Natural Product Micheliolide (MCL) Irreversibly Activates Pyruvate Kinase M2 and Suppresses Leukemia

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Compound Synthesis

1. General information of synthesis:

Unless otherwise mentioned, all reactions were carried out under a nitrogen atmosphere with dry solvents under anhydrous conditions. The used solvents were purified and dried according to common procedures. Yields refer to chromatographically and spectroscopically (1H NMR) homogeneous materials, unless otherwise stated. Reactions were monitored by thin-layer chromatography carried out on 0.25 mm Tsingdao silica gel plates (60F-254). Visualization was achieved using UV light, phosphomolybdic acid in ethanol or potassium permanganate in water, each followed by heating. Tsingdao silica gel (60, particle size 0.040 – 0.063 mm) was used for flash column chromatography. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br. = broad, m = multiplet), coupling constants and integration.

2. Synthesis and information of micheliolide (MCL), S-MCL, DMAMCL, biotion-S-MCL and biotin-MCL:

MCL were prepared in high yield using our modified method. S-MCL, DMAMCL were prepared according to our previous report.

Synthesis of compound 6. 5-Hexynoic Acid (1.12 g, 10 mmol) was dissolved in anhydrous CH₂Cl₂ (20 mL), and oxalyl chloride (3.7 mL, 60 mmol) were added to the solution. The reaction mixture was stirred under reflux for 1.5 h, cooled to room temperature, concentrated on a rotary evaporator to remove the solvent and residual oxalyl chloride. The acid chloride 5 thus obtained was then re-dissolved in anhydrous CH₂Cl₂ (30 mL), pyridine (2.6 mL, 32 mmol) was added to the solution, cooled to 0°C, micheliolide (2.8 g, 11.6 mmol) was added to the solution followed by 4-dimethylaminopyridine (19 mg, 0.16 mmol) and stirred at room temperature overnight. Washed successively with H₂O, 10% K₂CO₃, saturated citric acid, H₂O, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure to give a crude residue, which was purified by silica gel column chromatography to afford the product 6 as white solid (2.2 g) in 64% yield. ¹H NMR (400 MHz, CDCl₃): δ6.19 (d, J = 3.3 Hz, 1H), 5.46 (d, J = 3.1 Hz, 1H), 3.78 (t, J = 10.1 Hz, 1H), 3.12 (d, J = 10.1 Hz, 1H), 2.73-2.63 (m, 1H), 2.50-2.40 (m, 4H), 2.30-2.23 (m, 4H), 2.13-2.05 (m, 1H), 1.99-1.90 (m, 2H), 1.90-1.79 (m, 2H), 1.71 (s, 3H), 1.57 (s, 3H), 1.40-1.23 (m, 2H); ¹³C NMR (100MHz, CDCl₃):
δ172.4, 170.3, 139.4, 131.7, 130.2, 118.8, 88.8, 83.6, 83.0, 69.0, 56.7, 50.1, 36.5, 34.9, 34.1, 30.4, 25.9, 24.2, 23.7, 18.8, 17.8.

Synthesis of Biotin-MCL (3). The known compound biotin-azide 7 (110 mg, 0.25 mmol) and the alkyne 6 (126 mg, 0.37 mmol) were dissolved in tert-butanol (5 mL) and the solution was slowly diluted with water (2.5 mL). After addition of aqueous CuSO₄ solution (0.05 mmol, 1 M) and freshly prepared aqueous sodium ascorbate solution (0.05 mmol, 1 M), the reaction mixture was stirred for 36 h at room temperature. Then water was added and the reaction mixture was extracted thrice with ethyl acetate. The combined organic layers were washed with water (3×), brine and dried over Na₂SO₄. After evaporation of the solvent in vacuum and flash chromatography (0-10% methanol/dichloromethane) the pure product Biotin-MCL (171 mg, 0.22 mmol) was obtained. ¹H NMR (400 MHz, CDCl₃): δ7.58 (s, 1H), 6.94-6.87 (m, 1H), 6.66-6.55 (m, 1H), 6.15 (d, J = 3.3 Hz, 1H), 5.75-5.64 (m, 1H), 5.45 (d, J = 3.0 Hz, 1H), 4.53-4.42 (m, 1H), 4.32-4.23 (m, 3H), 3.86 (t, J = 5.2 Hz, 2H), 3.77 (t, J = 10.1 Hz, 1H), 3.63-3.46 (m, 10H), 3.43-3.35 (m, 2H), 3.14-3.05 (m, 2H), 2.90-2.82 (m, 1H), 2.78-2.59 (m, 4H), 2.50-2.37 (m, 3H), 2.36-2.28 (m, 2H), 2.28-2.15 (m, 5H), 2.10-1.83 (m, 4H), 1.77-1.55 (m, 6H), 1.51 (s, 3H), 1.46-1.19 (m, 3H); ¹³C NMR (100MHz, CDCl₃): δ173.4, 172.6, 170.3, 164.1, 147.2, 139.4, 131.7, 130.1, 122.3, 118.8, 88.7, 83.1, 70.5, 70.4, 70.1, 69.9, 69.6, 61.8, 60.2, 56.7, 55.6, 50.0, 50.0, 40.5, 39.1, 36.5, 35.9, 34.9, 34.8, 30.5, 28.2, 28.1, 25.9, 25.6, 24.9, 24.7, 24.2, 18.8; HRMS (ESI-TOF) caleld for C₃₉H₅₉N₅O₉S [M + H⁺] 787.4059; found 787.4049.

Synthesis of Biotin-S-MCL (4). Biotin-S-MCL was prepared following the same procedure as S-MCL. HRMS (ESI-TOF) caleld for C₃₀H₅₀N₆O₉NaS [M + Na⁺] 811.4035; found 811.4039.

**MTT assay for DMAMCL**

Cells were seeded into 96 well plates and 24 hours after which, different concentrations of DMAMCL solution were added into corresponding wells. The cells were incubated with this compound for 72 hours. And for adherent cells, 20 µL 5 mg/mL MTT was added into each well and the culture medium was abandoned following 4 hours of incubation. 150 µL of DMSO was added and the plate was vibrated for 10 mins. The OD value of each well was measured by a microplate reader at 490
nm and the IC\textsubscript{50} value and IC\textsubscript{90} value were calculated. For suspension cells, a centrifugation was needed before the remove of the medium.

**Pull-down of biotin-MCL bound proteins**

HL-60 cells were harvested and lysed in RIPA buffer supplemented with protease inhibitors (Calbiochem, Darmstadt, Germany) with brief sonication. After centrifugation at 14,000 g for 10 minutes, the supernatant (1.5 mg/mL) was collected and equally divided into four parts, two of them were pre-incubated with unlabeled MCL (200 µM or 400 µM), then incubated with 20 µM of biotin-MCL(3). The other two samples were either incubated with 20 µM of biotin-MCL (3) or biotin-S-MCL (4) in RIPA buffer overnight at 4 °C, respectively. After incubation, Prewashed 20 µL streptavidin beads were added to each samples and incubated for another 4 hours at 4 °C. Then the streptavidin beads were washed three times with RIPA buffer, and the bead-bound proteins were eluted, separated by SDS-PAGE, and visualized by silver staining.

**Molecular modeling**

The initial apo tetrameric structure was obtained from the Protein Data Bank X-ray structure (PDB ID: 1ZJH). The initial FBP bound tetrameric structure was retrieved from the PDB ID code 1T5A\textsuperscript{5}. The missing N-terminal helical region (residues 13-14) in 1ZJH was modeled with Modeler 9.15\textsuperscript{6} using 1T5A\textsuperscript{5} as a template. The missing mono- and divalent ions (Mg\textsuperscript{2+} and K\textsuperscript{+}) in 1ZJH that are required for PKM2 activity were placed by aligning 1T5A onto 1ZJH. The crystal structure of PKM2 in complex with FBP (PDB ID: 1T5A) was used to model MCL-bound structure. To model MCL-bound PKM2, MCL was covalently docked to cysteine 424 in the tetrameric 1T5A using GOLD 5.1\textsuperscript{7,8}. GOLD defines a “link atom” both in the ligand and in the protein and forces the ligand link atom to occupy the same steric volume as the protein link atom to mimic the covalent binding event. The best scoring conformation was selected. Each monomer had one MCL bound. A total of two systems were created for further molecular dynamic (MD) simulations to properly evaluate the impact of MCL on PKM2. They are referred to as PKM2 (apo) and PKM2•MCL•FBP (MCL bound tetramer).

All energy minimizations and molecular dynamics simulations were performed with AMBER 14\textsuperscript{9}. The force fields used were ff14SB for the protein\textsuperscript{10} and gaff parameters for the ligands (MCL)\textsuperscript{11}. Hydrogen atoms were added to the models with the tLeap module of AMBER 14. Each system was
solvated with water molecules, with a minimum distance of 10.0 Å from the surface atoms of the complex to the edge of the periodic simulation box. Counter ions were added to neutralize the net charge of the complex. First, the water atoms and counter ions were minimized for 15000 steps of steepest descent and 10000 steps of conjugate gradient while restraining all other atoms with a force constant of 500 kcal/mol Å$^2$. Finally, the whole systems were successively allowed to move. The particle mesh Ewald (PME) method was used to treat long-range electrostatic interactions, with a cubic B-spline interpolation. The cutoff for nonbonded interactions was 10 Å. SHAKE was used to constrain all bonds involving hydrogen atoms (All bonds involving hydrogen atoms were constrained using the LINCS algorithm). The time steps were 1 fs during the equilibration. The temperature of the simulated systems was then gradually increased to 300 K over 50 ps in the NVT ensemble with the Langevin thermostat. After the system was heated to 300 K, equilibrating calculation was executed at 1 atm and at 300 K using the NPT ensemble for a total of 1.0 ns. Subsequently, 100 ns production runs for each system were carried out in the isothermal isobaric ensemble ($p = 1$ atm and $T = 300$ K). We utilized hydrogen mass repartitioning (HMR) as a method to increase the time step of the MD simulation by a factor of 2 during the production runs. NMR restraints were applied between Mg$^{2+}$ and K$^+$ and their coordinating ligands by maintaining the metal geometry in 1T5A. Data were analyzed using the CPPTRAJ code in AMBER14 and TCL scripts in VMD.

**Binding energy calculation**

To calculate binding energies for dimer (CC’), tetramer (AA’) interfaces ligand with PKM2, we used the molecular mechanics combined with the generalized Born and surface area continuum solvation (MM/GBSA) method. The MM/GBSA method combines molecular mechanics, generalized Born electrostatics for polar solvation free energy, nonpolar solvation energy based on solvent-accessible surface area, and normal-mode analyses for entropy to calculate the binding free energy for the protein complexes. For the purposes of MM/GBSA analysis, we sampled frames at 40 ps intervals from the last 40 ns of the MD trajectories (the first 60 ns were discarded as equilibration). In total, 1000 frames were used for averaging. MM/GBSA energy decomposition was further carried out to pinpoint residues (1D decomposition) contributing significantly toward the PKM2 AA’ and CC’ interface binding energy.
Western blot

Proteins were separated by SDS-PAGE before being transferred to PVDF membrane (Bio-Rad) using standard Western blotting procedures. All Western blots were blocked in TBST (0.1% Tween-20, 150 mM NaCl, 10 mM Tris, pH 8.0) containing 3% BSA for 2 h at RT. The blots were then incubated with the appropriate primary antibody: anti-PKM2 (Cell Signaling, 4053, dilution 1:1,000); anti-PKM1/PKM2 (Cell signaling technology, 3190, dilution 1:1,000); anti-biotin (Streptavidin-HRP antibody, Abcam, ab7403, dilution 1:15,000) in blocking buffer at 4 °C overnight. The blots were then washed three times in TBST for 10 min and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h in blocking buffer at RT. HRP-conjugated anti-rabbit (Abcam, ab6721) were used at 1:5,000 dilution. After being washed three more times with TBST for 10 min, the blots were developed using ECL reagents (Bio-Rad) and the ChemiDoc XRS+ molecular imager (Bio-Rad).

Immunofluorescence analysis

HL60 cells were fixed and incubated with anti-PKM2 (Cell Signaling, 4053), Alexa Fluor 647 conjugated goat anti-rabbit IgG (red), and Hoechst 33342 (blue) according to standard protocols. Cells were examined using a deconvolution fluorescent microscope (Zeiss, Thornwood, NY) with a 63- oil immersion objective. Axio Vision software from Zeiss was used to deconvolute Z-series images.

Preparation of recombinant proteins

The expression of PKM1 and PKM2 from *E. coli* were carried out as previously described. Plasmids pET28a-PKM1, pET28a-PKM2 and mutant pET28a-C424S-PKM2 were transformed to *E. coli* strain BL21. BL21 strains containing corresponding plasmids were cultured containing 100 µg/mL kanamycin at 37°C to an absorbance of 0.5-0.8 at 600 nm, and induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 18°C for 15-20 hours before being harvested by centrifugation. The cell pellets were suspended in lysis buffer (10 mM HEPES, pH 7.5; 300 mM KCl; 5 mM imidazole, 5 mM MgCl₂; 5% glycerol; 2.5 mM TCEP) and disrupted by sonication. After centrifugation, the supernatant was applied to a Ni-beads column (QIAGEN, Valencia, CA, USA) and washed 10 times with wash buffer (10 mM HEPES, pH 7.5; 300 mM KCl; 40 mM imidazole, 5 mM
MgCl$_2$; 5% glycerol; 2.5 mM TCEP). Proteins were eluted with elution buffer (10 mM HEPES, pH 7.5; 300 mM KCl; 300 mM imidazole, 5 mM MgCl$_2$; 5% glycerol; 2.5 mM TCEP). The fractions eluted from Ni-NTA column were pooled, concentrated, and loaded onto a Superdex 200 10/300 GL column (GE, 17-5175-01) pre-equilibrated with 10 mM HEPES, pH 7.5; 100 mM KCl; 5 mM MgCl$_2$; 5% glycerol; 2.5 mM TCEP. The fractions containing target proteins were collected, concentrated to 20 mg/mL using Amicon Ultra-15 centrifugal filter device (Millipore), and stored at -80°C until use.

**Gel filtration of cell fraction**

HL60 Cells were treated with DMSO, MCL (5 µM), pervanadate, and MCL combined pervanadate and then extracted in a lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.3% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 30 min and centrifuged (30 min at 13,000 rpm) to remove cell debris. The gel filtration column Hi Prep 16/60 Sephacryl S200 HR column (GE) was washed and equilibrated by cold PBS. Extracts were passed over the gel filtration column. The speed rate of flow is 0.5 ml/min. Fractions were collected every 0.3 ml per tube and analyzed by western blot. Molecular mass was determined by Gel Filtration Calibration Kit HMW (GE Healthcare).

**Recombinant PKM2 activity assay**

The activity of recombinant PKM2 (0.1 mg/mL) was determined by a lactate dehydrogenase coupled assay. The change in absorbance at 340 nm resulting from the oxidation of NADH was measured via a pyruvate kinase assay kit (Sigma, MAK072-1KT), according to the manufacturer's instructions. One unit of catalytic activity induces the oxidation of 1 µmol NADH per minute at 37°C at pH 7.6. For activator screen, compounds were pre-incubated with PKM2 enzyme in reaction buffer at 4°C for 30 minutes. According to the pyruvate standard curve, we calculated the normalized PKM2 activity.

**Protein identification by LC/MS/MS**

Pyruvate kinase immunoprecipitates were separated by SDS-PAGE, and the band corresponding to pyruvate kinase by molecular weight was excised, subjected to in-gel trypsin digestion and then analyzed by reversed-phase microcapillary LC/MS/MS analysis. MS/MS spectra were searched against the concatenated target and decoy (reversed) Swiss-Prot protein database using Sequest (Proteomics
Browser Software, Thermo Fisher Scientific). Peptides passing a false discovery rate threshold of 1% were accepted.

**Kinetic determination of PKM2-MCL interaction**

The specific interaction between PKM2 and biotin-MCL (3) follows the typical covalent binding scheme\(^{20-21}\), where PKM2 forms initial encounter complex PKM2: biotin-MCL and then form the irreversible covalent linked complex PKM2: biotin-MCL.

Two-step model of covalent activation:

\[
\begin{align*}
\text{PKM2} + \text{MCL} & \xrightarrow{k_{\text{on}}} \text{PKM2-MCL} \\
& \xrightarrow{k_{\text{act}}} \text{PKM2-MCL}
\end{align*}
\]

\(K_i = k_{\text{off}} / k_{\text{on}}\)

**step 1:** non-covalent binding  \hspace{1cm} **step 2:** covalent bond formation

To determine the values of \(k_{\text{act}}\), the rate constant for PKM2: biotin-MCL formation at saturating [biotin-MCL], and \(K_i\), the apparent dissociation constant for the initial PKM2: biotin-MCL complex, PKM2 was incubated with large excess of biotin-MCL for different time periods and the covalent linked PKM2: biotin-MCL complexes were analysed by SDS-gel and subsequent western blot using streptavidin-HRP. The protein bands on the gels were quantified by densitometry. Scanning was performed at optimal exposure time where band intensity was proportional to the concentration of protein present. Gel photographic images were stored as GRAYSCALE pictures in the TIFF format and were processed using Quantity One software. Under the pseudo-first order experimental conditions, the reaction follows as the equations:

\[
[\text{PKM2}] = [\text{PKM2}]_0 * e^{-k_{\text{obs}} * t} \quad (1)
\]

\[
[\text{PKM2-} \text{biotin-MCL}] = [\text{PKM2}]_0 - [\text{PKM2}]_t = [\text{PKM2}]_0 *(1-e^{-k_{\text{obs}} * t}) \quad (2)
\]

\[
k_{\text{obs}} = k_{\text{act}} * \text{[biotin-MCL]} / ([\text{biotin-MCL]}+K_i) \quad (3)
\]

The reaction rate constants of \(k_{\text{obs}}\) at different biotin-MCL concentration were obtained by fitting to equation (1) and (2), where \([\text{PKM2}]_0\) is the total concentration of PKM2 added to the solution and \([\text{PKM2}]_t\), PKM2 concentration at time t. The value of \(k_{\text{act}}\) and \(K_i\) were determined by fitting \(k_{\text{obs}}\) values to equation (3).

**Anti-cancer evaluation in HL60 cells xenografted zebrafish**
Xenograft zebrafish model and anti-cancer drug screening carried out as previously described. The details were as follows. The standard mating embryos of zebrafish were used for cell xenoplantation at 2 days after post fertilization (2dpf). HL60 cells were detached from culture dishes using 0.05% trypsin-EDTA and washed twice with PBS were stained with 2 mg/mL CM-Dil diluted in PBS and washed four times (once with FBS, twice with PBS and then once with 10% FBS diluted in PBS). Staining HL60 cells were counted by microscopy, suspended in 10% FBS. Embryos at 2df were developed into zebrafish with yolk sac. About 300 staining HL60 cells were microinjected into the perivitelline cavity of each zebrafish. 30 fishes were used per group.

Injected zebrafishes were transferred to 6-well plates (one embryo/well) 3 mL Holtfreter’s solution with series concentrations of DMAMCL (1, 3, 10, 20, 30, 40, 50 µg/mL), positive drug cytarabine with 200 µg/mL, and saline control as negative control at 35°C. After 4 days incubation, zebrafish were anesthetized with 0.64 mM tricaine and imaged under a confocal laser-scanning microscopy (CLSM; Zeiss LSM-510, Germany) to evaluate the tumor growth inhibition. Representative pictures were also captured using upright microscopy. The fluorescence intensity of tumor cells would be quantified to assess the tumor inhibition effect. Nikon NIS-Elements D 3.10 Advanced image processing software was used to capture and analyze the images.

\[
\text{Inhibition rate of proliferation (\%)} = (1 - \frac{S_{\text{drug}}}{S_{\text{control}}}) \times 100\% \\
(S_{\text{drug}}: \text{quantification of the fluorescent area of drug treated tumor xenograft}; S_{\text{control}}: \text{quantification of the fluorescent area of untreated tumor xenograft})
\]

\[
\text{Inhibition rate of metastasis (\%)} = (1 - \frac{L_{\text{drug}}}{L_{\text{control}}}) \times 100\% \\
(L_{\text{drug}}: \text{quantification of the largest fluorescent length of drug treated tumor xenograft}; L_{\text{control}}: \text{quantification of the largest fluorescent length of untreated tumor xenograft})
\]

Results are presented as mean ± SD of triplicates from at least 3 independently performed experiments. The student’s t- test was used for comparison between experimental groups. A value of \( p < 0.05 \) was considered to be statistically significant.
Supplementary Figures & Tables

Scheme S1. Synthesis of Biotin-MCL (3) and Biotin-S-MCL (4). 3 was obtained by acylation of MCL with acyl chloride 5 and a following click reaction with azide 7. Reduction of 3 with NaBH₄ afforded 4.
Figure S1. Concentration dependence of the DMAMCL-mediated inhibition of cancer cell lines. a) HL-60 (1.25–50 µM), b) HL-60/ADR (1.25–50 µM), c) K562 (1.25–50 µM), d) K562/ADR (1.25–50 µM), e) K562/Gi (1.25–50 µM), f) KG-1a (1.25–50 µM), g) SW1990 0.47–122 µM), h) SH-SY5Y (0.47–122 µM). The inhibition was determined for 48 h at 37°C. Representative data from 3 independent experiments.
Figure S2. Identified amino acids and peptides of PKM2. The resultant band in experiment for Fig.2b was excised and de-stained, subjected to in-gel tryptic digestion and analysis by MS, wherein the identified amino acids and peptides are shown in red characters.
Figure S3. Concentration dependence of the MCL-mediated activation of PKM2. Recombinant PKM2 was incubated with a series of MCL (0.001-10 µM) at 4°C for 30 min and then performed activity determination. Representative data from three independent experiments are shown as Eadie-Hofstee plots, and each point represents the mean ± S.D. (n = 3).
Figure S4. a) The chromatography profile of different concentration of the rPKM2; b) The chromatography profile of the effect of MCL on the recombinant PKM2; c) SDS-PAGE detection of the fraction of b)
**Figure S5.** Western blot detection of nucleic and cytoplasmic PKM2 of cells incubated with DMSO and MCL (5 µM). The blots were developed with anti-PKM2.
Figure S6. The structure of the PKM2 tetramer (PDB ID: 1ZJH and 1T5A). a) The cartoon representation of the PKM2 tetramer. The bound MCL and effector FBP are indicated as magenta and orange spheres. The four PKM2 monomers are represented in cartoon mode with different colors. The up left chain is colored according to different domains: domain A (grey), domain B (purple), domain C (red) and N-terminal (cyan). The large (AA’) and small (CC’) interfaces between monomers are shown as dashed lines. b, c) Surface representation of one PKM2 monomer. The secondary elements forming the tetramer and dimer interfaces are labeled. d) Binding energy contributions from individual residues at the AA’ interfaces. e) Binding energy contributions from individual residues at the CC’ interfaces. Per residue binding energies computed from the 1D MM/GBSA decomposition. Residues are colored according to the value of the binding energy in kcal/mol from red (positive) to blue (negative). The bound MCL are shown as magenta spheres. The large (AA’) and small (CC’) interfaces between monomers are presented as dashed lines. Only one monomer is shown.
Table S1. Proliferation inhibition rate of human leukemia cell-line HL60 transplantation tumor model in xenograft zebrafish

| Group               | Fluorescence area (Mean ± SD) | Proliferation inhibition (%) |
|---------------------|-------------------------------|------------------------------|
| Untreated           | 217948 ± 79974                | -                            |
| Cytarabine          | 123624 ± 50982**              | 43.28**                      |
| (200 µg/mL)         |                               |                              |
| DMAMCL (1 µg/mL)    | 154220 ± 36617*               | 29.24*                       |
| DMAMCL (3 µg/mL)    | 113719 ± 31331**              | 47.82**                      |
| DMAMCL (10 µg/mL)   | 94949 ± 57885**               | 56.44**                      |

Table S2. Migration inhibition rate of human leukemia cell-line HL60 transplantation tumor model in xenograft zebrafish

| Group               | Fluorescence area (Mean ± SD) | Proliferation inhibition (%) |
|---------------------|-------------------------------|------------------------------|
| Untreated           | 320 ± 136                     | -                            |
| Cytarabine          | 179 ± 21**                    | 44.04**                      |
| (200 µg/mL)         |                               |                              |
| DMAMCL (1 µg/mL)    | 197 ± 24*                     | 38.51*                       |
| DMAMCL (3 µg/mL)    | 173 ± 51**                    | 46.08**                      |
| DMAMCL (10 µg/mL)   | 134 ± 38**                    | 58.10**                      |
NMR spectra of compounds

Compound 6 $^1$H NMR (400 MHz, CDCl$_3$):
Compound 6 $^{13}$C NMR (100MHz, CDCl$_3$):
Biotin-MCL $^1$H NMR (400 MHz, CDCl$_3$):

Biotin-MCL $^{13}$C NMR (100MHz, CDCl$_3$):
REFERENCES

1. Morgan, H. P.; Zhong, W. H.; McNae, I. W.; Michels, P. A. M.; Fothergill-Gilmore, L. A.; Walkinshaw, M. D. Structures of pyruvate kinases display evolutionarily divergent allosteric strategies. *Royal Society Open Science* 2014, 1,140120.

2. Zhai, J.-D.; Li, D.; Long, J.; Zhang, H.-L.; Lin, J.-P.; Qiu, C.-J.; Zhang, Q.; Chen, Y. Biomimetic semisynthesis of arglabin from parthenolide. *The Journal of organic chemistry* 2012, 77, 7103-7107.

3. Zhang, Q.; Lu, Y.; Ding, Y.; Zhai, J.; Ji, Q.; Ma, W.; Yang, M.; Fan, H.; Long, J.; Tong, Z.; Shi, Y.; Han, B.; Zhang, W.; Qiu, C.; Ma, X.; Li, Q.; Shi, Q.; Zhang, H.; Li, D.; Zhang, J.; Lin, J.; Li, L. Y.; Gao, Y.; Chen, Y. Guaianolide sesquiterpene lactones, a source to discover agents that selectively inhibit acute myelogenous leukemia stem and progenitor cells. *J. Med. Chem.* 2012, 55, 8757-8769.

4. Yang, P. Y.; Liu, K.; Ngai, M. H.; Lear, M. J.; Wenk, M. R.; Yao, S. Q. Activity-based proteome profiling of potential cellular targets of Orlistat—an FDA-approved drug with anti-tumor activities. *J. Am. Chem. Soc.* 2010, 132, 656-656.

5. Dombrauckas, J. D.; Santarsiero, B. D.; Mesecar, A. D. Structural basis for tumor pyruvate kinase M2 allosteric regulation and catalysis. *Biochemistry* 2005, 44, 9417-9429.

6. Marti-Renom, M. A.; Stuart, A. C.; Fiser, A.; Sanchez, R.; Melo, F.; Sali, A. Comparative protein structure modeling of genes and genomes. *Annual Review of Biophysics and Biomolecular Structure* 2000, 29, 291-325.

7. Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. Development and validation of a genetic algorithm for flexible docking. *Journal of Molecular Biology* 1997, 267, 727-748.

8. Hartshorn, M. J.; Verdonk, M. L.; Chessari, G.; Brewerton, S. C.; Mooij, W. T. M.; Mortenson, P. N.; Murray, C. W. Diverse, high-quality test set for the validation of protein-ligand docking performance. *Journal of Medicinal Chemistry* 2007, 50, 726-741.

9. DA Case, V. B., Josh Berryman, RM Betz, Q Cai, DS Cerutti, TE Cheatham Iii, TA Darden, RE Duke, H Gohlke, AW Goetz, S Gussarov, N Homeyer, P Janowski, J Kaus, I Kolossvary, A Kovalenko, Tai-Sung Lee, S LeGrand, T Luchko, R Luo, B Madej, KM Merz, F Paesani, DR Roe, A Roitberg, C Sagui, R Salomon-Ferrer, G Seabra, CL Simmerling, W Smith, J Swails, RC Walker, J Wang, RM Wolf, X Wu, PA Kollman. University of California. 2014.

10. Maier, J. A.; Martinez, C.; Kasavajhala, K.; Wickstrom, L.; Hauser, K. E.; Simmerling, C. ff14SB: Improving the accuracy of protein side chain and backbone parameters from ff99SB. *Journal of Chemical Theory and Computation* 2015, 11, 3696-3713.

11. Wang, J. M.; Wang, W.; Kollman, P. A. Antechamber: An accessory software package for molecular mechanical calculations. *Abstracts of Papers of the American Chemical Society* 2001, 222, U403-U403.

12. Jorgensen, W. L.; Jenson, C. Temperature dependence of TIP3P, SPC, and TIP4P water from NPT Monte Carlo simulations: seeking temperatures of maximum density. *Journal of Computational Chemistry* 1998, 19, 1179-1186.
13. Darden, T.; York, D.; Pedersen, L. Particle Mesh Ewald - an N.Log(N) Method for Ewald Sums in large systems. *Journal of Chemical Physics* **1993**, *98*, 10089-10092.

14. Loncharich, R. J.; Brooks, B. R.; Pastor, R. W. Langevin Dynamics of Peptides - the Frictional Dependence of Isomerization Rates of N-Acetyltalanyl-N'-Methylamide. *Biopolymers* **1992**, *32*, 523-535.

15. Hopkins, C. W.; Le Grand, S.; Walker, R. C.; Roitberg, A. E. Long-time-step molecular dynamics through hydrogen mass repartitioning. *Journal of Chemical Theory and Computation* **2015**, *11*, 1864-1874.

16. Humphrey, W.; Dalke, A.; Schulten, K. VMD: Visual molecular dynamics. *Journal of Molecular Graphics & Modelling* **1996**, *14*, 33-38.

17. Miller, B. R.; McGee, T. D.; Swails, J. M.; Homeyer, N.; Gohlke, H.; Roitberg, A. E. MMPBSA.py: An efficient program for end-state free energy calculations. *Journal of Chemical Theory and Computation* **2012**, *8*, 3314-3321.

18. Gohlke, H.; Kiel, C.; Case, D. A. Insights into protein-protein binding by binding free energy calculation and free energy decomposition for the Ras-Raf and Ras-RalGDS complexes. *Journal of Molecular Biology* **2003**, *330*, 891-913.

19. Anastasiou, D.; Yu, Y.; Israelsen, W. J.; Jiang, J. K.; Boxer, M. B.; Hong, B. S.; Tempel, W.; Dimov, S.; Shen, M.; Jha, A.; Yang, H.; Mattaini, K. R.; Metallo, C. M.; Fiske, B. P.; Courtney, K. D.; Malstrom, S.; Khan, T. M.; Kung, C.; Skoumbourdis, A. P.; Veith, H.; Southall, N.; Walsh, M. J.; Brimacombe, K. R.; Leister, W.; Lunt, S. Y.; Johnson, Z. R.; Yen, K. E.; Kunii, K.; Davidson, S. M.; Christofk, H. R.; Austin, C. P.; Inglese, J.; Harris, M. H.; Asara, J. M.; Stephanopoulos, G.; Salituro, F. G.; Jin, S.; Dang, L.; Auld, D. S.; Park, H. W.; Cantley, L. C.; Thomas, C. J.; Vander Heiden, M. G. Pyruvate kinase M2 activators promote tetramer formation and suppress tumorigenesis. *Nat Chem Biol* **2012**, *8*, 839-847.

20. Warner, D. R.; Hoffman, J. L. Suicide inactivation of thioether S-methyltransferase by ethyl sulfide. *Biochemistry* **1996**, *35*, 4480-4484.

21. Lopez-Garcia, M. P.; Dansette, P. M.; Mansuy, D. Thiophene derivatives as new mechanism-based inhibitors of cytochromes P-450: inactivation of yeast-expressed human liver cytochrome P-450 2C9 by tienilic acid. *Biochemistry* **1994**, *33*, 166-175.