



Published in final edited form as:

Angew Chem Int Ed Engl. 2020 March 02; 59(10): 3896–3899. doi:10.1002/anie.201914391.

A chemical proteomic probe for the mitochondrial pyruvate carrier complex

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Abstract

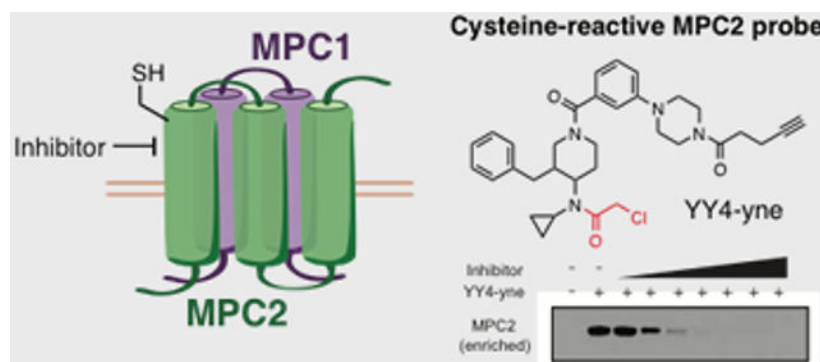
Target engagement assays are crucial for establishing the mechanism-of-action of small molecules in living systems. Integral membrane transporters, due to their specialized biophysical properties and activity assays, can present a challenging protein class for assessing cellular engagement by small molecules. Here, we describe the chemical proteomic discovery of alpha-chloroacetamide (α CA) compounds that covalently modify cysteine-54 (C54) of the MPC2 subunit of the mitochondrial pyruvate carrier (MPC). We leverage this finding to create an alkyne-modified α CA, YY4-yne, that serves as a versatile cellular target engagement probe for MPC2 in click chemistry-enabled western blotting or global mass spectrometry-based proteomic experiments. Using YY4-yne, we demonstrate that UK-5099, an alpha-cyanocinnamate inhibitor of the MPC complex, first discovered more than 30 years ago, but still with a poorly defined mechanism-of-action, engages MPC2 with remarkable selectivity in human cells. These findings support a model where UK-5099 inhibits the MPC complex by binding to C54 of MPC2 in a covalent reversible manner that can be quantified in cells using the YY4-yne probe.

Graphical Abstract

We report the chemical proteomic discovery of covalent, irreversible ligands for the human mitochondrial pyruvate carrier (MPC) and demonstrate their utility as cellular engagement probes for this important integral membrane metabolite transporter.

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Keywords

Chemical proteomics; mitochondrial pyruvate complex; Target engagement; α -chloroacetamide; cysteine; reversible covalency

As part of a broader research program aimed at characterizing the protein targets of electrophilic compounds in primary human T cells^[1] using a mass spectrometry (MS)-based chemical proteomic method that quantifies cysteine reactivity on a global scale^[2], we discovered herein an α -chloroacetamide (α CA) YY4 (**1**; Figure 1A) that strongly engages cysteine-54 (C54) in subunit 2 of the mitochondrial pyruvate carrier (MPC2) (Figure 2A, B). This interaction was of interest because, to our knowledge, covalent, irreversible ligands have not been described for MPC2. Additional C54 of MPC2 was not sensitive to structurally related α CA compounds (e.g., EV-93 (**3**); Figures 1A and 2A, B and Supplementary Table 1) or broadly reactive electrophilic fragments (Figure S1)^[1]. The restricted structure-activity relationship (SAR) displayed by C54 in MPC2 was suggestive of residence in a specific small molecule-binding pocket. Motivated by the important role of the MPC in human metabolism^[3], combined with the dearth of assays available to evaluate endogenous MPC activity and pharmacological inhibition in cells^[4], we set out to further investigate the YY4-MPC2 interaction.

The MPC is an integral membrane transporter composed of two subunits, MPC1 and MPC2^[5], that facilitates the transport of cytosolic pyruvate into the mitochondrial matrix and consequently plays a crucial role in cellular metabolism^[6]. Small-molecule inhibitors have been described for the MPC^[7], including an α -cyanocinnamate UK-5099 (**4**; Figure 1B) that is speculated to act by a covalent reversible mechanism^[8] and has been used to study MPC involvement in pyruvate-related metabolic processes^[9]. However, so far, monitoring MPC activity and inhibition in human cells has depended on technically intricate metabolic labeling assays or the introduction of exogenous MPC fusion proteins as BRET-based biosensors^[4].

Recognizing that covalent ligands have been converted into versatile target engagement probes for other protein classes, including, for instance, hydrolases^[10], kinases^[11], glycosidases^[12], and GTPases^[13], we set out to investigate whether the YY4-MPC2 interaction could be similarly leveraged for developing a cellular engagement probe for the MPC complex. MPC2 is a small protein (14 kDa) with only a single cysteine – C54 (Figure

S2) – the site of covalent modification by YY4 mapped in our chemical proteomic experiments. Given the small size of MPC2, we wondered whether covalent modification by YY4 would cause a band shift in the protein migration by SDS-PAGE. Consistent with this hypothesis, MPC2 migrated as a higher molecular weight (HMW) species in YY4-treated vs DMSO-treated human T cells, as detected by Western blotting with commercial anti-MPC2 antibodies (Figure 2C). Curiously, however, the signal intensity for MPC2 was also substantially lower in YY4-treated cells (Figure 2C). Neither the HMW shift nor reduction in signal intensity in MPC2 was observed in T cells treated with UK-5099 (Figure 2C).

The epitope for the commercial anti-MPC2 antibody is unclear, only stated as being a synthetic peptide corresponding to residues surrounding N33 (#46141, Cell Signaling Technology). We initially assumed that YY4 modification of C54 in MPC2 would not disrupt antibody recognition and alternatively considered that the reduction in MPC2 signal could reflect loss of MPC2 protein in cells treated with YY4. We tested this hypothesis by developing an alkyne analogue of YY4 – YY4-yne (**2**; Figure 1A) – suitable for direct labeling and enrichment of MPC2 from human T cells by conjugation to an azide-biotin tag^[14] using copper-catalyzed azide alkyne cycloaddition (CuAAC) chemistry^[15]. We performed time-course studies comparing MPC2 signals enriched from T cells treated with YY4-yne measured by MS-based proteomics (Figure 3A) or Western blotting (Figure 3B), which revealed a time-dependent increase and maintenance of YY4-yne-enriched MPC2 signals throughout the treatment period (Figure 3A, B), despite substantial reductions in total MPC2 signal observed by Western blotting in unenriched lysates from YY4-yne-treated cells (Figure 3B). These results indicate that modification of C54 by YY4-yne does not alter MPC2 content in cells, but rather impairs recognition of this protein by the commercial anti-MPC2 antibody.

Despite the apparent perturbation of antibody recognition of MPC2 caused by YY4(-yne)-reactivity, sufficient signal intensity was still observed for streptavidin-enriched MPC2 in YY4-yne-coupled Western blotting to furnish a convenient method to measure the engagement of MPC2 by small molecule inhibitors in cells. Pre-treatment of human T cells, for instance, with YY4 or UK-5099, but not the inactive compound EV-93, blocked YY4-yne-enriched MPC2 protein (Figure 3C). We also note that UK-5099 blocked the reduction in total anti-MPC2 signal intensity observed in lysates of T cells treated with YY4-yne (Figure 3C, input). This result is consistent with the proposed covalent, reversible mechanism of action for UK-5099, which would not be expected to permanently modify MPC2 after denaturation of the protein prior to Western blotting. Finally, we found that similar results were obtained *in vitro*, where T cell lysates were pre-treated with compounds prior to exposure to YY4-yne, although the extent of engagement of MPC2 by YY4(-yne) appeared lower, as reflected in the total and enriched signals measured in standard or YY4-yne-coupled Western blotting, respectively (Figure S3). This result suggests that YY4(-yne) reacts better with MPC2 in cells, where the integrity of the MPC complex may be better maintained.

Despite the widespread use of UK-5099 as an MPC inhibitor, its precise mechanism of action and broader proteomic selectivity remain poorly understood. The latter item was of particular interest to us, as structurally related α -cyanoacrylamides have emerged as a class

of covalent reversible ligands for diverse proteins^[16]. We first assessed the relative cellular potencies of MPC2 engagement by UK-5099 and YY4 using YY4-yne-coupled Western blotting, revealing that both compounds exhibited sub- μM IC_{50} values (Figure 4A, B). We then evaluated the broader proteomic interactions profiles for UK-5099 and YY4 at concentrations >10X their respective cellular IC_{50} values for MPC2 engagement (10 μM of each compound). Following treatment with compounds for 3 h, human T cells were exposed to YY4-yne (10 μM , 1 h), lysed, conjugated to a biotin-azide tag by CuAAC, and biotinylated proteins enriched by streptavidin beads, digested on-bead with trypsin, and characterized by quantitative MS-based proteomics. These experiments confirmed that both UK-5099 and YY4, but not the inactive control compound EV-93 (10 μM), strongly engaged MPC2 in cells, as revealed by the blockade of YY4-yne enrichment of this protein (Figure 4C). UK-5099 further showed remarkable selectivity for MPC2 and did not block the enrichment of any other YY4-yne-reactive proteins in T cells (Figure 4C, y-axis, left plot). This selectivity profile contrasted with YY4, which decreased the enrichment of several additional YY4-yne-reactive proteins (Figure 4C, x-axis, left plot). MPC2 still showed an exceptional profile in YY4-treated cells, however, in that most of the other YY4-sensitive proteins were also blocked in their YY4-yne enrichment by EV-93 (Figure 4C, right plot). These data, taken together, indicate that UK-5099 shows excellent proteomic selectivity for MPC2, as readout through competitive profiling experiments with the YY4-yne cellular engagement probe.

In summary, we have described herein the first, to our knowledge, covalent, irreversible ligands for the MPC and demonstrated their utility as cellular engagement probes for this important metabolite transporter. Using the YY4-yne probe, we have provided evidence that UK-5099, a long-standing and putatively covalent reversible inhibitor of the MPC^[8], shows excellent potency ($\text{IC}_{50} \sim 0.02 \mu\text{M}$) and proteomic selectivity in human cells. UK-5099 has been previously postulated to engage an (as of yet unidentified) cysteine on MPC1^[8, 17], but our data suggest instead that this compound may bind to C54 of MPC2. We also note that we did not enrich MPC1 with YY4-yne, indicating that the αCA probes described herein only interact with the MPC2 subunit of the MPC. We acknowledge, however, that it remains possible UK-5099 inhibits the MPC by binding to MPC1, or at an MPC1-MPC2 interface, in a manner that indirectly blocks YY4-yne reactivity with MPC2. Possibly consistent with the binding site for UK-5099 involving both MPC1 and MPC2, MPC1 mutants have been identified that show resistance to UK-5099^[5b] and MPC2 homomeric complexes are apparently insensitive to UK-5099^[17], while, on the other hand, this compound has been shown to block radiolabeled thiazolidinedione binding to MPC2^[7b].

We should also emphasize that, while using irreversible chemical probes to monitor the cellular engagement of proteins by reversible compounds is well-documented^[18], these experiments require attention to the kinetics of irreversible probe reactivity, as reversible binding of competitor ligands can be underestimated, or even completely overlooked if incubation periods extend well-beyond the time required for complete probe reactivity with a protein target of interest in cells. Finally, our initial SAR data with αCA compounds support that C54 of MPC2 resides within a druggable pocket suitable for the future development of covalent, irreversible inhibitors of the MPC, which may offer advantages over covalent, reversible compounds like UK-5099 in terms of sustaining target engagement

in increasingly complex biological systems (e.g., *in vivo* animal models). More generally, our findings highlight how broad cysteine reactivity profiling of electrophilic compounds can identify target engagement probes for monitoring the cellular activity and small molecule interactions of challenging protein classes such as integral membrane metabolite transporters.

Experimental Section

Experimental details and compound characterization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by NIH CA231991 (B.F.C.), a Clinical Translational Science Award (UL1 TR001114), a Damon-Runyon Cancer Research Foundation fellowship to X.Z. (DRG-2341-18), and a Life Sciences Research Foundation Fellowship to E.V.V.

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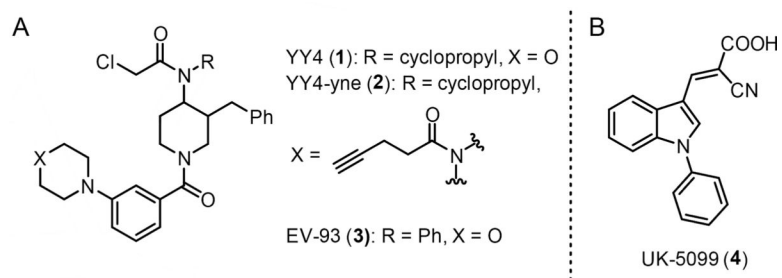


Figure 1. Chemical structures of compounds used in the study. A) Structures of α CAs YY4 (**1**) and corresponding alkyne probe YY4-yne (**2**), and inactive control compounds EV-93 (**3**). B) Structure of the MPC inhibitor UK-5099 (**4**).

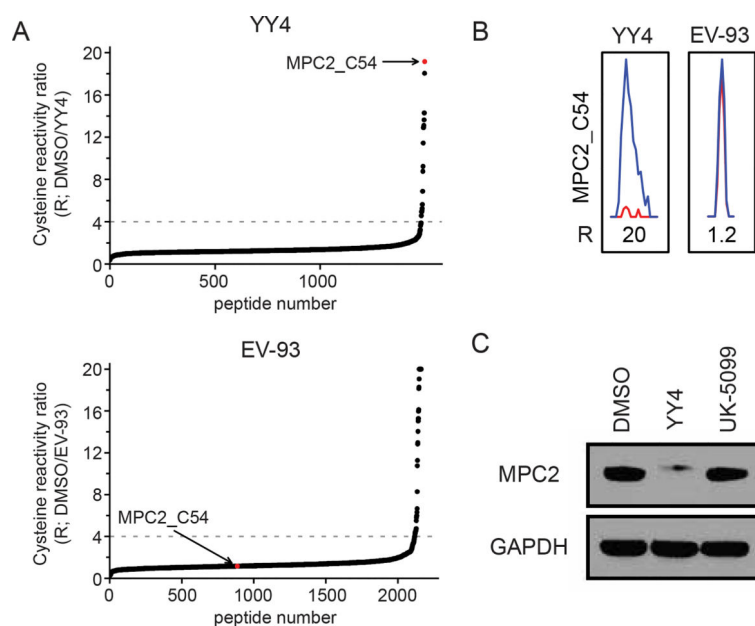
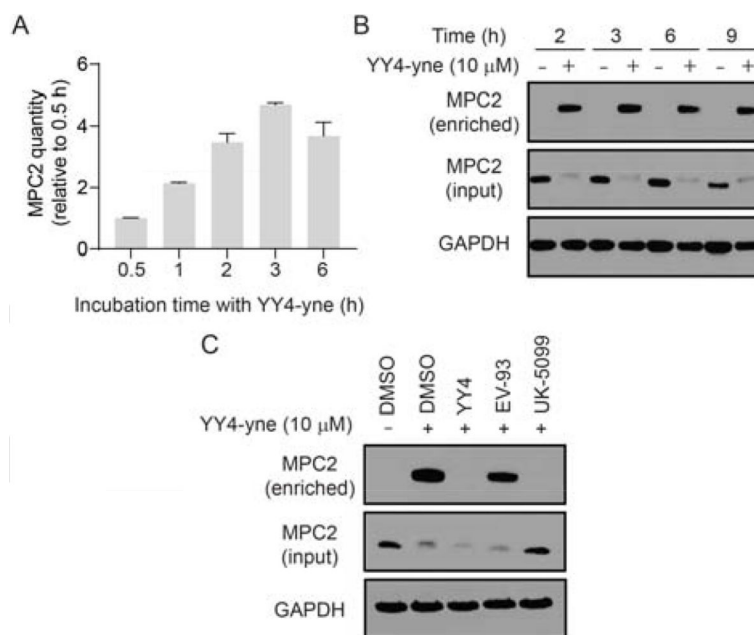
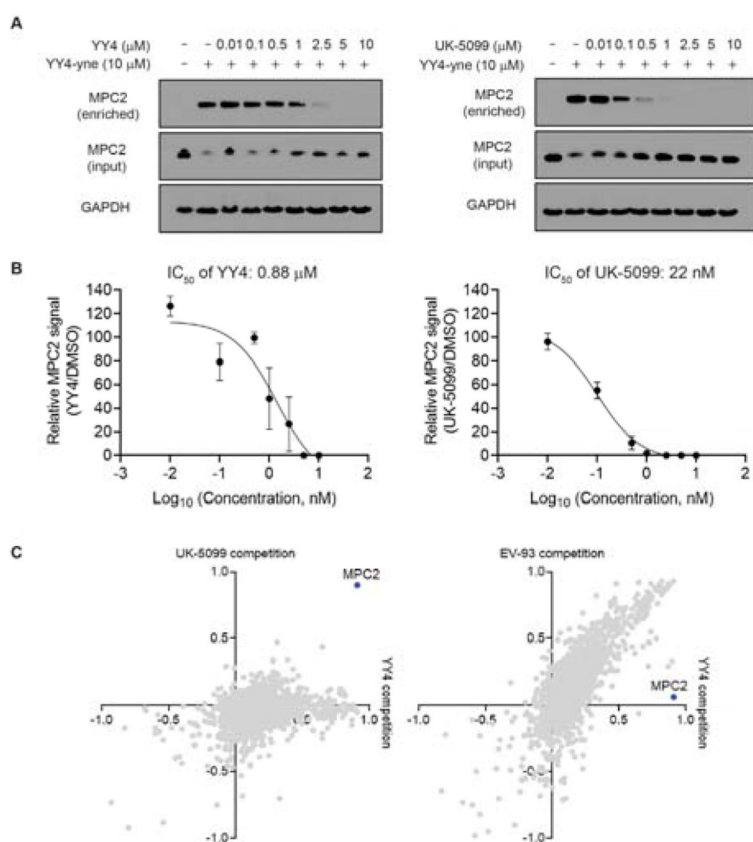


Figure 2. Cysteine reactivity profiles of primary human T cells treated with YY4 or EV-93 (10 μ M, 3 h). A) Waterfall plots showing reactivity ratios, or R values (DMSO/compound), for cysteines in the particulate fraction of YY4- or EV-93-treated cells, as measured by MS-based proteomics. C54 of MPC2 is marked in both graphs. B) Representative MS1 chromatograms for the MPC2 tryptic peptide containing C54 (K.WGLVC*AGLADMAR.P) from YY4- or EV-93-treated primary human T cells. C) Anti-MPC2 Western blot showing HMW band shift and reduced signal intensity for MPC2 from human T cells treated with YY4, but not UK-5099 (10 μ M of each compound, 3 h) or DMSO.

**Figure 3.**

Characterization of MPC2 interactions with the YY4-yne probe. A) MPC2 signals in human T cells treated with YY4-yne (10 μM) or DMSO for the indicated times, after which YY4-yne-labeled proteins were enriched by CuAAC to an azide-biotin tag and analyzed by multiplexed (isobaric tandem mass tagging) MS-based proteomics. Data represent average values \pm SD for two replicate experiments. B) YY4-yne-coupled Western blotting of MPC2 from streptavidin-enriched fractions of T cells treated with YY4-yne (10 μM) or DMSO for the indicated times. C) YY4-yne-coupled Western blotting from T cells treated with the indicated compounds (YY4, EV-93, and UK-5099; 10 μM, 3 h) or DMSO, followed by YY4-yne (10 μM, 2 h). Note that the stronger apparent Western blot signals for YY4-yne-labeled MPC2 in enriched samples likely reflect the larger amount of total cellular protein used as input for streptavidin enrichment experiments (see Supporting Information for more details).

**Figure 4.**

YY4-yne serves as a cellular target engagement probe for MPC2. A) YY4-yne-coupled Western blotting of human T cells treated with YY4 or UK-5099 (0.01–10 μM, 3 h) or DMSO followed by YY4-yne (10 μM, 2 h). B) IC₅₀ values calculated from YY4-yne-coupled Western blotting. YY4: 0.02 μM (13–37 nM, 95% confidence interval (CI)); UK-5099: 0.88 μM (0.4–1.8 μM 95% CI). Data represent average values ± SD for two independent experiments. C) Scatter plots showing competition ratios for proteins engaged by the indicated compounds, as measured by blockade of enrichment with the YY4-yne probe. Human T cells were treated with compounds (YY4, EV-93, and UK-5099; 10 μM, 3 h) or DMSO, followed by treatment with YY4-yne (10 μM, 1 h), after which YY4-yne-labeled proteins were enriched by CuAAC conjugation to an azide-biotin tag and analyzed by MS-based proteomics. Data are for one experiment representative of two replicate experiments.