Electronic Supplementary Information

iso-pencillixanthone A from a marine-derived fungus reverses multidrug resistance in cervical cancer cells through down-regulating P-gp and re-activating apoptosis

Li Chen,[a] Xinxin Li,[a] Miaomiao Cheng,[a] Siyuan Wang,[a] Qiuhong Zheng*[b] and Qinying Liu*[b]

[a]Institute of Biomedical and Pharmaceutical Technology, Fuzhou University, Fuzhou 350002, P. R. China

[b]Fujian Provincial Key Laboratory of Tumor Biotherapy, Fujian Cancer Hospital & Fujian Medical University Cancer Hospital, Fuzhou 350014, P. R. China. E-mails: zqh2858@foxmail.com; liuqy@fjmu.edu.cn; Tel.: +86-591-8366-0063

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Detailed Experimental Methods.

Figure S1. HR-(+) ESI-MS Spectrum of iso-PXA.
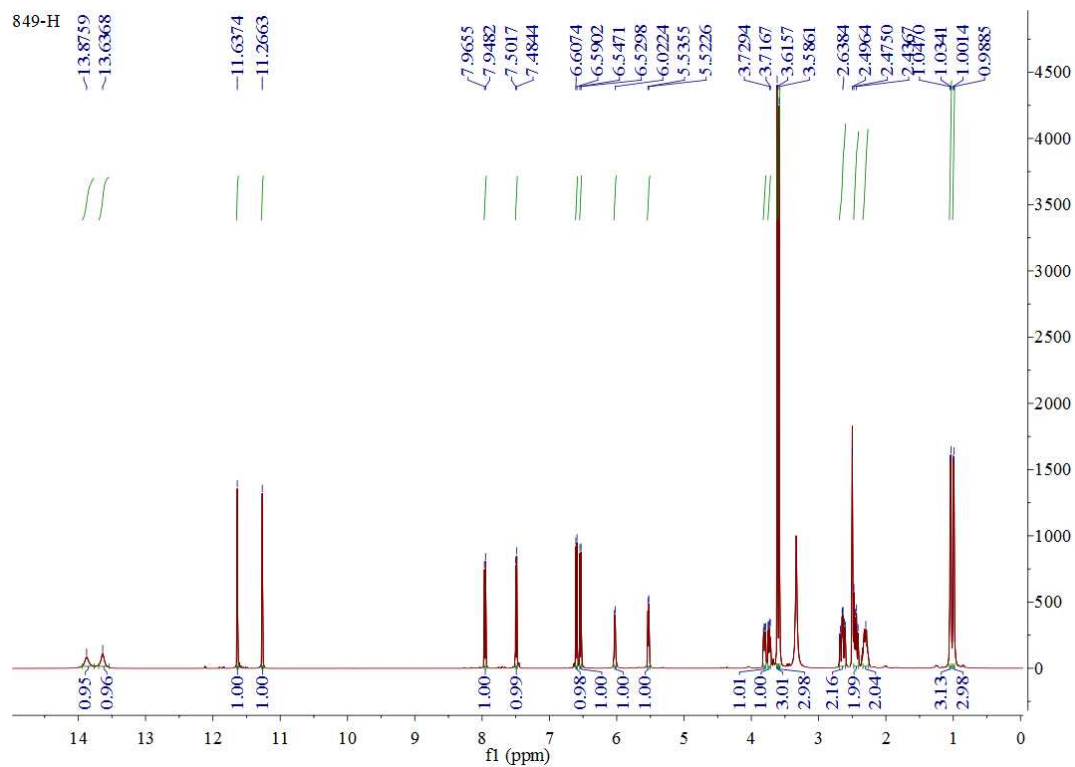
Figure S2. ^{1}H NMR (500 MHz, DMSO-d_6) spectrum of iso-PXA.
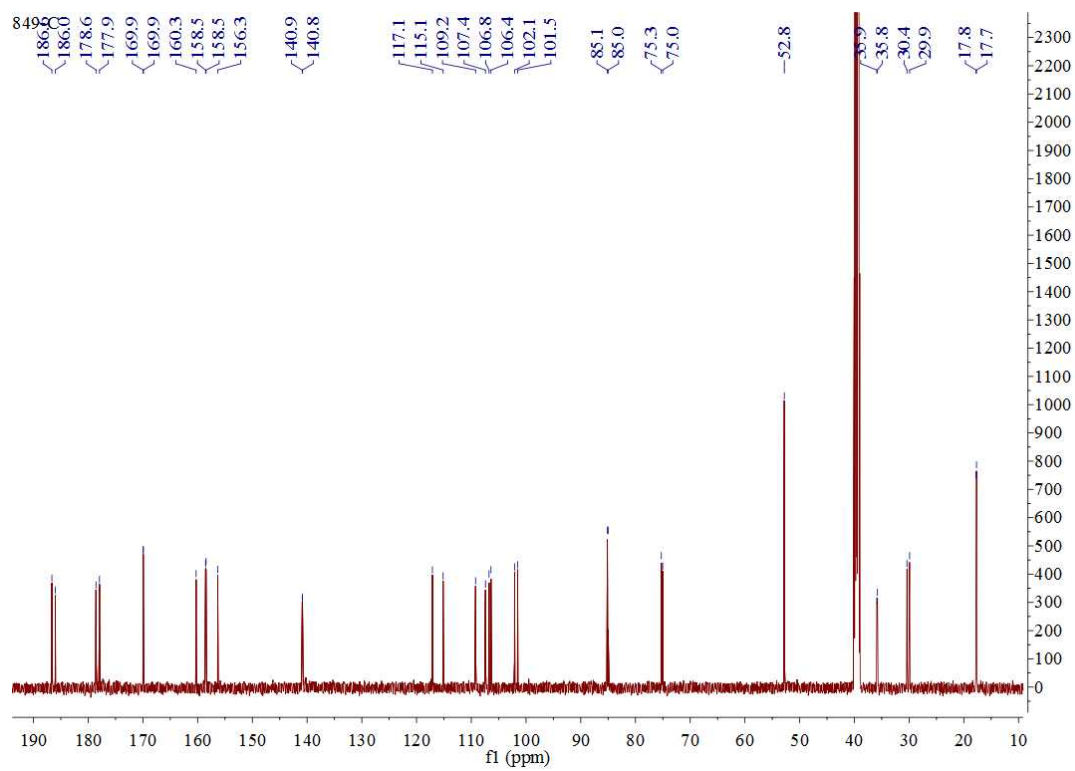
Figure S3. ^{13}C NMR (125 MHz, DMSO-d_6) spectrum of iso-PXA.
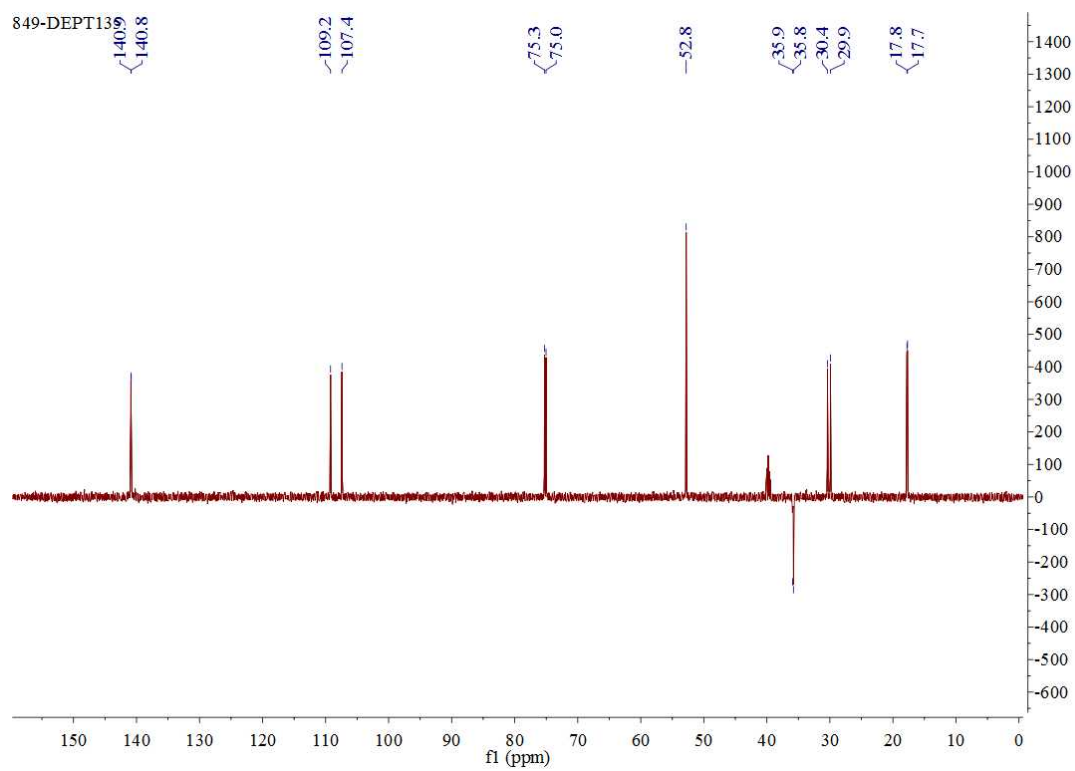
Figure S4. DEPT 135° Spectrum of iso-PXA (in DMSO-d_6).
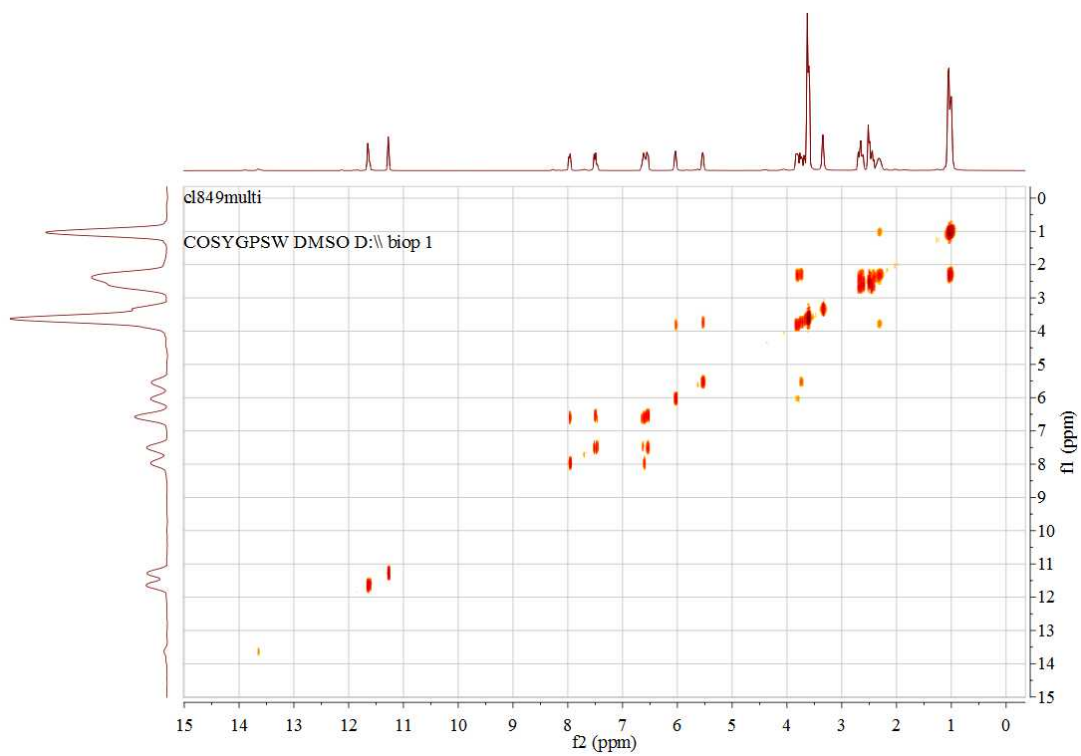
Figure S5. ^{1}H-^{1}H COSY Spectrum of iso-PXA (in DMSO-d_6).
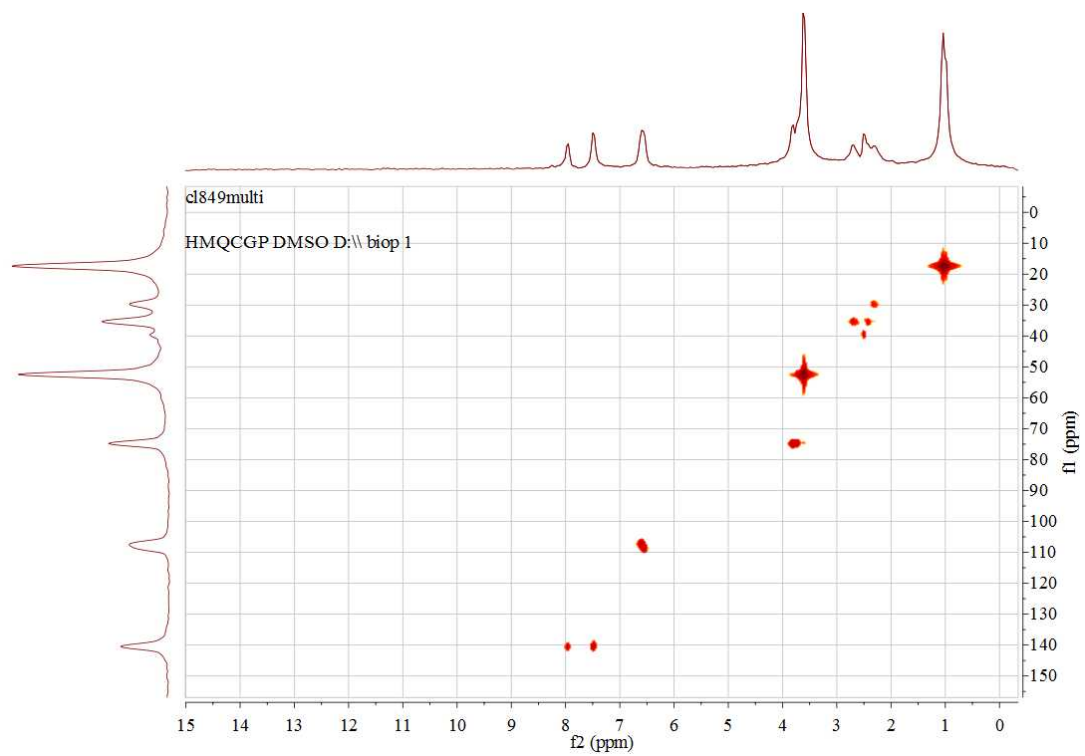
Figure S6. HMQC Spectrum of iso-PXA (in DMSO-*d₆*).
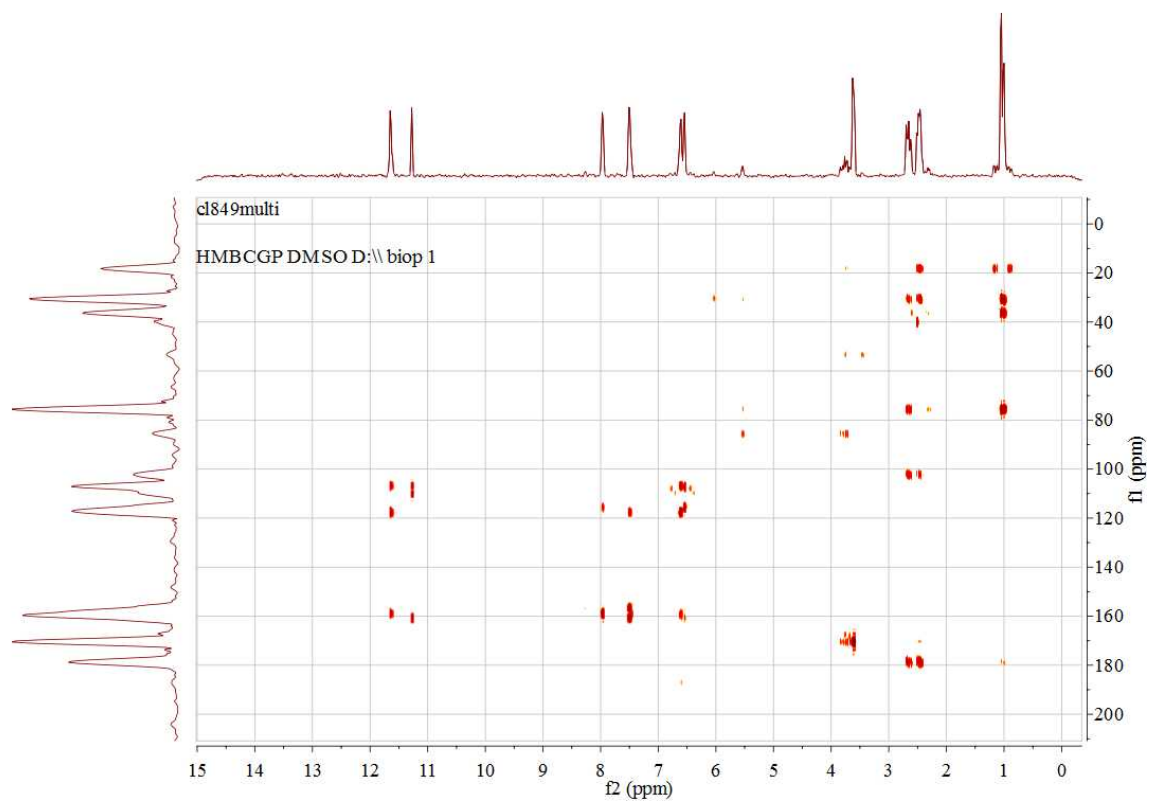
Figure S7. HMBC Spectrum of iso-PXA (in DMSO-d_6).

P. oxalicum

Static culture   28 °C/30 day/60L

Gauze filter

Mycelia

Acetone:H_2O Extracting three times
=4:1

   Vacuum concentration

Aqueous solution

EtOAc   Extracting three times

   Vacuum concentration

Crude extract (36.5 g)

Si gel CH_2Cl_2:MeOH

A   B   C (12.7 g)   D   E

LH-20 CH_2Cl_2:MeOH=1:2

C_1 C_2 C_3 (4.9 g) C_4

HPLC   55% CH_3CN, 0.1% TFA

iso-PXA (510 mg)

Figure S8. The progress chart of extraction and purification for iso-PXA.
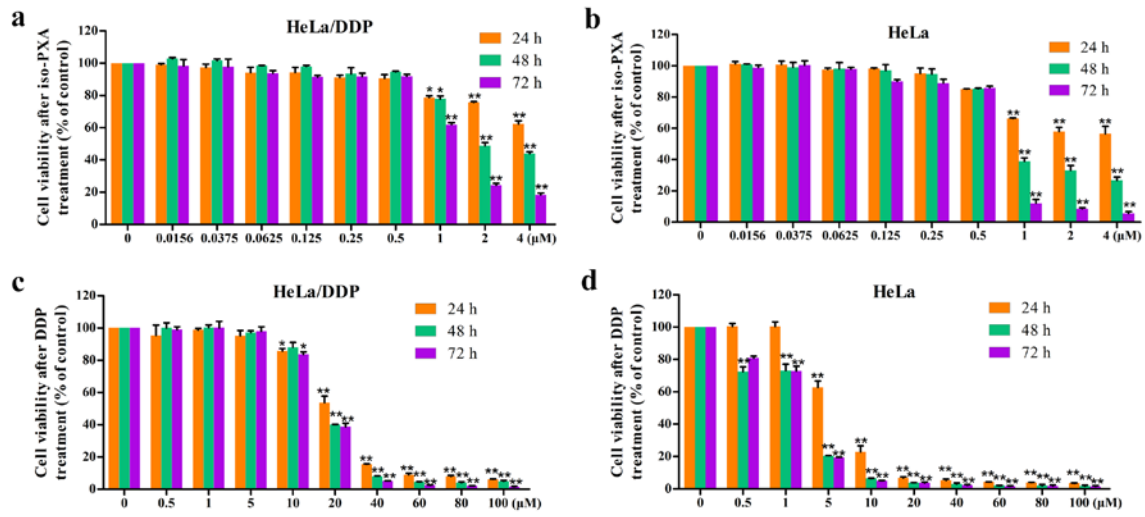
Figure S9. Cytotoxicity of iso-PXA and DDP on HeLa/DDP and HeLa cells was determined by WST-1 assay. a. Cytotoxicity of iso-PXA on HeLa/DDP cells. b. Cytotoxicity of iso-PXA on HeLa cells. c. Cytotoxicity of DDP on HeLa/DDP cells. d. Cytotoxicity of DDP on HeLa cells. Data are the means ± SD of at least three independent experiments.

Table S1 500 MHz ^{1}H and 125 MHz ^{13}C NMR Data for iso-PXA (in DMSO-d_6)

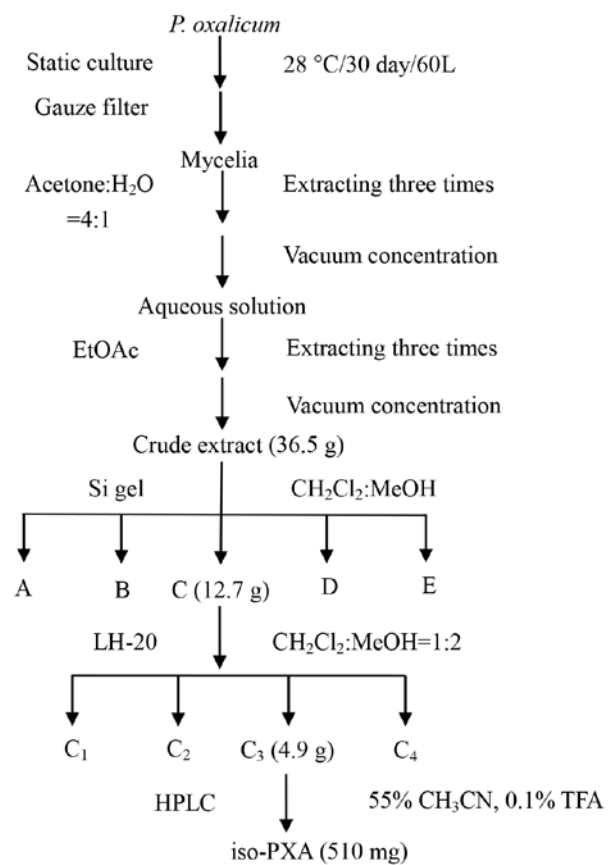| NO.	δ_C	δ_H (J in Hz)	NO.	δ_C	δ_H (J in Hz)
1	158.5 s		1'	160.3 s	
2	117.1 s		2'	109.2 d	6.54 (1H, d, 8.7)
3	140.9 d	7.96 (1H, d, 8.7)	3'	140.8 d	7.49 (1H, d, 8.7)
4	107.4 d	6.60 (1H, d, 8.7)	4'	115.1 s	
4a	158.5 s		4a'	156.3 s	
5	75.3 d	3.80 (1H, dd, 11.0, 5.5)	5'	75.0 d	3.73 (1H, dd, 11.0, 6.5)
6	30.4 d	2.30 (1H, m)	6'	29.9 d	2.30 (1H, m)
7	35.9 t	a 2.66 (1H, dd, 19.1, 6.2) b 2.48 (1H, dd, 19.1, 5.9)	7'	35.8 t	a 2.63 (1H, dd, 19.1, 8.2) b 2.44 (1H, dd, 19.1, 11.5)
8	177.9 s		8'	178.6 s	
8a	101.5 s		8a'	102.1 s	
9	186.6 s		9'	186.0 s	
9a	106.4 s		9a'	106.8 s	
10a	85.1 s		10a'	85.0 s	
11	17.8 q	1.04 (3H, d, 6.5)	11'	17.7 q	0.99 (3H, d, 6.5)
12	169.9 s		12'	169.9 s	
13	52.8 q	3.59 (3H, s)	13'	52.8 q	3.62 (3H, s)
OH-1		11.64 (1H, s)	OH-1'		11.27 (1H, s)
OH-5		6.03 (1H, d, 5.5)	OH-5'		5.53 (1H, d, 6.5)
OH-8		13.64 (1H, s)	OH-8'		13.88 (1H, s)

Table S2 Inhibitory activity of iso-PXA against cancer cell lines for 48 h

Cell line	IC_{50} (μM)	Cell line	IC_{50} (μM)
PLC/PRF/5	0.807 ± 0.038	A375	1.500 ± 0.002
Huh-7	1.110 ± 0.136	SW620	0.781 ± 0.043
SK-Hep-1	0.905 ± 0.034	SW480	1.573 ± 0.078
SPC-A1	0.979 ± 0.026	LOVO	2.096 ± 0.148
SK-MES-1	1.472 ± 0.063	CNE1	1.142 ± 0.055
95D	1.388 ± 0.069	CNE2	1.518 ± 0.075
U937	1.325 ± 0.002	BGC-823	1.455 ± 0.054
MDA-MB-231	1.995 ± 0.029	SGC-7901	1.200 ± 0.094
Raji	1.588 ± 0.070	HGC-27	1.358 ± 0.035
Jeko-1	1.067 ± 0.001	HFF-1	26.73 ± 1.002
KYSE450	1.186 ± 0.080	LO2	8.238 ± 0.158
EC9706	0.862 ± 0.045		

Data are the means $\pm$ SD of at least three independent experiments.

Table S3 The cytotoxicity of drugs to the corresponding resistant cell lines for 48 h

Drugs	Cell Line	IC$_{50}$ (µM)	Drugs	Cell Line	IC$_{50}$ (µM)
VCR	HeLa	0.013 ± 0.003	iso-PXA	HeLa	0.409 ± 0.019
	HeLa/VCR	1.199 ± 0.021		HeLa/VCR	0.341 ± 0.005
DDP	HeLa	1.805 ± 0.060	iso-PXA	HeLa	1.145 ± 0.017
	HeLa/DDP	17.77 ± 0.317		HeLa/DDP	2.633 ± 0.069
DDP	A549	26.18 ± 1.520	iso-PXA	A549	1.019 ± 0.008
	A549/DDP	82.81 ± 1.718		A549/DDP	0.924 ± 0.081
ADM	K562	0.216 ± 0.042	iso-PXA	K562	9.464 ± 0.049
	K562/A02	33.21 ± 1.203		K562/A02	3.605 ± 0.066
ADR	MCF-7	0.294 ± 0.023	iso-PXA	MCF-7	2.493 ± 0.070
	MCF-7/ADR	3.680 ± 0.197		MCF-7/ADR	3.325 ± 0.014

Data are the means ± SD of at least three independent experiments.

Table S4 Inhibition effect of iso-PXA on tumor growth of HeLa/VCR xenograft model

Group	Drug	Concentration (mpk)	Mice member (initial/end)		Body weight (initial/end)		Tumor weight (g)	Inhibition rate (%)
		0	5	5	19.2 ± 0.1	22.7 ± 0.6	3.971 ± 0.17	--
		2.5	5	5	19.0 ± 0.2	21.1 ± 0.9	$1.796\pm0.29^{**}$	54.7%
5	Iso-PXA	5	5	5	18.9 ± 0.3	21.6 ± 0.8	$0.880\pm0.19^{***}$	77.8%
		10	5	5	19.1 ± 0.2	21.4 ± 0.7	$0.435\pm0.29^{***}$	89.1%
	VCR	2.5	5	5	18.9 ± 0.3	22.9 ± 0.7	$2.465\pm0.16^{*}$	37.9%

The experiment was carried out using nude mice implanted subcutaneously (sc) with HeLa/VCR (1.2×10^{7}/0.1 ml) cells under the left hind legs. Animals were randomized into five groups including control, three desired dosages of iso-PXA and VCR, i. g, q. d. $\times$ 15. At the end of the experiment, body weight, tumor weight and tumor volume were recorded. Data are means $\pm$ SD of the tumor weight for each group of 5 experimental animals. *P < 0.05, **P < 0.01, ***P < 0.001 *v.s.* control group.

Table S5 Primers for the genes used in RT-qPCR test

Gene		Sequences
PARP	forward	5'- GGGATGGTCAGTGTTGATGGA -3'
	reverse	5'- GCTATCGTGGTGGCAAACAATA -3'
CyclinB1	forward	5'- GTACCATGGCGCTCAGGGTCAC -3'
	reverse	5'- CTAGATTATGCTTTTGTCACGGC -3'
caspase-3	forward	5'- GAAATTGTGGAATTGATGCGTGA -3'
	reverse	5'- CTACAACGATCCCCTCTGAAAAA -3'
caspase-8	forward	5'- TGTTGGAGGAAAGCAATCTG -3'
	reverse	5'- CCTGGTGTCTGAAGTTCCCT -3'
caspase-9	forward	5'- CTCAGACCAGAGATTCGCAAAC -3'
	reverse	5'- GCATTTCCCCTCAAACTCTCAA -3'
Bcl-2	forward	5'- CGACTTTGCAGAGATGTCCA -3'
	reverse	5'- ATGCCGGTTCAGGTACTCAG -3'
Bax	forward	5'- CCTTTTCTACTTTGCCAGCAAAC -3'
	reverse	5'- GAGGCCGTCCCAACCAC -3'
P-gp	forward	5′- GGGATGGTCAGTGTTGATGGA -3′
	reverse	5′- GCTATCGTGGTGGCAAACAATA -3′
Survivin	forward	5'- CGCGGGACCCGTTGGCAGAG -3'
	reverse	5'- GGAATTCGGCAGCTCCGGCCAGAGG -3'
FBW7	forward	5'- CGACGCCGAATTACATCTGTC -3'
	reverse	5'- CGTTGAAACTGGGGGTTCTATCA -3'
Mcl-1	forward	5'- GTGCCTTTGTGGCTAAACACT -3'
	reverse	5'- AGTCCCGTTTTGTCCTTACGA -3'
MAPK	forward	5′- TACACCAACCTCTCGTACATCG -3′
	reverse	5′- CATGTCTGAAGCGCAGTAAGATT -3′
PTEN	forward	5′- TTTGAAGACCATAACCCACCAC -3′
	reverse	5′- ATTACACCAGTTCGTCCCTTTC -3′
EGFR	forward	5′- TTGCCGCAAAGTGTGTAACG -3′
	reverse	5′- GTCACCCCTAAATGCCACCG -3′
β-catein	forward	5′- CATCTACACACTTTGATGCTGCT -3′
	reverse	5′- GCAGTTTTGTCAGTTCAGGGA -3′
JNK	forward	5′- TGTGTGGAATCAAGCACCTTC -3′
	reverse	5′- AGGCGTCATCATAAAACTCGTTC -3′
SAPK	forward	5′- GAAACTAAGCCGTCCTTTTCAGA -3′
	reverse	5′- TCCAGCTCCATGTGAATAACCT -3′
GAPDH	forward	5′-GAAGGTGAAGGTCGGAGTC-3′
	reverse	5′-GAAGATGGTGATGGGATTTC-3′

Detailed Experimental Methods

Reagents

iso-PXA (purity > 98%) was isolated from the metabolites of marine-derived *P. oxalicum*. Paclitaxel (PTX), doxorubicin (DOX), vincristine (VCR), cisplatin (DDP), adriamycin (ADR), nocodazole, verapamil (VRP), propidium iodide (PI), acridine orange (AO), ethidium bromide (EB) and rhodamine123 (Rh123) were purchased from Sigma Chemical (St. Louis, MO, USA). Paraformaldehyde (PFA) and WST-1 were purchased from Roche (Roche, Indianapolis, USA). Primary antibodies against P-gp, PARP, cyclin B1, caspase-3, caspase-8, caspase-9, Bax, Bcl-2, Mcl-1 and β-actin were supplied by Cell Signaling Technology (Danvers, MA, USA). Primary antibodies against Bid and cytochrome c were supplied by Abcam (Cambridge, MA, USA). FBXW7/Cdc4 antibody was purchased from R&D Systems (Minneapolis, MN, USA). The primary antibody against Cofilin and the HRP-conjugated secondary antibodies against rabbit and mouse were obtained from BOSTER Biological Technology Co., Ltd. (Wuhan, China). All reagent water used was pretreated with a Milli-Q apparatus (Millipore Corporation, Darmstadt, Germany). Other chemicals were purchased from Aladdin (Shanghai, China). All chemicals except iso-PXA were of the highest purity commercially available.

Culture of Cell Lines

All of the human sensitive cancer cell lines were purchased from Shanghai Cell Resource Center (Shanghai, China). The K562, K562/AO2 and HeLa/VCR cell lines were a generous gift from the Fujian Medical University. The resistant cell lines

(MCF-7/ADR and A549/DDP) were purchased from BoGu Biotech Co., Ltd. (Shanghai, China). HeLa/DDP was purchased from Fenghui Biotech Co., Ltd. (Hunan, China). All of the cell lines were cultured in DMEM or RPMI1640 (HyClone, USA) with 10% fetal bovine serum (GEMINI, China), penicillin (100 U/ml) and streptomycin (100 μg/mL) at 37 °C in a 5% (v/v) CO_2 humidified atmosphere.

Cytotoxicity Assay

Cell viability was assessed by the WST-1 cell proliferation assay kit (Roche, Indianapolis, IN, USA). Briefly, 100 μl of cell suspension were cultured in 96-well plate and treated with gradient concentrations of iso-PXA. After 48 h, one-tenth volume of WST-1 solution was added. After cells were incubated at 37 °C for 4 h, the absorbance at 450 nm was measured. The cell proliferation inhibition of 50% (IC_{50}) was analyzed by GraphPad Prism 5.0 software. The resistant index (RI) was calculated by the ratio of the IC_{50} of the resistant cells to that of parental cells.

Real-time Cell Proliferation Assay Analysis

Cell proliferation was determined using the DP version of the xCELLigence real-time cell analyzer (RTCA) (ACEA, San Diego, CA, USA), which recorded changes in impedance (reported as a cell index (CI)) over a prolonged time course in a noninvasive system. Briefly, the background impedance of RTCA DP E-Plates 16 (ACEA, SanDiego, USA) was performed using the standard protocol provided in the software with 50 μL of media. 5×10^3 cells were seeded with 100 μL of RPMI-1640 and left to equilibrate at room temperature for 30 min. Cells were allowed to grow for 24 h before adding iso-PXA to the cultures at the indicated concentrations in duplicate.

The cell index (CI) data of the proliferating cells were recorded and expressed as mean of CI normalized to the CI recorded at the time of cells treatment compared to untreated cells.

Cell Cycle Analysis

The cell cycle assays were performed by flow cytometry (Beckman Coulter FC500, Brea, CA, USA). Briefly, cells were incubated with gradient concentrations of iso-PXA (0, 0.25, 0.5 and 1 µM) and VCR (1 µM) for 24 h and then collected, fixed and permeabilized with cold 70 % (v/v) ethanol at 4 °C overnight. After being washed with cold PBS, cells were resuspended in 1.0 mL stained solution containing 100 µg/mL of DNase-free RNase A and 50 µg/mL of propidium iodide and then measured by flow cytometry (Beckman Coulter FC500, Brea, CA, USA) to analyze cell cycle. A minimum of 10,000 events were analyzed in each experiment.

Western Blotting Analysis

Cells (1×10^6 cells/well) were pre-incubated with iso-PXA for 24 h. Whole cells were then collected and suspended in lysis buffer (Boster Biological Technology Co., Ltd., Wuhan, China). Equal amounts of protein were separated by SDS-PAGE and transferred to an NC membrane (Bio-Rad, Richmond, CA, USA). The membrane was blocked with 5% skim milk-PBST and was then incubated overnight with primary antibodies (Primary antibodies against P-gp, PARP, cyclin B1, caspase-3, caspase-8, caspase-9, Bax, Bcl-2, Mcl-1, β-actin and Cofilin were diluted in 1:1000, FBXW7/Cdc4 antibody was diluted in 1:500) overnight at 4 °C. The secondary antibodies were peroxidase-conjugated anti-rabbit or mouse IgG (1:5000 dilution).

The protein bands were detected by a FluorChem E digital darkroom system (Protein Simple, Santa Clara, USA). Densitometry analysis was performed using Ipwin 32 software.