Preclinical profile of novel and potent c-Met Kinase inhibitors

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Swaroop Vakkalanka[ab] , Meyyappan Muthuppalaniappan[b] , Babu, G[b]., Prashant K. Bhavar[b], Kanthikiran VS Varanasi[b] , Sridhar Veeraraghavan[b], Sumalatha Kuppireddi[b], Srikant Viswanadha[b], and Dhanapalan Nagarathnam[ac]

[a]Rhizen Pharmaceuticals SA, Fritz Courvoisier 40, 2300 La Chaux de Fonds, Switzerland

[b]Incozen Therapeutics Private Limited, "Spectrum", Discovery Zone, SP Biotech Park, Shamirpet, Hyderabad, 500 078, India, [c]DNSK International LLC, 925 Sherman Avenue, CT, USA

Background: *c-Met* is a proto-oncogene that encodes the protein Met with intrinsic tyrosine kinase activity. Aberrant Met kinase activity triggers a series of unwarranted phosphorylation events and signalling processes that ultimately lead to the development of cancer. Alteration of the Met kinase signalling cascade represents an attractive approach aimed at blocking invasion and metastasis of cancer cells. Herein, we describe the biological and pharmacokinetic properties of representative molecules from a series of novel and small molecule inhibitors with scope to be further developed as clinical candidates for cancers mediated by dysregulated Met kinase activity.

Methods: Met Kinase activity of test compounds was determined using an HTRF® KinEASE assay kit (Cisbio, Bedford, MA) with modifications. Met-dependent anti-proliferative effect was determined in MKN-45 cells. Inhibition of constitutive Met kinase phosphorylation in MKN-45 and NCI-H441 cells was measured in an ELISA assay. Subsequently, effect of the compounds on Akt phosphorylation, a downstream marker in the Met signalling cascade, was determined. Metabolic stability of the compounds was evaluated in microsomes obtained from mouse, rat, dog, monkey, and human.

Results: Among the compounds evaluated, RP1236, RP1269, RP1316, and RP1400 demonstrated remarkable potency against the purified Met kinase enzyme as well as in an MKN-45 cell proliferation. In addition, the compounds caused a significant reduction in constitutive Met kinase phosphorylation in MKN-45 and NCI-H441 cells. Based on ADME and PK profile, RP1236 and 1400 were studied further in cellular assays. RP1236 demonstrated excellent anti-tumor activity in MKN-45 and U87MG xenograft mocels. Further, the compounds exhibited a favourable ADME profile across the species studied.

Conclusions: Our findings demonstrate that RP1236 and RP1400 are potent Met kinase inhibitors with efficacy values comparable to existing Met kinase inhibitors in development. On lines with selective inhibitors, the compounds display anti-proliferative effect only in cells with amplification of the Met kinase gene. The compounds are currently being tested for efficacy and target inhibition in various xenograft models. Clinical candidate shall be nominated in late 2012.

Introduction

Receptor tyrosine kinases (RTK) represent a class of high affinity cell surface receptors that play a critical role in the development and progression of several types of cancers. Among the several RTK currently under evaluation as druggable targets for cancer, Mesenchymal epithelial transition factor (c-Met), stands out due to its immense potential in regulating downstream events including cell proliferation, metastases, survival, and apoptosis. *c-Met* is a proto-oncogene that encodes the protein Met with intrinsic tyrosine kinase activity. Aberrant Met kinase activity triggers a series of unwarranted phosphorylation events and signalling processes that ultimately lead to the development of cancer. Abnormal activation of MET due to gain-of-function mutations or excessive stimulation by hepatocyte growth factor, an endogenous ligand of MET, is implicated in the progression of various tumours. Met being on the cell surface regulates several key oncogenic signalling pathways including ras/raf/MEK/ERK/PI3K thus controlling downstream events such as proliferation, metastases, motility, and cell death.

The current study describes the pharmacological and pharmacokinetic properties of a series of novel and potent Met kinase inhibitors with immense potential in the treatment of solid cancers.

Compound	Enzyme IC_{50} (nM)	MKN-45 proliferation GI_{50}	Met-Kinase in MKN-45 cells IC_{50}
RP1236	27.9	18.0	11.5
RP1269	20.2	18.0	11.5
RP1316	40.5	2.3	33.3
RP1400	8.9	29.4	28.6

Table 1. Inhibition of Met Kinase enzyme activity, MKN-45 cell proliferation, and Met kinase phosphorylation in MKN-45 cells for selected compounds.

c-Met Kinase activity was determined using an HTRF® Met Kinase Assay (Cisbio) with modifications.

Cell proliferation assays were carried out using the high Met expressing cell line (MKN-45) and viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide **(***MTT***)** *dye reduction test* .

Inhibition of Met phosphorylation was determined using a cell based ELISA assay. Optical density was measured on a microplate reader (BMG Labtech., Germany) at 450 nM.

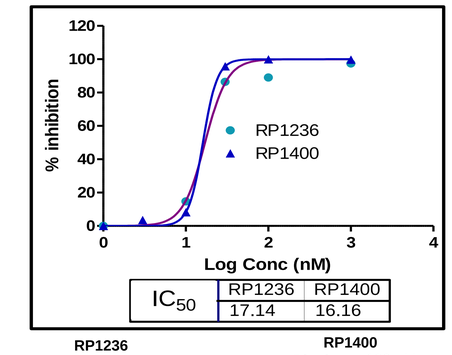

Fig. 1. Inhibition of Akt phosphorylation in MKN-45 cells. Cells were treated with compounds, lysed, and the proteins separated on a 10% SDS-PAGE. Following separation, proteins were transferred onto a nitrocellulose membrane and detected by chemiluminescence after incubation with pAkt S473 mAb (primary) and rabbit anti-mouse Ab (secondary) **.** Intensity of the bands was determined using ImageJ 1.42q (NIH, USA) and normalized to Actin (loading control).

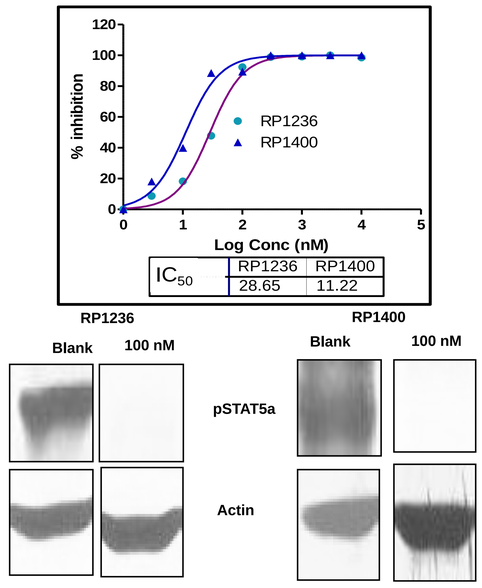

Fig. 2. Inhibition of Stat5a phosphorylation in MKN-45 cells. Cells were treated with compounds, lysed, and the proteins separated on a 10% SDS-PAGE. Following separation, proteins were transferred onto a nitrocellulose membrane and detected by chemiluminescence after incubation with pSTAT-5a mAb (primary) and rabbit anti-mouse Ab (secondary)**.** Intensity of the bands was determined using ImageJ 1.42q (NIH, USA) and normalized to Actin (loading control).

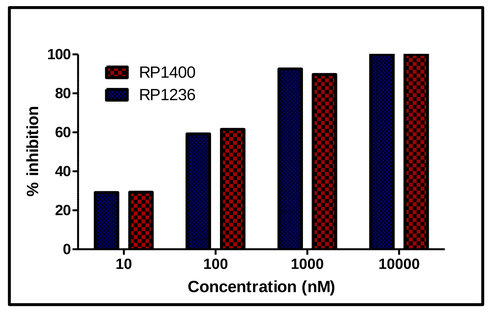

Fig. 3. Inhibition of HGF-induced trans-endothelial migration in MDA-MB-231 cells. Day-1- 0.1 x 10^6 Huvec/ 250 ul seeded in the 8 uM insert and incubated for 24 hrs. **Day-2**- Media added to 24 well plate, with/without 25 mg/ml HGF and inserts transferred to these wells. Huvec media in the insert was removed and 250 ul media 0.1 x 10^6 MDA-MB-231 cells with drug were added to inserts and incubated for another 24 hours. **Day 3**- Media removed from inserts, Swapped with cotton, Stained and quantified calorimetrically.

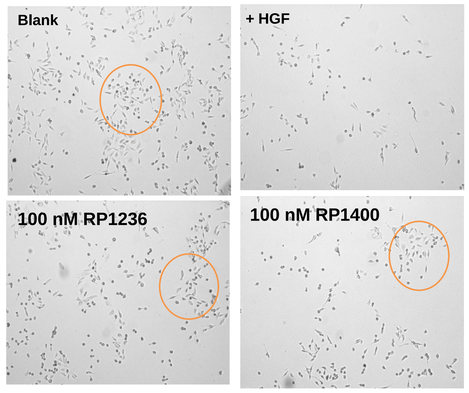

Fig. 4 Inhibition of HGF stimulated scatter in MKN-45 cells: Cells were plated in a 6-well plate. After 24 h, medium was replaced with serum-free media and starve O/N. Desired concentrations of inhibitor in DMSO for 2 h and scatter induced with 40 ng/ml HGF.

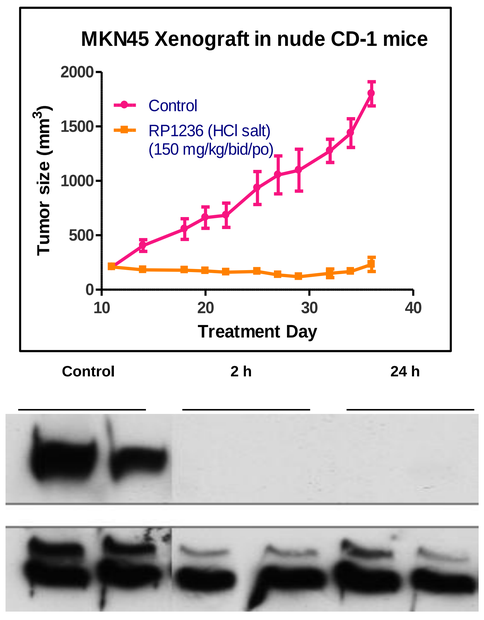

Fig. 5 MKN-45 (human gastric cancer) Xenograft. MKN-45 gastric carcinoma cells (3x10^6cells/mouse) were transplanted into nude CD-1 mice (8-10 mice/group). Mice were randomized when tumors reached approx 200 mg (8-10 mice/ group

***In vivo* target Inhibition: RP1236 was given three times (BID) at the dose of 150mg/kg and MKN45 tumors were collected 2hr and 24hrs after the last dose. pAKT status was analyzed by Western Blotting**

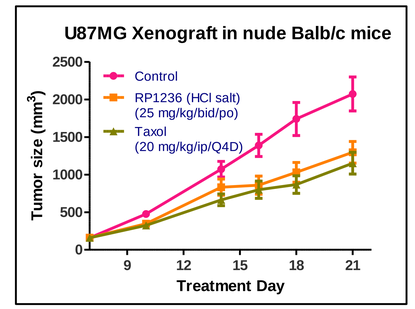

Fig. 6. U87MG (human glioblastoma) Xenograft . Female Balb/c nude mice were innoculated subcutaneously at the right flank with U87MG tumor cells in 1% PBS with matrigel (1:1) for tumor development. Treatments were started on d 7 after innoculation when the mean tumor size reached ~160 mm^3

Inset: In vivo target Inhibition: pAKT in tumor was analyzed by ELISA

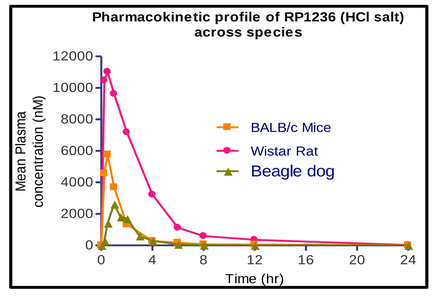

Fig. 7. Single dose Pharmacokinetic Profile of RP1236 (HCl salt) in mice, rat and dog @ 10mg/kg.bw

Summary

- RP1236 and 1400 identified as leads based on in vitro potency and cellular activity
- Additionally, RP1236 demonstrated significant inhibition of MKN-45 and U87MG tumor growth

Current status and future direction

Considering the therapeutic potential of RP1236 and 1400 as evidenced from *in vitro* pharmacological and pharmacokinetic studies, the compounds shall be evaluated in further mouse xenograft models of lung and breast cancer either in isolation or in combination with chemotherapeutic agents. Toxicological evaluation of the compounds shall be carried out concomitantly

For further information please contact at info@rhizen.com

rhizen Pharmaceuticals SA