Genome-wide CRISPR screen reveal targets of chiral gold(I) anticancer compound in mammalian cells

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Additional Supplemental Material.

An excel file labeled Awuah Supplemental Files contains additional data and analysis results.

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Tab 2: “Raw counts” contains the raw counts, for each sgRNA determined as described in methods for the three control samples and three treated samples at 14 days.

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Tab 4: “Resistant across methods” shows the consensus results across the three different methods. For Mageck, genes with FDR <0.1 are shown, for Deseq2, genes for which two or more sgRNA had a FDR <0.1 are shown, for EdgeR, genes for which two or more sgRNA had a FDR <0.1 are shown. The average LogFC determined by each method is shown. Candidates which were identified in two or more methods are bolded.

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Tab 22: Primers for NGS – contains the CRISPR amplification primers used in the study.

Tab 23: Oligos for sgRNA cloning: contains the individual sgRNAs used for the individual KO studies.
Materials and instrumentation. 1,10-Phenanthroline was from Sigma-Aldrich and used without further purification or drying. Tetrachloroauric acid (HAuCl4•3H2O) was purchased from NANOPARTZ and used as received. ACS grade solvents were purchased from Pharmco-Aaper and used without further purification or drying. Deuterated solvents were purchased from Cambridge Isotope Laboratories and used as received. Silica gel for column chromatography (Silicycle, P/N: R10030B SiliaFlash F60, Size: 40-63 μm, Canada) was purchased from Silicycle. Aluminum backed silica-gel plates (20 x 20 cm2) were purchased from Silicycle (TLA-R10011B-323) and utilized for analytical thin-layer chromatography (TLC).

All reactions were insensitive to air or moisture, as a result, they were carried out under standard atmospheric conditions without air-sensitive techniques or drying agents. Reactions were carried out in round-bottom flasks or scintillation vials equipped with Teflon-coated magnetic stir bars for stirring non-homogenous reaction mixtures. Reactions were monitored by NMR and TLC, and the TLC plates visualized under low-wavelength light (254 nm) or stained with iodine on Silica. All compound purification was performed using silica-gel chromatography, employing CombiFlash RF+ Lumen, Teledyne ISCO. Removal of solvents in vacuo was performed using a Büchi rotary evaporator and further drying was achieved by Schlenk line at ~120 mTorr using a dynamic vacuum pump.

$^1$H, $^{13}$C (1H-decoupled), and $^{31}$P (1H-decoupled) NMR spectra were recorded on a Varian Unity 400 MHz NMR spectrometer with a Spectro Spin superconducting magnet at the University of Kentucky NMR facility in the Department of Chemistry. Chemical shifts in $^1$H and $^{13}$C NMR spectra were internally referenced to solvent signals ($^1$H NMR: CDCl$_3$ at $\delta$ = 7.26 ppm; $^{13}$C NMR: CDCl$_3$ at $\delta$ = 77.16 ppm), and those in $^{31}$P NMR spectra, which were run in CDCl$_3$, were externally referenced to 85% H3PO4 in D$_2$O at $\delta$ = 0 ppm.

High-resolution mass spectra (HRMS) were obtained using a Waters Synapt G2 HD mass spectrometer. Samples were directly injected into the instrument at 50 μL/min and ionized with ElectroSpray Ionization (ESI) in the positive mode. The source parameters were: capillary = 2.8 kV, sampling cone = 40, extraction cone: 5.0, source temperature = 80 °C, desolvation temperature = 150 °C, and desolvation gas flow = 500L/h. Mass spectrometry experiments and analysis were conducted at the Central Analytical Laboratory at the University of Colorado, Boulder.

Experimental Details
ToxCRISPR sgRNA Library Preparation and Lentivirus production
**ToxCRISPR sgRNA Library construction.**

We prepared a custom-designed toxicant response-related sgRNA library (ToxCRISPR) that can be employed in the CRISPR knockout (KO) system to perform genetic screening in human cells. To create this novel panel, A. Sobh and C. Vulpe (UFL, Gainesville), Luoping Zhang (UC Berkeley) and Quan Lu (Harvard University) combined the environmentally responsive human “S1500+” gene set prioritized by NIEHS/NTP/Tox21 program with 647 Environmental Genome Project (EGP) genes and a few other selected toxicant response-focused genes, resulting in 3,675 genes. The ToxCRISPR library includes ~15000 sgRNAs, where each gene is targeted by 4 different sgRNAs, in addition to 500 non-targeting sgRNAs that can be used as negative controls. sgRNA guide sequences were designed using the MIT CRISPR design tool (Zhang lab). Pooled synthesis of the designed DNA oligonucleotides flanked by the appropriate cloning ends (79 nt) was performed by CustomArray. The oligonucleotides were amplified by PCR and cloned into the CRISPR lentiviral backbone vector (LentiCRISPRv2, Addgene #52961) using Gibson assembly® cloning (Macrolab, UC Berkeley). The vector library was transformed into electrocompeent cells with a transformation efficiency that ensures sufficient library representation (~250X) and plasmid DNA was extracted from the combined colonies (UC Berkeley, Macrolab). To check sequence quality and representation, library oligos were amplified from the plasmid pool by PCR and deep sequenced by next generation sequencing (NGS). Sequences were aligned to the ToxCRISPR guides using Bowtie. The histogram of sgRNA distribution and cumulative sgRNA read counts shows minimal bias and the difference in representation between the 90th and 10th percentile was low (~6 fold) which is considered excellent representation. The entire library with sequence of sgRNA primers and gene name is provided in the excel file labeled Awuah Supplemental Files.

**ToxCRISPR library screening**

**Lentiviral production and functional titration**

Lentivirus production was performed as previously described,1 with minor modifications. Briefly, HEK293T cells cultured in a T225 flasks were co-transfected with 20 µg of the plasmid library, 15 µg of the packaging plasmid (psPAX2, Addgene # 12260) and 10 µg of the envelope plasmid (pMD2.G, Addgene # 12259). Media containing the virus were collected 60 hrs post transfection and filtered through a Steriflip-HV 0.45 µm low protein binding PVDF membrane (Millipore). The lentiviral supernatant was concentrated 50 folds using Lenti-X Concentrator (Clontech) following the manufacturer’s protocol. Viral solutions were
aliquoted and stored at -80°C until use. To perform functional titration of the prepared viral solutions, K562 cells were suspended in transduction medium (RPMI 1640, 10% FBS, 1% PS + 8ug/ml polybrene) and seeded at a density of 1.25x10^6 cells/ml in 12-well plates (2.5x10^6 cells per well). Different volumes (0, 0.625, 1.25, 2.5, 5, and 10 µl) of the lentivirus were mixed with the cell suspension in each well and the plates were centrifuged at 1000g for 2 hrs at 33°C. Transduced cells from each well were suspended in fresh media and recovered for 48 hours. For each transduction volume, cells were seeded in 96-well plate at a density of 10^5 cells/ml (10^4 cells/well; 100 µl) with or without puromycin (2 µg/ml) and maintained for 7 days during which 25 µl of cell suspension from each well were added to 75 µl of fresh media in a new replica plate every 48 hours. Following puromycin selection, cell viability in each condition was evaluated by CellTiter Glo and the multiplicity of infection (MOI) corresponding to each transduction volume was calculated by dividing the average luminescence signal from wells with puromycin by the average luminescence signal from wells without puromycin. A transduction volume corresponding to a MOI 0.4-0.5 was used in the large-scale transduction.

**CRISPR/Cas9 screening**

50 million K562 cells were transduced as described above for the titration of the library with the lentiviral ToxCRISPR library using an estimated MOI of 0.4-0.5. 48 hours post-transduction, the cells were selected against 2 µg/ml puromycin for 6 days. The cells were allowed to recover for 48 hrs before beginning the exposures. Three aliquots of 20 million cells were frozen and stored to serve as reference of the representation of CRISPR mediated mutants at the first day of the screen to assess the loss of essential genes during the screen. The screen was performed in T75 cm2 flasks (Corning cat# 430641U), with 7.5 millions cells per replicate to achieve a 500X coverage, in 30 ml RPMI 1640 supplemented with 10% FBS and 1X penicillin/streptomycin.

The CRISPR screen conditions were untreated cells (control) and 0.4 µM JHK-21. Each condition was performed in triplicates. The medium was replaced every 48 hrs by spinning down the cells at 300g for 5 min, and reseeding 7.5 millions cells each time. The screening lasted 14 days which corresponds to 14 doublings of the untreated WT K562.
DNA extraction, library preparation and next generation sequencing

Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer instructions. We used 2 columns per samples. Amplicon libraries for NGS were prepared using the two-steps PCR method described in detail in Sobh et al., 2019.²

In brief, for each sample, the gRNA region was PCR amplified using the high fidelity Herculase II Fusion DNA Polymerase kit (Agilent) and primers CRISPR1-FOR and CRISPR1-REV (PCR1). The amplicon libraries for NGS were prepared and barcoded by carrying out a second PCR (PCR2) using a combination a common forward primer (CRISPR2-FOR) and a sample specific reverse primer (CRISPR2-REV) incorporating respectively the P5 and the P7+barcode adapters. For each sample, four 100 µl PCR1 reactions were performed using 10 µg genomic DNA template per reaction and 18 cycles. The PCR1 products were pooled for each sample followed by two 100 µl PCR2 reactions. PCR2 was carried out using 5 µl PCR1 product per reaction, and 20 cycles producing 358 bp amplicon libraries.

The amplicons were gel purified using the QIAquick Gel Extraction Kit (Qiagen) and quantified using the Qubit HS dsDNA assay (Thermoscientific). Equimolar amounts of each amplicon library were multiplexed in one pool. The library size and concentration were confirmed by TapeStation (Agilent). The sequencing was performed at the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida at Gainesville, using the NextSeq500 High Throughput single read 75 cycles platform (Illumina).

David analysis

Enrichment analysis of the 14 sensitive and 10 resistant consensus candidates was carried out using the David functional annotation enrichment analysis tool at https://david.ncifcrf.gov using default settings.

| Sensitive Candidates | Resistant Candidates |
|----------------------|----------------------|
| FASN                 | SPRED2               |
| SLC25A46             | SUV39H1              |
| Protein 1 | Protein 2 |
|----------|----------|
| NDUFS6   | KDM6A    |
| AIFM1    | RPL31    |
| NPRL2    | SLC25A25 |
| ATP5I    | CENPA    |
| SOD2     | RRAGA    |
| ABCC1    | HIST1H2BK|
| CUL3     | GALE     |
| GARS     | ASNA1    |
| NFU1     |          |
| SLC25A19 |          |
| TIMM17A  |          |
| KAT2B    |          |

**Stringdb analysis.**

Enrichment analysis of the 14 sensitive and 10 resistant consensus candidates was carried out using the Stringdb GO enrichment analysis tools at [https://string-db.org/](https://string-db.org/) using default settings.

**UV-Vis Stability in PBS and RPMI-1640 at 37 °C.** All spectra were recorded on a Shimadzu UV-1280 model instrument. PBS was used as received from Corning® (without calcium or magnesium). RPMI-1640 was purchased from Corning© and used as is. Each medium was warmed to 37 °C prior to dilution of the complexes. All complexes were freshly prepared prior to use as a 1 mM stock in 200 mL of DMSO and 800 mL of PBS or RPMI-1640. The solutions were then diluted to 25 µM with the respective biological medium. The amount of DMSO in each solution was <1%, therefore the instrument was blanked with either DMSO/(PBS or RPMI-1640) solution prior to each scan. The solutions were kept in an incubator at a controlled temperature of 37 °C.
In Vitro Biological Assays

Cell Lines and Cell Culture Conditions. All K562 and knocked-out K562 cells were maintained in the Roswell Park Memorial Institute (RPMI) 1640 medium. All cell lines were cultured in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% amphotericin. All cells were grown at 310K in a humidified atmosphere containing 5% CO2.

Cell viability assay.

Whole cell uptake studies. K562 and knocked-out K562 (5 x 10^5 cells) were seeded in a 6-well plate and incubated for 1 h at 37 °C. Cells were then incubated with the test compounds (5 μM) in fresh RPMI 1640 medium (5 mL) and subsequently incubated for a given period of time (~12 h) at 37 °C. And then centrifuged to get cell pellets. Cells were then washed with PBS (3 x 1 mL). The cells were digested by adding 0.5 mL of concentrated HCl and briefly placing them on an agitator. Cells were then transferred to a new tube containing 4.5 mL of DI water. The gold content was analyzed by ICP-OES to obtain the whole cell uptake after quantification.

Cell cycle analysis: Cells were seeded in 6-well plates (5 x 10^5 cells/well) and were treated with PBS and JHK-21 for 6, 12 and 24 h at a concentration of 0.2 μM. The cells were re-suspended in ice-cold PBS, fixed with 70% ethanol in PBS at 4 °C overnight, subsequently washed with ice-cold PBS (x2), and then incubated with RNase A (1 μg/mL) for 20 min at 37 °C. They were then stained with PI (10 μg/mL for 30 min in the dark) and their DNA content and cell cycle distribution were measured, using a FACSCalibur flow cytometry (BD Biosciences, USA) and as determined with ModFit software.

Apoptosis study. K562 cells were seeded in 6-well plates (5 x 10^5 cells/well), treated with PBS and JHK-21 (0.2 mM) for 48 h, but for H_2O_2 (2 mM) for 3 h, and then harvested. The Annexin V-FITC Apoptosis Detection Kit (BD Biosciences) was used to determine the fraction of cells (from a total of 1×10^5 cells) that underwent apoptosis, using fluorescence-activated cell-sorting sorting (BD Biosciences, USA) and by following the manufacturer’s protocol. Data were analyzed using FlowJo software.

Mitochondrial Membrane Potential (JC-1 assay). K562 cells were plated at a density of 5 x 10^5 cells/plate using a glass bottom petri dish fitted with a #1.5 cover slip with a final volume of 1.5 mL. JHK-21 was prepared as a stock in DMSO/RPMI (2:8) and added at a final concentration of 10 μM. The cells were treated for 6 h at this concentration. CCCP was prepared as a stock in DMSO and added at a final concentration of 100 μM and the cells treated for 1 h. This was used as a positive control. After the indicated
treatment time, a working solution of the JC-1 dye (Cayman Chemicals) was prepared by adding 100 µL of dye into 900 µL of RPMI. Note: the working solution of JC-1 should always be prepared fresh and not stored for long-term use. Then, 100 µL/mL of RPMI were added to the cells and incubated at 37 °C for 20 minutes. Prior to imaging, the media was removed and replaced with room temperature PBS (2 mL). The cells were then visualized using confocal microscopy on a Nikon A1R Inverted Confocal Microscope. J-aggregates were imaged with (excitation/emission: 510/590 nm) and J-monomers with (excitation/emission: 488/525 nm). Each image is representative of three technical replicates.

**Dichloro(1,10-Phenanthroline)gold(III) dichloroaurate(I)**

Under normal atmospheric conditions, 1,10-phenanthroline (0.217 g, 1.096 mmol) and HAuCl₄·3H₂O (0.436 g, 1.107 mmol) were dissolved in ethanol (100 mL). The reaction mixture was stirred for 22 hours at 82 °C. The precipitate was then vacuum filtered and washed with ethanol and diethylether to afford a yellow solid (0.323 g, 37 % yield), which could then be used without further purification. 

1H NMR (400 MHz, DMSO-d₆) δ 9.71 (d, J = 5.7 Hz, 1H), 9.36 (d, J = 8.2 Hz, 1H), 8.53 (s, 1H), 8.45 (dd, J = 8.2, 5.7 Hz, 1H).

**JHK-21: (1,10-Phenanthroline) [(R)-tert-butyl(3-((R)-tert-butyl(methyl)phosphanyl)quinoxalin-2-y1)(methyl)phoshine oxide]gold(I) chloride**

Under normal atmospheric conditions, Dichloro(1,10-Phenanthroline)gold(III) dichloroaurate(I) (0.054 g, 0.068 mmol) was placed in a 20 mL of scintillation vial and 10 mL of CH₂Cl₂ was added, the solution turned yellow. (R,R)-(−)-2,3-Bis(t-butylmethylphosphino)quinoxaline (0.023 g, 0.069 mmol) was added, the solution turned red-brick color instantly and then gradually turned into yellow. The solution was stirred for about 72 hours. The solution was monitored by TLC in 5% MeOH in CH₂Cl₂ as an eluent. Separation of compound was achieved via flash chromatography using CombiFlashR Rf+ Lumen with 2:98/MeOH:CH₂Cl₂ to afford the desired product (bright yellow, 0.013 g, 32.4 % yield), which could then be used without further purification.

1H NMR (400 MHz, Chloroform-d) δ 8.14 (ddd, J = 14.1, 5.8, 3.0 Hz, 2H), 7.99 – 7.89 (m, 2H), 2.19 (dd, J = 9.9, 3.8 Hz, 3H), 1.94 (dd, J = 35.2, 12.7 Hz, 3H), 1.50 (dd, J = 37.9, 16.7 Hz, 9H), 1.34 (dd, J = 19.6, 15.2 Hz, 9H). 13C NMR (101 MHz, Chloroform-d) δ 139.94, 139.83, 139.81, 139.56, 139.41, 139.39, 132.87, 132.84, 132.72, 132.68, 129.64, 129.62, 129.60, 129.56, 129.55, 129.53, 35.61, 35.39, 34.92, 34.71, 34.58, 34.24, 27.85, 27.83, 27.79, 27.78, 25.89, 25.63, 14.25, 13.60, 13.52, 12.86, 10.85, 10.51, 10.37, 10.02. 31P NMR (162 MHz, Chloroform-d) δ 51.10 (d, J = 5.1 Hz), 50.87 (d, J = 5.1 Hz), 37.41 (d, J = 5.0 Hz), 36.59 (d, J = 5.5 Hz). HRMS (ESI) (m/z): calcd. for
C_{18}H_{28}AuClN_{2}P_{2}O+Li\]^+ 589.1191, found: 589.1191. Anal. Calcd. for C_{18}H_{28}AuClN_{2}OP_{2}: C, 37.10; H, 4.84; N, 4.81. Found: C, 37.18; H, 4.92; N, 4.69

NMR spectra data

Figure S1. $^1\text{H}$ NMR spectrum of complex JHK-21 in CDCl$_3$ at 298K

Figure S2. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of complex JHK-21 in CDCl$_3$ at 298K
Figure S3. $^{31}$P$\{^1$H$\}$ NMR spectrum of complex JHK-21 in CDCl$_3$ at 298K
Figure S4. HRMS (ESI) of compound JHK-21.

Supplementary Figures

(a) 

(b) 

Figure S6. UV-Vis spectra for reactivity of JHK-21 (25 mM) in PBS (a) and RPMI (b).
Figure S7. FITC Annexin V/PI apoptosis dead cell assay. K562 cells were used. Plots of untreated cells (a, negative control), cells treated with JHK-21 (b, 0.5 mM for 48 h), and H$_2$O$_2$ (c, 2 mM for 3 h)

Figure S8. Bar charts representing the different phases of the cell cycle of K562 in the presence JHK-21 (0.2 µM) and vehicle control over the course of 24 h. 6 h untreated: G1: 40.98 %, S: 46.51 %, G2: 12.51 %, 12 h untreated: G1: 37.75 %, S: 49.68 %, G2: 12.57 %, 24 h untreated: G1: 35.88 %, S: 48.84 %, G2: 15.27 %. 6 h treated: G1: 37.78 %, S: 48.52 %, G2: 13.70 %, 12 h treated: G1: 33.62 %, S: 45.63 %, G2: 20.75, and 24 h treated: G1: 39.73 %, S: 54.71 %, G2: 5.56 %.
Figure S9. Whole cell (K562 and K562-ABCC1-G1) uptake results from auranofin (1 μM) and JHK-21 (1 μM). Cells were incubated with compounds for 15 h.

Figure S10. Images of K562 and K562-ABCC1-G1 cells with JC-1 dye, overlay of green and red fluorescence, a)-f). a) K562 cells, negative control (untreated), b) K562 cells, JHK-21 induced mitochondrial membrane potential (Δψm) loss, c) K562 cells, positive control, CCCP, d) K562-ABCC1-G1 cells, negative control (untreated), e) K562-ABCC1-G1 cells, JHK-21 induced mitochondrial membrane potential (Δψm) loss, and f) K562-ABCC1-G1 cells, positive control, CCCP.
Reference:

(1) Shalem, O.; Sanjana, N. E.; Hartenian, E.; Shi, X.; Scott, D. A.; Mikkelsen, T. S.; Heckl, D.; Ebert, B. L.; Root, D. E.; Doench, J. G.; Zhang, F. Genome-Scale CRISPR-Cas9 Knockout Screening in Human Cells. Science 2014, 343, 84-87.

(2) Sobh, A.; Loguinov, A.; Yazici, G. N.; Zeidan, R. S.; Tagmount, A.; Hejazi, N. S.; Hubbard, A. E.; Zhang, L.; Vulpe, C. D. Functional Profiling Identifies Determinants of Arsenic Trioxide Cellular Toxicity. Toxicol Sci 2019, 169, 108-121.