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Syntheses of a novel thiophene derivative that induces cancer cell apoptosis through modulation of AKT and MAPK pathways

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Materials and methods

1. Instrumentation and Chemicals

^{1}H NMR spectra were recorded on a Bruker AV-300 MHz spectrometer (Germany) at room temperature and TMS as the internal standard. Electrospray mass spectra (E-MS) were obtained from Finnigan MAT. Infrared spectra (IR) spectra were determined on a Bruker Equinox 55 spectrometer. Enzyme standard instrument were determined using a ELX800, Beckman Coulter Epics XL were bought from Miami, FL. Flash column chromatography was carried out using Kanto Chemical silica gel. Thiophene (AR, Aldrich), $AlCl_3$ (AR, Aldrich), P-aminopheno (AR, Aldrich) and succinyl chloride (AR, Sigma) were purchased commercially. ABTS (2,2′-azinobis(3-ethylbenzo- thiazoline-6-sulfonic), DPPH (1,1-di-phenyl-2-picryhydrazyl), thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma. Air-sensitive reagents were manipulated in an argon atmosphere. All solvents were dried and purified by standard procedures.

2. Experimental Procedure and Characterization Data for Products

2.1 Crystal structure determination of SDS

Crystal Data. $C_{12}H_{10}O_2S_2$, M = 250.23, monoclinic, a = 5.6345 (4), b = 6.2244 (3), c = 16.3779 (9) Å, U = 573.66(5) Å3, T = 293 K, space group $P2(1)/n$, Z = 2, 1982 reflections measured, 1010 unique (R_{int} = 0.0212) which were used in all calculations. The final $wR(F_2)$ was 0.190.

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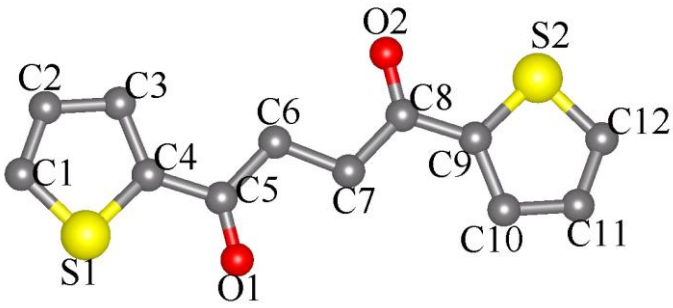

Fig. S1 Crystal structure of SDS

2.1 Synthesis of N-(4′-hydroxyphenyl)-2,5-di(2″ -thienyl)pyrrole (SNS-OH)

The N-(4′-hydroxyphenyl)-2,5-di(2 ″ -thienyl)pyrrole (SNS-OH) was synthesized by using the Knorr-Paal reaction[1] from 1,4-di(2-thienyl)-1,4-butanedione (DSD) and p-aminophenol in the presence of catalytical amount of Para Toluene Sulfonic Acid (PTSA). Synthetic route of SNS-OH as **Scheme. S1**. Refluxing DSD (0.25 g, 1 mmol), p-toluidine (0.109 g, 1 mmol), PTSA (0.034 g, 0.2 mmol) and toluene (15 mL) in a round bottom flask equipped with an Argon inlet and mechanical stirrer for 48 h yielded the crude product. Then, toluene was evaporated and SNS-OH was obtained via column chromatography. Elution with dichloromethane: hexane (1:1) of SiO_2 flash column gave the desired compound at the solvent front. Yields, 25%. ^{1}H-NMR (DMSO-d_6), δ = 4.21 (s, 1H; hydroxy), 6.54 (s, 2H; pyrrole), 6.70 (dd, 2H, J = 3.0 Hz; thiophene), 6.85 (dd, 2H, J = 6.0 Hz thiophene), 6.88 (dd, 2H, J=3.0 Hz; phenyl), 7.13 (m, 2H), 7.26 (m, 2H; phenyl). ESI-MS (m/z): 322.1 $[M-H]^-$, IR (KBr) cm^{-1}:v= 3440.4, 1633.4, 1517.7, 1249.6, 703.9;

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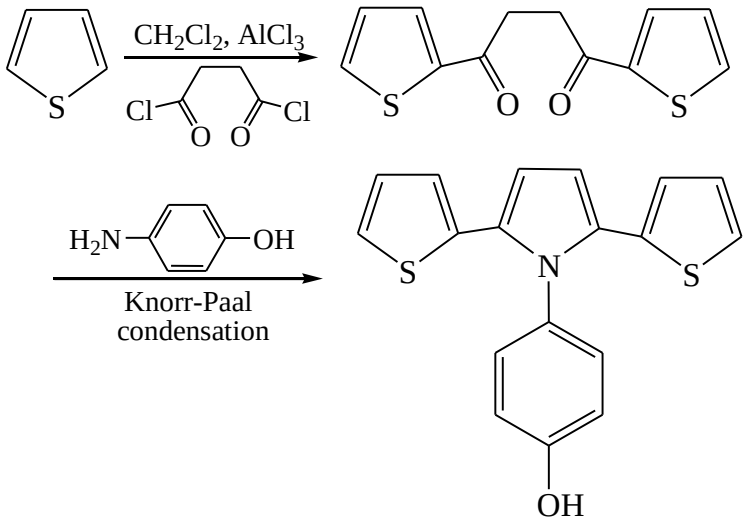

Scheme. S1 Synthetic route of SNS-OH

2.3. Cell lines and cell culture

Mouse neuroblastoma Neuro-2a cells, Human cancer cell lines (melanoma A375, human cerical cancer HeLa-299 cells, Osteosarcoma MG-63, and prostate carcinoma PC-3 cells) and human normal cell lines (kidney HK-2 cells) were all purchased from American Type Culture Collection (ATCC, Manassas, VA). All cell lines were maintained in either RPMI-1640 or DMEM media supplemented with fetal bovine serum (10%), penicillin (100 units/ml) and streptomycin (50 units/ml) at 37 °C in CO_2 incubator (95% relative humidity, 5% CO_2).

24. MTT assay

Cell viability was determined by measuring the ability of cells to transform MTT to a purple formazan dye[2]. Cells were seeded in 96-well tissue culture plates for 24 h. The cells were then incubated with the tested compounds at different concentrations for different periods of time. After incubation, 20 µl/well of MTT solution (5 mg/ml phosphate buffered saline) was added and incubated for 5 h. The medium was aspirated and replaced with 150 µl/well of acidic isopropanol (0.04 N HCl in isopropanol) to dissolve the formazan salt formed. The color intensity of the formazan solution, which reflects the cell growth condition, was measured at 570nm using a microplate spectrophotometer (SpectroAmax^{TM} 250).

2.5. Flow cytometric analysis

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The cell cycle distribution was analyzed by flow cytometry as previously described[3]. Treated or untreated cells were trypsinized, washed with PBS and fixed with 75% ethanol overnight at -20°C. The fixed cells were washed with PBS and stained with propidium iodide (PI) (1.21mg/ml Tris, 700U/ml RNase, 50.1μg/ml PI, pH8.0) for 4 h in darkness. The stained cells were analyzed with Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL). Cell cycle distribution was analyzed using MultiCycle software (Phoenix Flow Systems, San Diego, CA). Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G1 peak in the cell cycle pattern. For each experiment, 10,000 events per sample were recorded.

2.6. Caspase Activity Assay

Harvested cell pellets were suspended in cell lysis buffer and incubated on ice for 1 h. After centrifugation at 11000×g for 30 min, supernatants were collected and immediately measured for pro-tein concentration and caspase activities. The cell lysates were placed in 96-well plates and then specific caspase substrates (Ac-DEVD-AFC for caspase-3, IETD-AFC for caspase-8 and Ac-LEHD-AFC for caspase-9 substrate) were added. Plates were incubated at 37 °C for 1 h and caspase activity was determined by fluorescence intensity with the excitation and emission wavelengths set at 380 and 440 nm, respectively.

2.7. Western blot analysis

Total cellular proteins were extracted by incubating cells in lysis buffer obtained from Cell Signaling Technology and protein concentrations were determined by BCA assay. SDS-PAGE was done in 10% tricine gels loading equal amount of proteins per lane. After electrophoresis, separated proteins were transferred to nitrocellulose membrane and blocked with 5% non-fat milk in TBST buffer for 1 h. After then, the membranes were incubated with primary antibodies at 1:1,000 dilutions in 5% nonfat milk overnight at 4°C, and then secondary antibodies conjugated with horseradish peroxidase at 1:2,000 dilution for 1 h at room temperature. Protein bands were visualized on X-ray film using an enhanced chemiluminescence system (Kodak). To assess the presence of comparable amount of proteins in each lane, the membranes were stripped finally to detect the β-actin.

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2.8. Measurement of ROS Generation

The effects of SNS-OH on ROS-initi-ated intracellular oxidation were evaluated by DCF fluorescence assay[4]. Briefly, collected cells were incubated with DCFH-DA at a final concentra-tion of 10m M at 37 °C for 30 min. The loaded cells were then washed twice with PBS and ROS level was determined by measuring the fluorescence in-tensity on a Tecan SAFIRE fluorescence reader.

2.9. ABTS Free Radical Scavenging Assays

ABTS free radical scavenging activity of SNS-OH was measured according to the method as previously de-scribed[5]. Briefly, 1ml of ABTS reagent (5 mM) was mixed with 50 µl of SNS-OH at different concentrations. The decrease of absorbance at 517 nm was used to calculate the radical scavenging activity.

2.10. Statistics analysis

All the data are expressed as mean ± SD. Differences between two groups were analyzed by two-tailed Student's t test. One-way analysis of variance (ANOVA) was used in multiple group comparisons. These analyses were carried out by SPSS 12.0. Difference with $P<0.05$ (*) or $P<0.01$ (**) was considered statistically significant.

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[1] Nigel A. L and Jack M. *Synthetic Metals*, 2010,**160**,72-75.
[2] T. Chen and Y.S. Wong, *Biomed Pharmacother*, 2009, **63**, 105-113.
[3] T. Chen and Y.S. Wong, *Int J Biochem Cell Biol*, 2009, **41**, 666-676.
[4] Y. Luo and T. Chen, *Chem. Pharm. Bull.* 2011, **59** 1227-1223.
[5] T. Chen and Y.S. *Chem. Biol. Interact*., 2009, **180**, 54-60