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Approaches to selective fibroblast growth factor receptor 4 inhibition through targeting the ATP-pocket middle-hinge region†‡

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A diverse range of selective FGFR4 inhibitor hit series were identified using unbiased screening approaches and by the modification of known kinase inhibitor scaffolds. In each case the origin of the selectivity was consistent with an interaction with a poorly conserved cysteine residue within the middle-hinge region of the kinase domain of FGFR4, at position 552. Targeting this region identified a non-covalent diamino-pyrimidine series differentiating by size, an irreversible-covalent inhibitor in which Cys552 undergoes an SNAr reaction with a 2-chloropyridine, and a reversible-covalent inhibitor series in which Cys552 forms a hemithioacetal adduct with a 2-formyl naphthalene. In addition, the introduction of an acrylamide into a known FGFR scaffold identified a pan-FGFR inhibitor which reacted with both Cys552 and a second poorly conserved cysteine on the P-loop of FGFR4 at position 477 which is present in all four FGFR family members.

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1. Introduction

The fibroblast growth factor family consists of four fibroblast growth factor receptors (FGFRs), of the receptor tyrosine kinase family, which interact with eighteen fibroblast growth factor ligands (FGFs). With such a large number of ligand-receptor permutations the FGFs are key components of a wide range of signaling systems which are involved in regulating multiple aspects of development and normal physiology, as well as being implicated as the drivers of a number of malignancies.¹ Of particular interest in the context of cancer, the aberrant FGF19/FGFR4 signaling in hepatocellular carcinoma (HCC) has been shown to be both an initiating event in pre-clinical studies,² and additionally to be required for the maintenance of the disease.³ As a consequence, we became interested in the identifying FGFR4 inhibitors that could be used for the treatment of FGFR4-driven diseases, and in particular HCC. In this letter we describe a number of the hit-finding approaches that were taken to identify selective FGFR4 inhibitor starting points, and our rationale for prioritisation between them.

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A number of pan-FGFR inhibitors have been described which target the ATP binding site within the kinase domain of the receptor, and are characterised as being either: biased towards FGFR 1–3 inhibition, with >10-fold lower FGFR4 potency; or equipotent *versus* all four family members.⁴ The most advanced of these pan-FGFR inhibitors, infogratinib (NVP-BGJ398) and AZD4547,^{5,6} fall into the former FGFR 1–3 biased category, and are currently in PhII clinical studies for the treatment of cancer, Fig. 1. However, for all of these agents the robust inhibition of FGFR4 is anticipated to also lead to extensive inhibition of the other three isoforms, and as a result will be associated with the side effects resulting

Fig. 1 Structures of the pan-FGFR inhibitors infogratinib and AZD4547.

from FGFR 1–3 inhibition.⁷ Therefore, the opportunity to identify selective inhibitors of FGFR4 was investigated with the anticipation that it would provide better tolerated treatments, and as a result enable more robust inhibition of the target to be explored.

As part of the feasibility assessment for the project a sequence alignment was carried out across the human kinome looking for differences within FGFR4 that could be exploited to gain selectivity. This analysis revealed a poorly conserved cysteine residue within the middle-hinge region of the ATP binding site of the FGFR4 kinase domain at position 552. More specifically, this residue is situated two positions beyond the gate-keeper residue towards the C-terminus of the protein (GK+2 position), Fig. 2. Making a comparison based upon the size of the amino-acid side-chain at this position in FGFR4: only twelve other kinases in the kinome have similar sized, or smaller, residues at the GK+2 position. Additionally, the other three members of the FGFR family all contain a larger tyrosine residue at this position. Alternatively, the potential to target the nucleophilic character of Cys552, for a targeted covalent interaction, revealed only four other kinases sharing a cysteine at the GK+2 position.⁸ Therefore, either targeting the larger sub-pocket adjacent to the hinge, based upon size, or for covalent attachment, has the potential to gain selectivity over the other FGFR family members, and over 97%, or 99%, of the kinome respectively. Similar analyses for targeting FGFR4 selective inhibitors have also been reported by others.^{9–15}

The low conformational flexibility in this region of the kinase domain, adjacent to the hinge, was also anticipated to facilitate selectivity: substituents fitting well into this relatively rigid region of the protein should differentiate clearly, as any changes in protein conformation would be anticipated to be highly disfavoured.¹⁶ Additionally, for a covalent-binding approach the side chain of Cys552 occupies a position deep within the middle-hinge region of the protein. As a result, the shape and size of electrophile, and the possible approach vectors which could lead to the formation of a covalent

attachment, are anticipated to be limited, and to be very specific.

In addition to Cys552, the ATP-binding site of FGFR4 also contains a second poorly-conserved cysteine residue. Cys477 resides on the P-loop of FGFR4 and is also present at the equivalent position in FGFR 1–3, and five other kinases within the human kinome, Fig. 2.⁸ In contrast to the middle-hinge region, the P-loop is a relatively dynamic portion of the protein, and as a result Cys477 should be possible to target for a covalent interaction in multiple conformations.¹⁶

To date a number of groups have reported covalent inhibitors based upon variants of the pan-FGFR inhibitor scaffold PD 166866.¹⁷ Inhibitors which target the middle-hinge region for FGFR4 selectivity have been reported by five groups: the acrylamides BLU9931, BLU554, H3B-6527, 1 and 2 bind covalently to Cys552.^{10–14} A structurally unrelated series of imidazolopyridines, exemplified by 3, are reported to target the size difference in this region of the ATP pocket to achieve FGFR4 selectivity.¹⁵ In addition, the P-loop cysteine in the FGFR family has been targeted for pan-FGFR inhibition in an irreversible-covalent manner with the acrylamide containing inhibitors FIN-1 and PRN1371,^{18,19} and in a reversible-covalent manner with the structurally-related cyano-acrylamides, as exemplified by 4, Fig. 3.²⁰

2. Results and discussion

The differences within the middle-hinge region of the FGFR family are highlighted in Fig. 4, which shows the X-ray structure determined for infigratinib bound to FGFR1.⁵ In this figure the GK+2 tyrosine, present in FGFRs 1–3, and the cysteine in FGFR4 are overlaid.²¹ One hypothesis which was generated from this structure suggested that substitution of the phenyl ring of infigratinib in the position *ortho* to the secondary aniline, which resides in the hydrophobic channel of the ATP-pocket, would provide access to the GK+2 sub-pocket, as indicated in Fig. 4. Such a strategy was appealing as a number of kinase scaffolds have an aromatic group occupying this 'hydrophobic channel' region of the ATP pocket, and hence offered the opportunity to readily explore this hypothesis.

At the beginning of the project a number of hit-finding activities were initiated to identify FGFR4-selective starting points, including: a high-throughput screening campaign, in which hits were counter-screened against FGFR2 to gain an early insight into selectivity; data mining in combination with *de novo* design, to identify known ATP-pocket binders that could be modified to increase FGFR4 selectivity, and the rational incorporation of electrophiles for targeted covalent binding. One goal was to evaluate which type of starting point would provide the best opportunity to deliver a selective FGFR4 inhibitor candidate, by any means, but with a particular interest in those in which the selectivity involved an interaction with the middle-hinge region of the ATP-pocket. Such opportunities were considered to provide a high probability for kinome-wide selectivity based upon the above sequence and structural evaluation.

Fig. 2 Positions of Cys552 and Cys477 within the ATP-pocket of the kinase domain of FGFR4. Two possible conformations of the kinase P-loop are represented: in beige, the typical hairpin conformation and in magenta, a disordered conformation observed in the crystal structure of FGFR4 in complex with compound 8, *vide infra*.

Fig. 3 Structures of PD 166866, the FGFR4 selective inhibitors BLU9931, BLU554, H3B-6527, **1**, **2** and **3**, and the FGFR family P-loop cysteine targeting FIN-1, PRN1371 and **4**.

The analysis of the Novartis internal kinase panel revealed a small number of compounds exhibiting some level of selectivity for FGFR4 within the FGFR family, and the broader kinome. Of these potential starting points, the diaminopyrimidine **5**, Fig. 5, was of particular interest, having been originally prepared as part of a project targeting inhibitors of focal adhesion kinase,²² and possessing greater than 100-fold selectivity within the FGFR family. Biochemical data for the inhibition of the kinase activities of FGFRs 1–4 are shown in Tables 1 and 2.²¹

Starting from a well described ATP-competitive scaffold, such as the diaminopyrimidines, also enabled the binding

mode within the FGFR4 kinase domain to be assigned for **5** with a high degree of certainty.²³ Based upon this binding mode, the phenyl group in **5** would occupy the same position in the hydrophobic channel as shown for infigratinib in Fig. 4. As a result the isopropoxy group would be orientated into the larger sub-pocket created by the Cys552 residue. Thus, enabling the FGFR4 selectivity for **5** to be readily rationalised based upon this specific interaction, as shown in Fig. 4. Further support for this rationale was obtained with **6**, the methoxy analogue of **5**, in which the smaller methoxy residue can be accommodated in the middle-hinge region by all four FGFRs, and as a result the compound is a potent inhibitor across all the FGFR family members.

Taking the diaminopyrimidine **5** as the starting point, the structure activity relationships from the further optimisation of the series were consistent with the proposed binding mode, which included exploring a diverse range of substituents on the phenyl ring targeting the GK+2 sub-pocket. This

Fig. 4 Comparison of Cys versus Tyr at the GK+2 of the FGFR family based upon the X-ray structure of infigratinib (shown in beige) bound into the ATP binding site of FGFR1 (PDB code: 3TT0): the GK+2 Tyr563 of FGFR1 is shown in white, the GK+2 Cys552 side-chain of FGFR4 is modeled into the structure and shown in magenta. The hydrogen-bonds forming the hinge interaction are shown as dotted lines. The *ortho* position of infigratinib, with access to the middle-hinge sub-pocket, is highlighted by the green arrow.

Fig. 5 Structures of the diaminopyrimidines **5**, **6** and **7**.

Table 1 Biochemical FGFR 1–4 activities

Compound	Biochemical IC ₅₀ (nM)			
	FGFR4 (388–802)	FGFR1 (407–822)	FGFR2 (406–821)	FGFR3 (411–806)
Infigratinib	71 ± 34	0.8 ± 0.4	1.2 ± 0.8	1.8 ± 1.2
AZD4547	56 ± 21	0.8	1.1 ± 0.1	5.2
BLU9931	14 ± 10	3200	1700 ± 0.42	780
5	29 ± 2.1	6350 ± 2665	4433 ± 1650	8000 ± 1709
6	n.d.	19 ± 6.3	n.d.	85 ± 50
7	2.2 ± 0.9	280	363 ± 172	3500
8	1.5 ± 1.0	4.5 ± 4.9	2.2 ± 2.0	1.7 ± 0.5
9	53 ± 18	>10 000	>10 000	>10 000
10	57 ± 10	>10 000	>10 000	>10 000

n.d. = not determined; data are from single experiments, or expressed as mean ± SD, where 2 to 9 repeated experiments were performed.

Table 2 Biochemical FGFR4 activities with the non-phosphorylated wild-type and C552A/C477A variants

Compound	FGFR4 biochemical IC ₅₀ values (nM)		
	Wild-type (442–753)	C552A (442–753)	C477A (442–753)
Infigratinib	62 ± 23	0.82	64 ± 21
AZD4547	103 ± 46	655 ± 118	67 ± 17
BLU9931	11 ± 1	>10 000	9.4 ± 0.5
6	16 ± 3	1920 ± 200	20 ± 3
7	6.9 ± 3.6	8.4 ± 3.2	7.5 ± 0.5
8	0.8 ± 0.2	3.6 ± 1.2	443 ± 94
9	32 ± 11	>10 000	21 ± 1
10	65 ± 49	>10 000	43 ± 10
11	>10 000	>10 000	>10 000
12	>10 000	>10 000	>10 000
13	>10 000	>10 000	>10 000
14	>10 000	>10 000	>10 000

Data are from single experiments, or expressed as mean ± SD, where 2 to 8 repeated experiments were performed.

optimisation yielded analogues with improved FGFR4 potency and a high level of kinase selectivity, including >100-fold selectivity *versus* the other FGFR family members, as exemplified by 7.²¹ The isopropoxy starting point for the GK+2 targeting moiety in 5 was found to be close to optimal for this diaminopyrimidine series, and the phenyl ring in the optimised analogue 7 is substituted with the closely related isobutoxy substituent. Compound 7 was shown to be a useful non-covalent FGFR4 inhibitor tool compound, and is shown

modelled into the kinase domain of FGFR4 in Fig. 6. Key interactions include: hydrogen bonds to the hinge residue Ala553; and key hydrophobic contacts between the isobutoxy group and Cys552, and the methylene-bridge of the bicyclic moiety and a hydrophobic cleft formed between Val481 and Gly474.

The optimisation leading to the identification of 7 highlighted that the differences in the size of the GK+2 residue could be exploited by a non-covalent inhibitor to gain an acceptable level of selectivity for FGFR4 within both, the FGFR family, and also the broader kinome. In parallel, hit-finding activities for potential targeted-covalent binders revealed a number of putative electrophile-containing hits, suggesting a 'reactive-cysteine' residue, or residues, were present within the FGFR4 kinase domain. Of these, the three hits 8, 9 and 10, shown in Fig. 7, proved to be the most interesting and demonstrated the breadth of covalent-binding interactions that were identified with FGFR4, *vide infra*.

To better understand any putative covalent-interactions with FGFR4 biochemical assays were established in which either the middle-hinge Cys552, or the P-loop Cys477, were replaced by alanine and compared to the wild-type protein. These data are included in Table 2, and also differ from the FGFR4 data in Table 1 in that shorter non-phosphorylated

Fig. 6 Model of 7 bound to the kinase domain of the FGFR4.

Fig. 7 Structures of the FGFR4 covalent binders 8, 9 and 10.

kinase-domain constructs were used in these assays.²¹ Comparison of the IC_{50} values between the wild-type FGFR4 assays in Tables 1 and 2 indicated comparable behavior for the two proteins, across all the compounds. Additionally, both FGFR4 alanine variants in Table 2 showed similar biochemical behavior to the wild-type enzyme with respect to: ATP-binding affinity and the ability to phosphorylate the peptide substrate.²¹ Thus, supporting no gross changes having occurred to the kinase domain as a result of these cysteine to alanine modifications.

One striking difference with the alanine modified FGFR4s was the greatly reduced activity of the methoxy-substituted diaminopyrimidine 6 with the C552A variant, when compared to the isobutoxy analogue 7. Molecular modeling revealed a plausible hypothesis for this difference.²¹ In the case of 6, in which the smaller alanine side-chain residue is interacting with the smallest ether residue, the hydrophobic surface shielding the hinge binding interaction is interrupted. As a result this key element of the binding interaction is weakened due to an increased exposure of the hydrogen bonding network to solvent. In contrast, the larger isobutoxy moiety in 7 maintains the hydrophobic surface, shielding the hinge interaction with the Ala552 residue present, and as a result exhibits no decrease in potency.

The acrylamide 8 was identified by the rational introduction of an electrophile into a known FGFR ATP-site-binding pharmacophore. Starting from the pan-FGFR inhibitor AZD4547, the phenyl ring of the benzamide moiety resides in the hydrophobic channel of the ATP-binding site in a similar position to the phenyl ring of 7, as shown in Fig. 6.²⁴ Thus, the introduction of an acrylamide moiety into either position *ortho* to the carbonyl group on the phenyl ring was anticipated to present an electrophilic center proximal to Cys552, or to Cys477 if the P-loop adopted a disordered conformation. These two alternatives are freely interchangeable *via* an approximately 180° rotation about the amide carbonyl to phenyl-ring bond. However, the conformer in which the acrylamide is orientated towards Cys477 is the most stable due to an intramolecular hydrogen-bond between the acrylamide N-H and benzamide carbonyl groups.²⁵

Based upon biochemical activity, 8 showed a 50-fold increase in the IC_{50} value for the inhibition of wild-type FGFR4

when compared to the non-covalent parent-compound AZD4547, Table 1, strongly supporting a covalent interaction. Furthermore, comparing the data between the wild type and FGFR4 variants in Table 2, a 400-fold loss in activity with the C477A variant strongly supported this cysteine to be the primary site of reaction with the acrylamide. In contrast, only a 5-fold lower IC_{50} was determined for the middle-hinge C552A variant which suggested a much lower level of covalent interaction, if any, at this position for 8.

To confirm the putative covalent-interactions of 8, a series of mass spectrometry (MS) studies were conducted with the wild type and the cysteine to alanine FGFR4 variants.²¹ Compound 8 was found to covalently bind to all three proteins to give the mass of the kinase domain plus 420 Daltons, consistent with the anticipated Michael addition reaction, when compound and protein were incubated at equivalent concentrations. When higher concentrations of 8 were used, no multiple addition products were observed (highest ratio: 1 to 10). Therefore, confirming that reaction had taken place, likely with either the GK+2, or P-loop, cysteine. However, five other cysteines are present in the shorter FGFR4 442–753 kinase-domain constructs which are anticipated to be ‘unreactive cysteines’ based upon their involvement in interactions defining the secondary and tertiary protein structure. To establish if either, or both, the anticipated sites in wild-type FGFR4 were reacting with 8 a trypsin digest was made following the incubation with protein.²¹ From these studies both the anticipated compound-modified oligo-peptides could be detected, supporting that both sites were contributing to the observed activity of 8. However, although not quantitative, the signal strength for the P-loop modified oligo-peptide was much stronger, with the GK+2 modified oligo-peptide being close to the lower limit of quantification with the wild-type protein. This observation was in line with the biochemical IC_{50} values in Table 2, indicating that 8 inhibits FGFR4 mainly through binding to the P-loop cysteine, with only a small contribution from binding at the GK+2 position.

Although biochemical assays remain a satisfactory way to compare the activity of irreversible-covalent binding compounds, the IC_{50} values determined in this way are highly dependent on the assay conditions, and the ratio of k_{inact}/K_i represents a superior, and invariant, measure of potency in this

setting.²⁶ Applying this approach with **8**, by comparing the wild-type and C477A kinase domain variant, indicated a 1000-fold faster reaction with the P-loop cysteine compared to the middle-hinge cysteine, data are shown in Table 3.²¹

To further characterise the covalent binding of **8**, co-crystallisation studies were performed following reaction with the wild-type FGFR4 kinase domain. Purification of the major addition-product prior to crystallization provided the X-ray co-crystal structure in which the anticipated Michael addition with **8** had occurred with the P-loop Cys477, as shown in Fig. 8.²¹ This structure shows that **8** remains bound within the ATP-pocket following covalent attachment, and continues to make the key hinge, and back-pocket, interactions made by the parent compound AZD4547 consistent with the design hypothesis.²⁴

The dipyridylamine **9** was identified as a singleton from a high throughput screening campaign and was suspected to be a covalent binder due to such a relatively small molecule delivering a high level of FGFR4 potency and selectivity. Manual docking of **9** into the ATP pocket of FGFR4 led to a hypothesis in which the 5-trifluoromethyl pyridyl group constituted the hinge-binding element. As a consequence, the 6-chloropyridyl moiety would be positioned proximal to Cys552, due to an internal hydrogen bond between the 2-aminopyridyl N-H and nitro groups, and ideally situated for a nucleophilic aromatic substitution (S_NAr) reaction in which the 6-chloro substituent would be displaced by the thiomethyl side-chain. Strengthening this hypothesis, the inhibitory activity of **9** was completely lost with the C552A middle-hinge variant of FGFR4, when the putative nucleophile was removed.

MS studies were consistent with the anticipated S_NAr reaction between **9** and Cys552: the mass of kinase domain plus 352 Daltons was observed when equivalent concentrations of protein and compound were used. No covalent adducts were observed under these conditions with **9** and the C552A variant, confirming the primary site for reaction with the FGFR4 kinase domain. When higher compound concentrations were used (10-fold excess over protein), a double addition of **9** was observed with the wild-type protein, indicating some non-specificity for this electrophile. In contrast, no further change was seen with the C477A variant, beyond the mono-addition product, when the compound concentration was increased to the same extent. Additionally, at the higher compound concentrations a mono-addition product started to form with the C552A variant at a similar rate to the second addition to the wild-type protein. Taken together these data support the primary site of reaction for **9** to be Cys552, and that the compound can react in a non-specific manner with 'reactive cys-

Fig. 8 X-ray crystal structure of **8** covalently bound to the P-loop Cys477 of the FGFR4 kinase domain (PDB ID: 5NWZ).

teines'. The P-loop cysteine was classed as a 'reactive cysteine' based upon it being positioned on a flexible region of the protein, and not being involved in strong interactions within the protein structure. Interestingly, **9** is unable to impact the kinase activity of the C552A variant, Table 2, even though the MS studies indicate covalent modification of the P-loop cysteine would have likely occurred in the biochemical assay. Consistent with this observation, molecular modeling of the P-loop modified protein identified no interactions that would retain **9** within the ATP-site, to prevent ATP binding, and no interactions that would impede the phosphoryl transfer when the P-loop is in the active hairpin-conformation.²¹

For **9**, the k_{inact}/K_i ratio was determined to be $3.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ with the wild-type FGFR4 construct and indicated a 170-fold slower rate of reaction compared to the acrylamide **8**, although each compound targets primarily a different cysteine residue within the ATP-pocket for inhibition.

The X-ray co-crystal structure of **9** with the wild-type FGFR4 kinase domain was also obtained in the same manner as described above for compound **8**, and is shown in Fig. 9. The structure shows the anticipated S_NAr reaction with Cys552 having taken place at the 2-pyridyl position to displace chloride. Following covalent attachment the bound

Fig. 9 X-ray crystal structure of compound **9** covalently bound to the middle-hinge Cys552 of the FGFR4 kinase domain. Hydrogen bonds to the hinge residues are indicated by dotted lines (PDB ID: 5NUD).

Table 3 k_{inact}/K_i measured for **8** with the non-phosphorylated wild-type and C477A FGFR4 kinase domain variants

k_{inact}/K_i values ($\text{M}^{-1} \text{ s}^{-1}$)	
FGFR4 wild-type	FGFR4 C477A
5.1×10^6	5.1×10^2

dipyridylamine retains the hypothesised interactions within the hinge region: a hydrogen bond with residue Ala553, and an aromatic C–H pseudo hydrogen bond with the backbone carbonyl group of residue Glu551, both interactions involving the trifluoromethylated pyridine ring.

Somewhat surprisingly this mode of covalent binding has only been sparsely reported to date,²⁷ even though S_NAr reactions offer multiple opportunities to modulate reactivity by changing the nature of the aromatic group, the leaving group, and the steric and electronic properties of the ring substituents.

A small set of closely related 2-formylquinoline amides were identified as part of the screening campaign, of which **10** is a representative member. Once more, the high potency for the inhibition of FGFR4 from a relatively small inhibitor, combined with excellent FGFR 1–3 selectivity, suggested an interaction with the middle-hinge region was driving the activity, and likely a covalent interaction with Cys552. Additionally, the wider kinase-selectivity for **10** was also excellent when measured against a panel of >50 kinases.²¹ MK2 (MAPKAPK2) was the kinase inhibited to the greatest extent, when screened at a concentration of 10 μ M, and for which an IC_{50} value of 2.3 μ M was determined. This observation further implicated a key interaction with Cys552 for **10**, as MK2 is one of the four other kinases within the human kinome containing a GK+2 cysteine residue.⁸

A binding hypothesis for **10** was generated in which the 2-aminopyridyl moiety constituted the hinge-binding element, with an equivalent hydrogen-bonding interaction to that shown in Fig. 6 for **7**. Due to the strong intramolecular hydrogen bond in **10**, between the amide N–H and quinoline nitrogen, the 2-formyl quinoline group would then be ideally positioned for an addition reaction to occur with the thiol of Cys552 to generate a hemithioacetal adduct.²⁵ The topography of the protein in the middle-hinge region ensures that this addition reaction can only occur with a single face of the aldehyde when the hinge interaction is in place, or being formed. Fig. 10 shows the single crystal X-ray structure of **10**, in which the intramolecular hydrogen bond is highlighted.²¹

Fig. 10 ORTEP plot of **10** determined by X-ray crystallography. The quinoline-nitrogen to amide N–H distance in this structure is determined to be 1.9 Å, consistent with an intramolecular hydrogen-bond.

Additionally, in this X-ray structure the 2-formyl group adopts an *anti*-conformation with respect to the quinoline ring-nitrogen which was calculated to be the lowest energy conformation, and 4.9 kcal mol^{−1} more stable than the *syn* conformer. Although ground state conformations do not control reaction outcomes,²⁸ in this instance the addition of the lowest energy *anti*-conformer of **10** results in the epimer with the (*R*)-configuration at the newly formed hemithioacetal center. However, both epimeric addition products could be modeled with a hydrogen bond between the newly formed hydroxyl group and Val500, and these hydrogen-bonding interactions were also anticipated to stabilise both transition states. Therefore, an *a priori* prediction of the stereochemistry of the major addition product could not readily be made. Fig. 11 shows a model of **10** bound to FGFR4 based upon the above binding hypothesis in which the hinge interaction involves a hydrogen bond between the pyridyl N and the backbone N–H of Ala553, and the hemithioacetal hydroxyl group is shown in the (*R*)-configuration making a hydrogen bond with the carbonyl of Val500. In addition, as observed in the crystal structure of compound **9** bound to FGFR4, the model suggests the possibility of an aromatic C–H pseudo hydrogen bond between the pyridyl ring and the backbone carbonyl of Glu551.

Support for the hypothesised covalent-binding mode of **10** was derived from the complete loss of FGFR4 activity with the C552A variant, Table 2. This result highlights the critical role of the Cys552 thiol functionality for the binding of FGFR4 to **10**. Additionally, analogues **11**–**13**, Fig. 12, in which the 2-quinoliny aldehyde was replaced by a proton, carboxylic acid, or a hydroxymethyl group were all found to be inactive. These results highlight the critical role of the 2-formyl group for the interaction of **10** with FGFR4. The 2-formylquinoline analogue **14**, in which the hinge-binding interaction was destabilised by exchanging the pyridine for the corresponding phenyl analogue, also resulted in a complete loss of FGFR4 activity. Taken together, the data with analogues **10**–**14** provided consistent support for the hypothesised covalent interaction between **10** and FGFR4.

Fig. 11 Model of **10** covalently bound to the middle-hinge Cys552 of the FGFR4 kinase domain. Hydrogen bonds are represented as dotted lines.

Fig. 12 Structures of the analogues 11–14 exploring the key elements of the pharmacophore of 10.

Previously a number of aliphatic aldehydes have been described to react with cysteine thiol groups, in particular in the area of protease inhibitors.²⁹ The C–S bond formed between inhibitor and protein in these hemithioacetal intermediates constitutes a relatively weak covalent-bond, typically considered to be involved in reversible interactions.³⁰ Consistent with the notion of reversibility, mass spectral studies with the 2-formylquinoline 10 revealed no evidence for stable addition products under the same ionisation conditions in which covalent adducts had been observed with the irreversible inhibitors 8 and 9. Taken together the above observations strongly supported a reversible-covalent interaction to be occurring between compound 10 and FGFR4.

In parallel to the identification of covalent starting points for lead optimisation, the resynthesis-rates of FGFR4 in two FGF19/FGFR4 positive HCC cell lines (HUH7 and Hep3B) were determined. Using a stable isotope labelling with amino acids (SILAC) approach revealed a relatively rapid resynthesis half-life of <2 h in both cell lines.^{21,31} The inclusion of a fully efficacious concentration of a selective FGFR4 inhibitor in these SILAC experiments was found not to change the resynthesis rates, suggesting the half-life of FGFR4 is also independent of the level of pathway activation in these HCC cell lines.

This relatively rapid FGFR4 protein resynthesis rate in combination with an emerging pharmacodynamic (PD)/efficacy relationship, which suggested complete and continuous inhibition of FGFR4 would be required for maximum anti-tumor efficacy,³² resulted in a re-evaluation of the suitability of the irreversible-covalent approach for the treatment of HCC. The concern being that to achieve this level of inhibition *in vivo* would minimally require an efficacious level of compound to be maintained throughout the dosing interval, to continually engage newly synthesised protein. Achieving this exposure profile was considered to be challenging for an irreversible-covalent modality. However, acrylamide based inhibitors have been described with respectable *in vivo* half-lives, including BLU554.¹¹ Irreversible-covalent inhibition was also considered to be the situation most likely to incur undesirable side-effects due to non-specific reactivity.³³ In particular, in the gut for compounds administered orally at the relatively high doses that were anticipated to be required to achieve sustained PD modulation. In this setting even highly selective irreversible-covalent binders are anticipated to bind non-specifically due to the initial high gut-levels, with

the potential for protein adducts to remain long after the compound concentration has been depleted. Additionally, one further benefit of reversible-covalent inhibitors is that they can be envisioned to be less immunogenic. In contrast to irreversible-covalent binders, the cysteine affinity of reversible-covalent binders will be greatly reduced for the oligo peptides that arise following protein degradation.²⁰

With no possibility to disconnect efficacy from exposure in a favorable way, none of interesting irreversible-covalent starting points, as exemplified by 8 and 9, were evaluated further. However, the non-covalent and the reversible-covalent opportunities remained as interesting possibilities for further study. In particular, 10 highlighted the potential for high FGFR4 potency, and excellent selectivity, in combination with the expectation that efficacy would be regulated following a standard exposure effect relationship. Although some questions remained concerning the presence of the aryl aldehyde group in 10,³⁴ the favorable potency and selectivity made this an interesting series for further investigation.

Conclusion

In conclusion, structurally diverse series of selective FGFR4 hits have been identified through screening and *de novo* design. Although the screening approaches were unbiased, the origin of the selectivity for the most interesting examples can be rationalized to arise from key interactions within the middle-hinge region of the ATP pocket. This observation was consistent with a selectivity hypothesis generated at the start of the project from a kinase-domain sequence-alignment which was centered on the cysteine residue at the GK+2 position of FGFR4. This hypothesis also enabled the rational introduction of acrylamides into known FGFR inhibitor scaffolds to generate covalent inhibitors targeting both of the reactive cysteines within the ATP pocket of FGFR4. Of greater interest, potent and highly selective FGFR4 inhibitors were identified through screening which interacted covalently with Cys552 through less well established mechanisms of action. However, the rapid FGFR4 resynthesis-rate, and temporal requirements for FGFR4 inhibition in HCC, made irreversible-covalent inhibition a less attractive approach compared to the non-covalent and reversible-covalent options. Of these options the 2-formylquinoline amides, represented by 10, proved to be particularly interesting. The further evaluation of this hit series, which is presumed to be inhibiting through a reversible-covalent mechanism of action, will be the subject of future disclosures.

Author contributions

R. A. F., T. K., C. L., N. B., C. G., J. B., J. T., J. V., C. G., F. B., D. G-P. and P. F. analysed data and designed the experiments. R. A. F., T. K., C. L., N. B. and C. G. conducted the synthetic and medicinal chemistry. P. F. carried out the computational chemistry. J. B., I. G. and J. T. developed and conducted the biochemical assays. C. Z. conducted the

FGFR4 co-crystallisation studies. C. G., P. B-L. and F. B. designed and conducted the mass spectroscopy studies. J. V. designed and analysed the SILAC experiments. R. A. F., T. K., N. B. and P. F. wrote and reviewed the manuscript.

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References

- 1 E. P. Carter, A. E. Fearon and R. P. Grose, Careless talk cost lives: fibroblast growth factor signaling and the consequences of pathway malfunction, *Trends Cell Biol.*, 2015, 25, 221–233.
- 2 K. Nicholes, S. Guillet, E. Tomlinson, K. Hillan, B. Wright, G. D. Frantz, T. A. Pham, L. Dillard-Telm, S. P. Tsai, J.-P. Stephan, J. Stinson, T. Stewart and D. M. French, A mouse model of hepatocellular carcinoma, *Am. J. Pathol.*, 2002, 160, 2295–2307.
- 3 C. Heinze, Z. Erdem, J. Paur, B. Grasl-Kraupp, K. Holzmann, M. Grusch, W. Berger and B. Marian, Is fibroblast growth factor receptor 4 a suitable target of cancer therapy?, *Curr. Pharm. Des.*, 2014, 20, 2881–2898.
- 4 S. Lemieux and M. K. Hadden, Targeting the fibroblast growth factor receptors for the treatment of cancer, *Anti-Cancer Agents Med. Chem.*, 2013, 13, 748–761.
- 5 V. Guagnano, P. Furet, C. Spanka, V. Bordas, M. Le Douget, C. Stamm, J. Brueggen, M. R. Jensen, C. Schnell, H. Schmid, M. Wartmann, J. Berghausen, P. Drucekes, A. Zimmerlin, D. Bussiere, J. Murray and D. Graus-Porta, Discovery of 3-(2,6-dichloro-3,5-dimethoxy-phenyl)-1-{6-[4-(4-ethyl-piperazin-1-yl)-phenylamino]-pyrimidin-4-yl}-1-methyl-urea (NVP-BGJ398), a potent and selective inhibitor of the fibroblast growth factor receptor family of receptor tyrosine kinase, *J. Med. Chem.*, 2011, 54, 7066–7083.
- 6 P. R. Gavine, L. Mooney, E. Kilgour, A. P. Thomas, K. Al-Hadhim, S. Beck, C. Rooney, T. Coleman, D. Baker, M. J. Mellor, A. N. Brooks and T. Kinowska, AZD4547: an orally bioavailable, potent, and selective inhibitor of the fibroblast growth factor receptor tyrosine kinase family, *Cancer Res.*, 2012, 72, 2045–2056.
- 7 R. Dienstmann, J. Rodon, A. Prat, J. Perez-Garcia, B. Adamo, E. Felip, J. Cortes, A. J. Iafrate, P. Nuciforo and J. Tabernero, Genomic aberrations in the FGFR pathway: opportunities for targeted therapies in solid tumors, *Ann. Oncol.*, 2014, 25, 552–563.
- 8 E. Leproult, S. Barluenga, D. Moras, J.-M. Wurtz and N. Winssinger, Cysteine mapping in conformationally distinct kinase nucleotide binding sites: application to the design of selective covalent inhibitors, *J. Med. Chem.*, 2011, 54, 1347–1355.
- 9 E. Lesca, A. Lammens, R. Huber and M. Augustin, Structural analysis of the human fibroblast growth factor receptor 4 kinase, *J. Mol. Biol.*, 2014, 426, 3744–3756.
- 10 M. Hagel, C. Miduturu, M. Sheets, N. Rubin, W. Weng, N. Stransky, N. Bifulco, J. L. Kim, B. Hodous, N. Brooijmans, A. Shutes, C. Winter, C. Lengauer, N. E. Kohl and T. Guzi, First selective small molecule inhibitor of FGFR4 for the treatment of hepatocellular carcinomas with an activated FGFR4 signaling pathway, *Cancer Discovery*, 2015, 5, 424–437.
- 11 C. V. Miduturu, M. Hagel, M. Sheets, N. Rubin, W. Weng, N. Bifulco, L. V. Dipietro, J. Kim, N. Brooijmans, B. L. Hodous, N. Stransky, K. Hoeflich, V. J. Kadambi, N. Kohl, C. Lengauer and T. Guzi, *Discovery and development of BLU-554: a potent, highly selective covalent inhibitor of fibroblast growth factor receptor 4 (FGFR4) in development for the targeted treatment of advanced hepatocellular carcinoma (HCC) patients with amplified and overexpressed FGF19*, Presented at the 253rd National Meeting, San Francisco, April 5th, 2017, American Chemical Society, MEDI 351.
- 12 A. Selvaraj, E. Corcoran, H. Coffey, S. Prajapati, M.-H. Hao, N. Larsen, J. Tsai, T. Satoh, K. Ichikawa, J. J. Joshi, R. Hurley, J. Wu, C.-L. Huang, S. Bailey, C. Karr, P. Kumar, V. Rimkunas, C. Mackenzie, N. Rioux, A. Kim, S. Akare, G. Lai, L. Yu, P. Fekkes, J. Wang, M. Warmuth, P. Smith and D. Reynolds, *H3B6527, a selective and potent FGFR4 inhibitor for FGF19-driven hepatocellular carcinoma*, Presented at the AACR Annual Meeting, Washington, April 1–5, 2017, American Association for Cancer Research, abstract 3126.
- 13 C. Mo, Z. Zhang, C. P. Guise, X. Li, J. Luo, Z. Tu, Y. Xu, A. V. Patterson, J. B. Smaill, X. Ren, X. Lu and K. Ding, 2-Aminopyrimidine derivatives as new selective fibroblast growth factor receptor 4 (FGFR4) inhibitors, *ACS Med. Chem. Lett.*, 2017, 8, 543–548.
- 14 Y. Wang, Z. Chen, M. Dai, P. Sun, C. Wang, Y. Gao, H. Zhao, W. Zeng, L. Shen, W. Mao, T. Wang, G. Hu, J. Li, S. Chen, C. Long, X. Chen, J. Liu and Y. Zhang, Discovery and optimization of selective FGFR4 inhibitors via scaffold hopping, *Bioorg. Med. Chem. Lett.*, 2017, 27, 2420–2423.
- 15 K. G. Pike, D. Buttar, J. Tucker and C. Jones, *Identifying selective inhibitors of FGFR4 kinase*, Presented at the 23rd Int. Symp. Med. Chem., Lisbon, Sep. 7–11, 2014, European Federation for Medicinal Chemistry, LE098.
- 16 M. Huse and J. Kuriyan, The conformational plasticity of protein Kinases, *Cell*, 2002, 109, 275–282.

- 17 J. C. Connolly, J. M. Hamby, M. C. Schroeder, M. Barvian, G. H. Lu, R. L. Panek, A. Amar, C. Shen, A. J. Kraker, D. W. Fry, W. D. Klohs and A. M. Doherty, Discovery and structure activity of a novel series of pyrido[2,3-d]pyrimidine tyrosine kinase inhibitors, *Bioorg. Med. Chem. Lett.*, 1997, 7, 2415–2420.
- 18 W. Zhou, W. Hur, U. McDermott, A. Dutt, W. Xian, S. B. Ficarro, J. Zhang, S. V. Sharma, J. Brugge, M. Meyerson, J. Settleman and N. S. Gray, A structure-guided approach to creating covalent FGFR4 inhibitors, *Chem. Biol.*, 2010, 17, 285–295.
- 19 K. A. Brameld, *Discovery of PRN1371: a highly selective, irreversible inhibitor of FGFR1-4 in clinical development for the treatment of solid tumors*, Presented at the 253rd National Meeting, San Francisco, April 5th, 2017, American Chemical Society, MEDI 352.
- 20 J. M. Bradshaw, J. M. McFarland, V. O. Paavilainen, A. Bisconte, D. Tam, V. T. Phan, S. Romanov, D. Finkle, J. Shu, V. Patel, T. Ton, X. Li, D. G. Loughhead, P. A. Nunn, D. E. Karr, M. E. Gerristen, J. O. Funk, T. D. Owens, E. Verner, K. A. Brameld, R. J. Hill, D. M. Goldstein and J. Taunton, Prolonged and tunable residence time using reversible covalent kinase inhibitors, *Nat. Chem. Biol.*, 2015, 11, 525–531.
- 21 Further details are included in the ESI.†
- 22 C. Garcia-Echeverria, T. Kanazawa, E. Kawahara, K. Masuya, N. Matsuura, T. Miyake, O. Ohmori, I. Umemura, R. Steensma, G. Chopiuk, J. Jiang, Y. Wan, Q. Ding, Q. Zhang, N. S. Gray and D. Karanewsky, Preparation of 2,4-pyrimidinediamines useful in the treatment of neoplastic diseases, inflammatory and immune system disorders, *WO 2005/016894*, February 24th 2005.
- 23 B. Roth and C. C. Cheng, Recent progress in the medicinal chemistry of 2,4-diaminopyrimidines, *Prog. Med. Chem.*, 1982, 19, 269–331.
- 24 J. A. Tucker, T. Klein, J. Breed, R. Overman, C. Phillips and R. A. Norman, Structural insights into FGFR kinase isoform selectivity: diverse binding modes of AZD4547 and ponatinib in complexes with FGFR1 and FGFR4, *Structure*, 2014, 22, 1764–1774.
- 25 B. Kuhn, P. Mohr and M. Stahl, Intramolecular hydrogen bonding in medicinal chemistry, *J. Med. Chem.*, 2010, 53, 2601–2611.
- 26 M. C. Noe and A. M. Gilbert, Targeted covalent enzyme inhibitors, *Annu. Rep. Med. Chem.*, 2012, 47, 413–439.
- 27 K. X. Chen, C. A. Lesburg, B. Vibulbhan, W. Yang, T.-Y. Chen, S. Venkatraman, F. Velazquez, Q. Zeng, F. Bennett, G. N. Anilkumar, J. Duca, Y. Jiang, P. Pinto, L. Wang, Y. Huang, O. Selyutin, S. Gavalas, H. Pu, S. Agrawal, B. Feld, H.-C. Huang, C. Li, K.-C. Cheng, N.-Y. Shih, J. A. Kozlowski, S. B. Rosenblum and F. G. Njoroge, A novel class of highly potent irreversible hepatitis C virus NS5B polymerase inhibitors, *J. Med. Chem.*, 2012, 55, 2089–2101.
- 28 J. I. Seeman, The Curtin-Hammett principle and the Winstein-Holness equation, *J. Chem. Educ.*, 1986, 63, 42–48.
- 29 J. A. Clearly, W. Doherty, P. Evans and J. P. G. Malthouse, Quantifying tetrahedral adduct formation and stabilisation in the cysteine and the serine proteases, *Biochim. Biophys. Acta*, 2015, 1854, 1382–1391.
- 30 R. Caraballo, H. Dong, J. P. Ribeiro, J. Jiménez-Barbero and O. Romström, Direct STD NMR identification of β -galactose inhibitors from a virtual dynamic hemithioacetal system, *Angew. Chem., Int. Ed.*, 2010, 49, 589–593.
- 31 B. Schwanhäusser, M. Gossen, G. Dittmar and M. Selbach, Global analysis of cellular protein translation by pulsed SILAC, *Proteomics*, 2009, 9, 205–209.
- 32 R. A. Fairhurst, T. Knoepfel, P. Furet, N. Buschmann, C. Leblanc, R. Mah, M. Kiffe, D. Graus-Porta, A. Weiss, J. Kinyamu-Akunda, M. Wartmann, J. Trappe, T. Gabriel, F. Hofmann and W. R. Sellers, *FGF401: A reversible-covalent inhibitor of FGFR4 for the treatment of hepatocellular carcinoma*, Presented at the 253rd National Meeting, San Francisco, April 5th, 2017, American Chemical Society, MEDI 353.
- 33 R. A. Bauer, Covalent inhibitors in drug discovery: from accidental discoveries to avoided liabilities and designed therapies, *Drug Discovery Today*, 2015, 20, 1061–1073.
- 34 R. M. LoPachin and T. Gavin, Molecular mechanisms of aldehyde toxicity: a chemical perspective, *Chem. Res. Toxicol.*, 2014, 27, 1081–1091.