A Quantitative Chemical Proteomics Approach to Profile the Specific Cellular Targets of Andrographolide, a Promising Anticancer Agent that Suppresses Tumor Metastasis

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Drug target identification in live cells.

Abbreviations:

Activity based protein profiling- ABPP, Andrographolide- Andro; Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine- TBTA; Tris(2-carboxyethyl) phosphine- TCEP; dithiothreitol- DTT, SCX- Strong cation exchange; IPA- Ingenuity Pathway Analysis; FDR- False discovery rate

Summary:

Drug target identification is a critical step towards the understanding of the mechanism of action of a drug, which will help to improve the current therapeutic regime and to expand the drug’s therapeutic potential. However, current in vitro affinity chromatography-based and in vivo activity-based protein profiling (ABPP) approaches generally face difficulties discriminating specific drug targets from non-specific ones. Here we describe a novel approach combining isobaric tag for relative and absolute quantitation (iTRAQ) with Clickable ABPP, named ICABPP, to specifically and comprehensively identify the protein targets of andrographolide (Andro), a natural product with known anti-inflammatory and anti-cancer effects, in live cancer cells. We identified a spectrum of specific targets of Andro, which furthered our understanding of the mechanism of action of the drug. We found that Andro has a potential novel application as the tumor metastasis inhibitor, which was validated through cell migration and invasion assays. Moreover, we have unveiled the target binding mechanism of Andro with a combination of drug analogue synthesis, protein engineering and mass spectrometry-based approaches and determined the drug-binding sites of two protein targets, NF-κB and actin.
Introduction:

As most drugs exert pharmacological effects by interacting with their target proteins, identification of which is a critical step in unravelling the mechanisms of drug action. It is also imperative for our understanding of the pharmacodynamics of a known drug, suggesting the potentially unrevealed actions and thus refining its future clinical applications. Traditional approaches to identify protein targets of a drug typically utilize immobilized drug affinity chromatography coupled with mass spectrometry (MS). These methods can only be applied to cell lysates but not an in vivo setting, due to the requirement of a solid support. Thus, the in vitro target profiling may not accurately reflect the drug’s actions in the physiological environment in vivo. To overcome this limitation, several groups have used the activity-based protein profiling (ABPP) combined with bio-orthogonal click chemistry to identify drug targets both in vitro and in vivo (Fig. S1 in Supporting Information). ABPP probes exert their functions by a covalent reaction with the target proteins or photoaffinity-based labeling via incorporation of photoreactive groups. With the increasing sensitivity of modern MS platform, low abundance protein targets can be successfully identified. Although both the conventional affinity chromatography and recent ABPP-based methods allow us to detect a set of candidate protein targets for a drug, it remains difficult to discriminate specific interactions from non-specific ones. Hence, more time and effort are needed for subsequent validation due to the presence of a large number of non-specific binders. Therefore, there is an urgent need to develop comprehensive unbiased methods for specific target identification. Quantitative proteomics has been used to profile enriched kinases using cell lysate-based kinobead pull-down. However, these types of experiments are mainly suitable for studying kinase inhibitors. Recently, SILAC-based proteomics methods have been applied to determine the specific binders of small molecules or proteins with cer-
tain post-translational modifications. These studies shed light on how quantitative proteomics can improve the specificity of the target protein identification. Nevertheless, due to the inherent limitation of SILAC, such an approach takes a long time for complete incorporation of isotopic amino acids. Furthermore, it is also extremely difficult to apply the SILAC approach to tissue and body fluid samples, which are of particular relevance to biomedical research.

Here we introduce an isobaric tag for relative and absolute quantitation (iTRAQ)-basedClickable ABPP (ICABPP) approach for unbiased, specific and comprehensive identification of target proteins in live cells. iTRAQ is a stable-isotope labeling approach for multiplexed quantitative proteome profiling. An overview of the technique is illustrated in Fig. 1a. In this assay, cells are first incubated with a clickable probe or with DMSO, which serves as a negative control. After the probe permeates the cell and covalently binds to its dedicated in situ targets, the washed cells are lysed, clicked with biotin-N₃ tag and enriched through avidin pull-down in parallel. The beads are washed thoroughly and the bond proteins are directly digested on beads with trypsin. The resulting peptides are labeled with respective iTRAQ reagents, and pooled together for further identification and quantification by LC-MS/MS. This technique enabled us to discriminate specific protein targets from non-specific and endogenously biotinylated proteins. Biological replicates of probe- or DMSO-treated samples are included to overcome experimental variations. As shown in Fig. 1b, non-specific binding proteins’ iTRAQ reporters have equal or similar intensities, whereas specific target proteins enriched by the probe show highly differential intensities compared to DMSO-treated control samples (as illustrated by the significantly higher reporter intensities of 116 and 117 vs. 113 and 114 shown in Fig. 1b). The multiplexing nature of iTRAQ-based chemical proteomics method allows replicated enrichments to be compared within
a single LC-MS/MS analysis, hence increasing the accuracy of identifying specific targets and minimizing experimental errors.

In this context, ICABPP approach was applied to identify protein targets of andrographolide (Andro, Fig. 2), a natural product with known anti-inflammation and anti-cancer effects, (21–25) in live cancer cells. A spectrum of 75 potential Andro targets was identified with high confidence, which suggested that Andro may exert anti-cancer effects by acting on multiple targets to interfere with several cellular signaling pathways. Two targets, NF-κB and β-actin were validated by \textit{in vitro} binding assay and direct binding site mapping. Furthermore, our data revealed a novel mechanism of Andro in suppressing tumor metastasis.
Experimental Procedures:

Synthesis of the Andro-based analogue and probes:

The detailed materials and methods of the synthesis of the Andro-based analogue and probes were provided in supporting information.

Materials and Reagents:

Andrographolide (98%), Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine(TBTA), Tris(2-carboxyethyl)phosphine(TCEP), streptavidin beads, acetonitrile (ACN), Trifluoroacetic acid (TFA), urea, dithiothreitol (DTT), phosphoric acid, and iodoacetamide (IAA) were purchased from Sigma-Aldrich. Antibody against NF-κB p50 (SC-7178) was from Santa Cruz Biotechnology, Inc. Antibody against actin (1:4000) was from BD Transduction Laboratories. Migration assay and matrix gel invasion assay chamber were purchased from BD Biosciences, San Jose, CA. Cy3-Azide (CL K-CCA-9294-1) and biotin-Azide were from Jena Bioscience, Inc. β-actin from human platelet (>99% pure) was obtained from Cytoskeleton, Inc. Sequencing grade trypsin was obtained from Promega. Chymotrypsin, protease and phosphatase inhibitors cocktail were purchased from Roche. Ultrapure water used for all experiments was purified with an ELGA water system. Unless otherwise indicated, all the other reagents used for the biochemical methods were purchased from Sigma-Aldrich.

Cell Culture:

HCT116, HepG2, HeLa and MV4-11 were purchased from ATCC (Manassas, VA). HCT116 was maintained in modified McCoy’s 5A medium with L-glutamine (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1x anti-
otic/antimycotic (Invitrogen, Carlsbad, CA) at 37°C in 5% (v/v) CO₂. HepG2 and HeLa cell lines were cultured in Dulbecco’s Modified Eagle Medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1x antibiotic/antimycotic (Invitrogen, Carlsbad, CA). MV4-11 cell line was cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1x antibiotic/antimycotic. Cells were maintained at 37 °C in a humidified incubator supplemented with 5% CO₂.

**Inhibition of Cancer Cell Proliferation:**

HCT116, HeLa and HepG2 Cell Lines: 20,000 cells was seeded into 96 well plate and allowed to attach for 24 hrs. Cells were then treated with Andro at various concentrations for 48hrs. At the end of the treatment, media was removed and the wells were washed once with PBS. The cells were then stained with 0.5% crystal violet in 20% methanol for 10 min. Excess crystal violet was washed off with PBS and the wells were allowed to dry. Solubilization was done using 1% SDS for 30 min and the absorbance measured at 550nm.

MV4-11 Cell Lines: Cells were treated with increasing concentration of Andro for 48 hrs. Following the intended treatment, the cells were centrifuged and resuspended in 2 mL medium. A volume of cell suspension containing 1x10⁵ cells was centrifuged into a pellet. After dislodging the pellet, the samples were added with 10 μL of 50 μg/mL propidium iodide (PI) and subjected to flow cytometric analysis using the Beckman Counter (EPICS-XI MCL).

**In situ Fluorescence Labeling Experiments:**

HCT116 cells were grown to 80-90% confluence in 6-well plates. After the media was removed, cells were washed twice with PBS. P1 or P2 (100 μM) in 2 ml medium with a final DMSO concentration of 1% was added and cells were incubated for 4 hrs at 37 °C and 5% CO₂. Equal vol-
ume of DMSO was used as a negative control. For concentration optimization experiment (Fig. S3 in Supporting Information), increasing concentrations (20-200 μM) of P2 were used to culture the cells for 4 hrs. Subsequently, the medium was removed and cells were washed with PBS and detached with trypsin. The cell pellet was resuspended in PBS, washed, followed by sonication in 150 μl of PBS to lyse cells. The resultant cell lysate was cleared by centrifuging at 13,000 rpm for 30 min. Protein concentrations of the cell lysates were determined using the Bradford assay. Equal amounts (100 μg) of different treatment samples were used for subsequent fluorescent labeling. For each reaction, Cy3-azide (20 μM), Tris(2-carboxyethyl) phosphine (TCEP) (1mM, 100×fresh stock in water), Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA ligand) (100 μM, 100×stock in DMSO), and CuSO4 (1mM, 100×stock in water) were added to the lysate. The samples were incubated at room temperature for 2 hrs. Next, clicked proteins were precipitated by acetone and air dried. 100 μL 1× SDS loading buffer was added to dissolve the sample and 50 μL of sample was separated by SDS gel electrophoresis on 10% polyacrylamide gel. After SDS-PAGE, gels were visualized using a Typhoon 9410 laser scanner (GE, health care) and images were analyzed by TotalLab software.

**Andro Target Identification using ICABPP:**

**Cells Labeling:**

In the subsequent ICABPP study, two biological duplicate of P2 treated and two DMSO treated samples were pulled down and digested in parallel. The two DMSO control samples were labeled with iTRAQ reagent and quantified by iTRAQ ratios. Briefly, HCT116 cells were grown to 80-90% confluence in T175 flasks. Spent medium was then aspirated and the cells washed twice with PBS. P2 (100 μM) in 20 ml medium with a final DMSO concentration of 1% was
added to the cells in the flasks and incubated for 4 hrs in the CO₂ incubator. Culture medium containing 1% DMSO was used as negative control. Subsequently, P2- and DMSO-containing media were removed, and then the cells were washed with PBS and detached with trypsin. The cell pellet was resuspended in PBS, washed and lysed by sonication in PBS. The cell lysates were clarified by centrifugation at 13,000 rpm for 30 min followed by Bradford protein assay. Equal amount (5 mg) of cell lysates (2 Andro probe treated and 2 DMSO treated samples) were used for subsequent click chemistry to conjugate proteins with the biotin tags separately. For each reaction, Biotin-azide (20 μM), Tris(2-carboxyethyl) phosphine(TCEP) (1mM, 100× fresh stock in water), Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine (TBTA ligand) (100 μM, 100×stock in DMSO), and CuSO₄ (1mM,100×stock in water) were added to the cell lysates and incubated at room temperature for 4 hrs. Next, clicked proteins subjected to precipitation with acetone and air dried. Subsequently, the pellet was dissolved in 1 mL PBS and incubated with 50 μL of Streptavidin beads (Sigma-Aldrich) under gentle mixing for 2 hrs at room temperature.

**On-beads Digestion:**

The beads were washed a total of 9 times; thrice with 1% SDS, followed by 3 times with 6M urea and thrice with PBS. The extensively washed beads were resuspended in 25mM ammonium bicarbonate (NH₄HCO₃) and 2 μL tris-(2-carboxyethyl) phosphine (TCEP, 100 mM stock solution) added. The beads were placed in a 65°C heat block for 60 min. Next, 1μL methyl methane-thiosulfonate (MMTS, 200mM stock solution) was added and the samples left in the dark and allowed to react for 15 min at room temperature. Following reduction and alkylation, trypsin (12.5 ng/ μL, Promega) was added and incubated at 37°C overnight. The digested peptides were separated from the beads using a filter-spin column (GE, healthcare). These digested peptides
could be stored at -20 °C for several months pending iTRAQ labeling and mass spectrometry analysis.

iTRAQ Labeling of the Digested Pull-Down Samples:

iTRAQ labeling was performed out using iTRAQ Reagent kit (AB SCIEX, Foster City, CA, USA) based on the vender’s instruction manual with minor modifications. The two biological replicates of the negative control pull-down samples were labeled with iTRAQ reagent 113 and 114, respectively. Similarly, two biological replicate of digested Andro pull-down samples were labeled with reagent 116 and 117, respectively. Briefly, the on-beads digested peptides were dried and reconstituted with equal volume of dissolving buffer (0.5M TEAB). The peptides were then labeled with the respective iTRAQ reagents and incubated at room temperature for 2 hrs before all the samples were pooled together. The iTRAQ workflow is shown in Fig. 1 in the main text.

Strong Cation Exchange (SCX) Chromatography, C18 Desalting of Labeled Samples:

To remove interfering substances like SDS, isopropanol, dissolution buffer (TEAB), reducing agent (TCEP), alkylating agent (MMTS), calcium chloride and excess iTRAQ reagents etc., the pooled iTRAQ-labeled peptides sample was subjected to strong cation exchange chromatography (SCX) using the iTRAQ Method Development Kit (AB SCIEX, Foster City, CA, USA). The bound peptides were eluted with 5 % ammonium hydroxide (NH₄OH) in 30 % methanol. The eluate was desalted using a Sep-Pak C₁₈ cartridge (Waters, Milford, MA), dried and then reconstituted with 100 μL of diluent (98% water, 2% acetonitrile, 0.05% formic acid).

Proteins Identification and Quantification:

Nano LC–ESI-MS:
The detailed methods for LC-MS/MS was described previously. Briefly, separation of the iTRAQ labeled peptides was carried out on an Eksigent nanoLC Ultra and ChiPLC-nanoflex (Eksigent, Dublin, CA) in Trap Elute configuration. A volume of 5 μL of the sample was loaded to the LC system. Peptides were separated by a gradient formed by 2% ACN, 0.1% FA (mobile phase A) and 98% ACN, 0.1% FA (mobile phase B): 5–12% of mobile phase B (20 min), 12–30% of mobile phase B (90 min), 30–90% of mobile phase B (2 min), 90% of mobile phase B (5 min), 90–2% of mobile phase B in 3 min, and 50–5% of mobile phase B in 13 min, at a flow rate of 300 nL/min. The MS analysis was performed on a TripleTOF 5600 system (AB SCIEX, Foster City, CA) in information dependent mode.

**ProteinPilot Analysis:**

The detailed method of ProteinPilot analysis was described previously. Briefly, the protein identification and iTRAQ quantification were performed with ProteinPilot™ 4.5 (AB SCIEX, Foster City, CA) which uses the Paragon™ algorithm to perform database searches. The database used includes the International Protein Index (IPI) v3.87 human protein sequences (total 91,468 entries). The search parameters used were as follows: Cysteine alkylation of MMTS; Trypsin Digestion; TripleTOF 5600; Biological modifications. Redundancy was eliminated by the grouping of identified proteins using the ProGroup algorithm in the software. A decoy database search strategy was used to determine the false discovery rate (FDR) for peptide identification. A corresponding randomized database was generated using the Proteomics System Performance Evaluation Pipeline (PSPEP) feature in the ProteinPilot™ Software 4.5. In this study, a strict total score cut-off >1.3 was adopted as the qualification criterion, which corresponded to a peptide confidence level of 95%. The identification and quantification results were then exported into Microsoft Excel for manual data analysis.
Data Analysis:

To determine the cut-off threshold for the fold change of proteins identified from the iTRAQ study to be considered as significantly regulated, two equal amounts of six-protein mixtures (Applied Biosystems) were trypsin-digested and labeled with the iTRAQ reagents. The standard deviation (S.D) of all the ratios of the labeled peptides was computed to be 0.15. Thus by using a 1 + 2 S.D formula the fold-change cut-off thresholds were set as 1.3 for up-regulated proteins and reciprocally 0.77 for down-regulated proteins. This strategy was adopted for our quantitative study. This cut-off was used to eliminate protein targets where the two biological replicate samples showed significant change (ratio >1.3 or <0.77). Basing on this strategy, 208 proteins were considered to be the statistically reliable hits (Fig. 3d), and the distribution of the enrichment ratios of these proteins were further presented as the colored heat map as illustrated in Fig. 3b. The 4 set ratios of Andro pull-down vs. DMSO pull-down were presented as colored heatmap, using the MultiExperiment Viewer (MeV). Proteins with enrichment ratio closed to 1 are denoted in blue and likely to exhibit non-specific binding. In contrast, proteins labeled in red showed enrichment ratio above 2 or close to 3, suggesting that they are likely the specific binding targets.

To reduce the likelihood of selecting the false positive drug targets, we chose a stringent ratio equivalent to 2 as the cut-off to identify specific protein targets for subsequent experiments. Meanwhile, proteins identified based on a single peptide are considered unreliable and were removed. Using these criteria, 75 proteins were identified and selected (Fig. 3d). The full list of the 75 potential targets is shown in Table S1 in supporting information.
Pathway Analysis of Andro Targets:

The specific Andro targets identified using the ICABPP approach were analyzed using the Ingenuity Pathway Analysis software (Ingenuity® Systems, Redwood city, CA, USA). A spreadsheet containing the list of Andro targets was uploaded into IPA. The software mapped each of the proteins to the repository of information in the Ingenuity Pathways Knowledge base. Molecular networks and canonical pathways regulated by these drugs targets were obtained using IPA core analysis.

Validation of Drug Target using Western Blot:

Andro probe affinity pull-down sample was separated by 1D-SDS PAGE together with DMSO pull-down sample. After SDS-PAGE, the proteins were transferred onto PVDF membranes (BioRad). The blots were blocked with 5% (w/v) BSA in PBS with 0.1% Tween 20 (PBS-T) for 4hrs at room temperature. The membranes were incubated with rabbit anti- NF-κB p50 (1:1500), from Santa Cruz Biotechnology, Inc. as well as mouse anti- β-actin (1:4000) from BD Transduction Laboratories. HRP-conjugated from Pierce Biotechnology, or HRP-conjugated anti-mouse IgG (1:5000) from GE Healthcare were used as secondary antibodies and incubated for 2 hrs at room temperature. The membrane was washed 3 times in PBS-T between each antibody incubation step. Subsequent visualization was performed using ECL substrate (Pierce Biotechnology).

Validation of NF-κB p50 as the Target of Andro:

Expression of Wild Type and Mutated NF-κB p50:

DNA insert of P50 Rel homology domain (aa 39-364), a gift from Prof. G. Ghosh of UCSD, was subcloned into pET-M expression vector. P50-C62A mutant was generated by PCR-based site-directed mutagenesis method. The plasmids were transformed into E. coli BL21 (DE3) cells for
protein expression. A single colony of *E. coli* BL21 (DE3) cells containing the desired plasmid was cultured overnight in 10 ml LB medium containing 100 μg/ml ampicillin. Two milliliters of overnight cultures were inoculated into 1L LB broth culture medium containing 100 μg/ml ampicillin. The culture was grown at 37°C until OD₆₀₀ of 0.6 was reached. Protein expression was induced with 0.35 mM of IPTG at 37 °C for overnight. The cell suspension was harvested and resuspended in Ni binding buffer containing 20 mM Tris-Cl pH 8.0, 0.5 M NaCl, 5 mM Imidazole. The cell suspension was lysed by sonication on ice and subsequently centrifuged at 18,000x g for 30 min. The supernatant of the cell lysate was loaded on the Ni column and the column was washed 10 times with a total volume of 500 ml Ni washing buffer containing 20 mM Tris-Cl pH 8.0, 0.5 M NaCl, 30 mM imidazole to remove unbound proteins. Recombinant p50 with Histidine tag was eluted with 20 mM Tris-Cl pH 8.0, 0.5 M NaCl, 500 mM Imidazole. The eluted p50 was dialyzed overnight against 1xPBS pH 7.3 to remove imidazole. This protein was subsequently further purified using gel filtration column Superdex 200 (Amersham).

**In vitro Labeling of Human Recombinant NF-κB p50:**

Recombinant NF-κB p50 was reconstituted with PBS as 1 mg/ml. 1 μL protein solution was diluted with 42 μL PBS and incubated with 1 μL P2 at a final concentration of 0, 10, 20, 40, 80, 160 μM for 4 hrs, followed by Click reaction, SDS-PAGE and fluorescence scanning. For heat denatured sample, 1 μL protein solution was diluted with 40 μL PBS and 2 μL of 25% SDS and the sample was heated at 96 °C for 10 min. Then the heated sample was cooled to room temperature and reacted with 80 μM P2 at. Competition assay was carried out with the pre-treatment of 10× excess free Andro for 4 hrs, and then labeled with P2. 1mM DTT or 5mM BME co-treatment together with Andro were also included in our experiment. Mutated C62A p50 was labeled with 80 μM to test this critical amino acid in Andro reaction.
Andro Binding Site Mapping of NF-κB p50:

To map out the exact binding site of Andro in NF-κB p50, the recombinant p50 was treated with Andro or DMSO. After trypsin digestion, the resulting peptides were analyzed by MS/MS. Briefly, 1 µL of 10 mM Andro or 1 µL DMSO was incubated with 50 µg NF-κB in 100 µL PBS for 4 hrs at room temperature. The labeled samples were buffer exchanged against 50 mM NH₄HCO₃ to remove unbound drug using a filter spin column (10 kDa cut-off). The final solution volume was adjusted to 50 µL using 50 mM NH₄HCO₃. This was followed by the addition of 1 µg trypsin to the NF-κB samples and incubated for 16 hrs at 37°C. The digested samples were analyzed by high resolution LC-MS/MS. The MS/MS spectra of the native NF-κB peptide containing Cys62 are shown in Fig. 5d (top). The MS/MS spectra of the Andro labeled NF-κB peptide containing Cys62 are shown in Fig. 5d (bottom). C* (red) represents the Andro modified Cys. Peptide ions containing modified Cys are indicated ^* in red.

Molecular Docking:

Autodock is an automated procedure for predicting optical conformations and orientations for the ligand, protein or DNA with the target proteins at the binding site.(31)(32) NF-κB-Andro docking simulations were performed using Autodock version 4.2 (http://autodock.scripps.edu/) with the Lamarckian Genetic algorithm (LGA) method. Polar hydrogen atoms were added to the target protein p50 and its nonpolar hydrogens were merged. The molecule Andro was treated as flexible ligand and only torsions of freedom were explored, keeping both bond angles and lengths constant. The grid box was centered on p50 (PDB: 2V2T) with a dimension of 100×100×100 points. Each docking simulation was repeated 20 times using different random generator seeds. The interactions of complex p50-Andro conformations were analyzed using Pymol.
Validation of β-actin as the Target of Andro

In vitro Labeling of Human Recombinant β-actin:

β-actin from human platelet (≥99% pure), Cytoskeleton, Inc.) were reconstituted as 1mg/ml in G-buffer (2 mM Tris-HCl, 0.2 mM ATP, 0.2 mM CaCl₂, 0.2 mM DTT, 0.005% NaN₃, pH 8.0) or F-buffer (2 mM Tris-HCl, 2 mM MgCl₂, 100 mM KCl, 0.2 mM DTT, 0.2 mM CaCl₂, 0.5 mM ATP, pH 7.5), incubated for 18 hrs at room temperature. 1 μL protein in G-buffer or F-buffer was diluted with respective buffers and incubated with 1 μL P2 at a final concentration of 0, 10, 20, 50 or 100 μM for 4hrs, followed by Click reaction, SDS-PAGE and fluorescence scanning.

For heat-denatured sample, 1 μL protein in F-buffer was diluted with 40 μL F-buffer and 2 μL of 25% SDS and the samples were heated at 96 °C for 10 min. The heat-denatured control was subsequently cooled to room temperature and reacted with P2 at 100 μM.

Andro Binding Site Mapping of β-actin:

To indentify the exact binding site of Andro in β-actin, the polymerized actin was treated with Andro or DMSO. After chymotrypsin digestion, the resulting peptides were analyzed by MS/MS. Briefly, 1 μL of 10 mM Andro or 1 μL DMSO was incubated with 50 μg polymerized actin in F-buffer (2 mM Tris-HCl, 2 mM MgCl₂, 100 mM KCl, 0.2 mM DTT, 0.2 mM CaCl₂, 0.5 mM ATP, pH 7.5) for 4 hrs in room temperature. The labeled samples were buffer exchanged against 50 mM NH₄HCO₃ to remove unbound drug using a filter spin column (10 kDa cut-off). The final solution volume was adjusted to 50 μL using 50 mM NH₄HCO₃. 1 μg chymotrypsin was added to the β-actin samples and incubated for 16 hrs at 37°C. The digested samples were analyzed by high resolution LC-MS/MS. The MS/MS spectra of the native β-actin peptide containing Cys272 are shown in Fig. S9 in supporting information (top). The MS/MS spectra of
the Andro labeled β-actin peptide containing Cys272 are shown in Fig. S9 in supporting information (bottom). C^A* (red) represents the Andro modified Cys. Peptide ions containing modified Cys are indicated ^A* in red.

**Cell Cycle Determination by Flow Cytometry:**

Andro’s effect on cell cycle was evaluated by flow cytometry. Briefly, after the designated treatments, cells were detached using trpsin-EDTA. The cells were collected and washed 2× with PBS, then fixed and permeabilized in 70% pre-chilled ethanol for at least 2 hrs. Fixed cells were stained with PI staining solution (0.1% Triton X-100, 200 mg/ml RNaseA and 20 mg/ml PI in PBS) for 15 min at 37°C. Subsequently, the PI stained sample were detected by flow cytometry analysis.

**Transwell Migration Assay:**

Migration assay was carried out using 8.0 μm cell culture insert for 24 well (BD Biosciences, San Jose, CA) according to manufacturer’s protocol. Briefly, the inserts were coated with 10 μg/ml fibronectin (Sigma, St. Louis, MO) in PBS overnight at 4 °C. The coated inserts were washed once with PBS before using. HCT116 were seeded at a density of 50,000 cells into the top chamber of each insert with 5 μM of Andro or RA in media without FBS. Media containing 5 μM of respective drugs were supplemented with FBS and added to the bottom chamber. 1% DMSO was used as negative control. After incubation for 30 hrs, cells from the top layer of the inserts were removed by scrubbing. Subsequently, the inserts were fixed in 3% paraformaldehyde (Sigma, St. Louis, MO) in PBS for 20 min before staining with crystal violet for 30 min. Excess stain was washed off and the inserts were allowed to dry. Solubilization was done using 1% SDS for 2 hrs and the absorbance were measured at 550nm.
Matrigel Invasion Assay:

Matrigel invasion assay was carried out using matrigel coated inserts for 24-well plate (BD Biosciences, San Jose, CA) according to manufacturer’s protocol. 150,000 cells were seeded into the top chamber of each insert with Andro or RA in media without FBS. Media containing 5μM of respective drugs were supplemented with FBS and added to the bottom chamber. After 30 hrs incubation, cells from the top layer of the inserts were removed by scrubbing. Subsequently, the inserts were fixed in 3% paraformaldehyde (Sigma, St. Louis, MO) in PBS for 20 min before staining with crystal violet for 30 min. Excess stain was washed off and the inserts were allowed to dry. Cells from 5 randomly selected fields were counted using photographs taken under a light microscope at 200x magnification.
Results and Discussion:

<Figure 2>

Design and synthesis of Andro-based probes:

Ideally, the active moiety of Andro must be retained in the newly designed Andro-based ABPP probe and the attached tag should not affect its interaction with cellular targets during live cell labeling. However, the active moiety and precise mode of action of Andro remain largely elusive. Xia et al. previously reported that Andro binds covalently to Cys62 of NF-κB p50.(21, 33, 34) Based on this known interaction, we postulated that the α,β-unsaturated γ-lactones might be involved in the protein alkylation. The reduced form of Andro analogue RA (Figure 2, synthetic scheme shown in Scheme S1 in Supporting Information) was synthesized to test its biological activity against the colorectal cancer cell line HCT116, which is known to be sensitive to Andro treatment.(23, 25) Our results suggested that Andro had potent anti-cancer effect on HCT116 cell line with IC_{50} of 31 µM, as well as MV4-11, HeLa and HepG2 cell lines (Figure S2a in Supporting Information). When the C12-C13 double bond of Andro was reduced to a single bond, the analogue RA completely lost its cancer inhibitory potency even up to 100 µM concentration (Figure S2b in Supporting Information), suggesting that the α,β-unsaturated γ-lactones and the covalent protein binding of Andro are critical for its anti-cancer property. Previous structure-activity relationship studies suggested that the ester derivative of Andro at C14 hydroxyl group did not affect the C12-C13 moiety and the drug’s potency.(35–37) Therefore, we introduced an alkyne handle using the ester linkage at C14 position to synthesize the clickable probe P1 (Figure 2, synthetic scheme shown in Scheme S2 in Supporting Information). At the same time, to overcome the limitation of the instability of the ester bond, another probe P2 with amide bond link-
age was also synthesized. This probe would have better in vitro and in vivo stability, as it would be less prone to breakage or hydrolysis. (Figure 2, Synthetic scheme shown in Scheme S3 in Supporting Information). Because the introduction of amino group partially reverted the configuration of C14 during the synthesis, P2 was a mixture of two isomers with the ration of 1:0.8. We did not separate these two isomers as we found that the anti-cancer activity was still retained (vide infra). To investigate whether the structural modifications would influence the anti-cancer potency of the probe, we conducted in vitro growth inhibitory assay on HCT116 using P1 and P2. Our data confirmed that P1 and P2 still possess antiproliferative activity (Figure S2b in Supporting Information). Therefore, both P1 and P2 meet the essential criteria for target protein identification.

**In situ proteome profiling:**

To visualize the native cellular targets of Andro using fluorescence gel profiling (Figure S1 in Supporting Information), we treated live HCT116 cells with P1 or P2. The probe-labeled proteomes were reacted with Cy3 azide via click chemistry before being resolved on SDS-PAGE for fluorescence detection. As shown in Figure 3a, in sharp contrast to the DMSO control, P2 labeled proteome yielded high fluorescence intensity bands, signifying that there were proteins interacted with P2. As expected, P1 labeled proteome showed rather weak intensity bands, possibly due to the instability of the ester linker. These observations supported our hypothesis and were consistent with a recent review, which postulated that the elimination of the β-hydroxy group of Andro might occur during the Andro alkylation. Based on our results, the stronger labeling intensity of P2 in comparison to P1 suggests that the ester bond of P1 might be broken or hydrolyzed in the reaction, while the amide bond linkage in P2 is more stable. Therefore, P2 was chosen for subsequent analyses.
ICABPP and target identification:

We next performed ICABPP using P2 to identify specific cellular protein targets of Andro (Figure 1a), which yielded 4 sets of Andro probe vs. control pull-down ratios (116/113; 117/113; 116/114; 117/114). A total of 291 proteins was successfully identified and quantified in our experiment (Figure 3d). Outliers were identified using \( p \)-value >0.05 and 114/113 ratio >1.3 or <0.77. This resulted in 208 proteins being considered to be statistically reliable hits (Figure 3d). The distribution of the enrichment ratios of these proteins were further presented as a coloured heat map in Figure 3b. To reduce false positive targets, we chose a highly stringent ratio of 2 as the cut-off to differentiate specific (red) from non-specific (blue) binding targets. Meanwhile, proteins identified based on a single peptide are considered unreliable and were removed. Consequently, 75 proteins were regarded as the specific targets of Andro based on the above criteria (Figure 3d). The complete list of the 75 potential targets was shown in Table S1 in Supporting Information. NF-\( \kappa \)B p50, a known Andro target,\(^{(21, 33, 34)}\) was found to be enriched 2.8 fold in Andro pull-down when compared to DMSO control, validating our approach. The subsequent pathway analysis suggested that Andro may exert its anti-cancer effects through multiple targets and pathways: more than 30 targets were involved in cancer cell death pathways; 15 hits were involved in cell migration and metastasis (Figure 3c and Figure 4); 20 hits were related to inflammation and 10 to protein synthesis pathway (the respective pathway analyses are shown in Figure S5 and Figure S6 in Supporting Information). Previously, many studies have been carried out to investigate the effect of Andro in inducing cancer cell death and anti-inflammatory effects.\(^{(22–25)}\) Interestingly, our targets and pathway analysis revealed the anti-metastatic effects of Andro, which has not been extensively studied thus far.\(^{(38)}\) Among these target proteins (Fig-
MYH9 and NPM1 have been reported to be associated with regional lymph nodes tumor metastasis. YWHAZ has been reported to promote epithelial-mesenchymal transition in lung cancer. FLNA may promote metastasis by enhancing cell migration in melanoma. PFN1 is an actin-binding protein which was reported to be highly related to tumor cell metastasis. α/β-tubulin, known to be expressed at elevated levels in tumor cells, plays essential roles in maintenance of cellular shape and process of metastasis. HSPA1A and HSPD1 have been reported to play an important role in tumorigenesis and metastasis of breast cancer. Previous studies reported NF-κB p50 as a critical player in cancer metastasis by up-regulating MMP-9 and down-regulating anti-metastatic TIMPs and PAI 2. Actin, one of the most abundant cytoskeleton proteins, participates in cancer metastasis by regulating cell motility. The anti-metastatic effects of a variety of actin-binding agents have been extensively studied due to their therapeutic potential. In particular, we found NF-κB and β-actin were the top two target proteins with the highest differential ratios among all the other targets involved in the metastatic pathway. Thus, these two proteins were chosen for further validation of Andro’s anti-metastatic effect in view of their critical roles in tumor metastasis.

**Targets validation and functional analysis:**

Direct interaction of Andro with both NF-κB and Actin were first validated by pull-down followed by Western blot (Figure 3c). Recombinant NF-κB p50 protein was used for subsequent in vitro labeling experiments. The protein was incubated with different concentrations of P2 for 4 hrs with DMSO-treated p50 as the negative control (Figure 5a). The labeling only occurred to the native p50 in the presence of P2. Heat denatured p50 could not be labeled, suggesting the interaction is specific and physiologically relevant. Competition assay by pre-treating p50 with 10-fold excess of Andro resulted in drastically diminished fluorescent signal, further confirming the
specific binding between p50 and the drug. The presence of reducing agents DTT and BME dramatically reduced the labeling, indicating that the reaction site might be cysteine residues. Previous research has suggested Cys62 of p50 as the binding site of Andro.(34) Our in vitro labeling result confirmed the important role of Cys62 of p50 in Andro binding, which upon mutation to Ala significantly diminished the labeling efficacy (Figure 5a, lane 11).

We further explored the exact modification site of Andro on p50 using MS/MS sequencing. The Andro-treated p50 protein, together with the untreated control, were digested with trypsin and analysed by MS/MS. The peptide YVC\textsuperscript{\textasteriskcentered}EGPSHGG-LPGASEK (Figure 5d) was identified, in which Andro bound to the cysteine residue with the loss of one molecule of H\textsubscript{2}O (Figure 5b).(15, 34) Based on above results, we were able to confirm Andro’s binding site on p50 protein with great confidence.

To gain insight on how Andro alkylation inhibits DNA-binding activity of p50, a docking model was constructed. The thiol group of Cys62, which is located in the DNA-binding motif, is critical in DNA recognition of the p50 subunit. As shown in Figure 5c and Figure S7 in Supporting Information, the docking simulations presented a comprehensive model, in which Andro binds p50 at the active DNA-binding site through interaction with Cys62, the critical amino acid, thereby inhibiting transcription by blocking DNA binding of NF-\textkappaB. Our docking model thus provided further hint that the anti-cancer effects of Andro is at least in part through inhibiting the DNA binding of NF-\textkappaB protein.

Previous studies showed that several small molecules can bind to actin by forming a covalent bond with Cys via similar Michael addition reactions.(47, 51, 52) To validate the interaction between actin and Andro, \textbeta-actin in G-buffer and F-buffer were incubated with Andro, respectively.
The results demonstrated that Andro selectively bound to polymerized F-actin (Figure 6a). The binding site of Andro on actin was subsequently confirmed to be Cys272 by MS/MS, which was reported as a highly reactive cysteine due to its full solvent accessibility (Figure 6c)(53–55)

Finally we verified the anti-metastatic potential of Andro using cell migration and invasion assays. HCT 116 cells were treated with RA and Andro at a non-cytotoxic concentration of 5µM for 30 hrs. The results showed that Andro can effectively suppress the migration and invasion of HCT116 cells (Figure 7). These results further supported our novel finding on the role of Andro in inhibiting tumor metastasis, thus broadening its therapeutic applications as an anti-cancer agent.

As actin plays an important role in cytokinesis, we further examined Andro’s effect on cell cycle using flow cytometry. Our data showed that HCT116 cells were arrested at G2/M phase upon Andro treatment in a time-dependent manner (Figure 6b). We also analyzed the cell cycle using other cell lines including HeLa and HepG2. Significant cell cycle arrest was also observed at G2/M phase upon Andro treatment (Figure S8 in Supporting Information).

**Conclusion:**

In conclusion, our results demonstrated that the ICABPP method, which combines clickable ABPP and iTRAQ, is a powerful approach to identify specific drug targets in live cells. In this study, a spectrum of specific targets of Andro was identified using this new method. In particular, we have identified the novel anti-metastasis potential of Andro through targets and pathway analysis, which have been validated through subsequent migration and invasion assays. To the best of our knowledge, this is the first report that systematically combines the iTRAQ-based
quantitative proteomics with the clickable activity-based probe to profile specific drug targets in live cells.

In practice, ICABPP method can be easily optimized and used in all kinds of affinity chromatography or ABPP-based target identification. The multiplexing property of the iTRAQ enables the precise and accurate quantitation of up to 8 samples simultaneously, allowing the inclusion of the biological replicates or drug competition of pull-down samples. Moreover, it can be used to study drug targets using tissue or body fluid samples in clinical research. We anticipate this ICABPP approach to be widely applied in drug development and optimization to refine the therapeutic potentials of drugs by uncovering knowledge on their specific targets and mechanisms of action.

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Figure legends:

Figure 1. Identifying specific drug targets using ICABPP approach in live cells. a) Live cells were treated with DMSO and clickable probe in duplicates. Cells were lysed and tagged with biotin-N3 using click chemistry in parallel. The biotin bearing target proteins were enriched through avidin pull-down and directly digested on beads. The resulting peptides of the two biological replicates of control pulled-down samples were labeled with 113 and 114, respectively, and two probe pulled-down samples were labeled with 116 and 117, respectively. The labeled peptides were combined together to be identified and quantified by LC-MS/MS. b) For the non-specific targets, the iTRAQ reporters show similar intensities, while for the specific targets, the reporters show highly differential intensities.

Figure 2. Chemical structures of Andro, reduced Andro analogue RA and Andro-based clickable ABPP probe P1 and P2.

Figure 3. a) The in situ fluorescent labeling of HCT116 cells using P1 and P2 (100 µM) together with a DMSO-treated negative control. Probe labeled proteomes were visualized by click conjugation to the Cy3 azide tag, SDS-gel separation, and fluorescent scanning. b) Heat map of the enrichment ratio of potential Andro targets fulfilled the statistical requirement. The most enriched proteins are displayed in red, while the least enriched proteins are shown in blue. The enlarged figure is shown in Figure S4 in Supporting Information. c) The potential protein targets related to cell migration and metastasis identified by ICABPP, sorted by average enrichment ratios. d) Venn diagram showing the total numbers of proteins quantified by ICABPP (green rectangle), statistically reliable proteins (blue) and specific binding targets (yellow). e) Western-blot
validation of pulled-down fractions of HCT116 by P2 (or DMSO as negative control) with NF-κB p50 and β-actin antibodies.

**Figure 4.** Ingenuity Pathway Analysis (IPA) revealing that Andro affects the cell migration and metastasis. All proteins shown in green nodes were identified as the specific targets of Andro.

**Figure 5.** a) *In vitro* labeling of recombinant NF-κB p50 protein with P2: P50 is concentration-dependently labeled by P2 only in its native form, Δ=heat denaturation control; DTT and β-mercaptoethanol (BME) co-treatment, 10-fold excess Andro pre-treatment reduce the labeling; Drastically reduced labeling of p50 mutant shows Cys62 is the critical amino acid for Andro labeling. Coo=Coomassie staining; Flu=fluorescence scanning image. b) The schematic of the reaction of Cys with Andro. c) Docking simulation model showing Andro binding to the NF-κB p50 subunit through the critical amino acid Cys62 and Arg57. d) The MS/MS spectra of the NF-κB p50 peptide containing Cys62. P50 was incubated without (top) and with (bottom) Andro for 4hrs, and digested by trypsin. C A*=red represents the Andro modified Cys. Fragment ions containing modified Cys are indicated with A* in red.

**Figure 6.** a) *In vitro* labeling of purified β-actin protein using P2. G: actin in G-buffer; F: actin in F-buffer; Δ=heat denaturation prior to labeling. Coo=Coomassie staining, Flu=fluorescence scanning. b) Flow cytometry cell cycle analysis of HCT116 cells treated with Andro (31 µM). c) The MS/MS spectra of β-actin peptide containing Cys272. Polymerized actin in F-buffer was incubated without (top) and with (bottom) Andro for 4hrs, and digested by chymotrypsin. C A*=red represents the Andro modified Cys. Fragment ions containing modified Cys are indicated with A* in red.
**Figure 7.** Inhibition of cancer cell migration and invasion by Andro. a) Cell migration and invasion assays of HCT116 cells treated with DMSO, RA (5 µM) and Andro (5 µM), respectively. Cells were seeded in inserts coated with either fibronectin or matrigel and allowed to migrate and invade towards FBS containing medium for 30 hrs before imaging and quantification. b) & c) Histogram showing significant differences in cell migration and cell invasion, respectively. (**p-value<0.001**
Figures:

1. [Diagram of biological replicate process with iTRAQ labeling and m/z analysis]
2.

Andro  RA  P1  P2
3. 

![Image of a gel electrophoresis experiment with fluorescence and Coomassie staining. The gel shows bands at different molecular weights (kDa).]

| Name | % Cov | Peptides | 116:113 | 117:113 | 116:114 | 117:114 | Ave Ratio | p-value |
|------|-------|----------|---------|---------|---------|---------|-----------|---------|
| ACT8 | 60.7  | 27       | 2.71    | 2.75    | 2.88    | 2.92    | 2.81      | 1.1E-05 |
| NFkB1 | 10.1  | 4        | 2.88    | 3.40    | 2.34    | 2.72    | 2.80      | 8.9E-04 |
| HNRP2 | 17.3  | 4        | 2.16    | 2.16    | 2.90    | 2.82    | 2.48      | 1.5E-03 |
| MYH9  | 21.6  | 15       | 2.33    | 2.55    | 2.40    | 2.61    | 2.47      | 5.7E-05 |
| NPM1  | 26.2  | 6        | 2.43    | 2.36    | 2.57    | 2.50    | 2.46      | 2.0E-05 |
| TUBA1B| 39.3  | 10       | 2.25    | 2.28    | 2.52    | 2.56    | 2.40      | 1.2E-04 |
| YWHAZ | 42.7  | 8        | 2.26    | 2.48    | 2.12    | 2.32    | 2.29      | 1.3E-04 |
| PPIA  | 50.3  | 5        | 2.24    | 2.16    | 2.38    | 2.32    | 2.27      | 3.7E-05 |
| TUBB  | 46.4  | 17       | 2.15    | 1.67    | 2.78    | 2.19    | 2.16      | 5.1E-03 |
| PRDX5 | 56.1  | 5        | 2.24    | 2.22    | 2.08    | 2.07    | 2.15      | 4.6E-05 |
| HSPA1A | 29    | 9        | 2.25    | 2.08    | 2.05    | 1.90    | 2.07      | 2.3E-04 |
| PPI1  | 68.6  | 6        | 2.09    | 2.00    | 2.13    | 2.04    | 2.06      | 1.3E-05 |
| NME1  | 34.5  | 4        | 2.02    | 1.80    | 2.32    | 2.07    | 2.04      | 8.4E-04 |
| HSPD1 | 58.2  | 13       | 1.96    | 2.06    | 2.03    | 2.12    | 2.04      | 2.7E-05 |
| FLNA  | 16.8  | 7        | 2.09    | 2.15    | 1.85    | 1.92    | 2.00      | 2.9E-04 |

**d)** Total quantified proteins: 291 Consistent hits: 208 Specific Targets: 75

**e)** Images of Western blots with bands at 50 kDa and 42 kDa.
