Fatty Acid-Like Pt(IV) Prodrugs Overcome Cisplatin Resistance in Ovarian Cancer by Harnessing CD36

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Experimental details

General information. All reagents were purchased from Strem, Aldrich or Alfa and used without further purification. FALP1 was synthesized according to the reported literature.[1] All reactions were carried out under normal atmospheric conditions. GFAAS measurements were taken on a PerkinElmer PinAAcle 900Z spectrometer. Fluorescence images were acquired using an Olympus IX70 inverted epifluorescence microscope equipped with a digital CCD camera (QImaging). Images were processed and intensities were quantified with ImageJ software (NIH). Analytical HPLC was conducted on an Agilent 1100 system using C18 reverse-phase columns (Hypersil GOLD, 100 mm × 3 mm, 5 μm). Flow cytometry was carried out on a FACSAria™II flow cytometer. Cell confluence assays were carried out on a BioTek Cytation 5 plate reader.

Synthesis of Boc-FALP2. To FALP1 (105 mg, 0.15 mmol), HATU (57 mg, 0.15 mmol), was added 1.4 mL anhydrous DMF, stirred for 20 min, and then, N-Boc-ethylenediamine (26 mg, 0.16 mmol) in 0.4 mL anhydrous DMF was added under stirring. After 30 min of stirring at R.T., DIPEA (40 μL, 0.23 mmol) was added. The reaction mixture was stirred at R.T. for 5.5 hr. A total of 8 mL brine was added to the reaction mixture. Then, the precipitation was collected by centrifuge, washed with water (2 mLx2) and dried overnight under vacuum. Yield: 75 mg (60 %).^{1}H NMR (400 M Hz ,DMSO-d_6) : δ: 0.845 (5, t, 3H, J = 6.8 Hz), 1.226 (4, m, 28H), 1.365 (boc, s, 9H), 2.238-2.445 (1,2,3, m, 6H), 6.463-6.820 (6, 7, 8, 10, m, 8H), 7.938 (9, t, 1H, J = 5.2 Hz) ; ^{13}C NMR (500 MHz, DMSO-d_6) : δ: 180.4, 172.0, 164.4, 156.0, 78.075, 41.5, 39.3, 31.9, 31.7, 30.3, 29.5, 29.4, 29.3, 29.1, 28.7, 26.9, 22.5, 14.4; HR-MS (positive mode) for $[C_{28}H_{60}Cl_2N_5O_7Pt]^+$: m/z calc: 843.3514, obsd: 843.3513. Purity: 97% determined by HPLC.

Synthesis of FALP2. To 1.8 mL DCM solution of Boc-FALP2 (69 mg, 0.081 mmol), 0.2 mL TFA was added and the mixture was stirred at R.T. for 3.5 hr. Upon completion checked by TLC (5 % Methanol in DCM), reaction was stopped, and solvent was evaporated under reduced pressure and dried under vacuum overnight to obtain yellow solid. Yield: 58 mg (96 %). ^{1}H NMR (400 M Hz ,DMSO-d_6) : δ: 0.853 (5, t, 3H, J = 7.2 Hz), 1.216 (4, m, 28H), 2.272-2.509 (m, 4H, succinic Hs), 2.866-2.881 (1,3, m, 4H), 3.284-3.299 (2, m, 2H), 4.894 (NH_2, broad s, 2H), 6.672 (6,7, s, 6H), 8.220-8.234 (8, 9, m, 2H); ^{13}C NMR (500MHz,DMSO-d_6) : δ : 180.3, 172.7, 164.4, 41.4, 39.0, 36.8, 31.9, 31.7, 31.5, 30.3, 29.4, 29.3, 29.1, 29.0, 27.3, 26.9, 26.3,

22.5, 14.3; HR-MS (positive mode) for $[C_{23}H_{52}Cl_2N_5O_5Pt]^+$: m/z calc: 742.2966, obsd: 742.2965. Purity: 97% determined by HPLC.

Synthesis of FALP3. To FALP1 (70 mg, 0.1 mmol), HATU (38 mg, 0.1 mmol), was added 1 mL anhydrous DMF, stirred for 20 min, and then, (2-aminoethyl) triphenylphosphonium bromide (34 mg, 0.11 mmol) was added under stirring. After 30 min of stirring at R.T., DIPEA (30 µL, 0.16 mmol) was added. The reaction mixture was stirred at R.T. for 5.5 hr. A total of 8 mL brine was added to the reaction mixture. Then, the precipitation was collected by centrifuge, washed with water (2 mLx2) and dried overnight under vacuum. Yield: 96 mg (94 %).^{1}H NMR (400 M Hz ,DMSO-d_6) : δ: 0.850 (7, t, 3H, J = 7.2 Hz), 1.231 (6, m, 28H), 2.154-2.399 (succinic Hs, m, 4H), 2.910 (5, m, 2H), 3.728 (1, 2, m, 4H), 6.505-6.635 (4, 8, 9, m, 7H), 7.744- 7.926 (aromatic Hs, m, 15H), 8.271 (3, t, 1H, J = 5.8 hz) ; ^{13}C NMR (500MHz, DMSO-d_6) : δ : 180.0, 172.5, 164.4, 135.4, 134.2, 130.7, 130.6, 119.1, 118.3, 41.5, 33.2, 31.7, 31.5, 30.3, 29.5, 29.4, 29.2, 26.9, 22.6, 21.6, 21.1, 14.4; HR-MS (positive mode) for $[C_{41}H_{64}Cl_2N_4O_5PtP]^+$: m/z calc: 988.3637, obsd: 988.3648. Purity: 95% determined by HPLC.

Differentiation of adipocytes and preparation of the conditioned media. 3T3-L1 preadipocytes were cultured as normal to 70% confluency. Upon reaching this stage, DMEM was aspirated and 15 mL of MDI Induction media was added (DMEM 1g/L glucose, with L-glutamine & sodium pyruvate supplemented with 10% FBS and 1% Penicillin-Streptomycin containing 0.5 mM IBMX, 1 µM dexamethasone, and 10 µg/mL insulin. After 72 h incubation, MDI Induction media was removed and insulin media was added (DMEM 1g/L glucose, with L-glutamine & sodium pyruvate supplemented with 10% FBS and 1% PS containing 10 µg/mL insulin. Cells were again incubated for 72 h. After, cells were then cultured in fresh DMEM for 3 more days to allow for further differentiation. Conditioned media was collected after 3 days for 9 days. Conditioned media was stored at -80 ^{o}C.

Flow cytometric analysis of CD36 protein levels of SKOV3 cells treated with the conditioned media. SKOV3 cells were plated and allowed to attach overnight. Cells were then incubated in 5 mL of adipocyte conditioned media for 72 h. After incubation, Cells were treated with 5 µL Anti-human CD36-PE (BioLegend®, San Diego, CA, USA) 1 h at R.T. in the dark. Cells were washed. The cell pellet was then resuspended in PBS to 10^5 cells/ mL and analyzed via with at 10,000 events with FACSAria™II and data was processed by FlowJo software.

Flow cytometric analysis of BODIPY FL-C16 uptake. The cells treated with BODIPY FL-C16, conditioned media was changed to fresh DMEM and 1 µM BODIPY FL-C16 (Invitrogen™, Eugene, Oregon, USA) for 1 h at 37 ^{o}C under an atmosphere containing 5% CO_2. Cells were harvested and resuspended in PBS with 0.5% BSA to 10^5 cells/ mL and analyzed via with at 10,000 events with FACSAria™II (BD Biosciences, Franklin Lakes, NJ, USA) and data was processed by FlowJo software.

GFAAS analysis of cellular Pt contents of SKOV3 cells treated with conditioned media. SKOV3 cells were plated and pretreated with 5 mL of adipocyte conditioned media. After 72 h, media was replaced with fresh DMEM and the normal whole cell uptake protocol was followed. SKOV3 cells were plated in a 6-well and allowed to adhere overnight. Cells were then treated with 10 µM either cisplatin or FALP1 for 24 h. After incubation with the Pt compounds, cells were collected by trypsinization and washed with 1 mL PBS. Cells were counted, recollected via centrifuge and resuspended in 200 µL 65% nitric acid. Samples were

stored shaking at room temperature overnight. After the cell pellet was dissolved, the solution was either stored at -20 ^{o}C until analyzing by GFAAS or diluted 3 times with PBS. Samples were further diluted for analysis.

qPCR analysis of CD36 mRNA levels. A2780, A2780cis and HEK293 were seeded in a 6-well plate at a concentration of 4 x 10^5 cells/well. Cells were incubated at 37 ^{o}C, 5% CO_2 for 24hr. Then, cells were harvested, and total RNA was collected by the ReliaPrepTM RNA Cell Miniprep System (Promega, Madison, WI, USA). RNA concentrations were measured by the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Rochester, NY, USA) and RNA concentrations were diluted to 100 ng/μL RNA. After that, cDNA was formed by the qScript cDNA SuperMix (Quantabio, Beverly, MA, USA) in the PTC-100 thermal cycler (Bio-Rad, Hercules, CA, USA). Then, qPCR was ran via the Mastercycler® RealPlex2 (Eppendorf, Hamburg, Germany), and each reaction had the volume of 20μL mixture that contained 5μL of x50 diluted cDNA, 1.5 μL 5 μM primer, 3.5 μL water, and 10 μL PerfeCTa® SYBR® Green SuperMix (Quantabio, Beverly, MA, USA). The qPCR thermal cycles included an initial heating temperature of 95°C for 10min followed by 40 cycles of 95°C for 30 s, 60°C for 30s, and 72°C for 20s. qPCR was performed for CD36. The expression of mRNA was measured against housekeeping gene GAPDH and the sequences of primers are shown below: GAPDH: 5'-AGGTCGGTGAACGGA TTTG-3' (Forward) and 5'-GGGGTCGTTGATGGCAACA-3' (Reverse); CD36: 5'-GGCTGTGACCGG AACTGTG-3' (Forward) and 5'-TTCTGTGCCTGTTTTAACCCAA-3' (Reverse).

Flow cytometric analysis of CD36 protein levels of A2780 and A2780cis cells. A2780 and A2780cis were seeded in a 6-well plate at a concentration of 4 x 10^5 cells/well. Cells were incubated at 37 ^{o}C under an atmosphere containing 5% CO_2 for 24hr. Then, cells were harvested and counted populations. After counting, cells were centrifuged at 800 x g for 5min. The supernatant was aspirated, and 100 μL PBS with 0.5% BSA was added into the cells. Based on the cell populations, the use of 5 μL Anti-human CD36-PE (BioLegend®, San Diego, CA, USA) per million cells in 100 μL PBS 0.5% BSA and incubated at room temperature in the dark for 1hr. The cells were washed by 1 mL PBS with 0.5% BSA. The supernatant was aspirated, and the cells were resuspended by PBS with 0.5% BSA. Cells were then analyzed with PE channels on a FACSAria™II (BD Biosciences, Franklin Lakes, NJ, USA) and data was processed by FlowJo software.

GFAAS analysis of cellular Pt contents of A2780, A2780cis, and HEK293 cells. A2780, A2780cis and HEK293 cells were seeded in a 6-well plate at a concentration of 2 × 10^5 cells per well and incubated at 37 ^{o}C, 5% CO_2 overnight. These cells were treated with FALP1 or cisplatin ([Pt] = 10 μM) for 4 h at 37 ^{o}C, 5% CO_2. The remaining alive cells were harvested by trypsinization and counted. The cells were then digested in 200 μL 65% HNO_3 at r.t. overnight. The Pt contents in the cells were analyzed by GFAAS. All experiments were performed in triplicate.

GFAAS analysis of cellular Pt contents of A2780cis cells treated with SSO. The A2780cis cells treated with 200 μM Sulfo-N-succinimidyl Oleate (SSO, Cayman Chemical Company, Michigan, USA) in RPMI media without FBS and PS to avoid the fatty acid presence in FBS, and incubated 30 min at 37 ^{o}C, 5% CO_2. Then, the RPMI media was removed and fresh complete RPMI media was added in the wells. FALP1 or cisplatin (10 μM) was added into cells

and incubated 4 h at 37 ^{o}C, 5% CO_2. The Pt contents in the cells were analyzed by GFAAS following the abovementioned procedure.

MTT assays of SKOV3 cells treated with conditioned media. SKOV3 cells were plated at 2 x 10^4 cells/mL in a 96-well plate overnight. Media was aspirated and 125 µL of adipocyte-conditioned media was added. After 72 h, the conditioned media was replaced with 100 µL complete media and the normal MTT protocol was followed. MTT assays were completed to determine the cytotoxicity of prodrugs compared to cisplatin. 100 µL of a medium containing 4 x 10^4 cells/ mL was seeded in a 96-well plate. The plate was incubated for 24 h to allow for adherence of cells. 50 µL of cell medium with various concentrations of Pt compounds was added, and the cells were incubated. After 72 h, 0.8 mg/mL MTT in PBS was added to the cell medium. After 2-4 h, the medium was aspirated, and 200 µL of DMSO was added to each well. The plate was left on a shaker for 10 minutes. Then, the absorbance of purple formazan was recorded at 570 nm with a BioTek ELx800 plate reader. The IC_{50} values were determined using Origin software. All experiments were performed in triplicate.

MitoSOX assays. A2780cis cells were seeded in 2 mL of cell medium at a cell density of 5 x10^4 cells/ mL in 10 mm petri dishes and incubated overnight. Cells were then treated with 60 µM cisplatin, 10 µM FALP1, 1 µM FALP2, or 1 µM FALP3 for 24 h. The medium was aspirated, and cells were washed with 1 mL PBS. Then, the cells were incubated with 5 µM MitoSOX reagent in fresh medium for 60 min at 37 ^{o}C in the dark. The cells were trypsinized and collected. The cell pellet was washed 2 times with 1 mL PBS with 0.5% BSA. The cells were then resuspended with PBS with 0.5% BSA to 10^5 cells/ mL and analyzed 10,000 events with FACSAria™II and data processed with FlowJo.

Mitostatus assays. A2780cis cells were seeded at a cell density of 5 x 10^4 cells/ mL in 5 mL of medium in 6-well plate. After incubating overnight, 60 µM cisplatin, 10 µM FALP1, 1 µM FALP2, or 1 µM FALP3 for 24 h. The medium was removed, cells were washed 2 times with 1 mL PBS, and 5 mL medium was added to each well. 200 nM MitoStatus reagent was added to the cell medium and incubated in the dark at 37 ^{o}C for 30 min. The stain-containing medium was aspirated, and the cells were washed 2 times with 1 mL PBS. The cells were trypsinized, collected, and washed with 1 mL PBS. The cell pellet was then resuspended in PBS to 10^5 cells/ mL and analyzed via with FACSAria™II at 10,000 events and analyzed with FlowJo software.

MTT assays of cisplatin and FALP1 against A2780, A2780cis, and HEK293 cells. The cells (A2780, A2780cis, or HEK293) were seeded in 96-well microplates in 100 µL cell suspensions (2×10^4 cells per mL) per well to begin and were incubated for 24 h at 37 ^{o}C, 5% CO_2. Next, 50 µL volume of RPMI or DMEM with various concentrations of Pt compounds was added to each well of the microplates. The cells were then incubated for an additional 24 h at 37 °C, 5% CO_2. Next, a volume of 30 µL MTT (Alfa Aesar) (5 mg mL^{-1} in PBS) was added to the cells and then the cells were incubated for an additional 2-4 h at 37 ^{o}C, 5% CO_2. The solutions were then aspirated, leaving behind insoluble purple formazan. A volume of 200 µL DMSO was added to the wells and the plates were shaken for 10 min. Next, the microplates were analyzed for absorbance at 562 nm with an ELx800 absorbance reader (BioTek, Winooski, VT, USA). Finally, the data were analyzed using Origin software to produce dose-response curves and to determine IC_{50} values. All experiments were performed in triplicate.

MTT assays of cisplatin and FALP1 against SKOV3 cells treated with conditioned media. SKOV3 cells were plated at 2 x 10^4 cells/mL in a 96-well plate overnight. Media was aspirated and 125 µL of adipocyte-conditioned media was added. After 72 h, the conditioned media was replaced with 100 µL complete media, and cell viability was determined by following the abovementioned MTT assays protocol.

MTT assays of cisplatin and FALP1-3 against A2780cis cells. The A2780cis cells were incubated with cisplatin or FALP1-3 for 72 h in a 96-well plate. After 72-h incubation, cell viability was determined by following the abovementioned MTT assays protocol.

Cell confluence assays of cisplatin and FALP1-3 against A2780cis cells. Cytotoxicity profiles of cisplatin and FALP1-3 against A2780cis were evaluated by the cell confluence assays. A volume of 100 µL of RPMI containing 2 x 10^4 cells/mL was seeded in a 96-well plate. The plate was incubated for 24 h to allow for adherence of cells. A volume of 50 µL of RPMI with various concentrations of Pt compounds was added to each well of the microplates. After 72 h, a volume of 100 µL fresh medium was added to each well of the microplates, and the cell confluence was recorded with a BioTek Cytation 5 plate reader. IC_{50} values were determined using Origin software. All experiments were performed in triplicate.

Apoptosis assays. A2780cis cells seeded in a 6-well plate at a concentration of 2 x 10^5 cells/well and incubated for 24 h at 37 ^oC. Next, the cells were treated with cisplatin, while the fourth well was kept as a control. Cells were then incubated for 72 h at 37 ^oC. The medium was collected in clean 15 mL falcon tubes along with washed PBS solution. 1mL trypsin was added to the wells. After 5 min, cell suspensions were transferred to the falcon tubes that contained the media and PBS and centrifuged at 400–500x g at 4 ^oC for 5min. The cell pellet was re-suspended in 1 mL PBS and the cells were counted. The cell pellet was collected again and the appropriate amount of 1x binding buffer was added to reach a concentration of 10^6 cells/mL. 100-µL cell suspensions were added to new 2-mL Eppendorf tubes and 5 µL Annexin V-FITC was added to one tube and 5 µL of propidium iodide (PI) solution was added to other. Cells were gently vortexed and incubated at r.t. for 15 min in the dark. 400-µL 1x binding buffer was added to each Eppendorf tube and the cell suspensions were transferred to flow cytometry tubes. Flow cytometry analysis was done using FL-1 and FL-2 channels on a FACSAria™II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

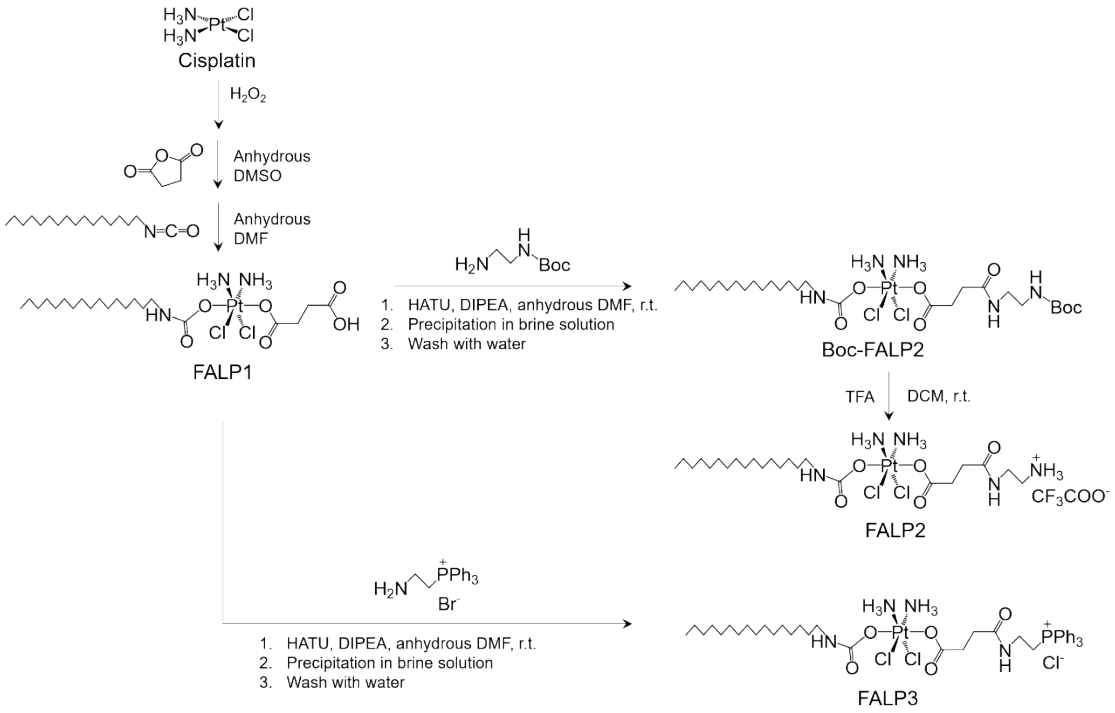


Fig S1. Synthesis of the fatty acid-like Pt(IV) prodrugs (FALP1-3).

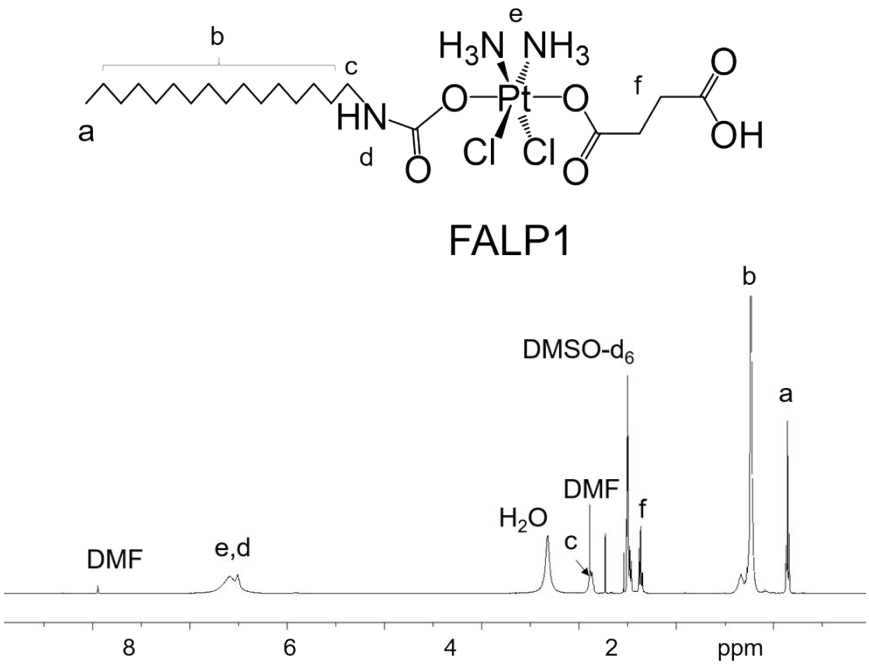


Fig S2. ^{1}H NMR spectrum of FALP1in DMSO-d_6.

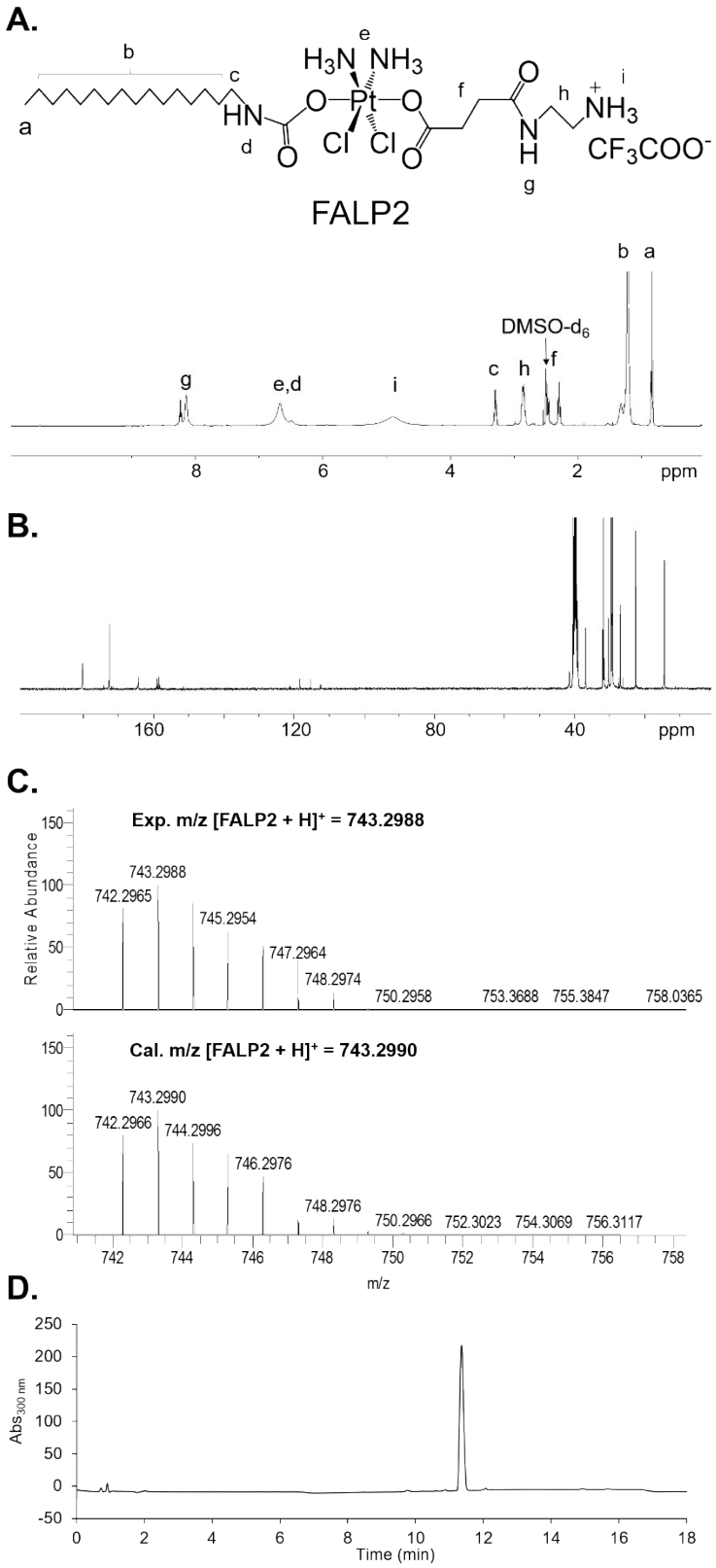


Fig S3. Characterization of FALP2: **A.** ^{1}H NMR spectrum in DMSO-d_6; **B.** ^{13}C NMR spectrum in DMSO-d_6; **C.** High resolution ESI-MS spectrum; **D.** Analytic HPLC analysis (Gradient: 0 min 5% B, 5 min 5% B, 10 min 95% B, 15 min 95% B, 18 min 5% B. solvent A is 0.1% TFA aqueous solution and B is acetonitrile).

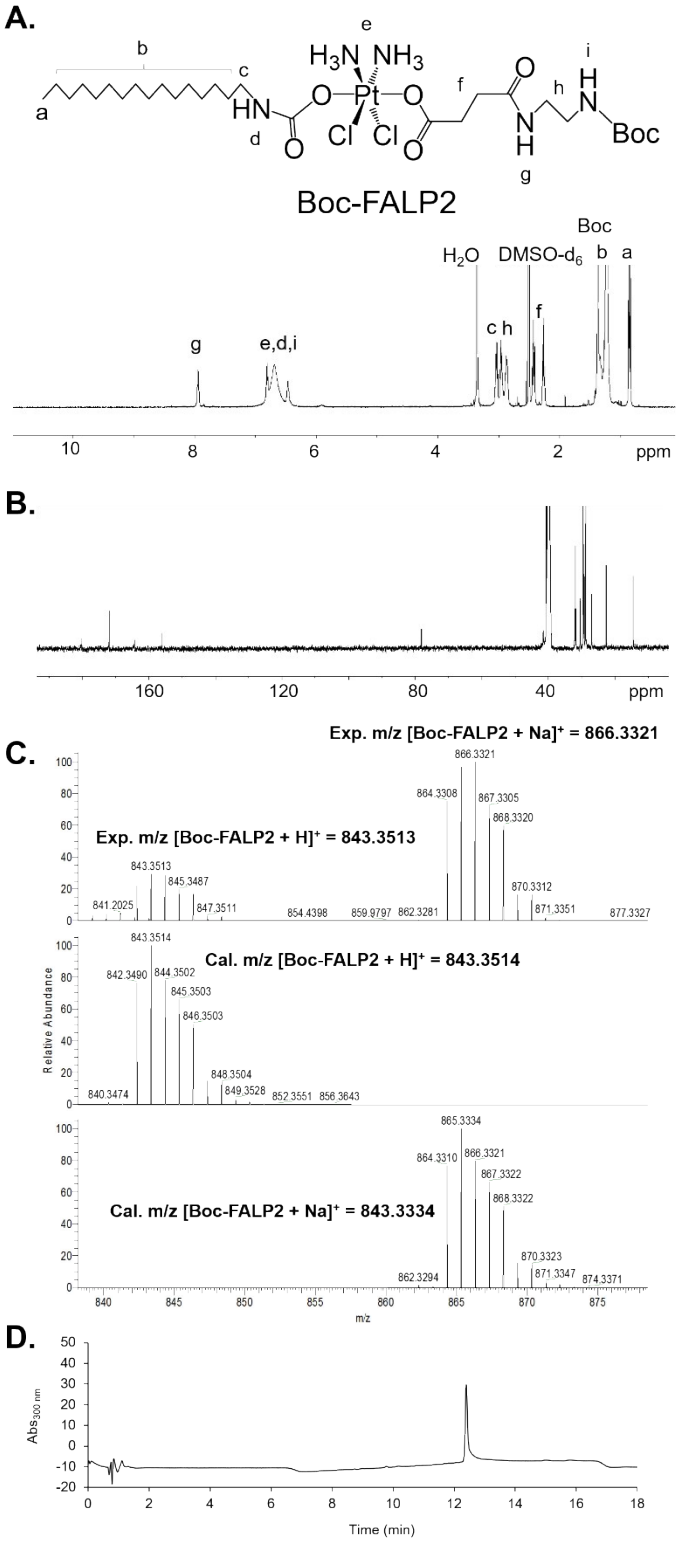


Fig S4. Characterization of Boc-FALP2: **A.** ^{1}H NMR spectrum in DMSO-d_6; **B.** ^{13}C NMR spectrum in DMSO-d_6; **C.** High resolution ESI-MS spectrum; **D.** Analytic HPLC analysis (Gradient: 0 min 5% B, 5 min 5% B, 10 min 95% B, 15 min 95% B, 18 min 5% B. solvent A is 0.1% TFA aqueous solution and B is acetonitrile).

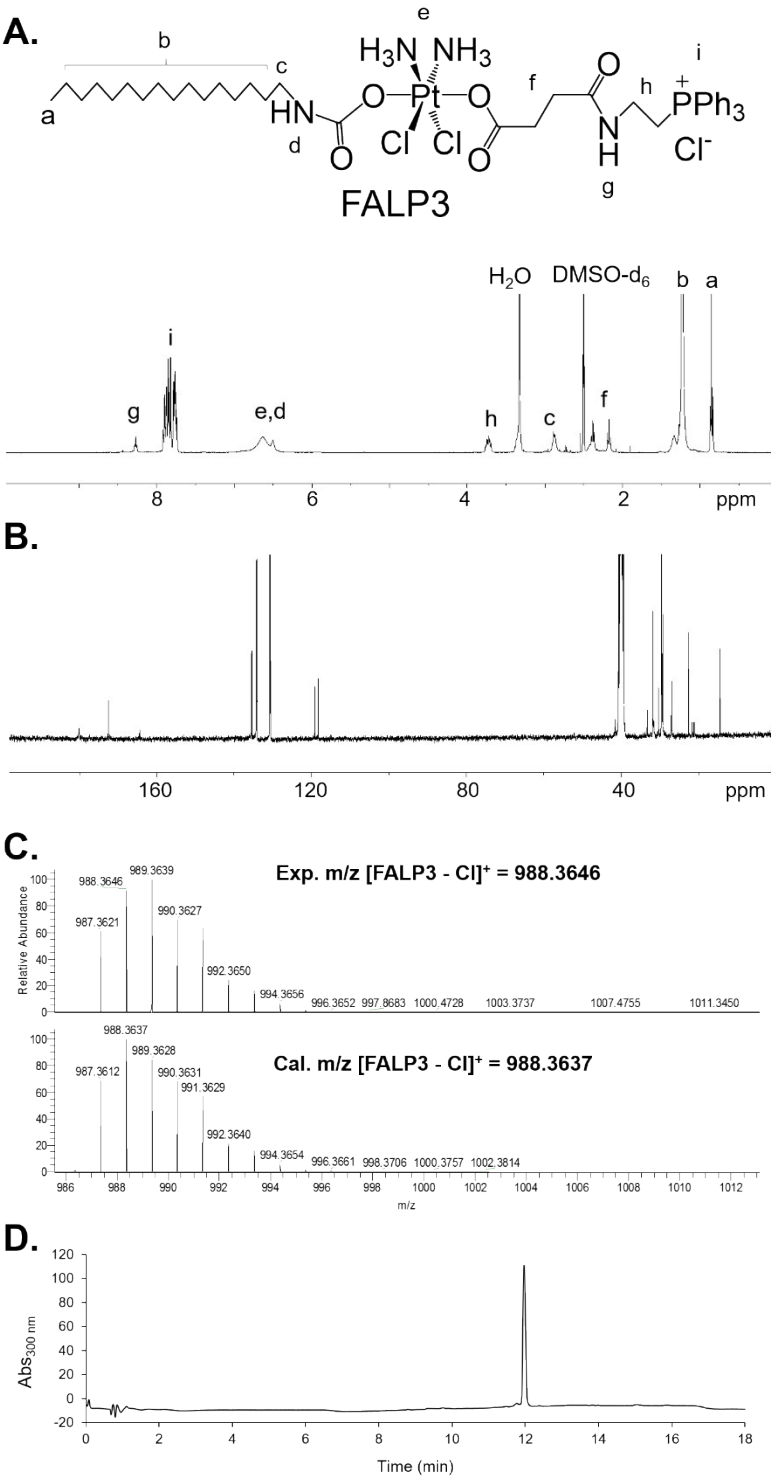


Fig S5. Characterization of FALP3: **A.** ^{1}H NMR spectrum in DMSO-d_6; **B.** ^{13}C NMR spectrum in DMSO-d_6; **C.** High resolution ESI-MS spectrum; **D.** Analytic HPLC analysis (Gradient: 0 min 5% B, 5 min 5% B, 10 min 95% B, 15 min 95% B, 18 min 5% B. solvent A is 0.1% TFA aqueous solution and B is acetonitrile).

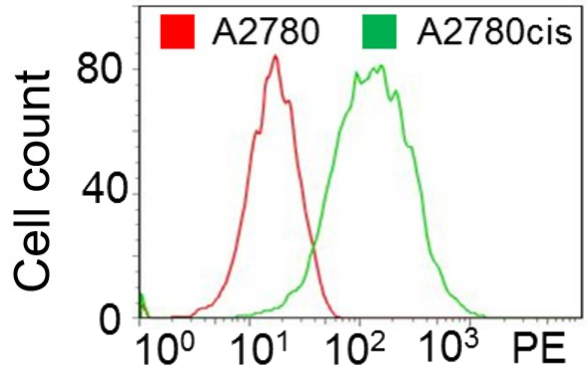


Fig S6. Flow cytometric analysis of CD36 protein levels in A2780 and A2780cis ovarian cancer cells.

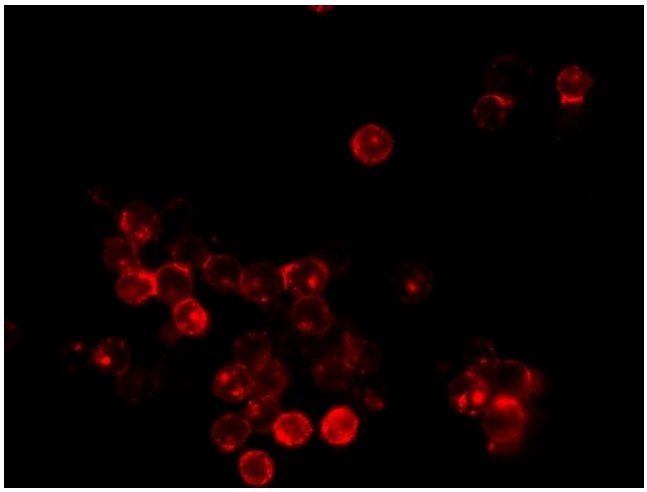


Fig S7. The cell image of the immunostained A2780cis cells using CD36-PE.

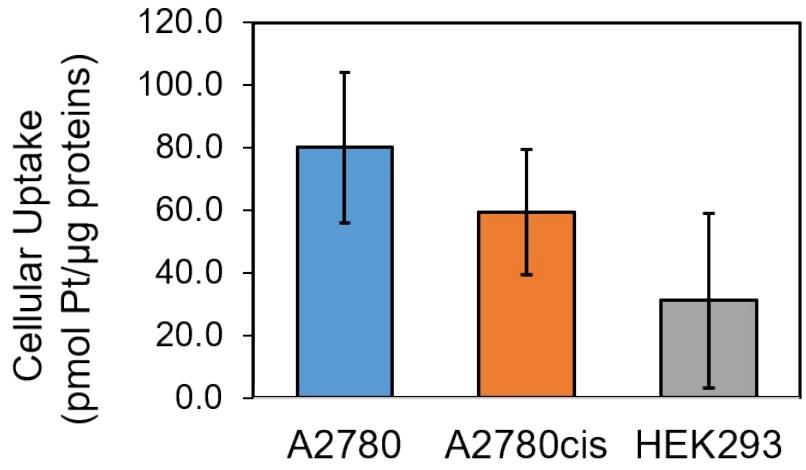


Fig S8. GFAAS analysis of cellular uptake of cisplatin (10 µM, 4 h) against A2780, A2780cis, and HEK293 cells.

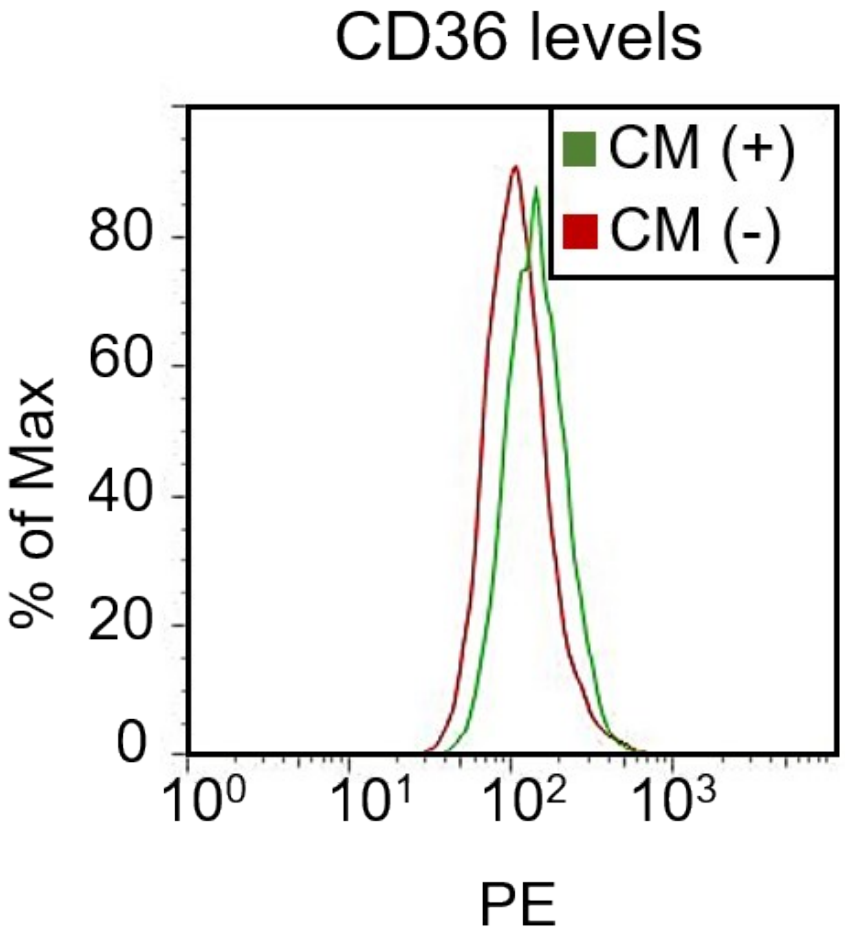


Fig S9. Flow cytometric analysis of protein levels of CD36 in SKOV3 cells treated with or without the conditioned medium (CM +) or (CM -) of the adipocytes differentiated from 3T3-L1 (72 h).

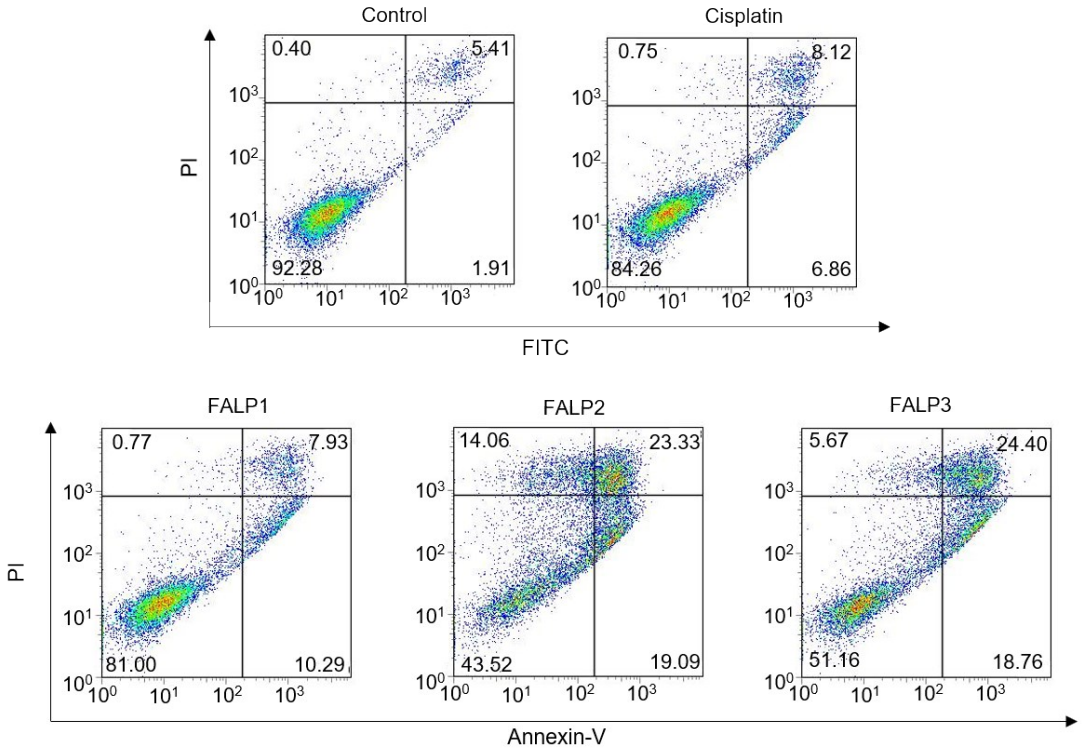
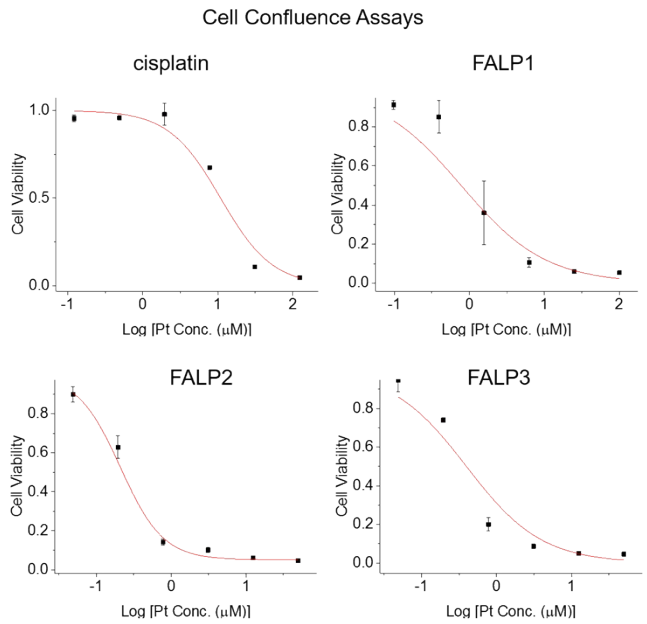


Fig S10. Flow cytometric analysis of apoptosis of the A2780cis cells treated with cisplatin (60 μM), FALP1 (10 μM), FALP2 (1 μM), and FALP3 (1 μM) for 72 h at 37 ^{o}C, 5% CO_2.

A

A2780cis Ovarian Cancer Cells	IC_{50} (μM) Cell Confluence Assays (72 h)	IC_{50} (μM) MTT Assays (72 h)
Cisplatin	17.35±1.40	9.16±1.84
FALP1	3.55±2.99	1.01±0.23
FALP2	0.80±0.75	0.24±0.03
FALP3	0.58±0.38	0.31±0.20

B



C

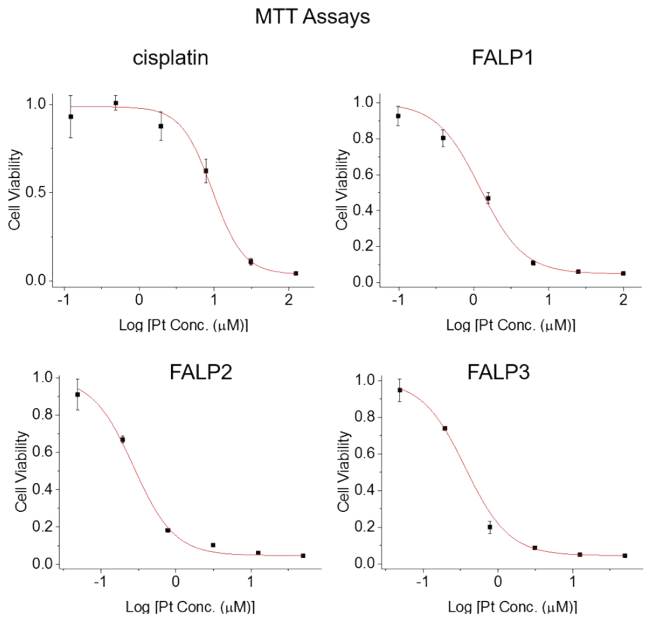


Fig S11. Cytotoxicity profiles determined by cell confluence and MTT assays: **A.** The table of IC_{50} values of cisplatin, FALP1, FALP2, and FALP3 against A2780cis cells with 72-h incubaiton at 37 ^{o}C, 5% CO_2; **B.** Representative killing curves determined by cell confluence assays; **C.** Representative killing curves determined by MTT assays.

Reference

(1) Zheng, Y.-R.; Suntharalingam, K.; Johnstone, T. C.; Yoo, H.; Lin, W.; Brooks, J. G.; Lippard, S. J. Pt(IV) Prodrugs Designed to Bind Non-Covalently to Human Serum Albumin for Drug Delivery. *J. Am. Chem. Soc.* **2014,** *136* (24), 8790.