Supporting Information

Anibamine, a Natural Product CCR5 Antagonist, as a Novel Lead for the Development of Anti Prostate Cancer Agents

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1. Chemistry

All reagents were used directly as obtained commercially unless otherwise noted. Melting points were determined with a Fisher scientific micro melting point apparatus and were uncorrected. All UV spectra were recorded on a UV-2401PC spectrophotometer. All IR spectra were recorded on a Nicolet Avatar 360 FT-IR Instrument. Proton (300 MHz) and carbon-13 (75M Hz) nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini-300MHz "Tesla" spectrometer or Varian Mercury-300MHz NMR spectrometer, with tetramethylsilane as the internal standard. LC-MS was performed on a Waters Micromass QTOF-2 instrument using ESI ion source. High-Resolution mass spectral analyses were performed on a Waters Micromass QTOF-2 instrument using ESI ion source operated in positive ion mode. TLC analyses were carried out on the Analtech Uniplate F254 plates. Chromatographic purification was carried out on silica gel columns (230~400 mesh, Merck). Preparative HPLC was preformed on a Varian Dynamax Microsorb 100-5 CN column (250 x 21.5mm), using Prostar 325 UV-Vis(254, 280nm) as the detector. Yields were not optimized.

Wittig reaction condition: LHMDS (3.6 mL, 1 M) in THF was added into the mixture of phosphonium bromide (1.90 g, 4.05 mmol) in toluene (10 mL) at room temperature under N_2

protection. After the bromide was dissolved, the resulting red solution was added dropwise into the solution of compound **1** (340 mg, 1 mmol) in toluene (10 mL) at -70 °C over 20 min. The resulting mixture was stirred at -70 °C for 10 min. and at room temperature for 30 min. The reaction was quenched by NH_4Cl (30 mL). The organic layer was separated out. The water layer was extracted with EtOAc (30 mL x 3). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated to give 1.8 g oil. Purification by chromatography on silica gel with a Hexane/EtOAc (10:1) solvent system as eluent to give 400 mg colorless oil in 92% yield as a mixture of four isomers with the (12Z, 22Z) isomer as the major one (65% yield). The NMR spectra was assigned according to the chemical shifts of the two methyl groups on position 4 and 6 on the pyridine ring with reference to the starting material NMR spectra and the reported anibamine spectra characterization.

2. Biology

2.1 SYBR-Based Real-time PCR

Gene expression of CCR5 and CCL5 was quantitated by SYBR-based Real-time PCR (qRT-PCR). The U6 gene was used as internal control. cDNA was synthesized using the iScript cDNA Synthesis Kit (Biorad) in a 20 μl reaction volume including 4 μl of iScript reaction mix, 1 μl of iScript reverse transcriptase and 1 μg of total RNA. The reactions were initiated by incubation first at 25°C for 5 min followed by incubation at 42°C for 30 min and inactivation at 85°C for 5 min. The CCL5, CCR5 and U6 genes were amplified by qRT-PCR in a 7900HT Sequence Detection System (Applied Biosystems). The 20-μl PCR mixture included 10 μl of the 2X Universal SYBR Master Mix (Applied Biosystems), 1 μl of primers (10 μM) and 3 μl of reverse transcription (RT) product. The reactions were incubated in a 96-well plate at 50°C for 2 min,

95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 55°C for 35 s and 68°C for 35 s. Primers used for the CCL5 gene were 5'-CTCATTGCTACTGCCCTCTGCGCTCCTGC-3' and 5'-GCTCATCTCCAAAGAGTTGATGTACTC-3'. Primers used for CCR5 gene were 5'-TTGAGTCCGTGTCACAAGCCC-3' and 5'-AATAATTGCAGTAGCTCTAACAGG-3' Primers used for U6 gene were 5'-CTCGCTTCGGCAGCACA-3' and 5'-AACGCTTCACGAATTTGCGT-3'. The threshold cycle (CT) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold (0.2). The relative quantity (RQ) of the target miRNAs was estimated by the ΔΔCT study using U6 expression as an endogenous control for each reaction. Relative expression was calculated using the comparative CT method.

2.2 Assessment of drug effects on prostate Cancer cell proliferation

All cell lines, PC-3, DU-145 and M12, were incubated at 37 ºC in the presence of 5% CO2. RPMI 1640 serum free media (GIBCO Invitrogen) containing 1 % L-glutamine, 0.1 % ITS (insulin, 5µg/ml; transferrin, 5µg/ml; and selenium, 5 µg/ml; Collaborative Research, Bedford) and 0.1 % gentamicin. The media for DU-145 and PC-3 cell lines included 10 % fetal bovine serum (FBS) whereas media for the M12 cell line contained 5 % FBS. Twenty-four hours after plating the M12 cells were switched to serum free media containing 0.01 percent epidermal growth factor (EGF).

The effect of anibamine on the prostate cancer tumor cell lines was assessed utilizing the WST-1 Cell Proliferation Reagent (Roche). Prostate cancer tumor cells (DU-145, PC-3, and M12) were plated in 96 well plates (BD Falcon, VWR) at a concentration of 2000 cells per well. For the purpose of this study all cells were plated in 100 µL of RPMI media (GIBCO Invitrogen)

containing 10 % FBS, 1 % L-glutamine, 0.1 % ITS and 0.1 % gentamicin and incubated overnight at 37 ºC and 5% CO_2. Various concentrations of drug were added the following day, maintaining a final total well volume of 150 μL. In wells containing only media or cells plus media, a volume of 50 μL of PBS was added to each well. The drug was dissolved in a minimum amount of dimethysulfoxide (Sigma-Aldrich) with further dissolution in PBS buffer (GIBCO Invitrogen). Incubation with the drug continued for 72 hours at 37 ºC and 5% CO_2.The next day 10 μL of WST-1 Cell Proliferation Reagent (Roche) was added to each well and incubated at 37 ºC and 5% CO_2 for 3 hours. The absorbance of each well was then measured using an EL 312e Microplate Bio-kinetics Reader (BIO-TEK Instruments). Calculations of % inhibition were performed utilizing a spreadsheet (Microsoft Office Excel 2007). The percent inhibition was calculated by using the following equation: percent inhibition = [(Acells – Adrug) / (Acells – Amedia)] x 100, where Acells is the absorbance of wells growing cells with no drug, Adrug is the absorbance of cells growing in the presence of drug and Amedia is the absorbance of just media in the absence of both cells and drug.

2.3 Adhesion assay

Adhesion assays were performed in 96-well microtiter plates coated with 10 μg/ml lamin-rich extracelluar matrix (IrECM). Cells were exposed to anibamine for 24 hrs and were then trypsinized and resuspended in RPMI-1640 with 1% BSA, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ at a concentration of 1x 10^6 cells/ml. 1x10^5 cells (100 μl) were added into each well and incubated for 30 min at 37°C and 5% CO_2 with humidified air incubation. Non-adherent cells were removed by gently washing the wells three times with phosphate-buffered saline (PBS) containing 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Adherent cells were fixed with 3.7%

paraformaldehyde for 10 min at room temperature, followed by rinsing with PBS, and stained with 0.4% crystal violet for 10 min. After extensive rinsing, the dye was released from the cells by the addition of 30% acetic acid, and the microtiter plates were read in a microplate reader (Molecular Devices, Berkeley, California) at 590 nm. M12 cells without any drugs were used as control.

2.4 In vitro invasion assay

Cell invasiveness was determined using Transwell chambers (Costar, Cambridge, MA, USA). The Transwell filters were first coated with 30ul of lrECM (diluted 1:10) and incubated at 37°C for 20-30 min. Cells (≅ 200,000) in RPMI1640 media were added to the upper chamber and the lower compartment was filled with 1 ml of RPMI 1640 medium containing 10% FBS, 20ng/ml EGF and 5ng/ml CCL5 as a chemoattractant. After 16 hr, the numbers of migrated cells in 15 random microscopic fields were counted and expressed as a percentage of the control. Results represent the mean ± S.E. of the percentage of invaded cells relative to the control (performed in triplicate).

2.5 Acinar morphogenesis

Commercially prepared EHS ECM extract, growth factor reduced, lrECM (Cultrex BME) was used for three-dimensional (3D) cultures. 3D cultures were prepared by growing prostate cancer cells to 80% confluence on plastic tissue culture dishes followed by trypsinization and collection by centrifugation. lrECM was prethawed on ice overnight. Cells (1×10^5) were mixed with 100μL undiluted lrECM and added to each well of a 96-well dish with drugs as indicated. Following incubation for 1 hr at 37°C to allow for polymerization of the lrECM, medium (200

μL) containing a specific drug as indicated was added on top of the solidified lrECM-cell mix. Medium was replaced every other day and cultures were grown for up to 8 days in lrECM.

2.6 Indirect Immunofluorescence

A sample (≅10 μL) of lrECM culture was spread on each well of a 4-well chamber slide, air dried, and fixed in 1:1 methanol/acetone at 20°C for 10 min as described [28]. The slides were washed by 1× PBS briefly followed by 400 μL of 1× IF buffer (130 mmol/L NaCl, 7 mmol/L Na_2HPO_4, 3.5 mmol/L NaH_2PO_4, 7.7 mmol/L NaN_3, 0.1% bovine serum albumin, 0.2% Triton X-100, and 0.05% Tween 20) with 10% goat serum and a secondary blocking in 200 μL of 1× IF buffer with 10% goat serum and 20 μg/mL goat anti-mouse $F(ab')_2$ fragment (Invitrogen) for 1 h sequentially. Slides were incubated with primary antibody overnight at 4°C followed directly by either FITC or Alexa-conjugated secondary antibody (1:200) for 45 min. The dilutions of antibodies used were as follows: α_6 integrin (1:200), β_1 integrin (1:200) and vimentin (1:200). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Sigma) overnight at room temperature. Slides were visualized under a LZM5100 confocal microscope according to the manufacturer's instructions. Representative pictures are shown from experiments with 20 to 40 morphologic structures analyzed.

2.7 Hemolysis Assay

6 ml of red blood cells was washed 3 times with NaCl (pH 7.4), the cells were re suspended in PBS and 200ul of the blood solution was added to 800ul of drug dissolved in PBS. The samples were incubated for either 1 hour or 24 hours, centrifuged for 5 min at top speed and the supernatants were collected and transferred to a 96 well microtiter plate. Absorbance was monitored at 541 nm on a microtiter plate reader. Positive control was blood dissolved in

distilled water. Blank was blood dissolved in PBS. % Hemolysis calculated as ((raw data-blank data)/positive control absorbance) x100.

2.8 In vivo study

Six athymic nude mice were injected subcutaneously with 2000 M12 cells. After the tumors became visible, three of the mice were injected intravenously via a lateral tail vein with 0.3mg/kg of anibamine. Three other control mice, were injected intravenously via a lateral tail vein with 0.3mg/kg of saline. Over a 16 day period each mouse received four injections of the anibamine or the solvent (injection administered every four days). As the average weight of the mice was 25 grams, each mouse received 75 ul of anibamine or the solvent control, as indicated. The length and width of each tumor was measured with a caliper. The size of the tumor was calculated as (length x width^2)/2.

2.9 Data analysis

All data are expressed as mean ± standard error. Data were statistically analyzed by ANOVA and p values lower than 0.05 were considered as indicative of significant difference, or as indicted otherwise.