 binding against these targets.

(a)

gene symbol	localization	protein function
FASN	cytoplasm	fatty acid synthase
CPT1A	mitochondria outer membrane	carnitine O-palmitoyltransferase activity
PDIA3	ER	catalyzing the rearrangement of -S-S bonds in proteins
TFRC	cell membrane	cellular uptake of iron
GAPDH	cytoplasm membrane nucleus	glyceraldehyde-3-phosphate dehydrogenase and nitrosylase
ASNS	cytosol	asparagine biosynthesis
HSP90AB2P	cytoplasm	molecularchaperone.
ALDOA	cytosol	glycolysis
LDHA	cytoplasm	glycolysis
CKAP4	membrane	receptor for Surfactant protein-A
MCCC1	mitochondrion	catalyzing carboxylation of 3-methylcrotonyl-CoA to 3-methylglutaconyl-CoA
ACADVL	mitochondrion inner membrane	acyl-CoA dehydrogenases
TXNRD1	ER membrane	formation of cell membrane protrusions
SOAT1	nucleus	catalyzing the formation of fatty acid-cholesterol esters
P4HB	cell membrane ER membrane	catalyzing the formation, breakage and rearrangement of disulfide bond
ADAR	cytoplasm	double stranded RNA specific adenosine deaminase

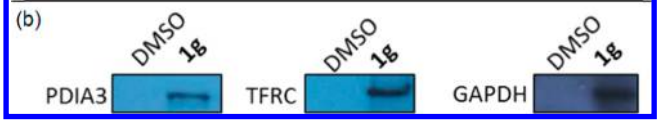

Figure 3. (a) Representative proteins identified from PD/LCMS experiments. (b) Pull-down/Western blotting validation by using **1g** (20 μM). (left lanes) negative PD/WB with DMSO.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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