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CS2164, a novel multi-target inhibitor against tumor angiogenesis, mitosis and chronic inflammation with anti-tumor potency

You Zhou, Song Shan, Zhi-Bin Li, Li-Jun Xin, De-Si Pan, Qian-Jiao Yang, Ying-Ping Liu, Xu-Peng Yue, Xiao-Rong Liu, Ji-Zhou Gao, Jin-Wen Zhang, Zhi-Qiang Ning and Xian-Ping Lu

Shenzhen Chipscreen Biosciences Ltd, Shenzhen, Guangdong, China

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Correspondence

Xian-Ping Lu and Zhi-Qiang Ning, Shenzhen Chipscreen Biosciences Ltd., BIO-Incubator, Suit 2-601, Shenzhen Hi-Tech Industrial Park, Shenzhen, Guangdong 518057, China.
Tel: +86-755-26711889; Fax: +86-755-26957291 and Tel: +86-755-26635958; Fax: +86-755-26957291;
E-mails: xplu@chipscreen.com and zqning@chipscreen.com

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Although inhibitors targeting tumor angiogenic pathway have provided improvement for clinical treatment in patients with various solid tumors, the still very limited anti-cancer efficacy and acquired drug resistance demand new agents that may offer better clinical benefits. In the effort to find a small molecule potentially targeting several key pathways for tumor development, we designed, discovered and evaluated a novel multi-kinase inhibitor, CS2164. CS2164 inhibited the angiogenesis-related kinases (VEGFR2, VEGFR1, VEGFR3, PDGFRα and c-Kit), mitosis-related kinase Aurora B and chronic inflammation-related kinase CSF-1R in a high potency manner with the IC_{50} at a single-digit nanomolar range. Consequently, CS2164 displayed anti-angiogenic activities through suppression of VEGFR/PDGFR phosphorylation, inhibition of ligand-dependent cell proliferation and capillary tube formation, and prevention of vasculature formation in tumor tissues. CS2164 also showed induction of G2/M cell cycle arrest and suppression of cell proliferation in tumor tissues through the inhibition of Aurora B-mediated H3 phosphorylation. Furthermore, CS2164 demonstrated the inhibitory effect on CSF-1R phosphorylation that led to the suppression of ligand-stimulated monocyte-to-macrophage differentiation and reduced CSF-1R$^+$ cells in tumor tissues. The *in vivo* animal efficacy studies revealed that CS2164 induced remarkable regression or complete inhibition of tumor growth at well-tolerated oral doses in several human tumor xenograft models. Collectively, these results indicate that CS2164 is a highly selective multi-kinase inhibitor with potent anti-tumor activities against tumor angiogenesis, mitosis and chronic inflammation, which may provide the rationale for further clinical assessment of CS2164 as a therapeutic agent in the treatment of cancer.

Tumor angiogenesis is an essential process in malignant tumor growth and facilitates tumor tissue invasion and metastasis. As one of the predominant pathways involved in tumor angiogenesis, the vascular endothelial growth factor (VEGF) signaling pathway has been considered as a promising target in controlling cancer progression.[1,2] Recently, a steadily growing number of small molecule inhibitors and antibodies against VEGF/VEGFR (VEGF receptors) pathway have entered the market for therapeutic interventions in various solid tumors, including non-small cell lung, renal, breast, prostate and liver cancers, as well as gastrointestinal stromal tumors.[3–5] However, clinical evidence for patients with anti-angiogenic treatment has shown relatively modest benefits at the initial stage of treatment. The acquired drug resistance or refractoriness to anti-angiogenesis monotherapy possibly reflects the heterogeneity of tumor cell populations or diversity of tumor evading mechanisms.[6,7] In contrast, the first generation of these anti-angiogenesis kinase drugs (e.g. sunitinib, sorafenib) with broad multi-kinase targets besides VEGFR elicited a range of "off-target" adverse effects unrelated to the blockade of VEGF/VEGFR signaling pathway.[8–10] Thus, devising safe new anti-angiogenic drugs with selected anti-tumor targets is necessary.

Because anti-angiogenics are generally cytostatic rather than cytoreductive, their anti-tumor activities could be enhanced by restricting the proliferation of tumor cells *per se* (i.e. mitosis). The key regulators of mitosis in mammalian cells are the conserved Aurora family of serine/threonine kinases, comprising three members: Aurora A, B and C.[11] These three kinases differ in sub-cellular localization and perform distinct functions during mitosis. Although overexpression of Aurora A and B have been detected in various tumor types and their expression negatively correlates with patient survival and prognosis,[12,13] gene expression levels of Aurora B but not Aurora A have been reported to be associated with overall survival in mesotheliomas[14] and metastatic colorectal cancer.[15] In addition, Aurora B overexpression predicted worse 5-year survival in hepatocellular carcinoma regardless of Aurora A expression status,[16] suggesting that Aurora B could be a better therapeutic target for controlling tumor mitosis. Recently, some

selective or pan-Auroras kinase inhibitors have entered into clinical trials with promising therapeutic benefits.[17,18]

Tumor-associated macrophages (TAM), which are essential components of the chronic inflammatory tumor microenvironment (TME), have recently drawn much attention as an emerging anti-tumor target. These cells functionally promote tumor progression through a variety of mechanisms, such as enhancing tumor cell proliferation, stimulating angiogenesis and suppressing effective anti-tumor immunity.[19,20] Importantly, clinical data have shown that more accumulation of TAM in the tumor tissues correlates with a poor prognosis for the majority of cancer patients.[21,22] Among the strategies for TAM modulation, targeting the colony-stimulating factor-1 (CSF-1)/CSF-1R (CSF-1 receptor) axis is particularly attractive due to its indispensable, non-redundant function in TAM survival/activation.[23] To date, many preclinical and early clinical studies on CSF-1/CSF-1R-targeting therapies have manifested encouraging results with anti-tumor efficacy and acceptable tolerability when used either alone or combined with standard treatment modalities,[24,25] which further validate the importance of TAM in the regulation of tumorigenesis and resistance to anti-tumor drugs.

In our efforts to find a small molecule potentially targeting several key pathways for tumor development, we designed, discovered and evaluated a novel multi-kinase inhibitor, CS2164. In this study, we show evidence that CS2164 selectively and potently inhibits VEGFR/Aurora B/CSF-1R, the key kinases involved in tumor angiogenesis, mitosis and tumor inflammatory microenvironment, which collectively contribute to the *in vivo* efficient anti-tumor activity for this compound.

Materials and Methods

Compounds. CS2164 was synthesized by Shenzhen Chipscreen Biosciences (Shenzhen, China). Control compounds sorafenib (BAY 43-9006, Nexavar; Bayer Healthcare, Whippany, NJ, USA) and sunitinib (SU11248, Sutent; Pfizer, New York, NY, USA) were purchased commercially. All compounds were dissolved in sterile DMSO for *in vitro* experiments. For *in vivo* administration, compounds were suspended in 0.5% (w/v) CMC-Na suspension for oral gavage.

Cell lines. Platelet-derived growth factor receptor β (PDGFRβ) overexpressed NIH3T3 cells, CSF-1R-transfected 293A cells, human colon cancer cell line HCT-8 and mouse breast cancer cell line 4T1 were cultured at 37°C with 5% CO_2 in DMEM (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS (Gibco-Invitrogen). HUVEC were cultured in endothelial cell medium (ScienCell Research Laboratories, San Diego, CA, USA) supplemented with FBS (5%), heparin (50 μg/mL) and endothelial cell growth supplement (ECGS) (50 μg/mL). Human acute lymphoblastic leukemia cell line Molt-4, human hepatoma cell line SMMC-7721, human gastric cancer cell line MGC-803, and human colon adenocarcinoma cell line COLO-320 were cultured in RPMI1640 (Gibco-Invitrogen) containing 10% FBS. Human non-small cell lung carcinoma cell line A549 were cultured in F12 (HyClone, Logan, UT, USA) containing 10% FBS.

Docking study. The X-ray co-crystal structures of human VEGFR2 (PDB code: 1YWN with a resolution of 1.71 Å), Aurora B (PDB code: 2BFY with a resolution of 1.80 Å) and CSF-1R (PDB code: 3LCO with a resolution of 3.40 Å) were taken from the Protein Data Bank (PDB) as docking templates. Charges and protonation states were assigned according to the standard SYBYL-X 1.1 (Tripos, St. Louis, MO, USA) procedure. The Molegro Virtual Docker (MVD 2010.4.0.0, Molegro Aps, Aarhus, Denmark) program was employed for docking simulation of flexible CS2164 into the target proteins. The docking spaces for the binding sites in Aurora B, VEGFR2 and CSF-1R co-crystal structures were defined as a sphere 10 Å in radius with center positions at *X* 10.64, *Y* −0.54, *Z* 4.22, *X* 0.46, *Y* 35.30, *Z* 16.08, and *X* −4.66, *Y* 18.74, *Z* −16.76, respectively. The final docking simulations were performed in the following settings: MolDock SE algorithm, number of runs being 10, Max iterations being 1500 and Max poses being 5. The docking poses used for prediction of protein-ligand interactions were ranked by energy-based criteria in MolDockScore.

Kinase inhibition assay. To determine the kinase selectivity of CS2164 *in vitro*, the customized Invitrogen's SelectScreen Kinase Profiling Services were used.[26] First, 50 cancer-related kinases were primarily screened for an inhibition spectrum with a single concentration (500 nM) of CS2164 for three repeats. The kinases with over 60% inhibition rate were selected, and the serial concentrations of CS2164 were used to obtain the final IC_{50} values for each kinase. For an early safety assessment, Millipore's drug discovery services were applied to test the effects of CS2164 on kinases/phosphatases, GPCR and ion channels.[27] A higher concentration of CS2164 (10 μM) was used in this experiment to determine whether there was any off-target liability.

Ligand-dependent cell growth inhibition assay. HUVEC and PDGFRβ overexpressed NIH3T3 cells were seeded in 96-well plates at 5×10^3 cells/well. After 6 h culture for attachment, the cells were cultured in FBS-free DMEM overnight. HUVEC and PDGFRβ overexpressed NIH3T3 cells were, respectively, treated with recombinant human VEGF 165 (rhVEGF165; R&D Systems, Minneapolis, MN, USA) and recombinant human PDGF-BB (rhPDGF-BB; PeproTech, Rocky Hill, NJ, USA) at 100 ng/mL together with CS2164 (or sunitinib as a reference drug) at indicated concentrations. Growth inhibition (GI_{50}) that reduces the activity of mitochondrial aldehyde dehydrogenases by 50% compared to control at 72 h was calculated by MTS (Promega, Madison, WI, USA) test, as described previously.[28]

Western blotting. For determination of CS2164 inhibition of VEGF, PDGF or M-CSF stimulated phosphorylation of each receptor kinase, HUVEC, PDGFRβ overexpressed NIH3T3 and CSF-1R-transfected 293A cells (1.5×10^6) were seeded in 6-well plates for 24 h followed by incubation with FBS-free medium overnight. The cells were pre-treated with CS2164 at indicated concentrations for 1 h before the addition of 100 ng/mL of VEGF165, rhPDGF-BB or recombinant human M-CSF (rhM-CSF; PeproTech). The whole cell lysates were collected 15 min later and analyzed by western blotting with corresponding antibodies specific for phospho-VEGFR2, phospho-PDGFRβ (Cell Signaling Technology, Beverly, MA, USA) or phospho-CSF1R (AbCam, Cambridge, MA, USA). Antibodies specific for VEGFR2, PDGFRβ (Cell Signaling Technology), CSF1R and β-actin (Santa Cruz Biotechnology, San Diego, CA, USA) were used for detection of internal controls.

For p-H3 inhibition, Molt-4 cells (5×10^6) were seeded in 9-cm culture dishes and cultured overnight. Cells were treated with CS2164, sorafenib or sunitinib at indicated concentrations for 24 h. The histone proteins were extracted and analyzed by western blotting with antibodies for phospho-H3 (Ser 10) and H3 (Santa Cruz Biotechnology).

Tube formation assay. To investigate the effect of CS2164 on tube formation by HUVEC, Matrigel (BD Biosciences, San

Jose, CA,USA) was placed into pre-chilled 96-well plates (80 μL/well) and polymerized for 45 min at 37°C. HUVEC (4×10^4) in complete media were seeded with vehicle or CS2164 at indicated concentrations in Matrigel-coated plates. After 24 h incubation, tubular structures were observed under light microscopy. Network formation was finally quantified by the mean number of loops per field as topological parameters and the total length (pixels) of the branches by using the ImageJ image analysis software (http://rsbweb.nih.gov/ij/).

Cell cycle analysis. Logarithmically growing Molt-4 cells (1×10^6) were seeded in 6-well plates and cultured overnight. After treatment with CS2164, sorafenib or sunitinib at indicated concentrations for 24 h, cells were gently fixed in cold 70% ethanol overnight, then re-suspended in PBS with 0.1 mg/mL RNase A and 0.04 mg/mL PI, and incubated at 37°C for 30 min. The percentages of cell populations in each cycle phase were determined by flow cytometry and the results were analyzed using WinMDI software (The Scripps Research Institute, San Diego, CA, USA).

***In vitro* monocyte-to-macrophage differentiation assay.** Human peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers by Ficoll-Hypaque gradient centrifugation. After 2 h incubation, non-adherent lymphocytes were discarded and the remaining adherent monocytes were stimulated by M-CSF at 100 ng/mL together with CS2164 at indicated concentrations. At day 6, the differentiated macrophages were observed under light microscope.

Tumor xenograft models. For the present study, 6-week old female BALB/c athymic (nu+/nu+) mice were purchased from the Animal Experimental Center (Guangzhou, China). Human cancer cell lines HCT-8, SMMC-7721, MGC-803 and A549 in 200 μL of FBS-free media were inoculated s.c. into BALB/c athymic mice, respectively. When the tumors reached approximately 100–150 mm^3 in volume, mice were randomly grouped and treated orally with indicated dosages of CS2164, sunitinib or vehicle once daily. Tumor development were monitored every 3–4 days until the termination of the experiments. The percentages of tumor growth inhibition in tumor volumes from CS2164-treated or sunitinib-treated mice at the last treatment were calculated when compared to those in vehicle controls.

In some experiments, human colon adenocarcinoma cells, COLO-320, were inoculated s.c. into SCID mice and mouse breast cancer cells, 4T1, into wild type BALB/c mice. Mice with established tumors were treated orally with CS2164 for 12 days. Half of the collected tumor tissues were used for histone protein extraction, and others were fixed in 10% formaldehyde solution buffered, then embedded in paraffin for immunohistochemistry assay.

Immunohistochemistry. Paraffin-embedded tissues were used for identification of CD34, p-H3 (Ser10), Ki67 and CSF-1R. After de-paraffinization and antigen retrievals, the sections were incubated with 3% hydrogen peroxide and 10% goat serum for 15 min, followed by an overnight incubation with primary antibodies to CD34 (AbCam), p-H3 (Ser10), CSF-1R (Santa Cruz Biotechnology) and Ki67 (Bioss, Woburn, MA, USA), respectively. The specific primary antibody binding was detected by HRP-conjugated secondary antibody using a PowerVision Two-Step Histostaining Reagent kit (ZSGB-BIO, Beijing, China) according to the manufacturer's protocol.

Statistical analysis. All data were analyzed statistically using the *t*-test. The difference is considered statistically significant at $P < 0.05$.

Results

CS2164 is a novel multi-kinase inhibitor of VEGFR, Aurora B and CSF-1R. We used a computer-aided rational drug design approach that led to synthesize and characterize CS2164 (*N*-(2-aminophenyl)-6-[(7-methoxy-4-quinolinyl)oxy]-1-naphthalenecarboxamide, Fig. 1a). Results from molecular docking studies showed that CS2164 interacts with each active ATP-binding pocket of VEGFR2, Aurora B and CSF-1R kinases, respectively. As shown in Figure 1(b), CS2164 binds to the ATP pocket of VEGFR2 by forming two hydrogen bonds of the carboxyl group of amide moiety with LYS-866 and the 2-amino group in the phenyl ring with ASP-1044 (Fig. 1b). In addition, the oxygen atom of the 7-methoxyl group in CS2164 forms a key hydrogen bond with GLU-177 at the hinge region of the Aurora B ATP-binding site, while the carboxyl group of amide moiety forms another hydrogen bond with LYS-122. Furthermore, the interaction of CS2164 with Aurora B might be stabilized by additional hydrogen bond between the 2-amino group in the phenyl ring and ASP-234 (Fig. 1c). Finally, while the nitrogen atom in the quinoline ring and two hydrogen atoms of *o*-diamino groups in the phenyl ring of CS2164 forms two key hydrogen bonds against CYS-666 and GLU-633 in the ATP-binding pocket of CSF-1R, respectively, it is more interesting to note that the hydrogen atom of *N*-(2-amino) in the phenyl ring of CS2164 forms another significant hydrogen bond with ASP-796 in the aspartate-phenylalanine-glycine (DFG)-out binding site, which likely contributes to the potent inhibition of CS2164 against CSF-1R (Fig. 1d). Taken together, these structure-based analyses strongly suggest that CS2164 could have highly potent and stabilized molecular interactions with each corresponding active site of VEGFR2, Aurora B and CSF-1R kinases.

To characterize an overall profile of CS2164 in kinase selectivity, we performed kinase inhibition assays against a panel of relevant kinases in a cell-free system. As shown in Table 1, CS2164 inhibited VEGFR2, Aurora B and CSF-1R kinases with very high potency (IC_{50} = 7, 9 and 7 nM, respectively). CS2164 also displayed inhibitory activities with single digital nanomolar IC_{50} against several angiogenesis-related kinases, including VEGFR1, VEGFR3, PDGFRα and c-Kit. The IC_{50} for PDGFRβ inhibition was tested as 93 nM. CS2164 only showed moderate inhibitory activities (100 nM < IC_{50} < 500 nM) in 4 kinases (c-RAF, DDR2, PLK1 and PLK3), little activity (IC_{50} > 500 nM) in 33 kinases, and almost no activity (IC_{50} > 10 μM) in over 120 kinases, including 76 GPCR and 8 ion channels tested. Thus, these results indicate that CS2164 is a novel small molecule inhibitor with selective and potent inhibitory activities in VEGFR/Aurora B/CSF-1R kinases.

CS2164 exhibits anti-angiogenesis activity. The *in vitro* cellular activity of CS2164 was first evaluated in ligand-stimulated receptor activation and cell proliferation models. Under serum-free conditions, HUVEC cells and NIH3T3 cells that exogenously overexpressed PDGFRβ were treated with CS2164 before the addition of each corresponding ligand, VEGF and PDGF, respectively. As shown in Figure 2(a), CS2164 induced the concentration-dependent inhibition of ligand-stimulated VEGFR2 tyrosine phosphorylation and PDGFRβ phosphorylation. Furthermore, both VEGF-induced proliferation of HUVEC cells and PDGF-induced proliferation of NIH-3T3 cells that exogenously overexpressed PDGFRβ were concentration-dependently inhibited by CS2164 with GI_{50} values of 20.70 and 44.16 nM, respectively (Fig. 2b). CS2164 also exhibited concentration-dependent growth inhibition in

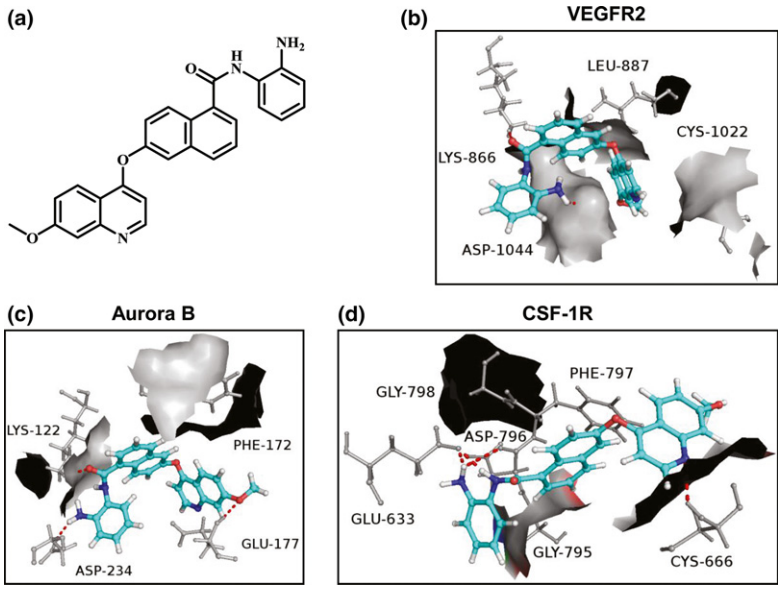

Fig. 1. Characterization of CS2164 as a multi-target inhibitor. (a) Chemical structure of CS2164, *N*-(2-aminophenyl)-6-[(7-methoxy-4-quinolinyl)oxy]-1-naphthalene-carborxamide. (b–d) Results from molecular docking studies of CS2164 (cyan stick) inserted into each ATP catalytic active pocket of VEGFR2 (b), Aurora B (c) and CSF-1R (d) were shown, respectively. The predicted hydrogen bonding interactions (dashed red lines) and key ATP pocket residues as grey ball-sticks and molecular surfaces were displayed with the indicated amino acids.

Table 1. The inhibition profile of CS2164 in kinase selectivity

Kinases	Biochemical IC_{50} (nM)
VEGFR1/FLT1	8
VEGFR2/KDR	7
VEGFR3/FLT4	9
PDGFRα	1
PDGFRβ	93
c-Kit	4
Aurora B	9
CSF-1R	7
c-RAF, DDR2, PLK1, PLK3	>100
AKT1, AKT2, ALK, AURKA, AURKC, BRAF, BTK, EGFR, EPHB4, ERBB2, FES, FGFR1, FGR, FLT3, FYN, IGF1R, JAK1, JAK2, JAK3, LCK, LYNA, MAPK8, MAPK9, MAPK10, MAPK11, MAPK14, MET, MST1R, PRKCB2, PRKACA, RET, SRC, SYK (33 kinases)	>500
Abl, AMPKα1, CaMKIIβ, CaMKIIγ, CaMKIIδ, CaMKIV, CDK1/cyclinB, CDK2/cyclinA, CDK2/cyclinE, CDK3/cyclinE, CDK5/p25, CDK5/p35, CDK7/cyclinH/MAT1, CDK9/cyclin T1, Flt3, GSK3β, IR, LKB1, MAPK1, MAPK2, p70S6K, PhKγ2, PKA, PKBβ, PKCα, PKCβI, PKCβII, PKCγ, PKCδ, PKCε, PKCη, PKCι, PKCμ, PKCθ, PKCζ, PKG1α, PKG1β, ROCK-II, SAPK2A, PTP-1B, TCPTP (39 kinases and 2 phosphotases)	>10, 000
GPCRs (76 members), and ion channels (Nav1.5, Kv4.3/KChIP2, Cav1.2, hKv1.5, KCNQ1/minK, hERG, HCN4, Kir2.1)	>10,000

Shown are the representative data from one of three independent repeats.

mVEGF-treated mouse endothelial cells bEnd.3 with GI_{50} value of 35.53 nM (Fig. S1). Results from *in vitro* capillary tube formation showed that, compared to the intense capillary tube networks formed by HUVEC plated onto BD Matrigel in the control group, treatment of the cells with CS2164 at 1.5 or 5 μM induced significant reductions in the number of loops and the total branch lengths of tubular network structures (Fig. 2c). In the xenograft model established by s.c. inoculation of the human colon adenocarcinoma cells COLO-320 into the SCID mice, daily treatment with oral CS2164 at either 2.5 or 20 mg/kg resulted in decreased vascularization in tumor tissues. Consistently, as a specific endothelial cell marker commonly used for microvessel quantification, CD34 staining was remarkably reduced in tumor tissues from CS2164-treated mice (Fig. 2d). Collectively, these results indicate that CS2164 is a potent inhibitor of tumor angiogenesis through targeting the corresponding tyrosine receptor signaling pathways.

CS2164 induces G_2/M cell cycle arrest by inhibition of Aurora B/p-H3. The inhibitory activity of CS2164 on Aurora B was determined by measuring the nuclear levels of Ser10-phosphorylated histone 3 (p-H3), a direct substrate of Aurora B,[29] in Molt-4 cells. As shown in Figure 3(a), treatment with CS2164 yielded a substantial reduction in the level of p-H3 in Molt-4 cells in a concentration-dependent fashion. In contrast, two VEGFR tyrosine kinase inhibitors (TKI), sorafenib and sunitinib, showed little inhibitory activity in histone H3 phosphorylation even at the higher concentration up to 6 μM (Fig. 3a). Interestingly, treatment with CS2164 in Molt-4 cells for 72 h induced many enlarged cells (data not shown), which might be polyploid (4N or even more) cells, likely due to the cell cycle arrest. Flow cytometry analysis verified that CS2164 induced the pronounced cell cycle arrest in the G_2/M phase at 3 μM (59.5% of cell cycle phases compared to 14.9% in control group, Fig. 3b). However, both sorafenib and sunitinib did not reveal activity in G_2/M arrest at the same concentration (13.3% and 20.4%, respectively; Fig. 3b). The *in vivo* anti-mitotic effect of CS2164 on Aurora B inhibition was further validated in a tumor xenograft mouse model. The histological analyses by staining for tissue p-H3, and Ki67, another cell proliferative marker, showed a substantial dose-dependent decrease of both markers in tissues from CS2164-treated compared to vehicle-treated animals (Fig. 3c). In addition, only tumor tissue but not normal colonic tissue showed the reduced p-H3 levels in a dose-dependent manner after treatment of the animals with CS2164 (Fig. 3d). CS2164 showed concentration-dependent growth inhibition of both Molt and Colo-320 cells *in vitro*, with GI_{50} values of 2.14 and 0.84 μM, respectively (Fig. S2). Thus, these results suggest that the anti-mitotic

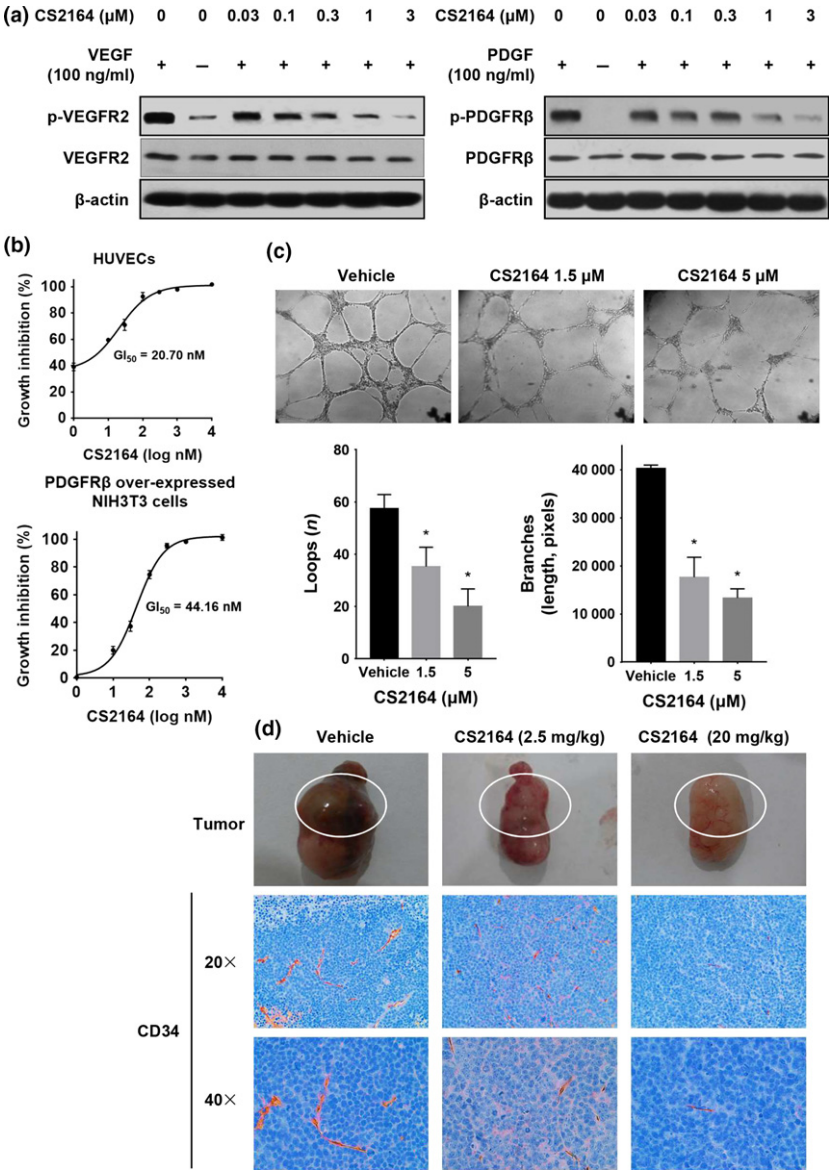

Fig. 2. The inhibitory effect of CS2164 on angiogenesis. (a) The representative western blotting results of VEGFR2 phosphorylation in HUVEC and PDGFRβ phosphorylation in PDGFRβ overexpressed NIH3T3 cells treated with CS2164 at indicated concentrations followed by VEGF or PDGF at 100 ng/mL are shown together with the levels of VEGFR2, PDGFRβ and β-actin as internal controls. (b) The kinetics of concentration-dependent growth inhibition by CS2164 on *in vitro* ligand-dependent proliferation of HUVEC and PDGFRβ overexpressed NIH3T3 cells. (c) The representative photographs of *in vitro* angiogenesis in HUVEC on Matrigel treated with CS2164 at the indicated concentrations for 24 h. Tubular network structures were evaluated as both number of loops and length of branches. Data are mean ± SD of five separate experiments. $^*P < 0.01$ versus vehicle group. (d) COLO-320 tumor-bearing SCID mice were treated orally with vehicle or CS2164 at 2.5 or 20 mg/kg/day for 12 days. The representative photographs of tumor tissues with the vascularization areas (in white circles) in the upper panel and immunohistochemistry results for CD34 staining at 20× and 40× magnifications in the lower panel are shown. Red-brown, stained microvessels; blue, hematoxylin counterstain. Shown data are from three independent experiments with similar results.

activity elicited by CS2164 in tumor cells is most likely due to its inhibitory effect on Aurora B-mediated H3 phosphorylation, which is independent of the blockade of VEGFRs or other angiogenesis-related TKI.

CS2164 inhibits CSF-1R signaling pathway and decreases tissue CSF-1R$^+$ cells. To investigate the inhibitory effect of CS2164 on CSF-1R signaling pathway, CSF-1R-transfected 293 cells were pre-treated with CS2164 followed by M-CSF stimulation. As shown in Figure 4(a), CS2164 treatment induced a concentration-dependent inhibition of CSF-1R phosphorylation induced by M-CSF. Using an *in vitro* ligand-induced monocyte-to-macrophage differentiation assay,[30] differentiation, as well as proliferation, of human blood-derived monocytes induced by M-CSF were inhibited by CS2164 in a concentration-dependent manner, and an almost complete inhibition could be achieved at a concentration as low as 0.3 μM (Fig. 4b). Similar results were also observed in mouse monocyte-to-macrophage differentiation (Fig. S3). To further study *in vivo* CSF-1R inhibition by CS2164 during tumor development, immune competent mice were implanted s.c. with 4T1 breast cancer cells and treated with CS2164 at either 2.5 or 20 mg/kg. CS2164 caused a dramatic reduction of CSF-1R$^+$ cells in tumor tissue (Fig. 4c), which was also correlated with the decreased tissue CD11b$^+$ macrophage infiltration (Fig. S4). Thus, CS2164 may regulate monocyte/macrophage tissue infiltration through CSF-1R signaling inhibition.

CS2164 exhibits broad and potent *in vivo* anti-tumor activities. The *in vivo* anti-tumor activities of CS2164 were assessed in multiple murine xenograft models inoculated with human cancer cell lines, including the cells derived from colon, lung, liver and stomach cancers. As shown in Figure 5, animals orally administrated with CS2164 demonstrated dose-dependent inhibition of tumor growth in all four models. For example, in human colon cancer cell line HCT-8 and hepatocellular carcinoma cell line SMMC-7721-derived xenograft models, daily treatment with CS2164 in the dose range of 2.5–20 mg/kg reduced tumor growth dose-dependently, and the 50% tumor growth inhibition (TGI_{50}) could be achieved at 2.5 mg/kg of CS2164 in both models. Interestingly, CS2164 at 10 mg/kg produced similar or greater efficacy than the VEGFR TKI

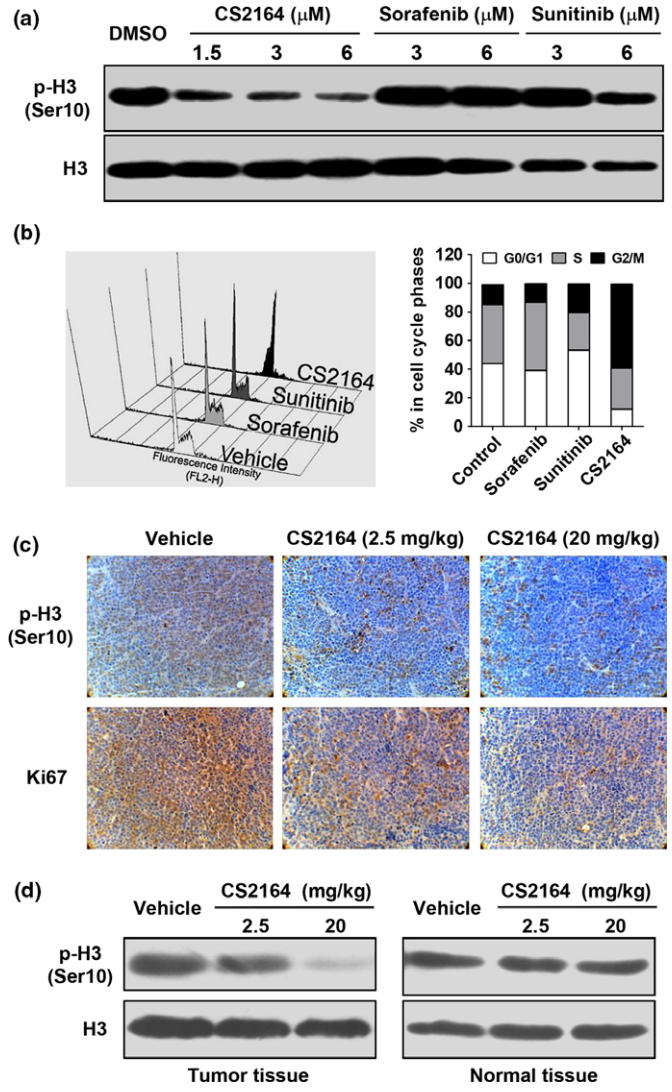

Fig. 3. CS2164 induced cell cycle arrest through p-H3 inhibition. (a) The representative western blotting results of phosphorylated H3 at Ser-10 and total H3 in Molt-4 cells treated with CS2164 compared with sorafenib and sunitinib at indicated concentrations are shown. (b) The representative FACS data and accumulative results of cell cycle phases in Molt-4 cells treated with CS2164 compared with sorafenib and sunitinib at 3 μM for 24 h were from two or three independent repeats. (c, d) COLO-320 tumor-bearing SCID mice were treated orally with vehicle or CS2164 at 2.5 or 20 mg/kg/day for 12 days. The representative immunohistochemistry results for p-H3 and Ki67 staining (c) at 20× magnification in tumor tissues as well as western-blotting analyses for phosphorylated H3 and total H3 in tumor tissues compared with normal colon tissues (d) are shown.

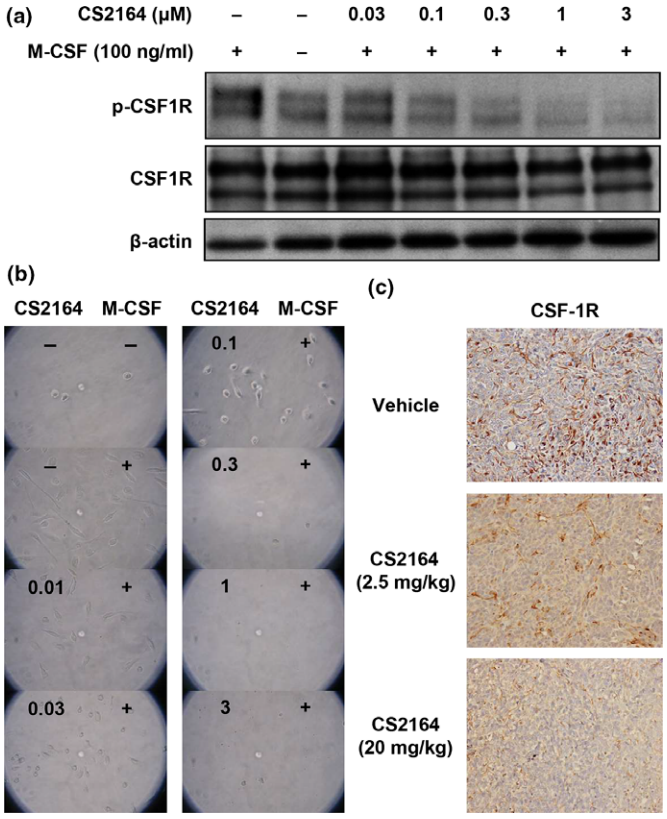

Fig. 4. CS2164 inhibits CSF-1R signaling and reduces tissue CSF-1R expression. (a) The representative western blotting results of phosphorylated CSF-1R in CSF-1R-transfected 293A cells treated with M-CSF at 100 ng/mL and CS2164 at indicated concentrations are shown with CSF-1R and β-actin as internal controls. (b) Human blood-derived monocytes were stimulated with M-CSF at 100 ng/mL and CS2164 at the indicated concentrations (ng/mL) for 6 days. The photographs of differentiated macrophages show the representative results from two independent repeats. (c) BALB/c mice were implanted s.c. with 4T1 breast cancer cells and treated orally with vehicle or CS2164 at 2.5 or 20 mg/kg/day for 12 days. The representative data of tumor tissue immunohistochemistry for CSF-1R staining at 40× magnification are shown.

sunitinib at 40 mg/kg in these two models. CS2164 at 1 mg/kg started to demonstrate tumor growth inhibition in the gastric cancer cell line MGC-803-derived model, and CS2164 at 5 mg/kg could induce near complete inhibition of tumor growth, which is comparable to the efficacy of sunitinib at 40 mg/kg. In another human non-small cell lung cancer cell line A549-derived xenograft model, treatment with CS2164 from 5 to 40 mg/kg induced dose-dependent inhibition of tumor growth, and the TGI_{50} was around 5 mg/kg. In addition, treatment with CS2164 at 5 or 10 mg/kg produced similar efficacy to sunitinib at 40 mg/kg in this model. No significant weight loss or signs of morbidity were observed during or at the end of the above experiments in the animals treated with CS2164 at indicated dosages, although the body weight gains in the highest dosage groups were slightly lower that may correlate to the maximal inhibition of tumor growth (Fig. S5). Overall, our results from *in vivo* experiments provide direct evidence of broad anti-tumor efficacy of CS2164.

Discussion

VEGF/VEGFR signaling pathway fulfills numerous functions in the regulation of the tumor vasculature, and induces abnormal angiogenesis in many tumor entities. Although several VEGFR inhibitors approved by FDA have shown the therapeutic effects of angiogenesis inhibition, more evidence indicates that VEGFR inhibition alone is usually not sufficient to block tumor progression with the emergence of resistance.[6,7] To overcome the possible mechanisms of resistance or escape mediated by tumor cells or by members of the tumor microenvironment, strategies for combination therapy or exploring multi-target drugs that simultaneously target the complementary and redundant pathways have been

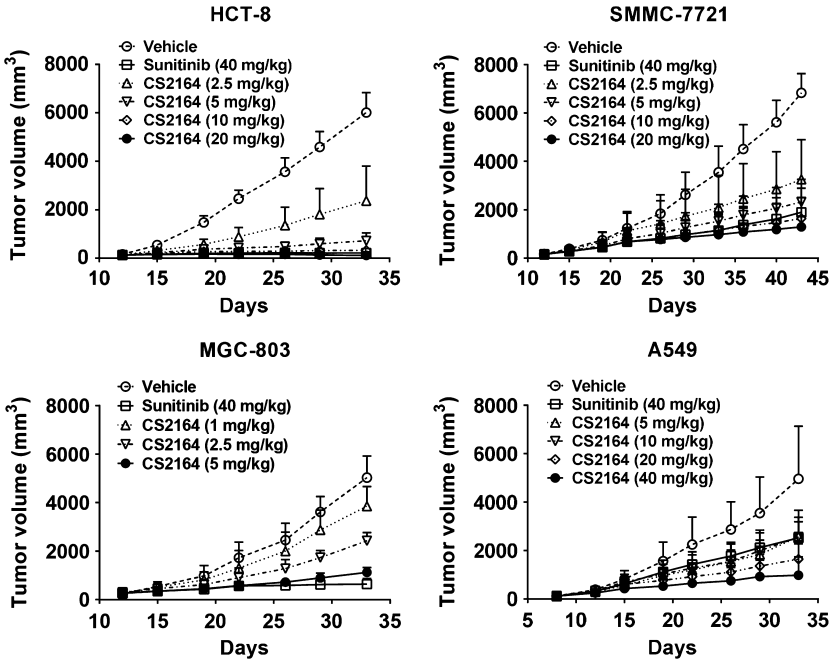

Fig. 5. *In vivo* anti-tumor activity of CS2164 in tumor xenografts. The anti-tumor potency of CS2164 were evaluated in HCT-8, SMMC-7721, MGC-803 or A549 cell line-derived mouse xenograft models. BALB/c athymic mice ($n = 8$ per group) were transplanted s.c. with indicated tumor cells and treated orally with CS2164, sunitinib or vehicle daily at the indicated dosages when tumors reached 100–150 mm^3 in volume. Tumor volumes (mm^3) were monitored for 33 or 43 consecutive days and are represented as mean ± SD.

suggested.[31] In fact, some multi-target inhibitors with cross-talk mechanisms involving VEGFR have recently demonstrated great success in clinical trials.[32] With this rationale, we designed a novel compound, CS2164, which simultaneously targets against VEGFR/Aurora B/CSF-1R, several key kinases involved in tumor angiogenesis, tumor cell mitosis, and chronic inflammatory microenvironment. Our results have shown that this novel compound displays potent anti-tumor activity through target-specific inhibition both *in vitro* and *in vivo*.

Given that VEGFR2 is critical for VEGF-induced signaling in vascular endothelial cells and plays a key role in tumor angiogenesis and hematopoiesis,[33] we chose VEGFR2 as the primary angiogenesis target for designing the small molecule inhibitor. Our results showed that CS2164 directly bound to the ATP pocket of VEGFR2 and exhibited kinase inhibitory activity at the nanomolar level. Furthermore, the inhibition of capillary tube formation was well correlated with the diminished VEGFR2 phosphorylation levels. Because of the structural similarities between VEGFR and other receptor tyrosine kinases,[34] we found that CS2164 could also efficiently inhibit other angiogenesis-related kinases, including VEGFR1, VEGFR3, PDGFRα, PDGFRβ and c-Kit. Because VEGFR3 is often closely correlated with proliferation and survival of lymphovascular cells, VEGFR3 inhibition may provide additional efficacy of anti-angiogenic therapies.[35] Previous studies have demonstrated that PDGFR inhibition through regulating pericytes homeostasis could enhance the anti-vascular therapy using VEGFR inhibitor alone,[36,37] while c-Kit blockade suppressed tumor growth by inhibition of tumor angiogenesis regulated by hematopoietic cells.[38] Taken together, our compound CS2164 could provide more efficient inhibition of tumor angiogenesis by concurrently targeting multiple VEGFR and PDGFR family members.

Several clinical trial studies have reported that anti-angiogenic drugs in combination with conventional chemotherapy or radiotherapy could significantly increase the rate of response, progression free survival and overall survival in patients with advanced solid tumors.[39–41] The synergistic effects between anti-angiogenesis and cytotoxic treatment inspired us to include a mitotic checkpoint kinase, Aurora B, as another key target in our compound designation. Our results showed that CS2164 directly bound to Aurora B kinase and induced G2/M cell cycle arrest at cellular level. In addition, inhibition of Aurora B was confirmed by the decreased H3 phosphorylation and Ki67 expression in CS2164-treated tumor tissue. A recent study shows that Aurora B kinase phosphorylates and instigates the degradation of tumor suppressor p53 through ubiquitination.[42] Accordingly, inhibition of Aurora B kinase in cancer cells induces upregulation of p53 and p53-targeted genes to inhibit tumor growth.[43] Therefore, CS2164 could inhibit tumor cell mitosis through modulating Aurora B/p53 pathway, which may explain at least in part why CS2164 showed a better anti-tumor efficacy than the first-generation VEGFR TKI sunitinib.

Chronic inflammation is generally recognized to promote tumor initiation, progression and metastasis by providing a tumor-supportive microenvironment.[44] Because TAM are usually the most abundant tumor-infiltrating immune cells in the tumor microenvironment and play a protumoral role in most human cancers, targeting TAM has become an attractive approach in cancer therapy.[19,20] We showed that CS2164 directly bound to the ATP pocket of CSF-1R kinase and inhibited CSF-1/CSF-1R signaling, which is correlated with the decreased expression of CSF-1R in tumor tissue from CS2164-treated mice. As TAM also facilitate tumor angiogenesis, depletion of TAM by clodronate-loaded liposomes was found to enhance the inhibitory effects of sorafenib in hepatocellular carcinoma models.[45] Inhibition of CSF-1R also reduced the recruitment of TAM and myeloid-derived suppressor cells (MDSC), and augmented the anti-angiogenesis and anti-tumor effects of VEGFR2 antibody.[46] More interestingly, very recent studies have shown that inhibition of CSF-1R increased the number of $CD8^+$ T cells in tumor tissue while reducing TAM.[47, 48] Taken together, the *in vivo* anti-tumor activity of CS2164 could be also realized by

synergistic anti-angiogenesis and anti-infiltration of inflammatory macrophages with enhanced immune responses through CSF-1R inhibition.

In summary, CS2164 is a novel orally active multi-target inhibitor that simultaneously inhibits the angiogenesis-related kinases (VEGFR2, VEGFR1, VEGFR3, PDGFRα and c-Kit), mitosis-related kinase Aurora B and chronic inflammation-related kinase CSF-1R in a high potency manner with the IC_{50} at a single-digit nanomolar range. In particular, CS2164 showed very high selectivity in the kinase inhibition profile with little activity on off-target non-receptor kinases, proteins, GPCR and ion channels, indicative of a better drug safety profile in terms of clinical relevance. Because of its broad preclinical anti-tumor efficacy and the potential to improve conventional TKI kinase inhibitor therapy in various cancer indications, CS2164 has now entered phase I clinical trials.

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Disclosure Statement

All authors have no potential conflicts of interest to declare.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. The *in vitro* concentration-dependent growth inhibition by CS2164 on mouse endothelial cells.

Fig. S2. The growth-inhibition curves of Molt-4 and Colo-320 cells by CS2164.

Fig. S3. *In vitro* inhibition of CS2164 against M-CSF-stimulated differentiation of mouse macrophages.

Fig. S4. Inhibited infiltration of $CD11b^+$ macrophages in tumor tissue by CS2164.

Fig. S5. Body weight curves for different treatment groups in SMMC-7721 and A549 xenograft nude mice.