Preclinical evaluation of sulfatinib, a novel angio-immuno kinase inhibitor targeting VEGFR, FGFR-1 and CSF-1R kinases

Jinghong Zhou, Jun Ni, Min Cheng, Na Yang, Junqing Liang, Liang Ge, Wei Zhang, Jianxing Tang, Qiaoling Sun, Fu Li, Jia Hu, Dongxia Shi, Hongbo Chen, Jingwen Long, Junen Sun, Fang Yin, Xuelei Ge, Hong Jia, Feng Zhou, Yongxin Ren, Weiguo Qing and Weiguo Su

Hutchison MediPharma Ltd. Building 4, 720 Cai Lun Road, Z.J. Hi-Tech Park, Shanghai, China, 201203

Abstract #4187

Hutchison Medi Pharma

Introduction

- The vascular endothelial growth factor receptors (VEGFR-1,-2,-3) and fibroblast growth factor receptor 1 (FGFR-1) singling pathways are the key regulators of tumor angiogenesis, which promote tumor proliferation, survival and metastasis.
- The roles of the VEGFR, FGFR in regulation of T cells, tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) have also been demonstrated [1-3].
- Colony stimulating factor-1 receptor (CSF-1R) signaling controls the survival and differentiation of myeloid cell lineages, especially for tumor associated macrophages. It polarizes macrophages towards the M2-type, which promote tumor progression by secreting pro-angiogenic and growth factors, as well as by forming an immuno-suppressive tumor microenvironment [4].
- Therefore, blockade of tumor angiogenesis and tumor immune evasion by simultaneously targeting VEGFR, FGFR and CSF-1R kinases may represent a promising approach for anti-cancer therapy.
- Sulfatinib is a VEGFR, FGFR-1 and CSF-1R inhibitor and currently in Phase III trials against neuro-endocrine tumors.

Materials and methods

- **In vitro cell signaling inhibition:** VEGFR2 phosphorylation induced by its ligand, VEGFA, was detected in HEK293-VEGFR2 cell line (established in Hutchison) with DELFIA assay. M-CSF stimulated CSF-1R phosphorylation in Raw 264.7 cell (ATCC) was detected with Western blot.
- **HUVEC proliferation:** The proliferation of primary HUVEC cells (Allcell, cat#HUVEC-001F) was accessed by adding 10 μL of CCK-8 solution (Dojindo, CK04-13) and optical density was read at 450 nm and 630 nm, respectively on Labsystems Multiskan K3.
- **HUVEC tube formation:** The basement membrane matrix (BD Biosciences, 354234) were added into 96-well plates and incubated for 30 minutes at 37℃ to form gelling. Primary HUVECs were seeded and incubated in a 5%CO_2, 37 ℃ incubator for 18 hours. The result was recorded by photographing under a microscope with 40×magnification.
- **Chick embryo chorioallantoic membrane (CAM) assay:** Fertilized chicken eggs were incubated at 37 ℃ with 50% humidity for 24 hours. On the following day, a small window (1 x 1 cm^2) was made in the shell under aseptic conditions. The slides loaded with 10 μL of physiological saline containing various concentrations of sulfatinib were placed on the top of the growing CAMs. The window was resealed with an adhesive tape. Upon 48 hours of incubation, the CAMs were photographed.
- **In vivo target inhibition:** After treatment with a single oral dose of sulfatinib, inhibition on p-VEGFR2 expression in lung tissues of nude mice was determined with Western blot after stimulated by VEGF i.v. injection. FGF23, a biomarker of FGFR inhibition, was determined in the plasma of nude mice with ELISA.
- **In vivo anti-tumor efficacy studies:** Different human tumor lines, BGC823, HT29, H460 and Caki-1 cells were subcutaneously inoculated to the right flanks of Balb/c nude mice. Sulfatinib was orally administered twice a day for three weeks. Murine tumor CT26 cells, were injected into intradermal layer of the right flank of Balb/c mouse. Sulfatinib was orally administered twice a day for 10 days (Experiment 1) or for weeks until achieving end point (Experiment 2).
- **Immunohistochemistry (IHC) or immunofluorescence (IF) staining on CT-26 tumor sections:** IHC staining was done for detection of CD8, CD163, CD31 or iNOS. Briefly, sections were incubated with primary antibody and then biotinylated secondary antibody followed by visualizing with DAB chromogen and counter-stained with hematoxylin. For IF staining, tumor sections were treated with antibodies against CSF-1R and CD163 together, then followed by fluorophore-conjugated secondary antibodies.

Results

In vitro activity of sulfatinib

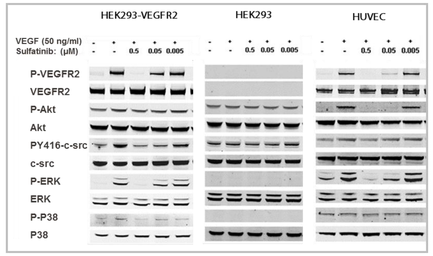
A. Selective inhibition on VEGFR, FGFR and CSF-1R in enzymatic and cell based assay

Kinase and Cell Assay	IC_{50} (μM)
Biochemical activities [5]	
VEGFR-2	0.024
VEGFR-1	0.002
VEGFR-3	0.001
CSF-1R	0.004
FGFR-1	0.015
FLT3	0.067
TrkB	0.041
278 other kinases	> 0.150
Cell-based activity	
VEGF-A stimulated p-VEGFR2 in HEK293-VEGFR2 cells	0.002±0.001
M-CSF stimulated p-CSF-1R in Raw 264.7	0.079
VEGF-A dependent HUVEC proliferation	0.016±0.007
bFGF dependent HUVEC proliferation	0.048±0.002
HUVEC, HEK293, Bcap-37 , H460, HT29 cells survival	≥ 5.0

Inhibition of sulfatinib against 285 kinases at 3μM was measured using [^{33}P-ATP] incorporation assay performed by Upstate Biotechnology Incorporated, now called Eurofin.

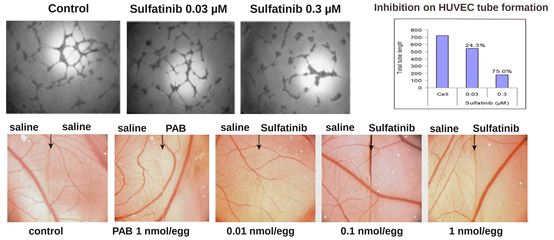
IC_{50} were obtained using FRET-based Z-lyte assay kits assay done by HMP

B. Inhibition on VEGFR signaling in HEK293-VEGFR2 cells and primary HUVECs



- In primary HUVECs and HEK293-VEGFR2 cells, sulfatinib inhibited VEGF stimulated VEGFR2 activation and downstream signaling in a concentration dependent manner.

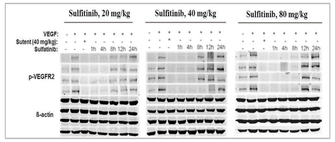
C. Effect on HUVEC tubule growth and CAM angiogenesis



- Sulfatinib suppressed the tube branching, tube length and area in a concentration-dependent manner. The tube length of primary HUVEC was decreased by 75% at 0.3 μM
- Sulfatinib reduced micro-vessel density in a dose-dependent manner, demonstrating significant inhibitory effect on micro-vessel sprouting at 0.1 and 1 nmol/egg concentration (Pseudolarix acid B (PAB) is a positive control)

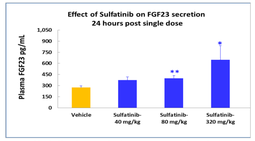
In vivo activity of sulfatinib

A. Effect on VEGF induced p-VEGFR2 in lung tissues of nude mice



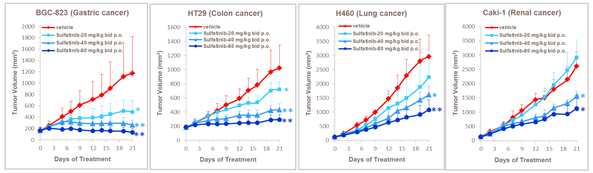
- Sulfatinib 20, 40 mg/kg completely inhibited p-VEGFR2 for 4 hours and 80 mg/kg for 8 hours.

B. Effect of sulfatinib on FGF23 level in plasma of nude mice



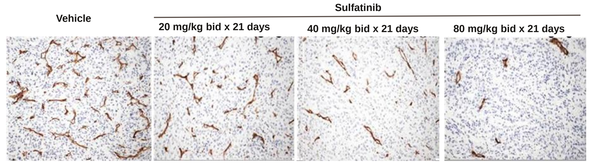
- Sulfatinib elevated plasma FGF23 levels in a dose dependent manner indicating its inhibition on FGFR-1 pathway.

C. Anti-tumor activities of sulfatinib in multiple human xenograft models in nude mice



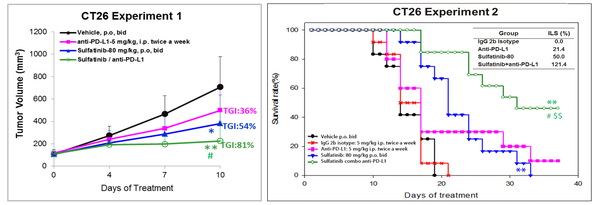
- The in vivo anti-tumor efficacy of sulfatinib was shown in a broad panel of human tumor xenograft. All data were expressed as Mean ± SD and statistical analysis was performed with using one-way ANOVA and Dunnett t test by JMP software. *p<0.05,**p<0.01 vs vehicle

D. Anti-angiogenesis activity in Caki-1 tumor tissue



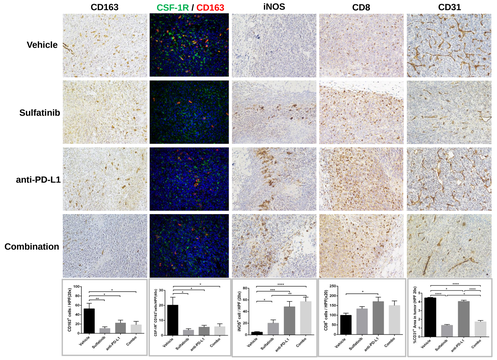
- Sulfatinib dose-dependently repressed CD31 expression in tumor tissue. CD31 staining was performed in the Caki-1 tumors collected at the end of efficacy study. The representative pictures are shown here.

E. Effect of sulfatinib combining with anti-PD-L1 in murine CT26 syngeneic tumor model



- Sulfatinib combined with anti-PD-L1 displayed improved tumor growth inhibition (experiment 1) and prolonged increase life-time span (ILS) (experiment 2, 1500 mm^3 was regarded as endpoint). Difference in tumor volume change was analyzed using student t test. Survival curves were drawn by the Kaplan-Meier method and analyzed by the log-rank test. * p<0.05, **p<0.01 vs vehicle; # p<0.05 vs anti-PD-L1; $^{\$\$}$, p<0.01 vs sulfatinib.

F. Effect of sulfatinib, anti-PD-L1 and their combination on the tumor infiltrated immune cells in CT-26 tumor tissue



- CT-26 subcutaneous tumors were collected at the end of Experiment 1. Sulfatinib significantly decreased M2 macrophages (CD163+), CSF-1R+M2 macrophages (CD163+CSF-1R+) infiltration in tumor tissues and resulted in the increased the infiltration of M1 macrophage (iNOS+) and CD8+ T cells. Sulfatinib significantly inhibited CD31 expression. Student unpaired t test was applied to detect difference among groups using GraphPad Prism 6.0 software. *, p<0.05; **, p<0.01, ***, p<0.001, ****, p<0.0001

Summary

- Sulfatinib is a novel angio-immuno kinase inhibitor targeting VEGFR, FGFR1 and CSF-1R kinases.
- Sulfatinib displayed anti-tumor efficacy in multiple tumor models in a dose dependent manner. The anti-tumor activity of sulfatinib may be partially mediated by anti-angiogenesis via inhibition of VEGFR and FGFR signaling.
- Sulfatinib decreased M2 TAMs and increased M1 TAMs. The immune-modulation effect of sulfatinib might result in enhanced anti-tumor effect when it combines with anti-PD-L1.
- Sulfatinib could simultaneously block tumor angiogenesis and modulate cancer immunity, which might support sulfatinib as an attractive candidate for exploration of possible combinations with checkpoint inhibitors against various cancers.

References

1) Voron T et al. J Exp Med 2015; 212(2):139-148
2) Voron T et al. Front Oncol 2014 ; 4: 1-9
3) Katoh M, et al. Int. J. of Mol Med 2016; 38: 3-1
4) Ries CH et al. Cancer Cell 2014; 25(6):846-859
5) Xu JM et al. Oncotarget 2017; Epub ahead of print