SUPPLEMENTARY MATERIAL

Valtrate from *Valeriana jatamansi Jones* induces apoptosis and inhibits migration of human breast cancer cells *in vitro*

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Abstract

Valtrate is a principle compound isolated from *Valeriana jatamansi Jones*, a traditional Chinese folk medicine originally used to treat various nervous disorders. Here, we found that valtrate exhibited significant anti-cancer activity *in vitro*, especially in human breast cancer cells, while displayed relatively low cytotoxicity to normal human breast epithelial cells (MCF 10A). Valtrate induced cell cycle arrest at G2/M stage and apoptosis in MDA-MB-231 and MCF-7 cells, with reduced expression of p-Akt (Ser 473), cyclin B1 and caspase 8, and increased expression of p21, p-cdc2, cleaved-caspase 3, cleaved-caspase 7 and poly (ADP-ribose) polymerase (PARP). In addition, valtrate inhibited cell migration through down-regulation of MMP-9 and MMP-2 expression. These results demonstrate that valtrate possesses anti-breast cancer activities via cell cycle arrest, apoptosis, and inhibition of cell migration, thus supporting valtrate as a potential antitumor agent.

Keywords: Valtrate; Breast cancer; Apoptosis; Migration

Figure legends：

Figure S1. Effects of valtrate on MDA-MB-231 and MCF-7 cell migration. (A and B) Images of the wound healing assays and percent of cell migration of MDA-MB-231 and MCF-7 cells. The wounded monolayer was incubated in FBS-free DMEM/F12 medium containing 0 and 5 μM of valtrate for 12 h and 24 h. Western blotting analysis of the

expression levels of MMP-2 and MMP-9 in MDA-MB-231 and MCF-7 cells. Values are represented as mean ± SD (n=3). $**p < 0.01$, $***p < 0.001$ vs untreated cells.

Figure S2. Effects of valtrate on the cell cycle distribution of MDA-MB-231 and MCF-7 cells. (A) MDA-MB-231 and MCF-7 cells were treated with 0, 5 and 10 μM valtrate for 24 h, fixed in 70 % ethanol at 4 ˚C overnight and stained with propidium iodide (PI). The cell cycle distribution as assessed by flow cytometry. Data are presented as the mean ± SD of three independent experiments, $*p < 0.05$ vs untreated cells. (B) The expression of the G2/M phase-related proteins, cdc2, p21 and cyclin B1 in the MDA-MB-231 and MCF-7 cells was analysed by western blot analysis.

Figure S3. Effects of valtrate on the cell apoptosis of MDA-MB-231 and MCF-7 cells. (A) MDA-MB-231 and MCF-7 cells were treated with 0, 5 and 10 μM valtrate for 24 h. Then the cells were stained with Annexin-V-FITC/7AAD and analysed by flow cytometry. Representative flow cytometry profiles are shown. The bottom-right quadrant indicates early apoptosis, and the top-right quadrant indicates late apoptosis. Data are presented as the mean ± SD of three independent experiments, $*p < 0.05$, $***p < 0.001$ vs untreated cells. (B) Western blot analysis of cleaved-caspase 3/7, caspase 8 and PARP and Akt signal pathway in the MDA-MB-231 and MCF-7 cells after treatment with valtrate.

Experiment

Plant Material

The root and rhizome of *Valeriana jatamansi Jones* were collected in TongRen, Guizhou Province, P. R. China, in October of 2012, and identified by Professor Ru-Song Zhang, College of Pharmaceutical Sciences, Zhejiang Chinese Medical University, Zhejiang, P. R. China. A voucher specimen (No. ZYGC-VJ-20121001) was deposited with the Laboratory of TCM Resources Engineering, Zhejiang Chinese Medical University (Yang et al. 2015).

Isolation and identification of valtrate

The dried rhizome and root of *Valeriana jatamansi Jones* (7.0 kg) were crushed into coarse powder, and then the powder was soaked with 95% EtOH for 48 h, and extracted with 20-fold 95% EtOH at r.t. by percolation. The combined extract was concentrated *in vacuo* to give crude extract, and the extract was suspended in 1 L of warm H_2O (30°C) and partitioned with CH_2Cl_2 (3 ×1 L). After evaporation under reduced pressure, the

CH_2Cl_2 extract (190 g) was subjected to CC (SiO_2) eluting with a gradient of PE/AcOEt (40:1, 20:1, 15:1, 12:1, 10:1, 8:1, 6:1, 4:1, 2:1, 1:1, 0:1) to give eleven fractions (*Frs. 1 – 7*). *Fr. 3* (5 g) was separated by prep. MPLC using MeOH/H_2O as the mobile phase (30%, 50%, 70%, 90%, each 2 L), flow rate 50 ml/min, some of the fractions eluted with 90% MeOH (150 mg) were subjected to prep.HPLC (65% ACN/H_2O, flow rate 13 ml/min) to obtain valtrate (85 mg, t_R 45.4 min). Approximately 10 mg of the sample was analyzed by ^{1}H NMR, ^{13}C NMR, and the compound was identified as valtrate (Salles et al. 2000).

Chemicals and reagents

Valtrate was maintained at -80 °C in the dark or dissolved by dimethyl sulfoxide (DMSO) and stored at -20 °C as stock solutions. The stock solutions were diluted to the desired concentrations in full medium immediately prior to each experiment. The final concentration of DMSO did not exceed 0.1 %. 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolinum bromide (MTT) were purchased from Sigma-Aldrich. The propidium iodide (PI) kit and AnnexinV-FITC apoptosis detection kit were purchased from BD Biosciences.

Cell lines and cell culture

The MDA-MB-231, MDA-MB-468, MDA-MB-453, MCF-7, MCF 10A, A2780, SK-OV-3, LNCaP and PANC1 cell lines were obtained from the cell bank of the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China). DMEM/F12 medium, trypsin, EDTA and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). All cells were cultured in DMEM/F12 medium, supplemented with 10 % heat inactivated fetal bovine serum and 1 % penicillin/streptomycin, and then incubated at 37 °C in humidified 5 % CO2, 95 % air atmosphere.

Cytotoxicity assay

The cytotoxicity was measured by MTT assay. Briefly, the MDA-MB-231, MDA-MB-468, MDA-MB-453, MCF-7, MCF 10A, A2780, SK-OV-3, LNCaP and PANC1 cells were seeded at a density of 3×10^3 cells/well in a 96-well tissue culture plate. After treatment with valtrate for 72 h, 20 μl MTT solution (5 mg/ml) was added to each well, and the cells were incubated for another 4 h at 37 ℃ in dark. Formed formazan crystals were dissolved in 150 μl DMSO and the relative cell viability was measured by

scanning with a cell Imaging Multi-mode Reader (BioTek, USA) using a 570 nm filter. IC_{50} value was calculated by GraphPad Prism 5.0 software (Graph Pad Software, La Jolla, CA, USA).

Wound-healing cell migration analysis

An *in vitro* wound healing assay was performed to measure unidirectional migration by MDA-MB-231 and MCF-7 cells, as described previously (with a slight modification) (Tian et al. 2014). Briefly, MDA-MB-231 and MCF-7 cells (2×10^4/mL) were seeded into 24-well plates and incubated at 37 ˚C until they attached. The monolayers of cells were scratch-wounded to a 1 mm depth in a straight line using a 10 μL pipette-tip. Then the cells were washed twice with PBS to remove floating cells and incubated with medium containing the indicated concentration of valtrate. The scratched areas were photographed at 0, 12 and 24 h after wounding under a microscopy and analyzed by Image J software to measure the wound width.

Flow cytometric analysis of cell cycle arrest and apoptosis

Cells were plated in 6-well plates (3×10^5 cells/well) and treated with various concentrations of valtrate for 24 h. For cell cycle analysis, cells were harvested, washed twice with ice-cold PBS and fixed in 70 % ethanol at 4 ˚C overnight, and stained with PI/RNase (0.5 ml/test, 1×10^6 cells) for 15 min at room temperature before analysis. To quantify the apoptotic cells, the treated cells were washed twice with ice-cold PBS and stained with Annexin-V-FITC/7AAD according to the manufacturer's instructions. Samples were subsequently analyzed by flow cytometer (Guava Technologies; Merck KGaA, Darmstadt, Germany) and DNA content was quantified using ModFit software.

Western blotting analysis

Western blotting was carried out as described previously (Tian et al. 2018). Cells were seeded into 6-well culture dishes (8×10^5 cells/dish) and treated with indicated concentrations of valtrate for 24 h. Cells were harvested, washed twice with ice-cold PBS, lysed by incubation in RIPA buffer containing protease inhibitors cocktail (1 mM phenylmethaesulfonyl fluoride and 1 μg/ml leupeptin) and a phosphatase inhibitors cocktail (1 mM sodium fluoride and 1 mM sodium orthovanadate) for 30 min on ice, and then centrifuged at 12,000 rpm 4 ˚C for 15 min. Supernatants were collected, and equal amounts of denatured proteins were separated by SDS-PAGE and transferred to PVDF

membranes (Millipore, Bedford, MA, USA), blocked with 5 % nonfat milk at room temperature for 1 h, and incubated with the respective specific primary antibodies overnight at 4 ˚C. The membranes were washed three times with Tris-Buffered Saline with 5 % Tween-20 (TBST) solution and incubated with a horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h. Chemiluminescent detection was performed by ECL (Bio-Rad, USA).

Statistical analysis

Data were given as mean ± SD. Statistical analysis was performed using Student's t-test, with $^{*}p < 0.05$, $^{**}p < 0.01$ being considered to indicate statistical significance.

References

Salles LA, A LS, Rech SB, Zanatta N, von Poser GL. 2000. Constituents of Valeriana glechomifolia Meyer. Biochemical systematics and ecology. Nov 1;28:907-910. Epub 2000/07/29.

Tian S, Chen Y, Yang B, Lou C, Zhu R, Zhao Y, Zhao H. 2018. F1012-2 inhibits the growth of triple negative breast cancer through induction of cell cycle arrest, apoptosis, and autophagy. Feb 22.

Tian SS, Jiang FS, Zhang K, Zhu XX, Jin B, Lu JJ, Ding ZS. 2014. Flavonoids from the leaves of Carya cathayensis Sarg. inhibit vascular endothelial growth factor-induced angiogenesis. Fitoterapia. Jan;92:34-40. Epub 2013/10/08.

Yang B, Zhang J-F, Song H-Z, Gu M-C, Zhao H-J, Xiong Y-K. 2015. Two New Iridoid Esters from the Root and Rhizome of Valeriana jatamansi JONES. Helvetica Chimica Acta. Sep;98:1225-1230.

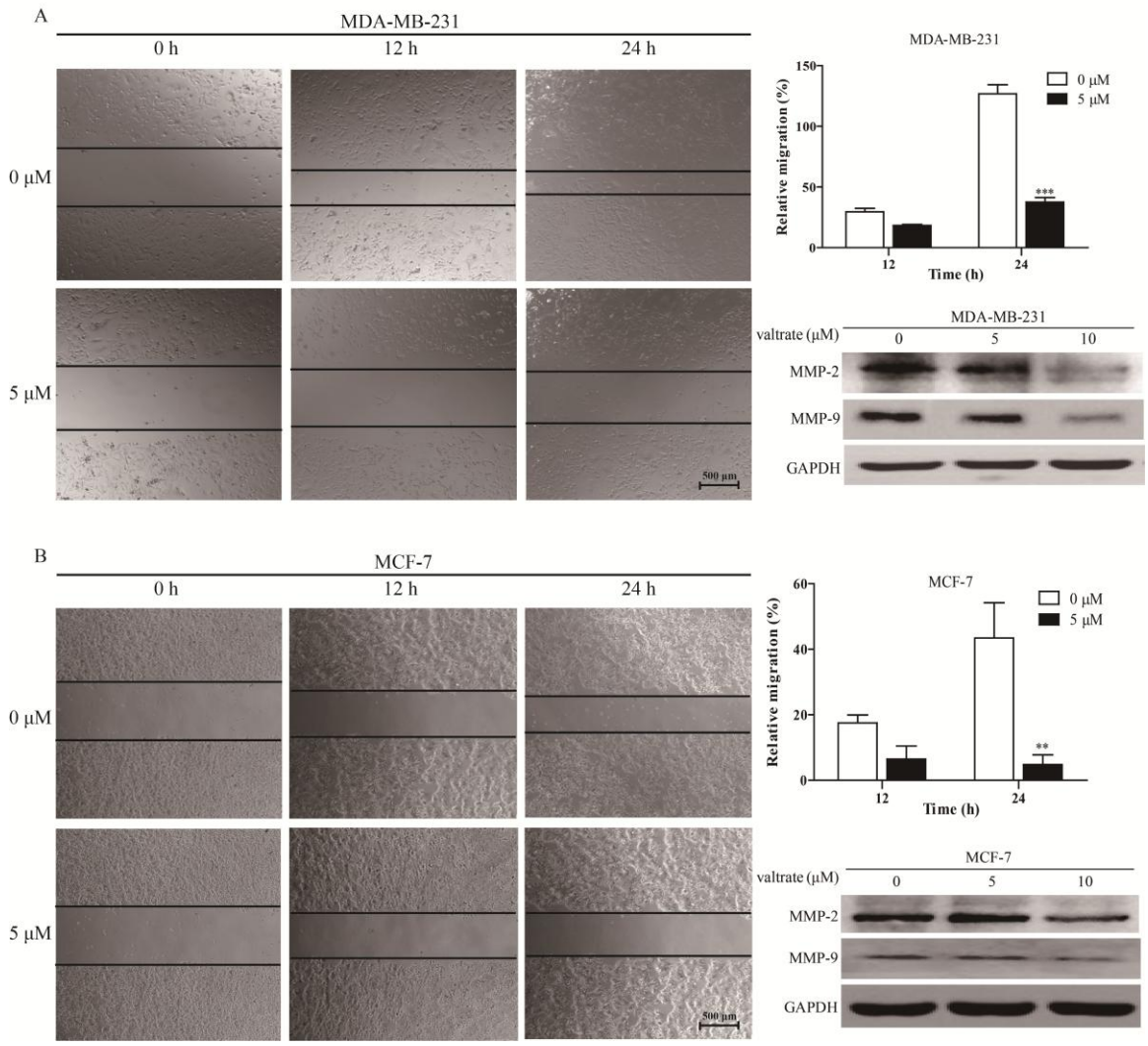

Figure S1. Effects of valtrate on MDA-MB-231 and MCF-7 cell migration

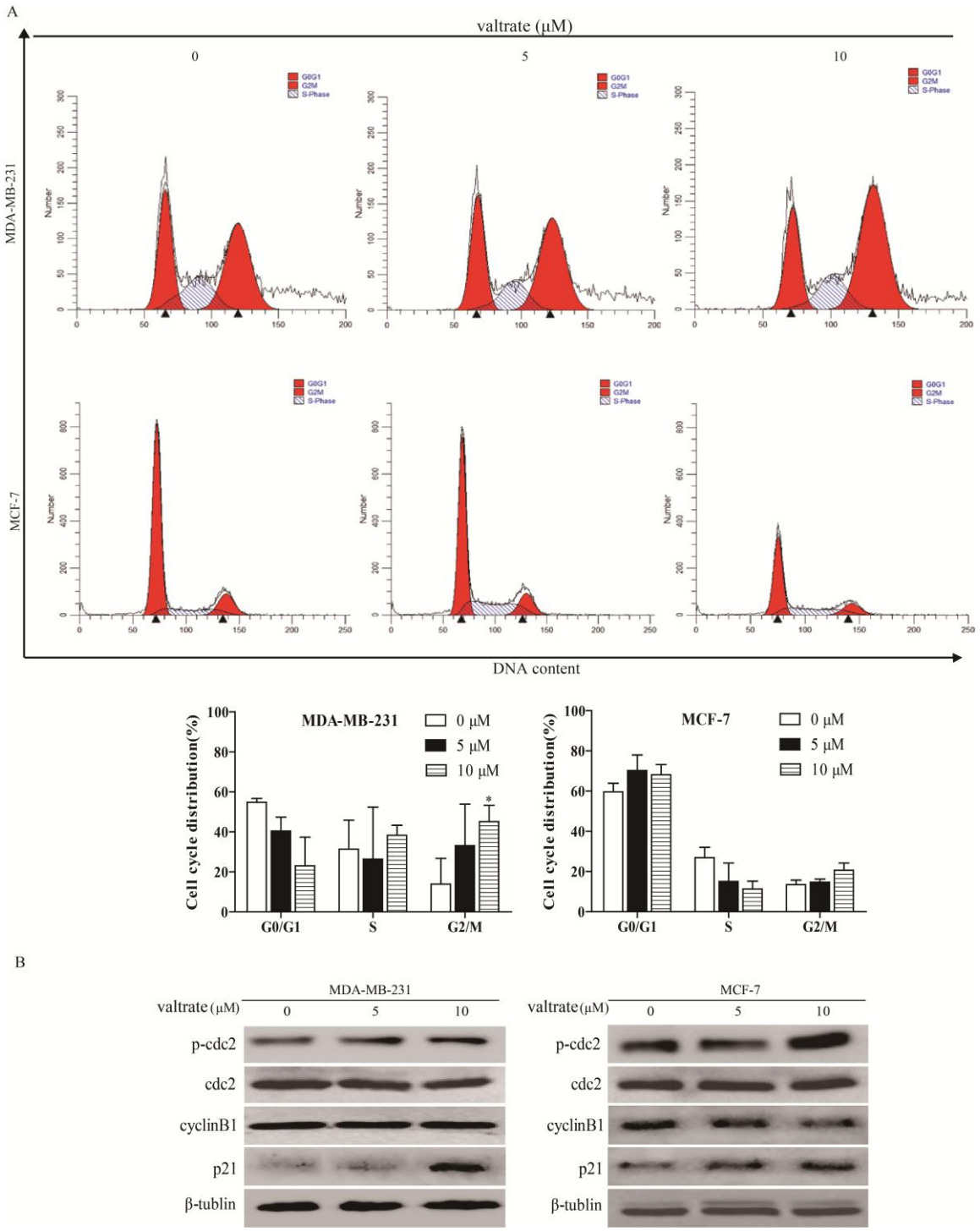

Figure S2. Effects of valtrate on the cell cycle distribution of MDA-MB-231 and MCF-7 cells

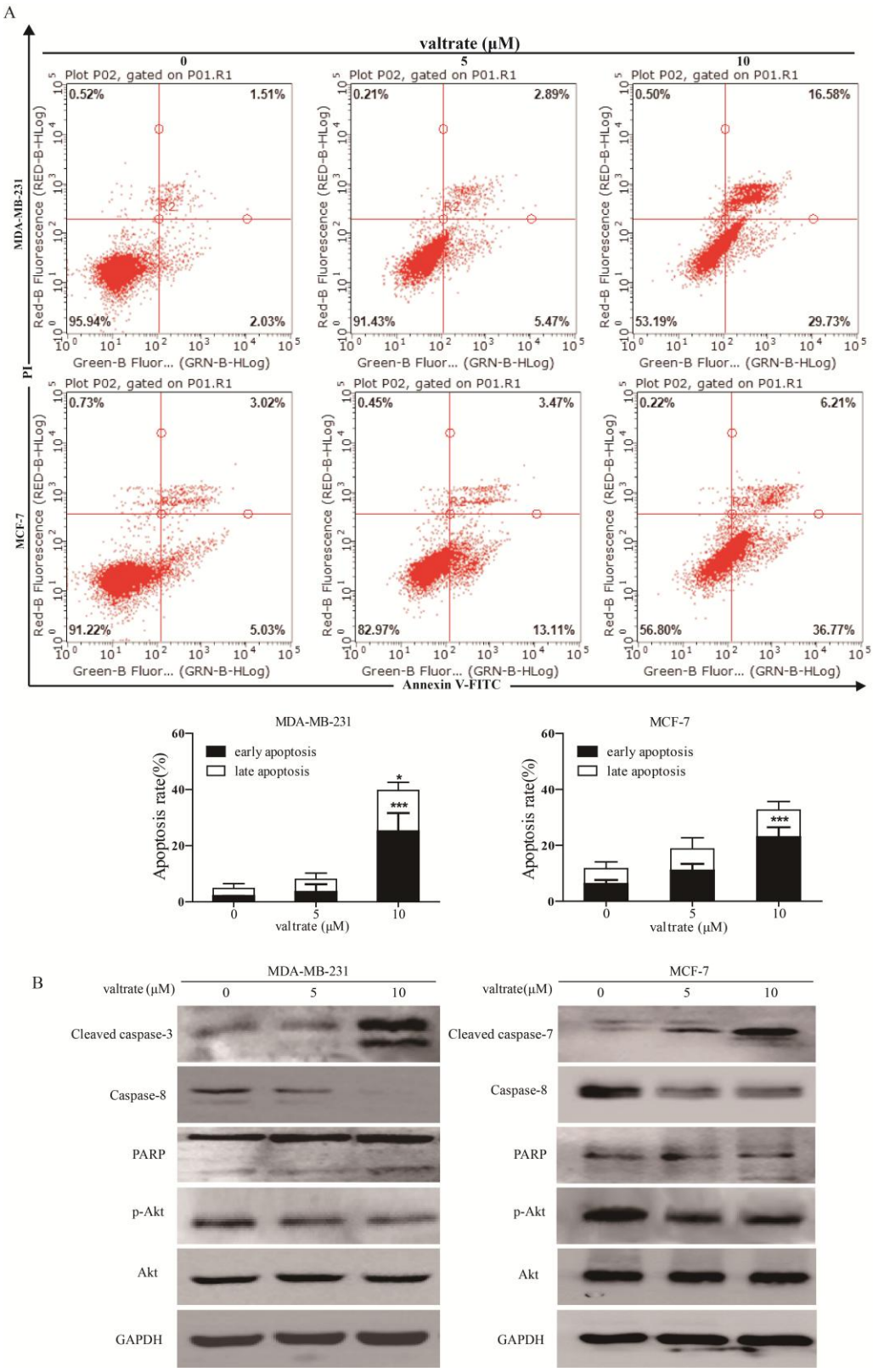

Figure S3. Effects of valtrate on the cell apoptosis of MDA-MB-231 and MCF-7 cells

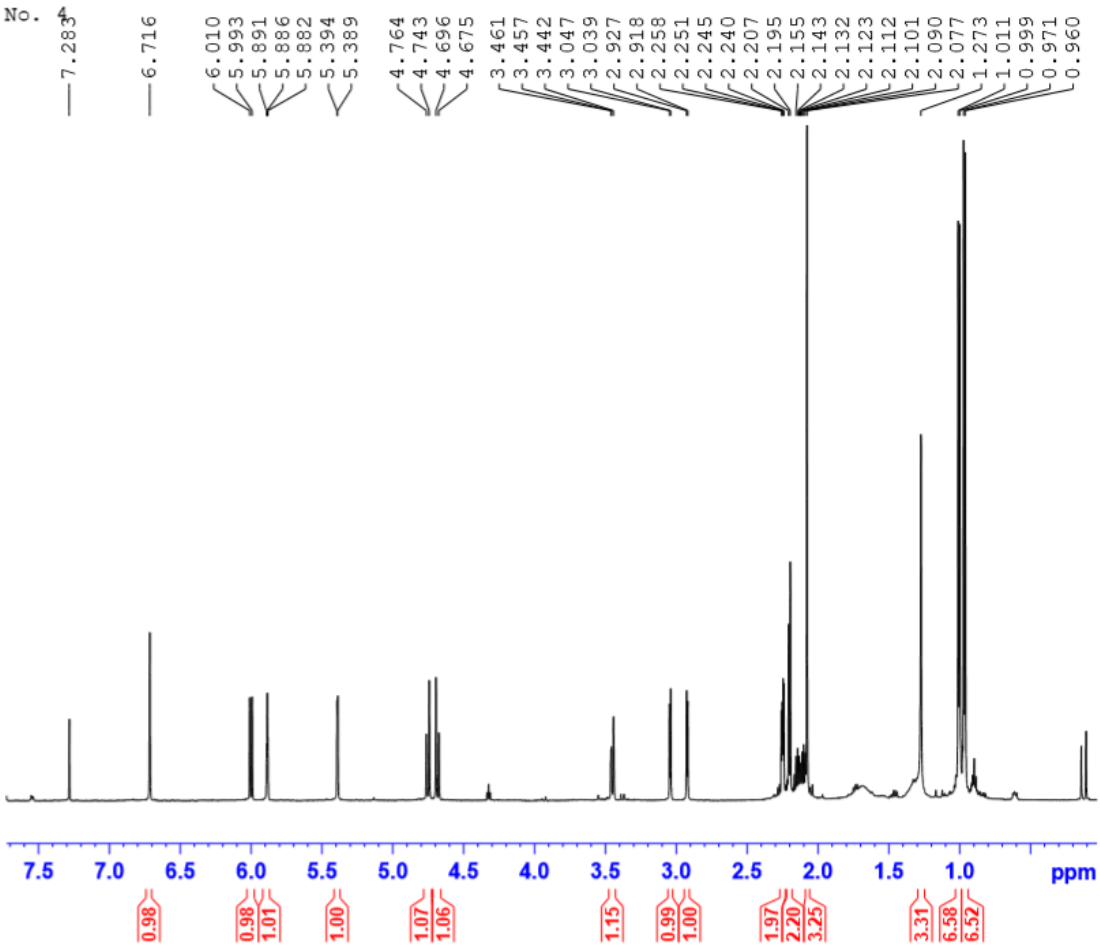

Figure S4. valtrate - ^{1}H NMR

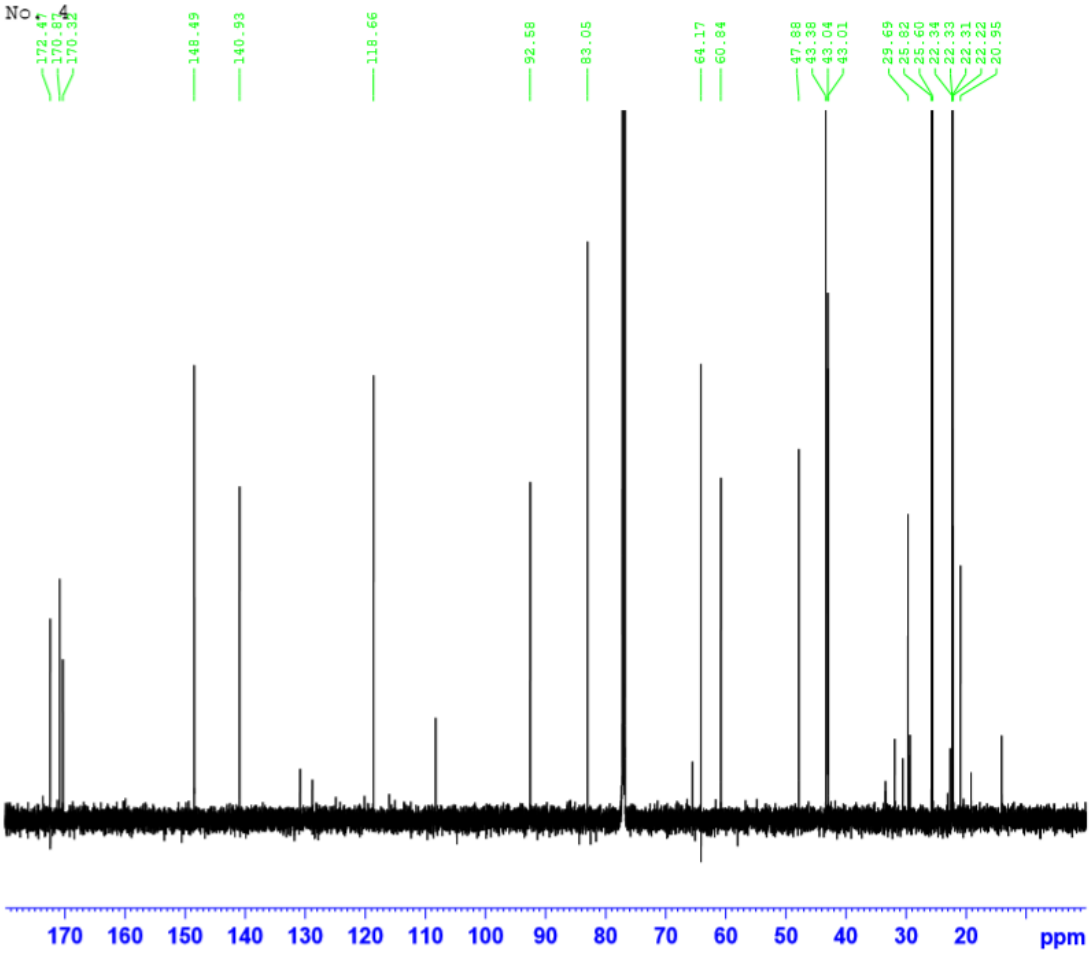

Figure S5. valtrate - ^{13}C NMR