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Synthesis and Biological Evaluation of Novel Substituted 4-Anilinoquinazolines as Antitumor Agents

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Abstract

Eleven novel 4-anilinoquinazoline derivatives were synthesized and evaluated for their *in vitro* antiproliferative activity. Among them, compound **9a** exhibited the best potency, with

IC_{50} values of 25−682 nM against various types of cancer cell lines. In addition, **9a** was confirmed that it could arrest the cell cycle at G_2/M phase and trigger apoptosis. Indirect immunofluorescence staining revealed its anti-tubulin property. Importantly, **9a** significantly inhibited tumor growths in SM-7721 xenograft models (57.0% tumor mass change) without causing significant loss of body weight, suggesting that **9a** is a promising new anticancer agent to be developed.

Introduction

Chemotherapy agents (1), including paclitaxel and docetaxel, play a predominant role in the treatment of human cancer, which is one of the most complicated and capricious diseases (2-3). They are clinically used to eliminate the remaining tumor population after surgery, or to cure patients when metastatic diseases were diagnosed. However, their narrow therapeutic windows and the emergence of drug resistance (4) have encouraged continued efforts to find safer and more effective agents capable of treating resistant cancer phenotypes (5).

The early discovery of the anticancer agents erlotinib (6) and gefitinib (7) prompted intensive research on 4-anilinoquinazoline compounds (8), leading to the development of new attractive compounds such as lapatinib (9), vandetanib (10), and afatinib (11) (Figure 1). These therapeutic molecules include the tyrosine kinase inhibitors (12), *β*-catenin/Tcf4 inhibitors (13), G9a inhibitors (14), and the like. Among them, verubulin (MPC-6827) was reported as a potent apoptosis inducer (Figure 1). Its corresponding hydrochloride salt is water-soluble and currently showing promising results in phase II human cancer clinical trials (15).

The success of previous research base on 4-anilinoquinazoline scaffold encouraged us to search for more potential drug candidates. In this paper, a series of substituted 4-anilinoquinazolines were synthesized and evaluated for their cytotoxicity in various types of tumor cell lines. And their pharmacological mechanism was subsequently investigated. The most promising compound was further tested for anti-tumor activity and toxicity *in vivo* (16–23).

Experimental Methods

Experimental apparatus and reagents

All chemicals were used without purification as commercially available unless otherwise noted. Column chromatography was performed on silica gel (300-400 mesh) eluting with ethyl acetate and petroleum ether. TLC was performed on 0.20 mm Silica Gel 60 F_{254} plates (Qingdao Ocean Chemical Factory, Shandong, China). UV light and I_2 were used to visualize products. NMR datas were obtained for ^{1}H at 400 MHz, and for ^{13}C at 100 MHz, on a Bruker Avance400 spectrometer (Bruker Company, Germany) or Varian spectrometer (Varian, Palo Alto, CA) , using TMS as an internal standard. Chemical shifts were given in ppm (parts per million). Mass spectra (MS) were measured by Q-TOF Priemier mass spectrometer (Micromass, Manchester, UK). The purity of each compound (>95%) was determined on an Waters e2695 series LC system (column, Xtimate C18, 4.6 mm×150 mm, 5μm; mobile phase, methanol (60%)/H_2O (40%); low rate, 1.0 mL/min; UV wavelength, 254−400 nm; temperature, 25°C; injection volume, 10 μL). The melting point of each compound was

determined on a SGWX-4A melting point instrument (±1°C, <200°C; ±2°C, 200～300°C. Shanghai Precision and Scientific Instrument Corporation, Shanghai, China).

General procedure for preparation of 4-anilinoquinazoline derivatives

The synthetic route to compounds **9a–k** is shown in **Scheme 1**. Commercial available **1** (*p*-nitrophenol) was the starting material for the synthesis. The phenolic hydroxyl group was first protected by chloromethyl methyl ether (MOMCl) to give **2**. Then reduction of **2** led to **3**. The resulting aniline **3** was further coupled with **4a** (2,4-dichloroquinazoline), **4b** (4-chloro-2-methylquinazoline) or **4c** (4-chloroquinazo- line), giving the intermediate compounds **5a**-**c**. Subsequently, **5a**-**c** were *N*-methylated by reacting with MeI to obtain the compounds **7a-c**. The intermediates **6a-c** and **8a-c** were prepared by removing hydroxyl-protected group. Ethylene carbonate was used to react with **8a**-**b** to obtain **9a**-**b**. 4-(2-Chloroethyl)morpholine was reacted with **6a**, **6b**, **8a** to prepare **9c**-**e**. Requisite boc-protected amino acids were coupled with **6c** to give **9f-h**. And **9i** was prepared by 3-(methylsulfonyl)propyl4-methyl-benzene- sulfonate coupling with **8a**. Then we used the **9a** to react with phosphorus oxychloride, directly converted to **9j** by treating with sodium methanolate-methanol solution and converted to **9k** by treating with sodium ethoxide-ethanol solution. (24, 25)

Cell culture and reagents

PI and caspase-3 (C9598) antibody were obtained from Sigma; PARP (AP102) antibody was purchased from Beyotime Co. (Nantong, Jiangsu, China). Cell lines were purchased from

American Type Culture Collection (ATCC; Manassas, USA) and cultured in DMEM or RMPI 1640 medium following ATCC's recommendations. The culture medium was supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, USA) and 1 unit/mL penicillin-streptomycin (Gibco). The cell lines were maintained at 37 °C under 5% CO_2.

Cell proliferation assay

The antiproliferation activities of the compounds were tested in the HepG2 cells, MCF-7 cells, SW620 cells, A549 cells, HCT116 cells, COLO 205 cells, HCT-8 cells, HCC827 cells, H1993 cells, H460 cells, BEL-7402 cells, SM-7721 cells, ZR-75-1 cells, SKOV-3 cells, PC-3 cells, DU145 cells, A375 cells and A2058 cells. Cells in logarithmic phase were seeded in 96-well plates and allowed to adhere (except the K562 cell line, which takes on a suspension property). Then the cells were incubated with indicated concentrations of the compounds for 24 h. MTT was subsequently added for an extra 2-3 h incubation. The MTT formazan precipitate was dissolved in DMSO, and the absorbance was measured at a wave length of 570 nm by a Spectramax M5 Microtiter Plate Luminometer (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry

SKOV3 cells were incubated with various concentrations of **9a** or DMSO vehicle for 24 or 48 h at 37 °C. The cells were collected and washed by PBS, then fixed in cold 70% ethanol overnight at 4 °C. The cells were washed again by PBS, then the cell DNA was stained with 50 μg/mL PI containing 1 mg/mL of DNase-free RNaseA for a minimum of 10 min. The samples were analyzed by a flowcytometer (BD FACS Calibur, Franklin Lakes, NJ, USA).

Western blotting

The cells were treated with **9a** by the indicated ways. Then the cells were collected and resuspended in RIPA lysis buffer (Beyotime Co., P0013B, components: 50 mM Tris, pH 7.4, 150 mM NaCl,1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, sodium fluoride, EDTA, and leupeptin). After 30 min incubation on ice, the lysates were collected by centrifuging at 12,000 g for 15 min at 4 ℃. The protein concentration was measured. Equivalent samples (30 μg of protein) were subjected to 10% or 12% SDS-PAGE, and then the proteins were transferred onto nitrocellulose membranes. After blocking by 5% non-fat milk for 2 h, the membranes were incubated with the indicated primary antibodies and subsequently probed by the appropriate secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized using enhanced chemiluminescence (Millipore, Billerica, USA). The molecular sizes of the proteins detected were determined by comparison with prestained protein markers (Invitrogen, California, USA).

Immunofluorescence staining

The immunofluorescence study of microtubule system was conducted generally as describe (26). SKOV3 cells were seeded into 6-well plates, and then treated as indicated for 16h. The cells were fixed with 4% paraformaldehyde and then penetrated with PBS containing 0.5% Triton X-100. After blocking for 30 min in 5% goat serum albumin at room temperature, cells were incubated with a monoclonal antibody (anti-α-tubulin) at room temperature for 1 h. Then the cells was washed three times by PBS following staining by fluorescence antibody

and labeling of Nuclei by 4, 6-diamidino-2- phenylindole (DAPI). Cells were finally washed thrice and visualized using a fluorescence microscope (OLYMPUS, Tokyo, Japan).

Animal Tumor Models and Treatment

To establish the SM-7721 *in vivo* model in a method similar as described (27), SM-7721cells (1×10^7 in 100 μl serum-free DMEM) were injected subcutaneously into the right flanks of 4-6 week old female nude mice. The tumors were allowed to form for 9 days. When the xenografts reached 100 mm^3, the mice were randomly divided (6 per group). The mice in the test group received intravenous (*i.v.*) injection of **9a** (10 mg/kg, dissolved in physiological saline containing 2.5% ethanol and 2.5% Tween-80) every 2 days. The mice in the vehicle group received *i.v.* injection of equal amount of physiological saline containing 2.5% ethanol and 2.5% Tween-80. Those in the adriamycin group (positive control) received *i.v.* injection of adriamycin (5 mg/kg, dissolved in physiological saline) every 2 days. Tumor burden was measured every 2 days with a caliper. Tumor volume (TV) was calculated using the following formula: $TV = length \times width^2 \times 0.52$. The very day that treatment started was defined as day 1 and the treatment ended at day 33. At the end of the experiment, mice were sacrificed and tumors were collected and weighed. This animal study was conducted in conformity with institutional guide for the care and use of laboratory animals, and all mouse protocols were approved by the Animal Care and Use Committee of Sichuan University (Chengdu, Sichuan, China).

Statistical analysis

The results are presented as mean ± SEM (or mean ± SD in the figures). The software *GraphPad Prism* 5 was used for statistical analysis. Data were statistically analyzed using One-way *ANOVA*. Differences were considered significant if $P < 0.05$.

Result and discussion

Chemistry

Preparation of 2-(4-((2-chloroquinazolin-4-yl)(methyl)amino)phenoxy)ethanol (9a)

To a solution of **8a** (1 mmol, 1.0 eq) in 5 mL DMF at 0 °C was added cesium carbonate (2 mmol, 2.0 eq) and the mixture stirred at 0 °C for 15 min. The reaction was warmed to 100 °C and ethylene carbonate (1.5 mmol, 1.5 eq) added. The resulting mixture was stirred at 100 °C for 4 h. The reaction was monitored by TLC. The solution was cooled to rt and 50 mL H_2O added. The resulting aqueous phase was extracted with ethyl acetate (3×40 mL).The organic phase was extracted with brine (3×40 mL), dried with $MgSO_4$, filtered and concentrated under reduced pressure. Yield: 63%; light yellow solid, melting point: 133.8~135.5 °C. ^{1}H NMR (400 MHz, CDCl3) δ 7.75 (d, *J* = 8.3 Hz, 1H), 7.56 (t, *J* = 7.5 Hz, 1H), 7.15 (d, *J* = 8.0 Hz, 2H), 7.05 – 6.99 (m, 1H), 6.97 (d, *J* = 8.0 Hz, 2H), 6.92 (d, *J* = 8.6 Hz, 1H), 4.13 (d, *J* = 3.7 Hz, 2H), 4.02 (d, *J* = 3.7 Hz, 2H), 3.61 (s, 3H), 2.27(s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 162.47, 158.33, 156.13, 152.95, 139.73, 133.49, 128.10, 128.10, 127.68, 126.54, 125.67, 116.45, 116.45, 114.85, 70.34, 59.99, 43.42. ESI HRMS: calcd. for $C_{17}H_{16}ClN_3O_2$+H^+ 330.1004, found 330.1003.

Preparation of 2-(4-(methyl(2-methylquinazolin-4-yl)amino)phenoxy)ethanol (9b)

To a solution of **8b** (1 mmol, 1.0 eq) in 5 mL DMF at 0 °C was added cesium carbonate (2

mmol, 2.0 eq) and the mixture stirred at 0 °C for 15 min. The reaction was warmed to 100 °C and ethylene carbonate (1.5 mmol, 1.5 eq) added. The resulting mixture was stirred at 100 °C for 4 h. The reaction was monitored by TLC. The solution was cooled to rt and 50 mL H_2O added. The resulting aqueous phase was extracted with ethyl acetate (3×40 mL).The organic phase was extracted with brine (3×40 mL), dried with $MgSO_4$, filtered and concentrated under reduced pressure. Yield: 68%; light yellow solid. melting point: 123.8~125.5 °C. ^{1}H NMR (400 MHz, DMSO) δ 7.65 (d, *J* = 7.9 Hz, 1H), 7.58 (dd, *J* = 11.0, 4.0 Hz, 1H), 7.18 (d, *J* = 8.8 Hz, 2H), 7.06 (t, *J* = 7.2 Hz, 1H), 6.98 (dd, *J* = 8.1, 5.8 Hz, 3H), 4.90 (t, *J* = 5.5 Hz, 1H), 4.00 (t, *J* = 4.8 Hz, 2H), 3.73 (dd, *J* = 10.0, 5.1 Hz, 2H), 3.49 (s, 3H), 2.59 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 162.46, 158.28, 156.11, 152.95, 139.83, 133.49, 128.10, 128.10, 127.70, 126.54, 125.69, 116.53, 116.45, 114.87, 70.34, 59.99, 43.42, 24.62. ESI HRMS: calcd. for $C_{18}H_{19}N_3O_2$+H^+ 310.1550, found 310.1549.

Preparation of 2-chloro-N-(4-(2-morpholinoethoxy)phenyl)quinazolin-4-amine (9c)

A mixture of compound **6a** (1 mmol, 1.0 eq) in 8 mL acetonitrile was added cesium carbonate (3 mmol, 3.0 eq) and the mixture stirred at 80 °C for 15 min and 4-(2-Chloroethyl)morpholine hydrochloride (1.5 mmol, 1.5 eq) added. The resulting mixture was stirred at 80 °C for 4 h. The reaction was monitored by TLC. The solution was cooled to rt and 50 mL H_2O added. The resulting aqueous phase was extracted with ethyl acetate (3×40 mL).The organic phase was extracted with brine (3×40 mL), dried with $MgSO_4$, filtered and concentrated under reduced pressure. Yield:64 %; light yellow solid, 122.8~124.9 °C. ^{1}H NMR (400 MHz, DMSO) δ 9.56 (s, 1H), 8.47 (d, *J* = 8.3 Hz, 1H), 7.77 (t, *J* = 8.6 Hz, 3H), 7.67 (d, *J* = 8.2 Hz, 1H), 7.52 (t, *J* = 7.5 Hz, 1H), 6.98 (d, *J* = 8.4 Hz, 2H), 4.10 (t, *J* = 5.5 Hz,

2H), 3.59 (m, 4H), 2.70 (t, *J* = 5.5 Hz, 2H), 2.64 (m, 4H). ^{13}C NMR (101 MHz, CDCl3) δ 158.76, 157.42, 156.28, 151.35, 133.75, 130.50, 128.22, 126.65 123.67, 123.67, 120.60, 115.12, 115.12, 113.37, 66.94, 66.94, 66.11, 57.66, 54.12, 54.12. ESI HRMS: calcd. for $C_{20}H_{21}ClN_4O_2$+H^+ 385.1426, found 385.1425.

Preparation of 2-methyl-N-(4-(2-morpholinoethoxy)phenyl)quinazolin-4-amine (9d)

A mixture of compound **6b** (1 mmol, 1.0 eq) in 8 mL acetonitrile was added cesium carbonate (3 mmol, 3.0 eq) and the mixture stirred at 80 °C for 15 min and 4-(2-Chloroethyl)morpholine hydrochloride (1.5 mmol, 1.5 eq) added. The resulting mixture was stirred at 80 °C for 4 h. The reaction was monitored by TLC. The solution was cooled to rt and 50 mL H_2O added. The resulting aqueous phase was extracted with ethyl acetate (3×40 mL).The organic phase was extracted with brine (3×40 mL), dried with $MgSO_4$, filtered and concentrated under reduced pressure. Yield:64 %; white solid, 142.8~145.1 °C. ^{1}H NMR (400 MHz, DMSO) ^{1}H NMR (400 MHz, DMSO) δ 9.56 (s, 1H), 8.47 (d, *J* = 8.3 Hz, 1H), 7.77 (t, *J* = 8.6 Hz, 3H), 7.67 (d, *J* = 8.2 Hz, 1H), 7.52 (t, *J* = 7.5 Hz, 1H), 6.98 (d, *J* = 8.4 Hz, 2H), 4.10 (t, *J* = 5.5 Hz, 2H), 3.59 (m, 4H), 2.70 (t, *J* = 5.5 Hz, 2H), 2.64 (m, 4H), 2.47 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.78, 157.46, 156.29, 151.36, 133.78, 130.55, 128.28, 126.66, 123.68, 123.68, 120.58, 115.11, 115.11, 113.37, 66.91, 66.91, 66.08, 57.62, 54.12, 54.12, 24.86. ESI HRMS: calcd. for $C_{21}H_{24}N_4O_2$+H^+ 365.1972, found 365.1973.

Preparation of 2-chloro-N-methyl-N-(4-(2-morpholinoethoxy)phenyl)-quinazolin- 4-amine (9e)

A mixture of compound **8a** (1 mmol, 1.0 eq) in 8 mL acetonitrile was added cesium carbonate (3 mmol, 3.0 eq) and the mixture stirred at 80 °C for 15 min and

4-(2-Chloroethyl)morpholine hydrochloride (1.5 mmol, 1.5 eq) added. The resulting mixture was stirred at 80 °C for 4 h. The reaction was monitored by TLC. The solution was cooled to rt and 50 mL H_2O added. The resulting aqueous phase was extracted with ethyl acetate (3×40 mL).The organic phase was extracted with brine (3×40 mL), dried with $MgSO_4$, filtered and concentrated under reduced pressure. Yield:64 %; light yellow solid, 128.8~130.2 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (d, *J* = 8.4 Hz, 1H), 7.56 (t, *J* = 7.7 Hz, 1H), 7.14 (d, *J* = 8.3 Hz, 2H), 7.01 (t, *J* = 7.7 Hz, 1H), 6.94 (t, *J* = 9.6 Hz, 3H), 4.16 (t, *J* = 5.5 Hz, 2H), 3.77 (m, 4H), 3.60 (s, 3H), 2.87 t, *J* = 5.5 Hz, 2H), 2.64 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 162.51, 158.08, 156.13, 152.96, 139.85, 133.52, 128.09, 128.09, 127.70, 126.54, 125.69, 116.53, 116.53, 114.87, 66.66, 66.66, 66.12, 57.46, 54.12, 54.12, 43.42. ESI HRMS: calcd. for $C_{21}H_{23}ClN_4O_2$+H^+ 399.1582, found 399.1584.

Preparation of 4-(quinazolin-4-ylamino)phenyl 2-((tert-butoxycarbonyl)amino)- acetate (9f)

Boc-glycine (0.45 mmol) was added to a stirred mixture of compound **6c** (147.3 mg, 0.4 mmol), EDCI (115.0 mg, 0.6 mmol), DMAP (24.4 mg, 0.2 mmol) in anhydrous CH_2Cl_2 (5 mL). The mixture was stirred at room temperature for 12 h. On completion, the slurry was partitioned between water (20 mL) and CH_2Cl_2 (20 mL), and the water was extracted with CH_2Cl_2 (3 × 10mL). The combined extract of CH_2Cl_2 and the solvent was removed under reduced pressure to yield a yellow solid. Chromatographic separation (petroleum ether-ethyl acetate, 10:1) gave the product as a pale yellow solid. Yield: 71%; light yellow solid, 191.2~193.5 °C. ^{1}H NMR (400 MHz, DMSO) δ 9.87 (s, 1H), 8.60 (s, 1H), 8.55 (d, *J* = 8.4 Hz, 1H), 7.96 – 7.84 (m, 3H), 7.80 (d, *J* = 8.2 Hz, 1H), 7.65 (t, *J* = 7.4 Hz, 1H), 7.41 (t, *J* = 5.7

Hz, 1H), 7.15 (d, *J* = 8.2 Hz, 2H), 3.98 (d, *J* = 5.8 Hz, 2H), 1.42 (s, 9H). ^{13}C NMR (101 MHz, DMSO) δ 170.06, 158.23, 156.41, 154.92, 150.12, 146.63, 137.29, 133.54, 128.27, 126.76, 124.01, 124.01, 123.45, 121.98, 121.98, 115.57, 78.93, 42.68, 28.63, 28.63, 28.63. ESI HRMS: calcd. for $C_{21}H_{22}N_4O_2$+H^+ 395.1714, found 399.1715.

Preparation of 4-(quinazolin-4-ylamino)phenyl 3-((tert-butoxycarbonyl)amino)-propanoate (9g)

Boc-beta-alanine (0.45 mmol) was added to a stirred mixture of compound **6c** (147.3 mg, 0.4 mmol), EDCI (115.0 mg, 0.6 mmol), DMAP (24.4 mg, 0.2 mmol) in anhydrous CH_2Cl_2 (5 mL). The mixture was stirred at room temperature for 12 h. On completion, the slurry was partitioned between water (20 mL) and CH_2Cl_2 (20 mL), and the water was extracted with CH_2Cl_2 (3 × 10mL). The combined extract of CH_2Cl_2 and the solvent was removed under reduced pressure to yield a yellow solid. Chromatographic separation (petroleum ether-ethyl acetate, 10:1) gave the product as a pale yellow solid. Yield:64 %; light yellow solid, 166.5~168.2 °C. ^{1}H NMR (400 MHz, DMSO) δ 9.87 (s, 1H), 8.60 (s, 1H), 8.55 (d, *J* = 8.4 Hz, 1H), 7.87(d, *J* = 8.4 Hz, 1H), 7.85 (t, *J* = 8.5 Hz, 2H), 7.80 (d, *J* = 8.2 Hz, 1H), 7.65 (t, *J* = 7.6 Hz, 1H), 7.18 (d, *J* = 8.5 Hz, 2H), 7.03 (s, 1H), 3.31 (t, *J* = 6.5 Hz, 2H), 2.71 (t, *J* = 6.5 Hz, 2H),1.40 (s, 9H). ^{13}C NMR (101 MHz, DMSO) δ 170.05, 158.23, 156.41, 154.92, 150.13, 146.61, 137.26, 133.52, 128.27, 126.77, 124.05, 124.05, 123.45, 121.93, 121.93, 115.55, 78.93, 36.68, 35.72, 28.61, 28.61, 28.61. ESI HRMS: calcd. for $C_{22}H_{24}N_4O_2$+H^+ 409.1870, found 409.1875.

Preparation of 4-(quinazolin-4-ylamino)phenyl 2-((tert-butoxycarbonyl)amino)-propanoate (9h)

N-(tert-butoxycarbonyl)-L-alanine (0.45 mmol) was added to a stirred mixture of compound **6c** (147.3 mg, 0.4 mmol), EDCI (115.0 mg, 0.6 mmol), DMAP (24.4 mg, 0.2 mmol) in anhydrous CH_2Cl_2 (5 mL). The mixture was stirred at room temperature for 12 h. On completion, the slurry was partitioned between water (20 mL) and CH_2Cl_2 (20 mL), and the water was extracted with CH_2Cl_2 (3 × 10mL). The combined extract of CH_2Cl_2 and the solvent was removed under reduced pressure to yield a yellow solid. Chromatographic separation (petroleum ether-ethyl acetate, 10:1) gave the product as a pale yellow solid. Yield: 70%; light yellow solid, 153.8~155.5 °C. ^{1}H NMR (400 MHz, DMSO) δ 9.89 (s, 1H), 8.60 (s, 1H), 8.55 (d, *J* = 8.3 Hz, 1H), 7.88 (t, *J* = 7.4 Hz, 3H), 7.84 – 7.76 (m, 1H), 7.65 (t, *J* = 7.5 Hz, 1H), 7.53 (d, *J* = 6.9 Hz, 1H), 7.13 (d, *J* = 8.4 Hz, 2H), 4.33 – 4.14 (m, 1H), 1.44(m, 3H), 1.40 (d, *J* = 16.9 Hz, 9H). ^{13}C NMR (101 MHz, DMSO) δ 170.08, 158.25, 156.47, 154.98, 150.21, 146.69, 137.31, 133.58, 128.32, 126.82, 124.07, 124.07, 123.45, 121.96, 121.96, 115.52, 78.95, 53.58, 28.67, 28.67, 28.67, 17.57. ESI HRMS: calcd. for $C_{22}H_{24}N_4O_2$+H^+ 409.1870, found 409.1875.

Preparation of 2-chloro-N-methyl-N-(4-(3-(methylsulfonyl)propoxy)phenyl)-quinazolin-4-amine (9i)

A mixture of compound **8a** (1 mmol, 1.0 eq), 3-(methylsulfonyl)propyl 4-methylbenzenesulfonate (1.1 mmol, 1.1 eq) in 5 mL DMF was added cesium carbonate (2 mmol, 2.0 eq) and the mixture stirred at 100 °C for 2 h. The reaction was monitored by TLC. The solution was cooled to rt and 50 mL H_2O added. The resulting aqueous phase was

extracted with ethyl acetate (3×40 mL).The organic phase was extracted with brine (3×40 mL), dried with $MgSO_4$, filtered and concentrated under reduced pressure. Yield: 64%; light yellow solid. 144.6~146.1 °C. ^{1}H NMR (400 MHz, CDCl3) δ 7.74 (d, *J* = 8.4 Hz, 1H), 7.56 (t, *J* = 7.7 Hz, 1H), 7.15 (d, *J* = 8.3 Hz, 2H), 7.02 (t, *J* = 7.7 Hz, 1H), 6.93 (d, *J* = 8.4 Hz, 3H), 4.16 (t, *J* = 5.7 Hz, 2H), 3.61 (s, 3H), 3.28 (dd, *J* = 17.2, 9.5 Hz, 2H), 2.97 (d, *J* = 7.6 Hz, 3H), 2.45 – 2.30 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 162.52, 157.85, 156.12, 152.96, 140.05, 133.53, 128.16, 128.16, 127.71, 126.53, 125.72, 116.55, 116.55, 115.06, 66.42, 50.96, 43.41, 40.71, 22.40. ESI HRMS: calcd. for $C_{19}H_{20}ClN_3O_3S+H^+$ 406.0987, found 406.1000.

Preparation of 2-(4-((2-chloroquinazolin-4-yl)(methyl)amino)phenoxy)ethyl dimethyl phosphate (9j)

Under argon atmosphere, $POCl_3$ (186 μL,2 mmol) was dissolved in anhydrous CH_2Cl_2(8 mL); the mixture was then cooled to 0 °C, and anhydrous triethylamine (700 μL, 5 mmol) was added. After 5 min of stirring, compound **9a** (111 mg, 0.39 mmol dissolved in 1 mL of anhydrous CH_2Cl_2) was added dropwise to the reaction mixture. The resulting mixture was stirred for 1 h at 0°C and then allowed to warm to room temperature overnight. The crude mixture was then evaporated to dryness under reduced pressure to remove the excess NEt_3 and $POCl_3$. The residue was dissolved in CH_2Cl_2 and evaporated to dryness again. The procedure was repeated twice. Followly, the residue was dissolved in 6 mL 30% sodium methoxide – methanol solution and the mixture was vigorously stirred for 1 h at 0°C. The crude mixture was then evaporated to dryness under reduced pressure. The residue was cooled to rt and 20 mL H_2O added. The resulting aqueous phase was extracted with ethyl acetate (3×20 mL).The organic phase was extracted with brine (3×20 mL), dried with $MgSO_4$,

filtered and concentrated under reduced pressure. Yield: 68%; light yellow solid. 110.5~111.8 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 8.3 Hz, 1H), 7.57 (t, *J* = 7.6 Hz, 1H), 7.16 (d, *J* = 8.3 Hz, 2H), 7.05 – 6.99 (m, 1H), 6.96 (d, *J* = 8.3 Hz, 2H), 6.92 (d, *J* = 8.5 Hz, 1H), 4.47 – 4.38 (m, 2H), 4.24 (s, 2H), 3.83 (s, 3H), 3.80 (s, 3H), 3.61 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 162.51, 157.68, 156.12, 152.96, 140.18, 133.52, 128.19, 128.19, 127.70, 126.53, 125.70, 116.55, 116.55, 114.85, 67.52, 66.21, 54.60, 54.56, 43.38. ESI HRMS: calcd. for $C_{19}H_{21}ClN_3O_5P+H^+$ 438.0980, found 438.0998.

Preparation of 2-(4-((2-chloroquinazolin-4-yl)(methyl)amino)phenoxy)ethyl diethyl phosphate (9k)

Under argon atmosphere, $POCl_3$ (186 μL, 2 mmol) was dissolved in anhydrous CH_2Cl_2 (8 mL); the mixture was then cooled to 0 °C, and anhydrous triethylamine (700 μL, 5 mmol) was added. After 5 min of stirring, compound **9a** (111 mg, 0.39 mmol dissolved in 1 mL of anhydrous CH_2Cl_2) was added dropwise to the reaction mixture. The resulting mixture was stirred for 1 h at 0°C and then allowed to warm to room temperature overnight. The crude mixture was then evaporated to dryness under reduced pressure to remove the excess Et_3N and $POCl_3$. The residue was dissolved in CH_2Cl_2 and evaporated to dryness again. The procedure was repeated twice. Followly, the residue was dissolved in 6 mL 30% sodium ethoxide-ethanol solution and the mixture was vigorously stirred for 1 h at 0°C. The crude mixture was then evaporated to dryness under reduced pressure. The residue was cooled to rt and 20 mL H_2O added. The resulting aqueous phase was extracted with ethyl acetate (3×20 mL).The organic phase was extracted with brine (3×20 mL), dried with $MgSO_4$, filtered and concentrated under reduced pressure. Yield: 72%; light yellow solid. 69.5~70.5 °C. ^{1}H NMR

(400 MHz, CDCl3) δ 7.76 (d, *J* = 8.2 Hz, 1H), 7.62 – 7.53 (m, 1H), 7.15 (t, *J* = 6.1 Hz, 2H), 7.07 – 6.99 (m, 1H), 6.99 – 6.89 (m, 3H), 4.46 – 4.36 (m, 2H), 4.27 – 4.21 (m, 2H), 4.21 – 4.10 (m, 4H), 3.61 (s, 3H), 1.36 (t, J = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ162.40, 157.54, 156.35, 152.66, 140.40, 132.78, 128.19, 128.19, 127.54, 126.35, 125.10, 116.15, 116.15, 114.65, 67.18, 65.47, 64.07, 64.01, 43.34, 16.19, 16.00. ESI HRMS: calcd. for $C_{21}H_{25}ClN_3O_5P+H^+$ 466.1293, found 466.1319.

Biological activities

Evaluation of the antiproliferative activity

Treating cells with a cytotoxic compound can result in a variety of cell fates. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis. The cells can also stop actively growing and dividing, or the cells can activate a genetic program of controlled cell death (apoptosis) (28). The synthesized derivatives **9a-9k** were evaluated for antiproliferative activities *in vitro* by MTT assay against HepG2 cells (human hepatocellular carcinoma), A549 cells (human lung cancer), MCF-7 cells (human breast cancer) and SW620 cells (human colon cancer) (Table 1). Compounds **9a-9b**, **9i-9k** displayed potent antiproliferative activities against above-mentioned tumor cell lines (IC_{50} values from 0.025 μM to 3.65 μM), particularly **9a**, which showed the strongest activity against HepG2 cells and MCF-7 cells (IC_{50} 0.025 μM and 0.032 μM). Howerver, Componds **9c-9h** showed weak activity. Compound **9a**, with a Cl group at the 2-positionin and a NMe linker, was found to be much more active than the other molecules, indicating that the Cl group at the 2-position of quinazoline and the NMe linker were important for in vitro activity.

Then we focused on the evaluation of the antiproliferative activity of **9a** across various tumor cells. As summarized in Table 2, the tumor cells were evaluated including cancer cell lines coming from colon, lung, liver, breast, ovarian, prostate and melanoma cancers. **9a** possessed potent inhibitory activity, with IC_{50} values ranged from 0.025 μM to 0.682 μM against all the tested tumor cells lines. In general, the wide range of cell killing across multiple tumor types may suggest that this kind of compounds induce tumor cell killing by inhibiting an intrinsically important process of tumor cell division. It is worth noting that **9a** showed potent activity in three human liver tumor cells, with IC_{50} 0.025 μM, 0.059 μM and 0.021 μM against HepG2, BEL-7402 and SM-7721 cells. **9a** was therefore selected for further analysis of its effects in SM-7721 cells.

9a caused a G_2/M arrest and induced apoptosis

Due to the best antiproliferation activities against SM-7721 cells (IC_{50} 0.025 μM), the effect of **9a** on cell cycle distribution was examined in SM-7721 cells by Propidium Iodide (PI) staining (Figure 2). The data indicated that **9a** treatment for 24h could cause a remarkable G_2/M arrest, which appeared in a concentration- dependent manner. In the vehicle control group, the percent of cells in G_2/M phase was just 5.4%, while the percent increased to 12.3%, 17.87%, 22.8%, 35.6% when cells were exposed to 1.25 nM, 2.5 nM, 5 nM, 10 nM of **9a**. When the dose changed to 20 nM, impressively, 51.8% of the cell population was blocked in G_2/M phase. As a result of the anti-mitotic drugs induced cell cycle arrest at G2/M phase, it seems that tubulin might be an effective target for **9a**.

The apoptosis-inducing effect of **9a** was further demonstrated by western blotting. After treatment for 48 h, it could be revealed that **9a** downregulated the protein level of

pro-caspase-3, which are regarded as important biomarkers of apoptosis (29), in a concentration-dependent way (Figure 3). Moreover, the protein level of α-tubulin was obviously downregulated after expose to at the corresponding concentration of **9a**. These trends were in accordance with the anti-mitotic drugs, further indicating that tubulin probably be an effective target for **9a.**

Immunofluorescence staining of microtubule

Blockage of the cell cycle in G2/M phase hinted that **9a** may carry on the tubulin-disrupting property, as the event of microtubule network turbulence often precedes G2/M phase arrest. To investigate whether the antiproliferative activities of **9a** were derived from an interaction with tubulin, we examined the effect on the cellular microtubule network. SM-7721 cells were exposed to vehicle control or 1 μM **9a** for 24 h, following visualizing of microtubule structures by staining for DNA (blue) and α-tubulin (green). In contrast with the vehicle control group, cells treated by **9a** exhibited disrupted microtubule organization. Therefor it could be indicated that **9a** acted as a microtubule destabilizer (Figure 4). The ability to disrupt microtubules could be a bonus to the value of **9a**, for this class of agents still stand as the best for cancer chemotherapy (30).

***In vivo* anticancer activity**

To determine whether the anti-tumor potency of **9a** could also be reflected *in vivo*, we investigated the effect of **9a** on the growth of SM-7721 cells in nude mice. Adriamycin (ADM), the agent for clinical treatment of liver cancer, was selected as a positive control drug. SM-7721 xenograft model was established. The mice in the test group received

intravenous (*i.v.*) injection of **9a** (10 mg/kg, dissolved in physiological saline containing 2.5% ethanol and 2.5% Tween-80) every 2 days. The mice in the vehicle group received *i.v.* injection of equal amount of physiological saline containing 2.5% ethanol and 2.5% Tween-80. Those in the ADM group (positive control) received *i.v.* injection of adriamycin (5 mg/kg, dissolved in physiological saline) every 2 days. In the vehicle control group, the mean tumor volume increased from 84.19 ± 12.88 to 1421.31 ± 209.38 mm^3 during a period of 33 days, whereas the change of that of **9a**-treated group was from 76.81 ± 12.47 to 433.45 ± 203.80 mm^3(Figure 5A). The mean tumor weight of **9a**-treated mice was 0.45 ± 0.20 g and the tumor mass change is 57.0%. As a comparison, the mean tumor volume change of ADM group was from 73.71 ± 5.97 mm^3 to 414.72 ± 154.88 mm^3 during the course, and the mean tumor weight of ADM group was 0.31 ± 0.11 g.

During the therapy, **9a** was dissolved well, reflecting its good aqueous solubility. In **9a**-treatment group, no serious side-effects, such as weight loss, feeding, abnormal behavior were observed (Figure 5B), suggesting **9a** is safe and convenient to administrate *in vivo*. On the contrary, the body weight of the positive control ADM group dropped severely during the treatment, consistent with reported high toxicity of this drug. All these results showed that **9a** could inhibit tumor growth significantly and seemed more safety than ADM when possessing a similar tumor suppression. The exact mechanism still needs further research.

Conclusion

In our research, we described the synthesis of a series of substituted 4-anilinoquinazolines, which induced apoptotic death of a wide variety of tumor cell lines in the double-digit

nanomolar range. Subsequently, a flow cytometric study showed that compound **9a** significantly induced cell cycle arrest in G2/M phase at 200 nM. Furthermore, *in vitro* immunofluorescence staining, **9a** was validated to maintain the microtubule disrupting ability. In the *in vivo* investigation, compound **9a** exhibited potent inhibitory activity in SM-7721 xenograft tumor models.

In the future, we will focus on discovering more potent compounds that can exhibit upgraded anticancer activity while remaining well solubility. Simultaneity, the precise molecular mechanisms of the microtubule disrupting ability of **9a** and its apoptosis-triggering efficiency will be studied.

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Conflicts of Interest

The authors declare no conflict of interest.

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SUPPORT INFORMATION

Supplementary data associated with this article can be found in the online version (The procedures for the synthesis of **2**, **3**, **5a**-**5c**, **6a-6c**, **7a-7c**, **8a-8c**).

Figure legends

Scheme Captions

Scheme 1.

Reagents and conditions: (a) MOMCl, NaH, DMF, 0 °C, 2 h; (b)10% Pd/C, H_2, EtOH, r.t., 12 h; (c) AcONa, THF/H_2O, 60 °C, 2 h; (d) MeI, NaH, DMF, 0 °C, 15 min, r.t. 1 h; (e) HCl, ethyl acetate, 0 °C, 1 h, r.t. 2 h; (f) EDCi, DMAP, DCM, r.t., 12h; (g) Cs_2CO_3, DMF, 100 °C, 2 h or Cs_2CO_3, CH_3CN, 80 °C, 4 h; (h) Cs_2CO_3, DMF, 100 °C, 4 h; (i) step A: Et_3N, $POCl_3$, 1 h at 0°C, r.t. 12 h; step B: 30% sodium ethoxide-ethanol solution or 30% sodium ethoxide-ethanol solution, 0 °C, 1 h.

Table Captions

Table 1. The antiproliferative activities of test compounds against four cancer cell lines.

Table 2. Activity of **9a** against various human tumor cell lines.

Figure Captions

Figure 1. Some reported anticancer agents.

Figure 2. 9a treatment of cancer cells results in G2/M arrest. SM-7721 cells were treated with **9a** at 1.25 nM, 2.5 nM, 5 nM, 10 nM, 20 nM for 24 h, and control group indicates the vehicle control.

Figure 3. Protein levels of pro-caspase-3, α-tubulin were determined by Western blotting. SM-7721 cell lines were treated with **9a** at 40 nM, 200 nM, 1000 nM for 48 h, with β-actin as a loading control. Control group indicates the vehicle control.

Figure 4. Effect of **9a** on the organization of cellular microtubule network. SM-7721 cells

were untreated (Control) and treated with **9a** at concentration of 1.0 μM for 24 h. Microtubules and unassembled tubulin are shown in green and the Nuclei in blue.

Figure 5. **9a** inhibited tumor growth in SM-7721 xenograft model. The *in vivo* experiment was done as described in the experimental section. (A) Tumor volume time curve. (B) Body weight time curve (C) The picture of the stripping tumor from mice. (D) Bar chart of tumor weight. *, P<0.05, **, P<0.01, and ***, P<0.001, significantly different compared with vehicle control by one-way *ANOVA*.

Table 1. The antiproliferative activities of test compounds against four cancer cell lines.

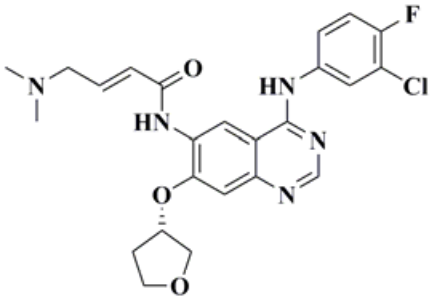
Compds	IC_{50} [a] (μM)			
	HepG2	**A549**	**MCF-7**	**SW620**
9a	0.025	0.056	0.032	0.089
9b	0.129	0.147	0.099	0.152
9c	>5.0	>5.0	>5.0	>5.0
9d	>5.0	>5.0	>5.0	>5.0
9e	>5.0	>5.0	>5.0	>5.0
9f	>5.0	>5.0	>5.0	>5.0
9g	>5.0	>5.0	>5.0	>5.0
9h	>5.0	>5.0	>5.0	>5.0

9i	1.25	2.36	1.12	3.56
9j	0.88	1.37	1.05	4.12
9k	0.92	1.90	0.99	3.65

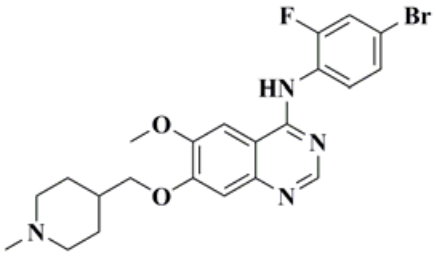
[a] IC_{50} = compound concentration required to inhibit tumor cell proliferation by 50%.

Table 2. Activity of **9a** against various human tumor cell lines.

Tumor type	Cell line	IC_{50} (μM)
Colon	HCT116	0.058
	COLO 205	0.143
	HCT-8	0.032
Lung	HCC827	0.165
	H1993	0.038
	H460	0.033
Liver	HepG2	0.025
	BEL-7402	0.059
	SM-7721	0.021
Breast	MCF-7	0.032
	ZR-75-1	0.123
	MDA-MB-231	0.682
Ovarian	SKOV-3	0.124
	A2780S	0.063
Prostate	PC-3	0.088
	DU145	0.033
Melanoma	A375	0.042
	A2058	0.037



Afatinib



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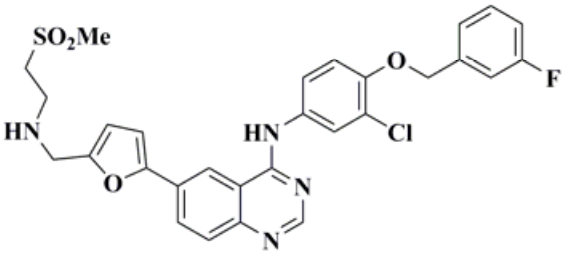

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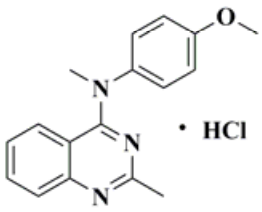

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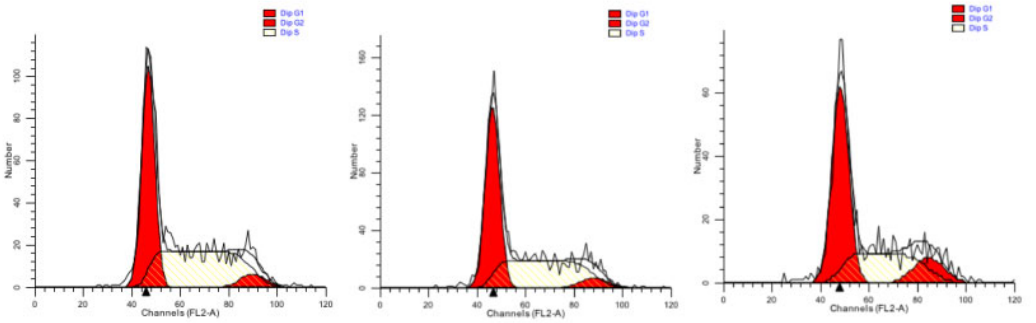
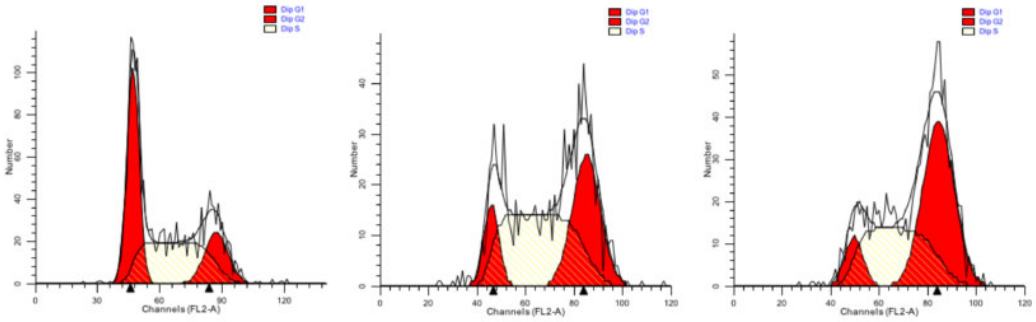


Control 1.25 nM 2.5 nM



5 nM 10 nM 20 nM