SUPPORTING INFORMATION

Antitumor Agents 295. E-ring Hydroxylated Antofine and Cryptopleurine Analogs as Antiproliferative Agents: Design, Synthesis, and Mechanistic Studies

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Materials and Methods

Cell culture. A549 (lung carcinoma), DU-145 (prostate cancer), KB (epidermoid carcinoma), and MRC-5 (lung fibroblast) cell lines (ATCC) were obtained from Lineberger Comprehensive Cancer Center (UNC-CH). KBvin (vincristine-resistant KB subline) was generously provided by Professor Y.C.Cheng, Yale University, CT. Cells were cultured in RPMI 1640 medium containing 25 mM HEPES and 2 mM L-glutamine (Mediatech), supplemented with 10% heat inactivated fetal bovine serum (Hyclone) , 100 IU penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Mediatech). KBvin cells were maintained in media containing 100 nM vincristine and were cultured for 7-10 days without vincristine before experiments were performed. Cells were maintained at 37 °in a humidified 5 % CO_2 atmosphere. The cells were passaged every 3-4 days. Other cell lines including PC9, PC9IR, CL1-0, and CL1-5 were grown in RPMI 1640 culture medium (GIBCO-Life Technologies, Inc., Gaithersburg, MD), supplemented with 1.5 g/L of $NaHCO_3$, 4.5 g/L glucose, and 10% fetal bovine serum (FBS; GIBCO-Life Technologies).

Cytotoxicity Assay. Cells (2×10^3/well) were plated in 24-well plates. After 24 h, cells were treated with drugs for three doubling times and then fixed and stained with 0.5% methylene blue in 50% ethanol for 2 h, followed by washing with tap water to remove excess color. Plates were dried and then resuspended in 1% sarkosyl and rotated at room temperature for 3 h. Cell growth was quantitated based on the amount of methylene blue adsorbed to the cells as measured by a spectrophotometer (Molecular Devices) at 595 nm. IC_{50} was defined as the concentration of drug that inhibited cell growth by 50% after continuous drug exposure for three doubling times.

DNA Microarray Analysis. Total RNA was extracted from the cells incubated with or without the drugs using RNAzol B solution (Life Tech, Gaithersburg, MD), and the mRNA was extracted using an mRNA isolation kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. Five micrograms of mRNA from each sample was used in each array. The microarray images were scanned, digitized, and analyzed using a flatbed scanner (PowerLook 3000; UMAX, Taipei, Taiwan) and GenePix 3.0 software (Axon Instruments, Union City, CA). When designing the microarray experiments, we adhered to the guidelines of the Microarray Gene Expression Data Society (www.mged.org/Workgroups/MIAME/miame_checklist.html).

Identification of Pathways Using the MetaCore Databases. The genes that are differentially expressed in the YXM110-treated cells will be analyzed by MetaCore to deduce the major signaling pathways affected.

Western Blot. Anti-HSP90, anti-β-catenin, anti-GSK3β and anti-β-actine antibodies were purchased from Santa Cruz Biotechnology. Anti-phosphorylated GSK3β (Ser9) and cyclinD antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Equal amounts (50 μg) of cell lysate were separated by 10% SDS–PAGE, and transferred to a

polyvinylidene membrane (Millipore, Billerica, MA). The membrane was probed with antibodies. Antibodies were diluted in TBS (pH 7.5) containing 0.05% (v/v) Tween 20 and 5% (w/v) dried milk. Blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Uppsala, Sweden). Bound antibodies were visualized by electrochemical luminescence staining with autoradiographic detection using Kodak X-Omat Blue film (PerkinElmer Life Science, Boston, MA).

HPLC Analysis of the Final Compounds

Compound purity was determined by two different HPLC conditions (MeOH/H_2O or ACN/H_2O).
System: Shimadzu LC-20AT prominence liquid chromatography
Detector: Shimadzu SPD-M20A at 254 nm
Column: Alltima 2.1 mm x 150 mm C-18 5u
Flow rate: 0.200 mL/min

Compound	MeOH%	Purity	Retention time (min)	ACN%	Purity	Retention time (min)
3a	60	100	7.84	85	97.3	5.94
3b	60	99.2	5.74	70	96.1	3.44
9a	70 (MeOH/ACN=7/3)	100	3.24	85	99.9	4.06
9b	70 (MeOH/ACN=7/3)	99.5	2.49	85	100	4.29
12	70	99.4	4.75	70	99.8	3.33
13a	90	98.9	6.70	90	99.5	5.99
13b	80	97.6	6.36	70	96.4	10.36
18	50	97.9	20.62	35	96.4	16.69
19a	90	96.0	4.33	90	100	8.40
19b	80	99.9	3.07	90	99.8	3.48
21a	90	95.3	5.15	90	100	10.38
21b	70	99.6	3.25	90	98.1	3.84
24a	90	96.3	3.65	90	95.1	5.49
24b	75	99.9	4.07	55	99.7	2.52
25	90	95.5	7.77	90	95.4	8.09
26a	90	98.9	4.12	90	99.0	4.22
26b	55	99.5	23.47	90	98.3	7.14