Aryl-hydrocarbon receptor-based drug-screening strategy for hepatocellular carcinoma based on dynamic fluorescence imaging

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Experimental Section

Materials and Reagents

All chemical reagents were purchased from Shanghai xianding biotechnology co., LTD. Analytical grade solvents were used without further purification. LX-2 cells were purchased from Boster Biological Technology co. ltd.

Instruments

UV-2600 UV-visible spectrophotometer (Shimadzu, Japan) was utilized to analyze absorption spectra. The F-4600 HITACHI fluorescence spectrophotometer was used to detect the fluorescence spectra. Utilizing the Bruker maXis ultra-high resolution-TOF MS equipment, mass spectra were identified. Using Bruker NMR spectrometers, ^{1}H NMR spectra were measured at 400 MHz, while ^{3}C NMR spectra were recorded at 100 MHz. Using a Leica TCS SP8 confocal laser scanning microscope, fluorescence imaging of cells was performed. Using the Perkinelmer IVIS Lumina III, in vivo NIR imaging of mice was measured.

Synthesis of ArP.

Synthesis of compound 1 refered to this work[1]. 1 mmol 362 mg compound 1, and 2 mmol 378 mg 4-hydroxyquinoline-2-carboxylic was dissolved to 10 mL tetrahydrofuran(THF), then stirred in room temperature under nitrogen protection for 10 min. 277 mg, 1 mmol 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride was added into the solution and reacted for another 3 h. Then, the THF was removed by vacuum distillation. ArP was purified by chromatography on silica gel with dichloromethane/ methanol(10:1) as faint yellow powder(9%). HRMS (ESI): m/z calcd for $C_{36}H_{27}N_3O_2$ $[M]^+$ 533.2175, found in 533.2186. ^{1}H NMR (400 MHz, Methanol-d_4) δ 9.71 (s, 1H), 8.49 (d, J = 0.8 Hz, 2H), 8.21 (dd, J = 8.0, 1.7 Hz, 7H), 7.54 (d, J = 2.6 Hz, 2H), 7.11 – 7.07 (m, 5H), 6.96 – 6.93 (m, 8H), 6.89 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) ^{1} δ 170.92, 168.81, 162.03, 158.27, 154.58, 149.61, 144.43, 141.55, 141.25, 137.92, 132.75, 132.52, 131.84, 131.39, 129.90, 128.72, 128.23, 127.82, 127.32, 126.35, 124.61, 122.81, 122.45, 121.60, 118.00, 117.82, 115.13, 113.62, 111.29, 104.89, 100.22, 72.21, 71.31, 65.66, 63.41, 42.85.

Fluorescent Spectra Measurement

The effect of viscosity on the AIE process was measured. Fluorescent spectra of ArP (5 μM) in methanol-glycerinum mixture with different glycerinum fractions. For the polarity, fluorescent spectra of ArP (5 μM) in methanol-PBS mixture with different water fractions. The F-4600 HITACHI fluorescence spectrophotometer was used to detect the fluorescence spectra.

Calculation of the LOD

The detection limit was calculated employing the following formula: Detection limit=3σ/k, where σ is the standard deviation of blank measurement, k is the slope between the fluorescence intensity and the concentration of AhR.

Cell Culture

Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin was used to culture Raw 264.7 and LX-2 cells. Cells were grown at 37 °C in a humidified environment of 5% CO_2. Medium replacements were made one or two days. LX-2 cells were collected using pancreatin and Raw 264.7 cells were collected by DMEM washing.

Cytotoxicity Assays

LX-2 cells were seeded in two 96-well plates at a concentration of 1×10^5 and cultured for 12 h. In one 96-well plates, cells were cultured with1 mM, 500 μM, 200 μM, 100 μM, 10 μM, 5 μM, 1 μM, 100 nM, 10 nM (concentrations calculated using ArP) AhRPH for 12 h. The medium volume of every well was 100 μL. Then, 10 μL CCK-8 solution was added to each well and incubated for another 1 h at 37 °C. After that, the absorbance at 450 nm was detected in a Triturus microplate reader.

Fluorescence Imaging of Cells In Vitro

LX-2 cells were seeded on the round coverslips in 6-well plates and then treated cells with different reagents. For AhR imaging, cells incubated with 100 μL 5 μM AhRPH (determinated by absorption of ArP) PBS solution (1% DMF) for 30 minutes. Then the cells were washed with PBS 3 times, and imaged under the confocal laser scanning microscope with a blue channel (λ_{ex}=405 nm (intensity=5%), λ_{em}=570 nm- 670 nm).

In Vivo Toxicity Assays

C57BL/6J mice (male, 4-6 weeks) were housed in a pathogen-free, temperature controlled facility with 12-hour light/dark cycles. Animals received food and water ad libitum. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals". Animal protocols were approved by the Ethics Committee of Linyi University (LYU20241002).

Mice were divided into two groups (6 mice per group). The mice in control group were given 100 μL PBS (pH=7.4) solution via intravenous injection per day for 7 days. The mice in experimental group were given 100 μL 100 μM AhRPH (calculated using ArP)) PBS solution (pH=7.4) via intravenous injection per day for 7 days. Their body weights were recorded every day.

Establishment of Orthotopic Mouse Model of HCC

C57BL/6J mice (male, 4-6 weeks) were subcutaneous injected with 1×10^7 HEPA 1-6 cells at the armpit of the right anterior limb. After 2-3 weeks, the mice were sacrificed by anaesthetic overdose. The grown tumors were separated and cut into small pieces (1-2 mm^3). Then, these pieces of tumor were transplanted to the livers of other healthy mice through surgical operation. To preserve the activity of tumor pieces, surgical operation time should be completed within 30 minutes.

Fluorescence Imaging of HCC Mice

The control group sham surgery group. Orthotopic mouse models of HCC were randomly divided into several groups (n=5). Mice in sorafenib group were treated with 60 mg/kg/day sorafenib (dissolved in 90% olive oil and 10 % DMSO) by intragastric administration for 14 days. Mice in lenvatinib group were intragastric administrated with 6 mg/kg/day lenvatinib for 14 days. Mice in regorafenib group were intragastric administrated with 60 mg/kg/day regorafenib for 14 days. 50 μL 50 μM AhRPH PBS solution (1% DMF) was injected intravenously into the mice and imaged using IVIS Spectrum in vivo imaging system after 30 min. λ_{ex}= 560 nm, λ_{em}=620 nm and 780 nm.

CYP1A1, CYP1B1, MRP1 and MDR1 Measurement

Excised tumor tissues (100 mg) or cells (1×10^6) were homogenized in protease inhibitor-containing protein extraction buffers (1 mL).CYP1A1, CYP1B1, MRP1 and

MDR1 were tested using ELISA kits in accordance with the manufacturer's recommendations.

Statistical Analysis

Values in this study were expressed as mean ± standard deviation (SD). Differences between two groups were analyzed by using two-tailed, unpaired Student's t test. P value < 0.05 was considered statistically significant.

Data Availability

All relevant data that support the findings of this study are available from the corresponding author upon reasonable request.

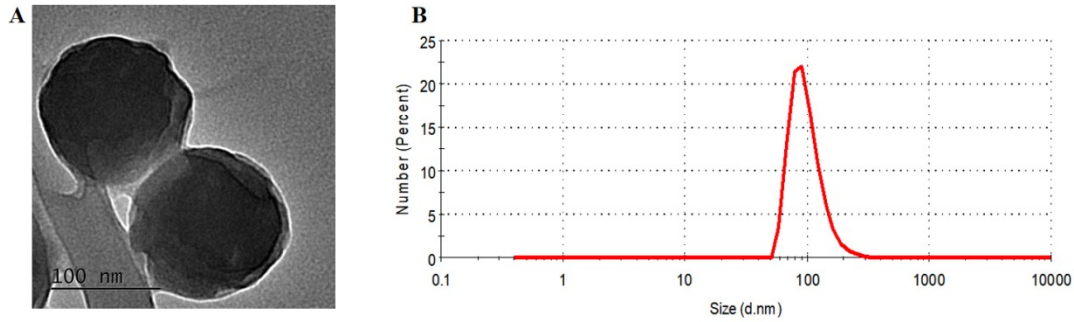


Fig. S1 AhRPH is a spherical material with a particle size of about 100 nm.

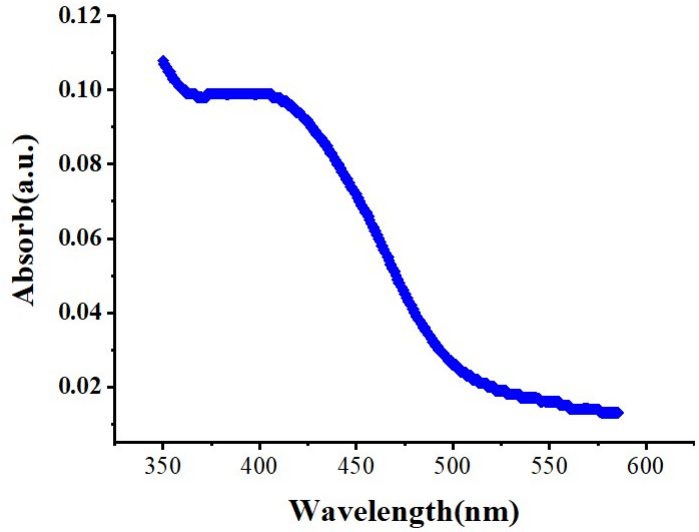


Fig. S2 Absorption of AhRPH.

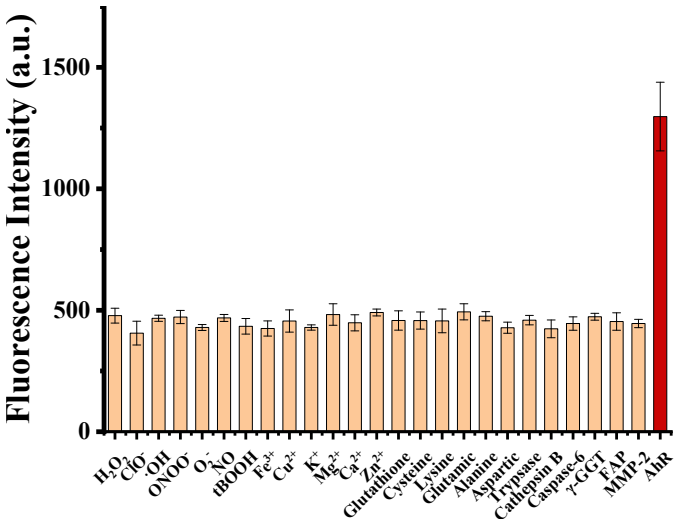


Fig. S3 Selectivity of AhRPH to several interferential factors. 5 μM ArP was introduced in these experiments. λex= 410 nm. The data are expressed as mean ± SD. 1 mM H_2O_2, 100 μM ClO^-, 100 μM •OH, 100 μM $ONOO^-$, 100 μM $O_2^{\bullet-}$, 100 μM NO, 100 μM ButOOH, 100 μM Fe^{3+}, 1 mM Cu^{2+}, 1 mM K^+ ,1 mM Mg^{2+}, 1 mM Ca^{2+}, 100 μM Zn^{2+}, 100 μM glutathione, 100 μM cysteine, 100 μM lysine, 100 μM glutamic, 100 μM alanine, 100 μM aspartic, 1 mg/mL Tyrpsase, 0.5 μg/mL Cathepsin B, 0.1 μg/mL Caspase-6, 1 U γ-GGT, 0.1 μg/mL FAP, 0.1 μg/mLMMP-2, 1 μg/mL AhR.

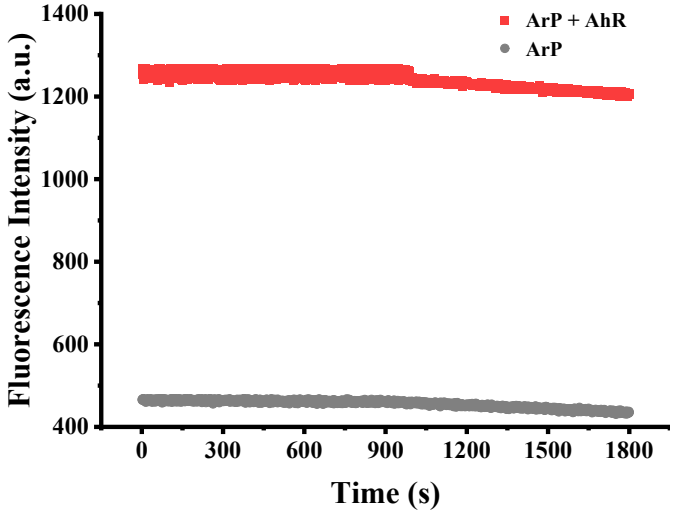


Fig. S4 Time-dependent fluorescence intensity of ArP (5 μM) with and withot AhR (750 ng/mL in PBS). The data are expressed as the mean ± SD. Error bars represent standard deviations of three separate measurements.

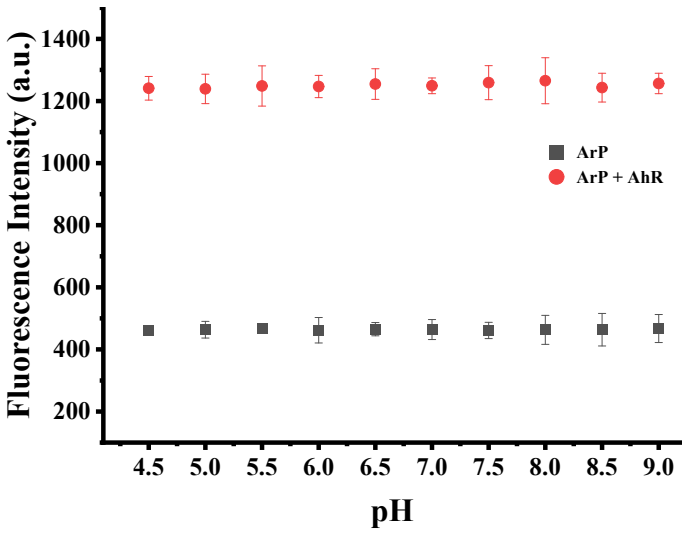


Fig. S5 Fluorescence enhancement of ArP (5 μM) with and without AhR (750 ng/mL in PBS) in different pH. The data are expressed as the mean ± SD. Error bars represent standard deviations of three separate measurements.

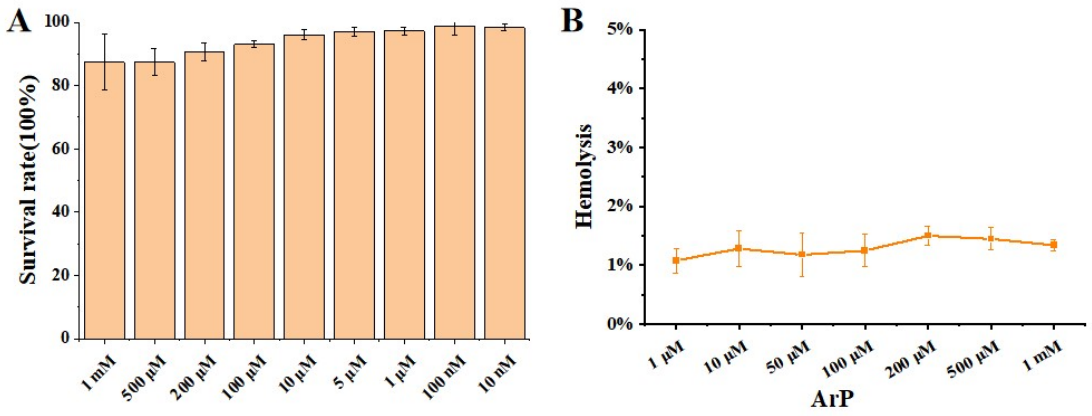


Fig. S6 The biocompatibility of AhRPH. (A) 1 mM, 500 μM, 200 μM, 100 μM, 10 μM, 5 μM, 1 μM, 100 nM, 10 nM (concentrations calculated using ArP) AhRPH was incubated with hepatic stellate cells(HSCs). (B) Hemolysis test of ArP from 1 μM to 1 mM.

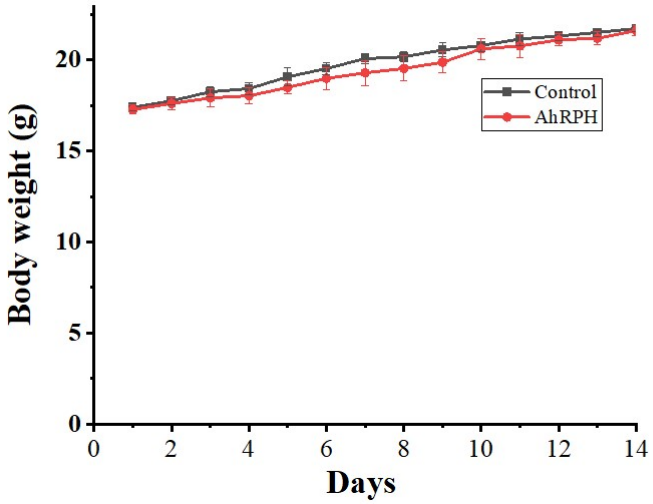


Fig. S7 Administer 10 nM AhRPH to mice and monitor their body weight changes over a two-week period.

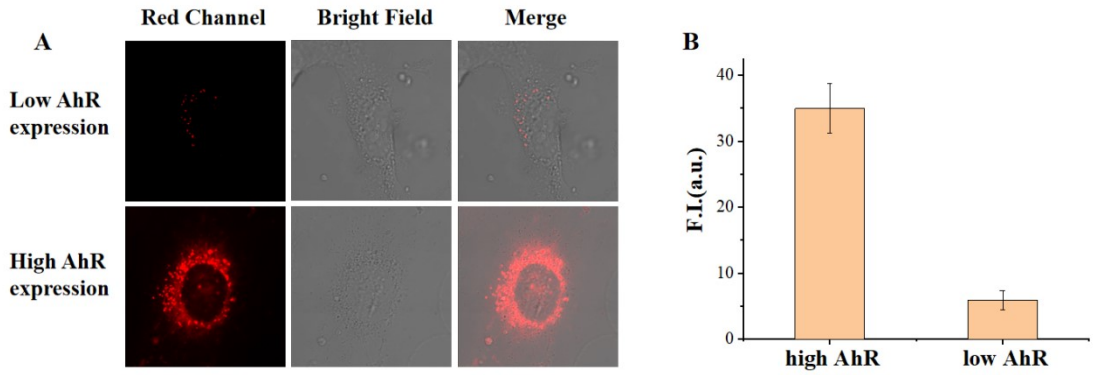


Fig. S8 AhR expression in HSCs. LX-2 cells with high AhR expression (2 ng/mL TGF-β for 24 hours) and low AhR expression(2 ng/mL TGF-β for 6 hour) was added with 5 μM AhRPH was and imaged under the excitation of 405 nm after 30 min..

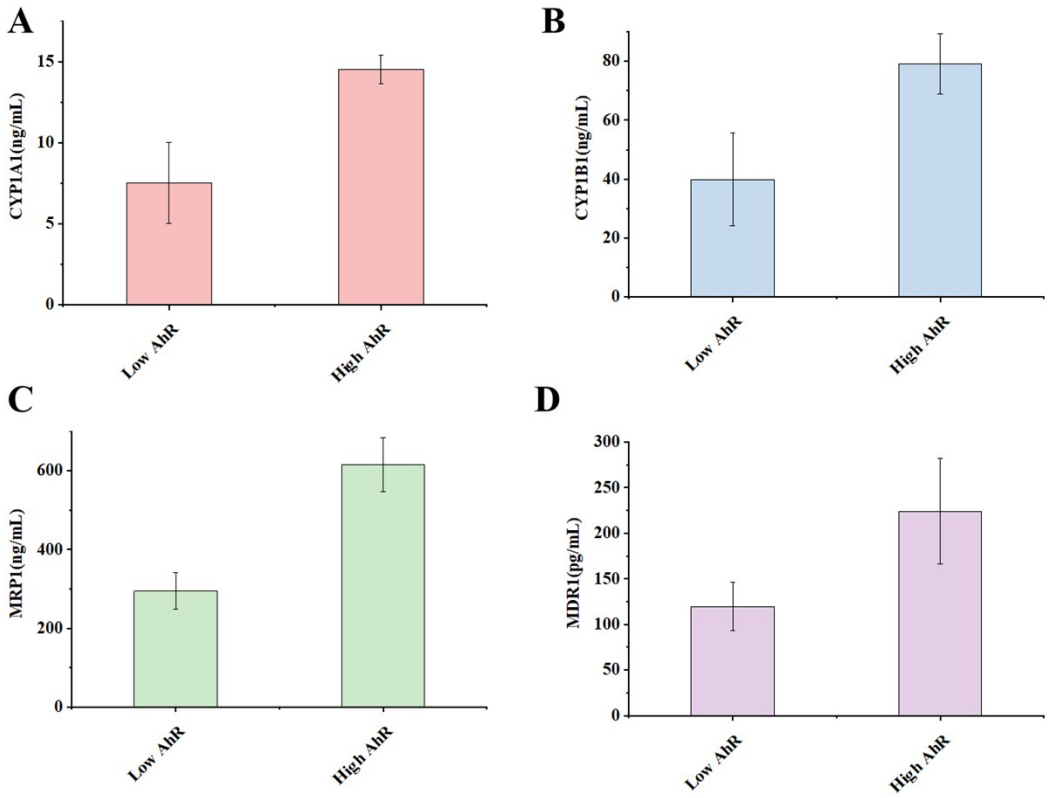


Fig. S9 (A)CYP1A1, (B)CYP1B1, (C) MRP1 and (D) MDR1 expressions in LX-2 cells with high AhR expression (2 ng/mL TGF-β for 24 hours) and low AhR expression(2 ng/mL TGF-β for 6 hour).

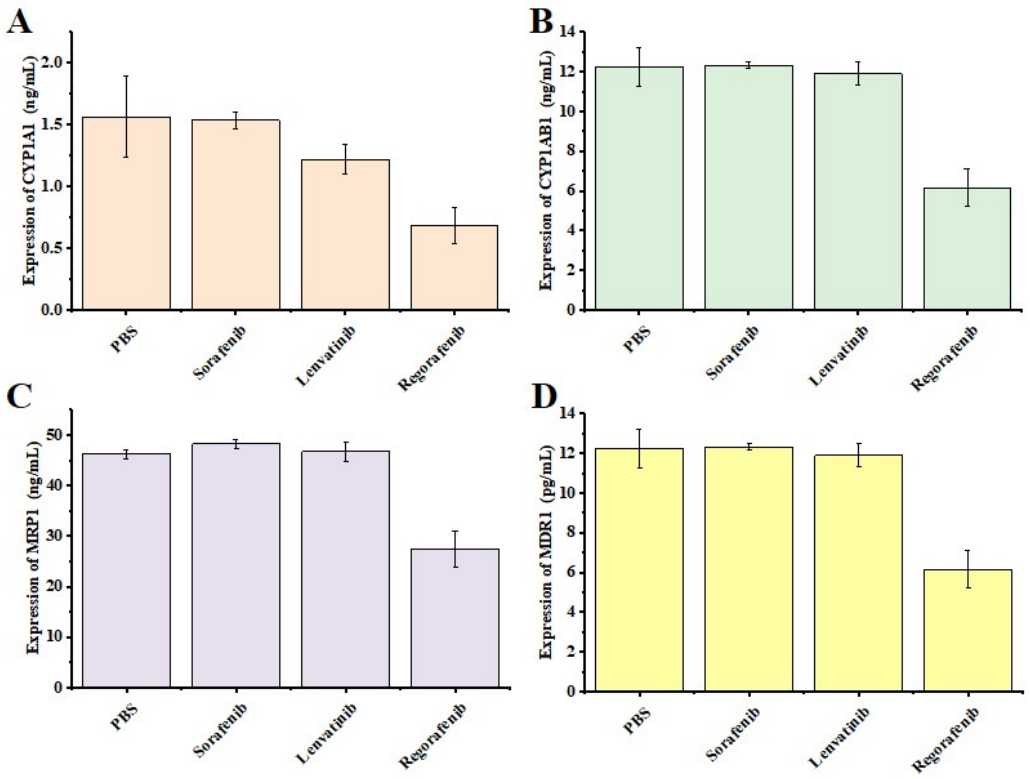
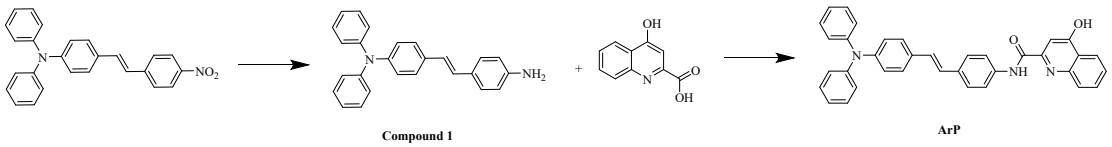
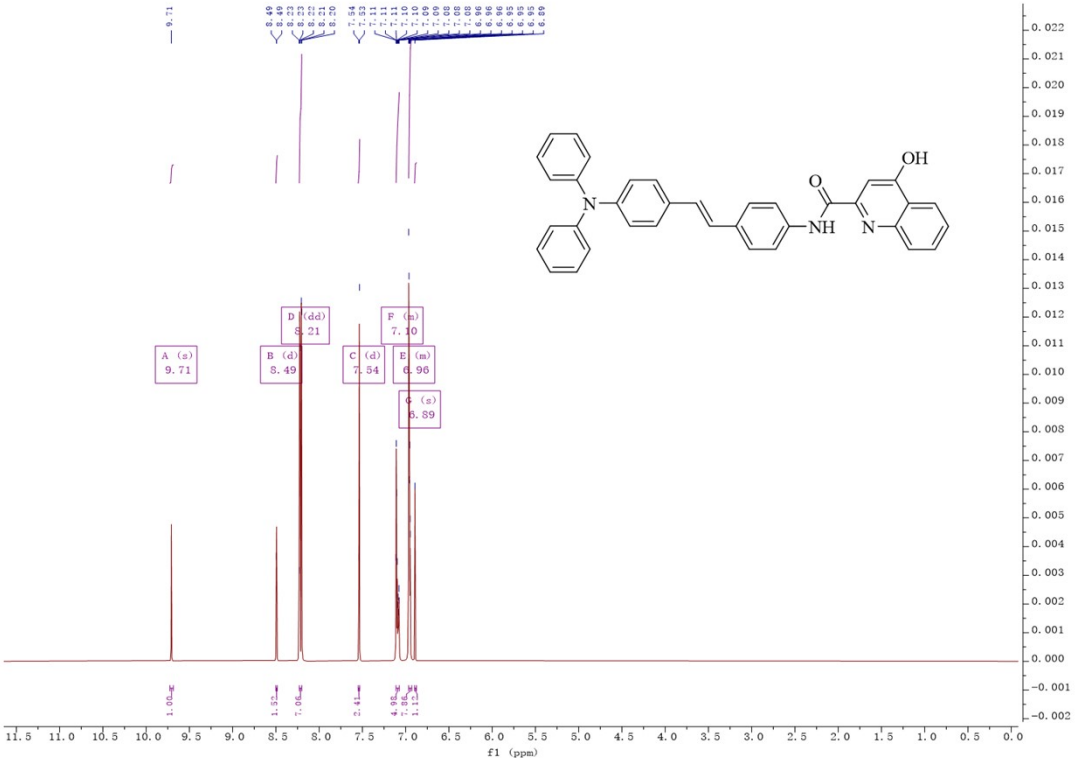


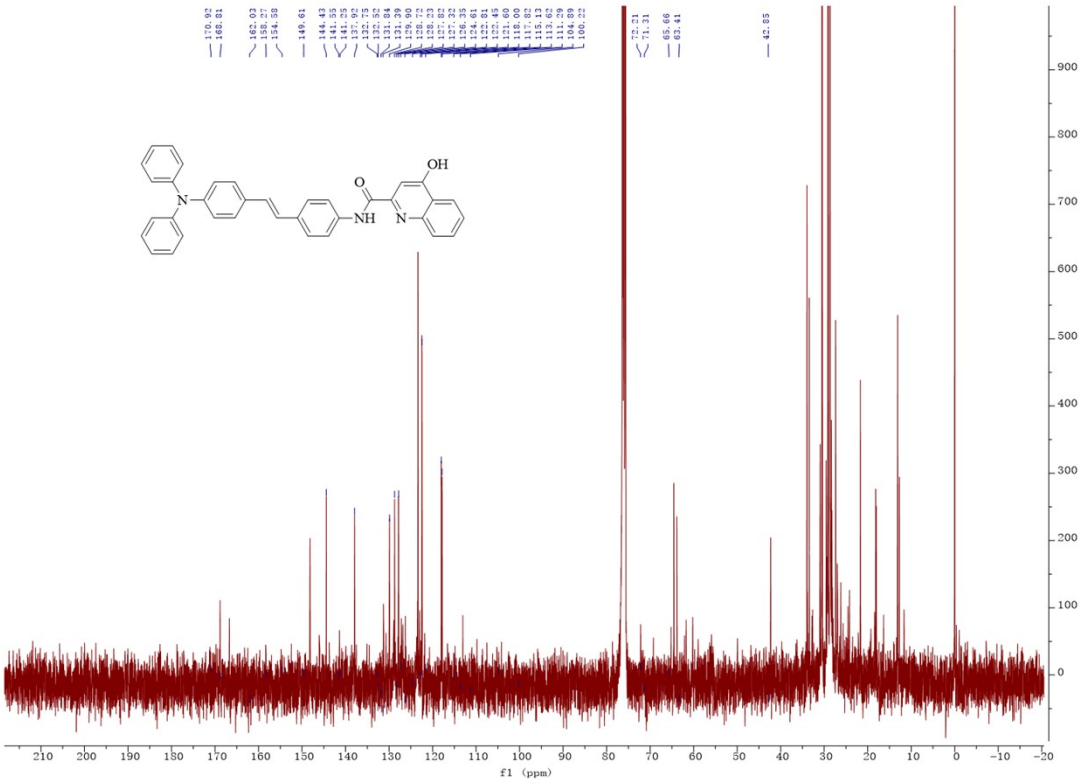
Fig. S10 The effects of therapeutic agents on the expressions of drug resistance-related markers in HCC mice. (A)CYP1A1, (B)CYP1B1, (C) MRP1 and (D) MDR1 expressions in HCC mice treated with PBS, sorafenib, lenvatinib, and regorafenib. The data are expressed as mean ± SD. n = 3.



Synthetic route of **ArP**



The ^{1}H NMR spectra of ArP.



The ^{13}C NMR spectra of ArP.

References

1. Zhou, H.; Zheng, Z.; Xu, G.; Yu, Z.; Yang, X.; Cheng, L.; Tian, X.; Kong, L.; Wu, J.; Tian, Y., 1, 3, 5-Triazine-cored derivatives dyes containing triphenylamine based two-photon absorption: Synthesis, optical characterization and bioimaging. *Dyes and pigments* **2012,** *94* (3), 570-582.