Title

Potent, selective inhibitors of FGFR define FGF dependence in pre-clinical cancer models.

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Abstract

We describe here the identification and characterisation of two novel inhibitors of the FGFR family of receptor tyrosine kinases. The compounds exhibit selective inhibition of FGFR over the closely related VEGFR2 receptor in cell lines and in vivo. The pharmacological profile of these inhibitors was defined using a panel of human tumour cell lines characterised for specific mutations, amplifications or translocations known to activate one of the four FGFR receptor isoforms. This pharmacology defines a profile for inhibitors that are likely to be of use in clinical settings in disease types where FGFR is shown to play an important role.

Introduction

The fibroblast growth factor (FGF) family and their four receptor tyrosine kinases, FGFR1/2/3/4, mediate numerous physiological processes including cell migration, proliferation, survival and differentiation. Given the importance of FGF/FGFR, it is unsurprising that aberrant FGFR signalling is found in many tumour types including multiple myeloma, gastric, endometrial, prostate and breast (1, 2). Gain of function mutations in FGFRs are the most common kinase abnormality in cancer with activation occurring via a range of mechanisms such as point mutation, amplification, chromosomal translocation and aberrant splicing (3). For example, the t(4;14)(p16;q32) chromosomal translocation found in 15% of multiple myeloma patients often, results in overexpression of FGFR3 (4, 5, 6). The over expressed FGFR3 is usually wild type and although somatic mutations are occasionally found the cells remain sensitive to FGF (7). Activated FGFR3 has a role in myelomagenesis and the ability of anti FGFR3 antibodies and kinase inhibitors, e.g. PD173074 and CHIR258 to inhibit MM cell growth, both in vitro and in vivo, validates FGFR3 as a therapeutic target (8-17). The FGFR2 gene is amplified in some cases of gastric cancer resulting in a highly over expressed and constitutively active receptor. Small molecule inhibitors and FGFR2
knockdown, reveal a critical role for FGFR2 amplification in gastric cancer cell growth both in vitro and in xenograft models (18, 19). Recent publicatons have identified FGFR1 amplification in ~20% of squamous NSCLC (20, 21) and around 10% of breast cancers (22). FGFR4 amplification has been observed in rhabdomyosarcoma and activating mutations characterised in 7% of cases (23). FGFs have a role in tumour angiogenesis and mediate resistance to vascular endothelial growth factor receptor 2 (VEGFR2) inhibitors (24). Together, these compelling data support the development of a specific, potent inhibitor of FGFR1-4 for cancer therapy.

The aim of this drug discovery programme was first to generate potent, selective, orally bioavailable inhibitors of the FGFR family of tyrosine kinases, to establish model systems capable of defining the appropriate pharmacology and to investigate the role of FGFR in cancer. Selectivity over closely homologous kinases including VEGFR2 was considered essential. Broad spectrum tyrosine kinase inhibitors exist but FGFR is never the most potently inhibited kinase and such mixed inhibitors fail to inhibit FGFR completely due to intervening kinase activities and their associated toxicities. Starting with fragment derived hits, we used structure-based design (25-27) to optimise lead molecules to potent FGFR inhibitors with selectivity against VEGFR2 which shares 57% sequence identity with the kinase domain of FGFR1-3 and 54% with that of FGFR4. Further optimisation of pharmacokinetic properties resulted in a series of imidazopyridine leads, the properties of which are described here. The profile in preclinical models presented here describes a paradigm for the response of cellular systems with a defined genetic background to a specific FGFR inhibitor.

**Materials and Methods**
**Compound 1 and compound 2 are** 1-{3-[7-(4-fluorophenyl)imidazo[1,2-a]pyridin-3-yl]phenyl}-3-(2,2,2-trifluoroethyl)urea and 1-{3-[7-(5-methyl-1,3,4-oxadiazol-2-yl)imidazo[1,2-a]pyridin-3-yl]phenyl}-3-(2,2,2-trifluoroethyl)urea respectively. These and additional compounds used were synthesised by Astex Therapeutics Ltd, Cambridge UK or Ortho Biotech, Turnhout, Belgium (28).

**Cell Lines**

KG-1, MFE-296 and RT112/84 cell lines were from the European Collection of Animal Cell Cultures. Snu-1, Snu-16, Kato-III, Hec-1A and AN3-CA cell lines were obtained from the American Type Culture Collection. RPMI-8226 and wild-type Ba/F3 cell lines were obtained from German Collection of Microorganisms and Cell Cultures. The KMS-11 cell line was obtained from the Japanese Collection of Research Bioresources. The Jim-1 cell line was obtained under licence from Cancer Research Technology, UK. Stably transfected Ba/F3 cell lines expressing TEL-FGFR1/3/4 were generated from fusion expression constructs of the TEL oligomerization domain linked to the 5’ end of FGFR kinase domains in pcDNA3.1 (Invitrogen) and electroporation into wild-type Ba/F3 cells. Selection of stable cell lines was performed with Geneticin (Invitrogen) in the absence of mouse IL-3. HUVECs were obtained from Clonetics and grown in EGM2 medium (Clonetics).

Antibodies were from Cell Signaling Technology, except total FGFR2 and total FRS2-α from Santa Cruz Biotechnology.

**Cloning, expression and purification of kinase domains for FGFR1 and VEGFR2**

A construct spanning residues 455-763 of the wild-type human FGFR1 with L455V, C486A and C582S mutations was expressed as a His-tagged protein in Sf9 insect cells.
Cell pellets were lysed by sonication in a buffer containing 25mM Tris/HCl at pH 8.0, 250mM sodium chloride, 10% glycerol, 10mM imidazole and 5mM BME. After centrifugation the supernatant was incubated with Ni NTA fast flow resin and eluted with an imidazole gradient. The eluate was incubated overnight with TEV protease to remove the His-tag and purified on a Ni column. Ion exchange and size exclusion chromatography were used to further purify the protein.

A construct encompassing residues 805 to 1171, but omitting 50 residues of the kinase insert domain was made for the kinase domain of VEGFR2. This construct was expressed and purified as described (29).

**Crystallization, diffraction data collection, structure determination and refinement**

Crystals were obtained by the hanging-drop method. FGFR1 crystals were obtained by seeding into a solution containing 10-15mg/ml of protein, 100mM Bis-Tris buffer at pH6.5, 300mM ammonium sulphate, 12-16% PEG10000, and 5% ethylene glycol.

VEGFR2 crystals were obtained from drops containing 7mg/ml of protein mixed with 100mM Hepes pH 7.2, 2.0-2.2M ammonium sulphate and 4% mPEG550.

Complexes of FGFR1 with Compound 1, 2 and 4 were prepared by soaking the crystals in saturated solutions in 10% DMSO and well solution for 3, 3 and 2 days respectively. The complex of VEGFR2 with Compound 4 was prepared by soaking the compound into the crystal for 3 hours in 10% DMSO and well solution.

All data for FGFR were collected using a Jupiter CCD detector mounted on an R200 rotating anode X-ray generator. Data were processed and scaled using D*trek. X-ray data for VEGFR2 were collected at the ESRF on ID23.1 and processed and scaled using MOSFLM.
All structures were solved using molecular replacement and difference Fourier methods, and refined using REFMAC.

**In vitro Kinase Activities**

Inhibition of kinase activity in vitro was performed as described in Supplementary Data.

**Proliferation Assays**

Cells were seeded into 96 well plates at 5x10^3 cells per well prior to addition of compound in 0.1% DMSO for 72h. 10% v/v Alamar Blue™ (Biosource International, Camarillo, CA.) was added following compound incubation and cells incubated for a further 6 hours. Plate fluorescence was read at \(\lambda_{ex}=535\text{nm}, \lambda_{em}=590\text{nm}\).

**Phospho-FGFR3 and Phospho-VEGFR2 ELISA**

Phospho-FGFR3 was measured in KMS-11 cell lysates using a DuoSetIC ELISA (R&D Systems). KMS-11 cells were plated out in serum-free medium (2x10^5 cells/well) and treated with compounds for 30 minutes before lysing in 125 µl of TG lysis buffer (20 mM Tris pH 7.6, 0.14 M NaCl, 1% (v/v) Triton-X-100, 10% (v/v) glycerol, 0.05 M NaF, 1 mM Na_3VO_4 + protease inhibitor tablet from Roche (Mini Complete™) (used at 1 tablet/10 ml)) for 30 minutes at 4 °C. Lysates (100 µl) were assayed according to the manufacturer’s protocol.

Phospho-VEGFR2 was measured using a DuoSetIC ELISA (R&D Systems). HUVECs (5x10^4 cells/well) were plated out in EGM2 medium (including 1% serum) and left to recover overnight. The cells were switched to serum-free medium and left for a further 16h then
treated with compounds for 30 minutes and stimulated with recombinant human VEGF165 (R&D Systems) at 100 ng/ml for 5 min at 37 °C, before lysing in 125 µl of TG lysis buffer for 30 minutes at 4 °C. Lysates (100 µl) were assayed according to the manufacturer’s protocol.

**Western Blotting**

Cell or xenograft lysates were prepared in TG lysis buffer (see above), cleared by centrifugation and normalised for total protein by BCA assay (Thermoscientific, Rockford IL). Equal quantities of total protein were denatured and run on SDS PAGE gels (Invitrogen, Carlsbad, CA.) and transferred to nitrocellulose blots (Invitrogen, Carlsbad, CA). Bound primary antibodies were detected using IR-labelled secondary antibodies (Li-Cor, Lincoln NE) and an Odyssey imager (Li-Cor, Lincoln, NE).

**Xenograft Models**

All animal studies were performed according to the relevant national regulatory guidelines and individual experiments approved by the appropriate institutional animal welfare committee. Cells from tissue culture were implanted subcutaneously in the right flank of 8 to 10 week old Balb/C nunu mice (Charles River UK) or BALB/c Hsd:athymic nude-Foxn1nu (Harlan UK) at 5x10^6 cells per animal in 50% matrigel basement media (BD Biosciences, UK) and 50% (v/v) RPMI 1640 media (Invitrogen, UK). Treatment commenced when tumours were palpable (approximately 5mm x 5mm, 10-14 days post implantation). Groups of tumour bearing animals (n=8) received dosing vehicle (control) by oral gavage (p.o.) or FGFR3 inhibitor as indicated in the schedule in a dosing volume of 10ml/kg. Tumour volume was calculated by calliper (Mitutoyo, Andover, UK.) measurements using the equation a2 x
b/2 where a is the smallest measurement and b the largest. Data are presented as mean relative tumour volume (RTV), where the tumour volume on the initial day of treatment (day 0) is assigned an RTV value of 1.

A complete regression was defined as a decrease in tumour volume to an undetectable size; <3 mm in any dimension. Tolerability was estimated by monitoring body weight loss, clinical signs and survival. Statistical significance between control and treatment was determined by using ANOVA with Dunnett’s post test for 3 or more groups; or Student’s t test for two groups.

**Pharmacodynamic Studies**

Sub-cutaneous xenograft tumours were removed from nude mice at the indicated times following a single oral administration of FGFR inhibitor. Tumour samples were ground to a fine powder under liquid nitrogen and protein extracted by addition of 1ml triton lysis buffer. Western blots were performed as outlined in Materials and Methods above.

**Results**

**Structure based design of compound 1 and compound 2, inhibitors of FGFR and basis for VEGFR2 selectivity**

Fragment screening against FGFR was performed using a combination of NMR, thermal denaturation and x-ray crystallography resulting in the identification of greater than thirty x-ray structures of fragments in FGFR1. One attractive starting point for medicinal chemistry was the imidazopyridine fragment (compound 3). Due to the low molecular weight of this fragment hit it had a relatively low potency with an IC$_{50}$ of 120uM versus FGFR3. However
when potency was normalised with respect to size an encouraging value of 0.38 kcal per heavy atom was obtained as a measure of the fragment’s ligand efficiency (30). The FGFR1 crystal structure shows that the imidazopyridine binds in the ATP site of the kinase forming a single hydrogen bond to the backbone NH of Ala564 (see Figure 1B). The binding site suggests that the agent acts as an inhibitor by competing for binding with ATP. The experimentally determined binding mode suggested how potency might be readily improved. Firstly, replacement of the chlorine with substituted aromatics could be used to form hydrogen bonds with the side chain of Asp641 and in addition, access to this part of the enzyme might be used to drive selectivity through interactions with Ala640 which in VEGFR2 is substituted by the larger amino acid, cysteine. Secondly, the crystal structure indicated that the ester group at the 6-position of the imidazopyridine template was probably not useful for affinity, whereas elaboration at the 7 position might facilitate good surface complementarity between the ligand and a region of the protein where affinity increases are often observed in kinases.

This structure-based drug design approach led to identification of the selective and potent FGFR inhibitor compound 1 which has an FGFR3 potency of 3nM and a selectivity of about 30-fold over VEGFR2 (Figure 1A and C and Table 1). Further optimisation led to compound 2 (Figure 1A and C) with reduced lipophilicity relative to compound 1. Both compounds were potent inhibitors of all four FGFR isoforms with greater selectivity over VEGFR2, PDGFRβ and the EGFR family compared to TKI-258 or brivanib (Table 1). Compound 1 in particular retained activity against VEGFR1 and 3 and Flt-3 although a screen performed against a larger panel of kinases, indicated that only MKNK and RIPK2 were sensitive to the compounds at 100nM and below. (Table S1). More details on the fragment screening, the structure-based drug design approach and the associated structure activity relationships will be reported elsewhere in due course.
Crystal structures of compounds 1 and 2 bound to FGFR1 were obtained (Figure 1C). Both compounds showed a positioning of the imidazopyridine template similar to compound 3 and the formation of the hydrogen bond with the N-H of Ala564 on the hinge of the kinase. As expected, the aromatic group at the 7 position of the imidazopyridine showed good surface complementarity with the protein. From the structure activity relationships of related compounds (data not shown), it is apparent that the urea group is important for both potency and selectivity of the compounds. The two N-H's of the urea formed a dual hydrogen bond with the carboxylate of Asp641 whilst the carbonyl of the urea forms a water mediated hydrogen bond with sidechain of Arg627. To our knowledge this positioning for Arg627 has not been seen before in FGFR1 crystal structures and it appeared to be induced by the water-mediated interaction with the urea.

The lack of potency of compounds 1 and 2 against VEGFR2 and their low solubility in the crystallography buffer system precluded us from obtaining a structure of either in complex with VEGFR2. However we did succeed in obtaining a VEGFR2 crystal structure for the relatively potent and selective compound 4 (Figure 1A) from the same series (FGFR3 IC50=12nM; VEGFR2 IC50=220nM). Figure 1D shows the experimentally determined binding mode of compound 4 in VEGFR2 superimposed on the experimental binding mode of the same compound in FGFR1. The two binding modes for this ligand are very similar and selectivity in the series must be driven by subtle energetic differences. One possible explanation for the selectivity is induced movement of Arg627 in the FGFR1 structure which does not occur with the corresponding residue (Arg1032) in VEGFR2. This lack of movement of Arg1032 is also seen in other VEGFR2 structures from this series suggesting a higher energetic penalty associated with this protein movement in VEGFR2. However it should be noted that our FGFR1 protein structures are obtained with non-phosphorylated protein whereas the VEGFR2 crystals are obtained from a mixture of mono and
diphosphorylated protein, so differences in conformational mobility associated with different phosphorylation states cannot be discounted. Another possible driver for selectivity is that in VEGFR2, the sulphur of Cys1045 (equivalent to Ala640 in FGFR1) is approximately 3.4 Å away from the NH and 3.5 Å away from the carbon of the urea group and that these close contacts are slightly unfavourable in VEGFR2.

**Compounds 1 and 2 inhibit proliferation and survival of a panel of FGFR-dependent Cell lines**

Compounds 1 and 2 were assayed for anti-proliferative activity against a panel of 14 cell lines comprising Ba/F3 cells engineered to express constitutively active forms of FGFR1, 3 and 4 and lines representative of a number of diseases in which FGFR signalling is known to be upregulated. In addition, three control cell lines were included (Snu1 a gastric line harboring mutant ras, HEC-1A a wild type FGFR2 endometrial line and RPMI-8226 a multiple myeloma line without the t(4;14) translocation). Finally wild type Ba/F3 cells that are dependent upon cytokine signalling for survival were included. Both compound 1 and compound 2 were potent inhibitors of FGFR-dependent cell survival with mean IC₅₀s in FGFR-dependent cells of 320nM and 670nM and FGFR-independent cells of 3500nM and >6500nM respectively. Examples of cell lines activated by each of the FGFR isoforms were included in the panel and cell proliferation was inhibited in all of these consistent with the pan-FGFR activity of the compounds (Table 1). Figure 2 is a graphical representation of the data with log IC₅₀s expressed as a fold change from the mean value. Brivanib alaninate, an example of a broad spectrum tyrosine kinase inhibitor, was significantly less potent in the panel overall and exhibited minimal selectivity for FGFR-dependent versus independent lines with a mean IC₅₀ of 2500nM and 6500nM respectively. These data clearly illustrate the selectivity of compounds 1 and 2 in FGFR dependent systems compared to those cell lines transformed by other mechanisms.
Compounds 1 and 2 selectively inhibit FGFR in cells

An ELISA was used to monitor levels of phospho-FGFR3 in KMS-11 multiple myeloma cells and phospho-VEGFR2 in HUVEC cells following incubation with a concentration range of compound 1, compound 2, the mixed VEGFR2/FGFR inhibitors TKI-258 and brivanib or the VEGFR inhibitor ZD6474. Both compounds 1 and 2 inhibited phospho-FGFR at concentrations around 10 fold lower than those required to inhibit phospho-VEGFR2 (Figure 3). This is consistent with the selectivity of these compounds in vitro (Table 1). The less selective compounds TKI 258, brivanib and ZD6474 were more potent against VEGFR2 in HUVECs (28, 27 and 190nM) than against FGFR in KMS-11s (300, 200 and >10000 nM) (Figure 3).

Inhibition of FGFR2 signalling in cells was confirmed in the FGFR2-amplified gastric cancer cell line Snu-16 (Figure 4). Snu-1 gastric lines were included as a control line as they express only low levels of FGFR2 and harbour a Ras mutation. Both compounds 1 and 2 inhibited phosphorylation of the FGFR2 receptor in Snu-16 cells at concentrations above 100nM. Inhibition of downstream signalling in the MAPK and AKT pathways was also observed at the levels of ERK, AKT and S6. Incubation of Snu-1 cells with the same concentrations had no inhibitory effect on these signalling pathways consistent with the lower anti-proliferative activity of the compounds in this cell line (Figure 2). Inhibition of phospho-FGFR3 and downstream signalling was observed in KMS-11 cells at concentrations below 100nM (Figure 4C). Inhibition of FGFR1 signalling in KG-1 cells, FGFR2 in AN3CA and MFE-296 and FGFR3 in 97/7, RT-4 and RT11284 bladder cell lines was also confirmed by the same methods (Figure S1).

Pharmacokinetic Characterisation of Compounds 1 and 2
Figure S2 shows the pharmacokinetic profiles of compounds 1 and 2 following oral dosing in the mouse. Compound 1 exhibits high oral bioavailability in the mouse (79%) and excellent dose linearity with respect to Cmax (19.5 and 28.6 ug/ml) and AUC (179 and 287 ug.h.ml for 50 and 100 mg/kg respectively). Compound 2 exhibits similarly favourable kinetics with an oral bioavailability of 100% and dose linearity (Cmax values of 16, 33 and 49 ug/ml and AUC values of 65, 130 and 330 ug.h.ml for 12.5, 25 and 50 mg/kg respectively).

Stability in liver microsomes suggested low clearance for both compounds (Table S2) and this was confirmed in vivo. Metabolic clearance was 2.3 and 5.1 ml/min/kg respectively for compound 1 in mouse and rat and 5.2 and 1.1 ml/min/kg for compound 2 following i.v. dosing. Low turnover in human microsomes suggested that therapeutic exposures should be achievable in human subjects.

**Pharmacodynamic studies in human tumour xenografts**

Inhibition of FGFR and downstream signalling was investigated in human tumour xenograft models (Figure 5). A single dose of either compound 1 or 2 at 50 mg/kg p.o. completely ablated phospho-FGFR3 in the Ba/F3-TEL FGFR3 model (Figure 5A). This inhibition was shown to be dose dependent for compound 1 (Figure S3). In Snu-16 (Figure 5B) and KMS-11 (Figure 5C) xenografts phospho-FGFR2 and phospho-FGFR3 were inhibited for more than 8h following a single dose of compound 1. Inhibition of downstream MAPK and Akt signalling and an increase in the levels of cleaved PARP, a marker of apoptosis, were observed at 8h in the Snu-16. Figure 6D shows RPMI-8226 tumours removed from animals 4 hours after treatment with the indicated doses of compound 1. In this FGFR-independent model no effect was observed on either MAPK or PARP.

**Efficacy in Human Tumour Xenografts**
The anti-tumour efficacy of both compounds 1 and 2 was investigated in FGFR3 dependent
multiple myeloma xenografts (Figure 6A and C). For this purpose we used KMS-11 cells that
express high levels of a mutant FGFR3 (Figure 4C) and Jim-1 cells that express physiological
levels of wild type FGFR3 (Figure S3). Compounds were administered orally, once daily at
doses which tolerability studies suggested were below the maximum tolerated doses.
Administration of 50mg/kg compound 1 for up to 21 days resulted in minimal body weight
loss and no observations of gross toxicity were made. For compound 2 no adverse toxicities
were observed at doses up to 100mg/kg once daily when dosed for up to 26 days. Compound
1 caused tumour growth inhibition at 12.5mg/kg p.o. in the Jim-1 model with regression of
tumour volume observed at both 25mg/kg and at 50mg/kg. At 50mg/kg all animals showed
tumour shrinkage by day 5, which persisted to day 10. By day 16, 50% of these tumours were
still smaller than on day 1. Compound 2 was less effective in the Jim-1 model at 50mg/kg
consistent with the lower potency of this compound. The higher dose of 100 mg/kg however
caused an equivalent growth inhibition in the KMS-11 model to that caused by 50mg/kg
compound 1. Both compounds were also efficacious in the endometrial xenograft model
AN3CA. Compound 1 was not efficacious even at the highest dose (50 mg/kg) tested in the
FGFR-independent RPMI 8226 model (Figure 6B), a dose and schedule that resulted in
complete growth inhibition in the FGFR positive Jim-1 model. Compound 2 had no efficacy
in the FGFR-independent MDA-MB-231 breast cancer xenograft a model known to be
dependent upon EGFR rather than FGFR for survival (Figure 6H). In contrast to compounds
1 and 2 the mixed FGFR/VEGFR2 inhibitor TKI 258 exhibited similar growth inhibitory
activity in both the Jim-1 and RPMI-8226 xenografts (Figure 6A and B). This suggests that a
proportion of the activity of this compound in xenograft models is due to inhibition of kinases
in addition to FGFR, most probably an effect on tumour angiogenesis due to VEGFR2
inhibition. The selective inhibition of FGFR-dependent systems by compounds 1 and 2 demonstrated the potential to exploit these selective compounds in a targeted fashion.

**Discussion**

Here we describe the characterisation of two fragment derived, specific inhibitors of the FGFR family of kinases. Compound 1 and compound 2 are potent inhibitors of all four isoforms of the FGFR family with no appreciable activity in a panel of related receptor tyrosine kinases including EGFR and PDGFRβ. The compounds were ~20 fold selective against the highly homologous VEGFR2 kinase. They were selective both in cell based assays and *in vivo* where this apparently modest level of selectivity *in vitro* translated to a highly specific tumour inhibitory effect in FGFR dependent xenografts with no activity in xenografts not dependent upon FGFR signalling. These data more than any other confirm that the compounds are acting in an FGFR specific manner when exerting their effects in these model systems.

This is the first detailed pharmacological characterisation of compounds that exhibit selectivity for FGFR family members over VEGFR2. With the growing interest in targeting FGFR, broad studies such as those presented here will help define the pharmacological profile of a specific FGFR inhibitor across a relevant panel of pre-clinical systems. Several studies in defined tumour types have been performed with PD173074 (12, 31, 32) but they predominantly use engineered cell lines or those representing a single disease type. A lack of small molecule and biological inhibitors, specific for FGFR and a lack of well characterised reagents have, to date, prevented similar studies.

There are several indications within oncology in which targeting the FGFR pathway specifically may be of benefit. These include tumour types with specific activating mutations in FGFR (5 - 7, 31), those with amplification of one of the receptor family members (2) or
more broadly as an anti-angiogenic therapy following VEGFR therapy in tumours in which FGF-2 has been upregulated (24). Several molecules are or have been investigated with the aim of potentially exploiting their FGFR activity. PD173074 demonstrated selectivity for the FGFR receptor in vitro and a number of reports described its activity in individual FGFR-dependent systems (12, 14) but it did not proceed into clinical development. Compounds explored clinically include brivanib (BMS, ref 29) in hepatocellular carcinoma (NCT 00355238), endometrial (NCT06888173) and colorectal cancer (NCT00207051) and TKI-258 (Novartis) in multiple myeloma (NCT 01058434), renal cell carcinoma (NCT00715182), urothelial cell carcinoma (NCT00790426) and breast cancer (NCT 00958971). Isoform specific antibody therapies are also in pre-clinical development. Two recent publications describe the potent activity of FGFR2-IIIb specific (34) and FGFR2-specific (35) antibodies in preclinical models of gastric cancer, harboring FGFR2 amplification. This further supports the important role of FGFR2 in this indication.

Data presented in here demonstrate broad spectrum compounds have in vivo activity against both FGFR-dependent and independent models most likely driven by their anti-angiogenic activity via VEGFR2 inhibition. Thus it is difficult to demonstrate FGFR specific effects attributable to these compounds either pre-clinically or in clinical studies. One possible limitation of these inhibitors in FGFR driven disease is the appearance intervening toxicities due inhibition of additional kinases which prevents maximal inhibition of FGFR in relevant tumour settings.

Compounds 1 and 2 described here exhibit very distinct activity profiles in FGFR-dependent and independent in vitro and in vivo systems. As such the compounds offer an exciting and innovative approach to targeting cancers dependent upon FGFR signalling.

Acknowledgements
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References


Figure Legends

Table 1.

In vitro assays were performed for the indicated kinases. IC₅₀ was calculated using Prism software and expressed as mean of at least 2 independent experiments performed in triplicate.

Figure 1.

Chemical structures of 1-3-[7-(4-fluoro-phenyl)-imidazo[1,2-a]pyridin-3-yl]-phenyl]-3-(2,2,2-trifluoro-ethyl)-urea (1); 1-3-[7-(5-methyl-[1,3,4]oxadiazol-2-yl)-imidazo[1,2-a]pyridin-3-yl]-phenyl]-3-(2,2,2-trifluoro-ethyl)-urea (2); 3-chloro-imidazo[1,2-a]pyridine-6-carboxylic acid methyl ester (3) (A). Figure 1B shows the initial fragment ligand (3) bound into FGFR-1 as a protein ligand co-complex with the Van-der-Waals protein surface (orange). Figure 1C shows the protein-ligand co-complex of both advanced compounds (1) (blue) and (2) (purple). Figure 1D shows the chemical structure of compound 4 (1-3-[7-(4-acetyl-piperazin-1-yl)-imidazo[1,2-a]pyridin-3-yl]-phenyl]-3-(2,2,2-trifluoro-ethyl)-urea). Figure 2B shows compound 4 bound to both FGFR1 (blue) and VEGFR2 (purple); Arg627, Ala640 and Tyr563 are labeled according to the FGFR sequence and numbering (the corresponding VEGFR2 residues are Arg1032, Cys1045 and Phe918). The water-mediated contact between the ligand and Arg627 is only present in the FGFR1 structure because in the VEGFR2 structure the corresponding arginine adopts a very different conformation.
Figure 2. Inhibition of proliferation and survival in FGFR-dependent cell lines

Antiproliferative activity of compounds 1 and 2 were assessed in a panel of human tumour cell lines as described in Materials and Methods. Cell lines known not to be dependent upon FGFR signalling were included (*). Experiments were performed in triplicate and data presented is comprised of the mean of at least 2 independent experiments and values expressed as the fold change from the mean log IC₅₀.

Figure 3. Compounds 1 and 2 exhibit selectivity for FGFR3 over VEGFR2 in cells.

Serum starved HUVEC cells (VEGFR2) or KMS-11 cells (FGFR3) were incubated with the indicated compounds for 30 minutes. HUVEC cells were stimulated by the addition of 100pg/ml VEGF165 for 5 minutes. An ELISA was used to monitor either pVEGF2 or pFGFR3. Curves are representative of at least 3 individual experiments in each case.

Figure 4. Inhibition of FGFR signalling in tumour cell lines.

The indicated cell lines were incubated with the indicated concentrations of compound 1 (A and C) and compound 2 (B) or 10uM PD173074. For KMS-11 cells FGF was added for the final 5 minutes. Western blotting was performed on cell lysates. Data are representative of at least 3 independent experiments.

Figure 5. Inhibition of FGFR signalling in xenograft tumours.

Mice bearing Ba/F3-TEL/FGFR3 (A), Snu-16 (B) or KMS-11 (C) xenografts were treated with a single dose of compound 1 or 2, at 50 mg/kg, as indicated. Triplicate tumour samples were removed at subsequent times and processed to determine levels of phospho-signalling by immunoblotting. Nude mice bearing RPMI-8226 xenografts (D) had compound 1
administered daily for 21 days at the indicated doses. Tumours were removed at 4h following the final dose.

**Figure 6. Compounds 1 and 2 are efficacious in FGFR-dependent tumour xenograft models.**

Compound 1 was administered orally to mice bearing (A) FGFR dependent (KMS11, Jim-1, AN3CA) or (B) FGFR independent xenografts (RPMI-8226) once daily at the indicated doses and time periods. For the Jim-1 and RPMI-8226 studies, TKI-258 was included as a positive control. Compound 2 was administered orally to mice bearing (C) FGFR dependent (KMS11, Jim-1, AN3CA) or (D) FGFR independent xenografts (MDA MB 231) once daily for the indicated doses and time periods. Groups were taken off study at the end of the study period or when tumours reached >1000 mm$^3$. 
Figure 1.

A

Compound 1

Compound 2

Compound 3

Compound 4

B

C

D

Arg627

Ala640

Tyr503
Figure 2.
Figure 3.
Figure 4.
A FGFR3 Inhibition in Baf3-TEL/FGFR3 model

B Compound 1 in Snu-16 xenograft

C Compound 1 in KMS 11 xenograft

D Compound 1 in RPMI 8226 xenograft

Figure 5.
**Figure 6.**

A. FGFR-dependent Xenografts

B. FGFR-independent Xenograft

C. FGFR-dependent Xenografts

D. FGFR-independent Xenograft
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Molecular Cancer Therapeutics

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