Discovery of a Potent and Highly Isoform-Selective Inhibitor of the Neglected Ribosomal Protein S6 Kinase Beta 2 (S6K2)

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Abstract

The ribosomal protein S6 kinase beta 2 (S6K2) is thought to play an important role in malignant cell proliferation but is understudied compared to its closely related isoform S6 kinase beta 1 (S6K1). To better understand the biological function of S6K2, chemical probes are needed but the high structural homology between S6K2 and S6K1 makes it challenging to selectively address S6K2 with small molecules. We were able to design the first potent and highly isoform-specific S6K2 inhibitor by merging a known S6K1-selective inhibitor with a covalent inhibitor known to engage a cysteine located in the hinge region in the fibroblast growth factor receptor kinase (FGFR) 4 via a nucleophilic aromatic substitution (S_NAr) reaction. The title compound shows high selectivity over kinases with an equivalently positioned cysteine as well as in a larger kinase panel. Good stability towards glutathione indicated a non-promiscuous reactivity pattern. Thus, the title compound represents an important step towards a high-quality chemical probe to study S6K2-specific signalling.

Introduction

Protein kinases have become highly important drug targets during the past 20 years with over 60 protein kinase inhibitors already being approved by the FDA.[1] Despite this success, many of the more than 500 protein kinases of the human kinome remain poorly characterized and most of the research being done focusses on a relatively small set of already well-understood and validated targets.[2] This narrow scope is not necessarily due to a lack of interest, but is often caused by a lack of enabling tools such as potent and highly selective inhibitors for understudied kinases. Such pharmacological modulators are required to complement genetic approaches for understanding the roles of neglected kinases and other proteins in signal transduction.[3, 4] The growing awareness about this need culminated in the goals set by representatives of academia, industry and public funders to generate pharmacological modulators for almost all human proteins until 2035 (*Target 2035*).[4]

The ribosomal protein S6 kinase beta 2 (S6K2 or p70S6Kβ) is an understudied member of the family of ribosomal protein S6 kinases (S6K), which are part of the group of AGC serine/threonine kinases and known to phosphorylate the 40S ribosomal S6 protein. It is encoded by the *RPS6KB2* gene on chromosome 11 and acts as a downstream effector of the AKT/mTOR and RAS/RAF/MEK/ERK pathways.[5] As such, it is involved in the regulation of cell growth and survival. S6K2 usually expresses in low levels but has been shown to be overexpressed in different forms of cancer including breast and prostate cancer.[6, 7] More specifically, amplification and gain of S6K2 correlates with breast cancer cells being estrogen receptor positive (ER-positive) and has been associated with chemoresistance and significantly reduced recurrence-free survival.[6] Silencing of the *RPS6KB2* gene provided some insight into its function in regulating malignant cell proliferation and suggested a potential role in cancer therapy. According to these findings, silencing of S6K2 was able to decrease cell viability of small cell lung cancer (SCLC) cells as well as non-small lung cancer (NSCLC) cells either via preventing formation of the FGF2-inducible PKCε/B-RAF/S6K2-complex or downregulation of the Hedgehog/GLI pathway, respectively.[8, 9] Similarly, knockdown of S6K2 promoted cell death in certain breast cancer as well as prostate cancer cell lines.[7, 10] However, the effects of pharmacological inhibition of S6K2 kinase activity

remain unknown. Apart from malignant disorders S6K2 showed increased phosphorylation suggesting increased activity during SARS-CoV-2 infection in a cell-based proteomics study.[11]

S6K2, which has been denominated the neglected member of the S6K family,[5] shares 80% of the amino acid sequence in the kinase domain with its closely related and more thoroughly studied isoform S6K1 (also known as p70S6K). Adding to that, other regions involved in regulation like the neighbouring kinase extension as well as the pseudosubstrate and inhibitory domain are also mostly conserved among the two enzymes.[6] Differences can be found mainly in their C- and N-terminal region. The only significant difference in the ATP binding site, however, is a non-catalytic cysteine (Cys_{150}) in the hinge region of S6K2 (Tyr_{151} in S6K1) only to be found in four other human protein kinases, namely MAPKAPK2, MAPKAPK3, FGFR4 and TTK.[6, 12]

It is believed that S6K1 and S6K2 share some biological functions but also have isoform-specific roles. S6K2 seems to be more involved in regulating cell death compared to S6K1 which has a more marked role in cell proliferation, invasion, and metastasis. In one example, knockdown of S6K2 led to cell death in breast cancer cells whereas knockdown of S6K1 led to the activation of other pathways and ultimately to inhibition of apoptosis, which corroborates the distinct roles of the underlying signalling networks.[6] The overall scarcity of S6K2 focused research prompted several authors to call for more extensive efforts in this field, especially focusing on pharmacological modulators and molecular probes.[6, 13, 14]

So far only two S6K1 inhibitors with notable selectivity over S6K2 in biochemical assays have been reported (FL772, PF-4708671) and no S6K2 selective inhibitor is known.[15] The minor structural differences in the kinase domain of S6K2 and S6K1 make it difficult to target either one of these isoforms by small molecules selectively. In our work we aimed to exploit the presence of Cys_{150} in the hinge region of S6K2 to achieve high inhibitory potency and excellent (isoform-)selectivity by means of a covalent reactive group ("warhead"). For this purpose, we wanted to make use of an electron-deficient heteroaryl system equipped with a leaving group to react with Cys_{150} via nucleophilic aromatic substitution (S_NAr). The S_NAr strategy extends the scope of cysteine-targeted warheads beyond the typical α,β-unsaturated carbonyl compounds and may be able to overcome some of their limitations in terms of tunability, sterics and metabolic properties.[16] However, such an approach has to the best of our knowledge only been reported once in the context of kinase inhibitor discovery with screening hit **1** (Figure 1A) from Fairhurst *et al.* targeting a cysteine in the middle-hinge region of fibroblast growth factor receptor kinase (FGFR) 4 via a unique binding mode.[16] The equivalent positioning of the FGFR4 cysteine compared to S6K2 Cys_{150} suggested that an analogous strategy may also be accomplished in our case.

Results and Discussion

Design. Our design strategy started from the co-crystal structure of known reversible S6K1 inhibitor PF-4708671 (**3**, Figure 1A, IC_{50} = 160 nM) which was reported to possess a weak off-target activity on S6K2 (IC_{50} = 65 μM).[15] We aimed at attaching the reactive chloronitropyridine-fragment of FGFR4 inhibitor **1** (Figure 1A) to the hinge-binding core of **3** via an amino linker to mimic the binding mode of the template

inhibitor. Since no Xray crystal structure of S6K2 is available so far, we created a hinge region-centred structure overlay of compound **1** bound to FGFR4 (PDB: 5NUD[17]) and PF-4708671 (**3**) bound to S6K1 (PDB: 3WE4[18]) (Figure 1B). The superimposition indeed showed a good structural alignment of the core scaffolds and indicated that the thiol group of Cys_{150} in S6K2 should be reachable by the chloronitropyridine warhead. This hypothesis was corroborated by covalent docking into a S6K1 Y151C mutant generated *in silico* (not shown). Interestingly, the distance between the *NH* of Ala_{563} in FGFR4 and the two pyridine nitrogen atoms of compound **1** (Figure 1 B) suggests the formation of a chelate-like hydrogen bond, which may be important to favour a reactive pre-orientation facilitating the displacement reaction.

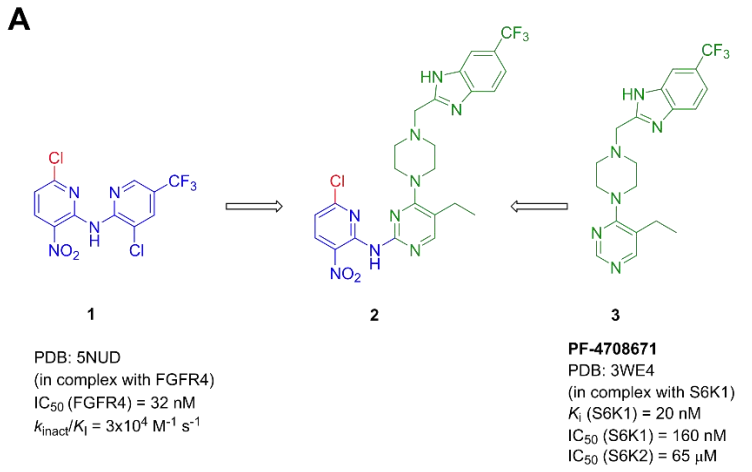
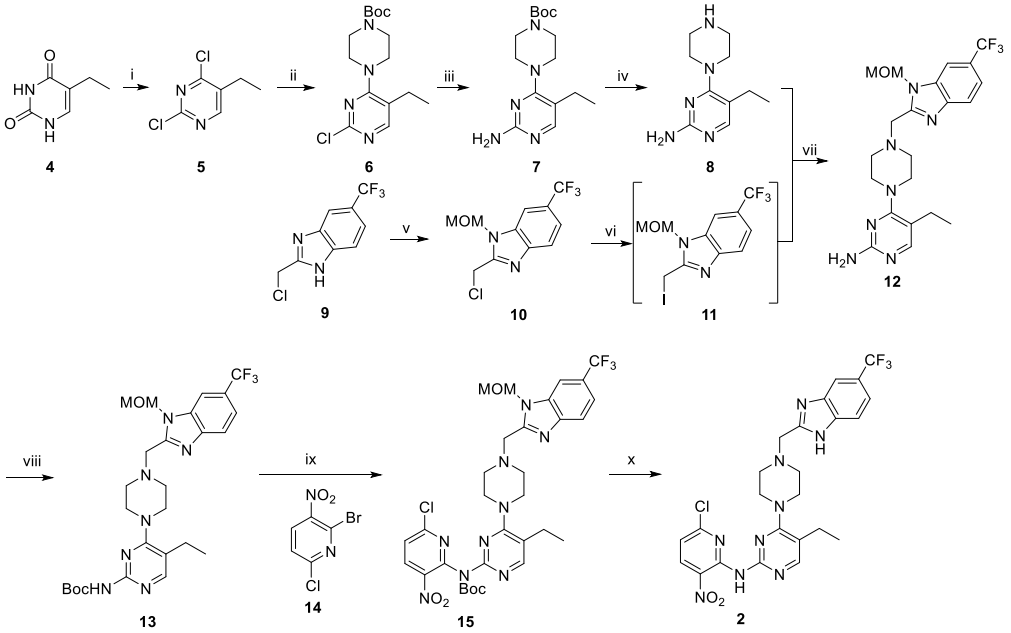


Figure 1: A: Merging of PF-4708671 (**3**, green)[18] with compound **1** (blue)[17] containing a reactive chloronitropyridine (leaving group in red). B: Crystal structure overlay of FGFR4 in complex with compound **1** (salmon; FGFR4 backbone in cyan) and S6K1 in complex with PF-4708671 (grey). The dotted yellow lines indicate the supposed chelate-type hydrogen bond formed by compound **1**. The FGFR4 cysteine (Cys552) is highlighted in magenta.

Synthesis. The synthesis of the title compound **2** is summarized in Scheme 1 and started from the commercially available uracil derivative **4,** which could be chlorinated by $POCl_3$ in the presence of DIPEA.[19] Attachment of the Boc-protected piperazine onto product **5** was achieved by S_NAr reaction under temperature-controlled conditions with almost exclusive formation of the desired regioisomer **6**. The introduction of the amino group to deliver the 2-aminopyrimidine **7** was possible via Buchwald-Hartwig arylamination with LiHMDS using the RuPhos Pd G4 precatalyst, after several common ammonia-surrogates have failed to provide useful amounts of the product under catalysed and non-catalysed conditions. Deprotection of intermediate **7** by means of acid led to free piperazine **8**. Chloromethylbenzimidazole **9** was equipped with a MOM-protecting group (**10**) prior to nucleophilic

displacement by piperazine **8** to prevent side reactions observed for unprotected benzimidazole **9** in the presence of base. The nucleophilic substitution was best carried out under Finkelstein conditions via iodomethylbenzimidazole **11** being generated *in situ*, with much lower yields being observed for direct substitution with the respective chloro-derivative **10**. Originally, we planned to directly connect the core scaffold **12** with the bromo-substituted proto-warhead **14** under Buchwald-Hartwig conditions. After screening a variety of different catalysts, we found XantPhos to be the only tested phosphine ligand suitable to enable the *C*-*N* bond formation. Surprisingly, we observed that the reaction furnished mainly the double-*N*-arylated product even when only one equivalent of proto-warhead **14** was used. This behaviour is thought to arise from the increased *NH*-acidity of the coupling product, which may overcompensate the decrease in reactivity caused by steric hindrance in the mono-*N*-arylated intermediate. We therefore hypothesized that the aminopyrimidine **13** carrying an electro-withdrawing Boc-protecting group may also react under these conditions. To our delight, we found that Buchwald-Hartwig coupling of **13** with the proto-warhead **14** delivered monoarylated product **15** in good yield which then could be globally deprotected to obtain final compound **2**.

Scheme 1: Synthesis of the title compound 2[a]

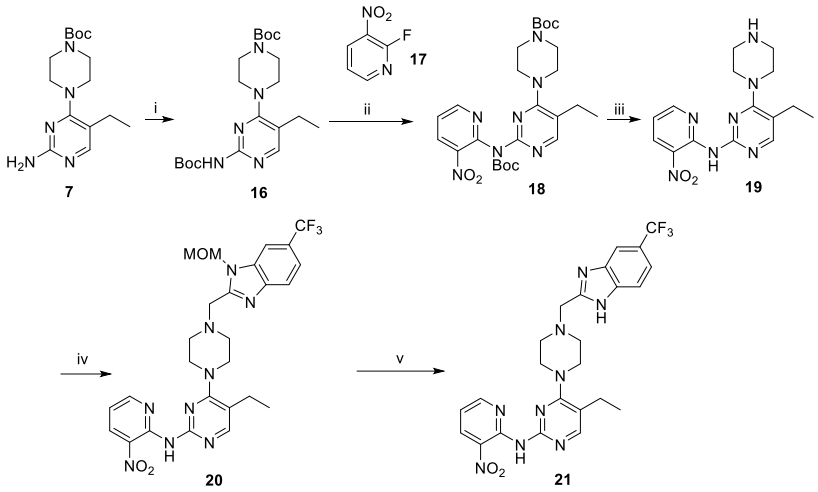


[a]Reagents and conditions: (i) $POCl_3$, DIPEA, reflux, 4 h, 98%; (ii) Boc-piperazine, EtOH, -20 °C, 17 h, 82%; (iii) LiHMDS, RuPhos Pd G4 2.5 mol%, 1,4-dioxane, 50 °C, 1 h, quant.; (iv): HCl, 1,4-dioxane, reflux, 1 h, quant.; (v) MOMBr, DIPEA, THF, -40 °C, 17 h, 68%, 10:8.7 regioisomer ratio; (vi) NaI, acetone, rt, 30 min *in situ*; (vii) DIPEA, acetone, 0 °C to rt, 17 h, 80% (viii) Boc_2O, *t*BuOH, 40 °C, 17 h, quant.; (ix) **14**, Cs_2CO_3, Xantphos Pd G4 5 mol%, toluene, 55 °C, 3 d, 69%; (x) HCl, 1,4-dioxane, reflux, 2 h, 88%.

The synthesis of the unreactive analogue **21** lacking the chloride leaving group started from the common precursor **7** but was modified so that the piperazine-bound benzimidazole could be attached at a later stage, allowing for easier derivatisation in future endeavours (Scheme 2). Aminopyrimidine **7** was Boc-protected to **16** and subjected to a S_NAr-reaction with 2-fluoro-3-nitropyridine (**17**) after deprotonation with sodium hydride to afford diarylamine **18**. Both Boc protecting groups were cleaved under acidic

conditions to give free the piperazine **19**. Then, the benzimidazole-derived side chain was attached via nucleophilic displacement under Finkelstein conditions as described before. Such late-stage introduction of the side chain was not possible for the title compound **2** since the piperazine would attach to the reactive warhead. Cleavage of the MOM-protecting group in **20** yielded the non-reactive control compound **21**.

Scheme 2: Synthesis of the unreactive analogue 21[a]



[a]Reagents and conditions: (i) Boc_2O, *t*BuOH, 40 °C, 2 h, 80%; (ii) NaH, DMF, 0 °C to rt, 1 h, then 2-fluoro-3-nitropyridine, DMF, rt, 7 d, 62%; (iii) HCl, 1,4-dioxane, 50 °C, 4 h, 89%; (iv) **10**, NaI, acetone, rt, 30 min then **19**, DIPEA, acetone, 0 °C to rt, 17 h, 68%; (v) HCl, 1,4-dioxane, 50 °C, 4 h, 80%.

Biochemical evaluation. In accordance with our design strategy, title compound **2** proved to be highly potent in an enzymatic assay with an apparent IC_{50} of 22 nM for S6K2 while being inactive for the closely related isoform S6K1 at a maximum tested compound concentration of 5 μM (Table 1). To investigate if the formation of a covalent bond between Cys_{150} and inhibitor **2** could drive the observed S6K2 potency, we tested unreactive analogue **21** lacking the leaving group. This compound showed negligible inhibitory activity for S6K2 (IC_{50} > 5000 nM). These findings were in line with inhibitor **2** being able to bind covalently to S6K2. A key factor contributing to the high isoform-selectivity of compound **2** might also be the increased steric hindrance of Tyr_{151} in S6K1 compared to Cys_{150} and the resulting repulsion of the warhead.

The IC_{50} values of compound **2** for MAPKAPK2, MAPKAPK3, FGFR4 and TTK were also determined (see Table 1). The compound did not show significant inhibitory activity on any of those kinases except FGFR4 (IC_{50} = 216 nM) confirming a good selectivity against kinases with an equivalent cysteine. To explore the broader kinome selectivity, a thermal shift screen (see supporting information Table S1 and Figure S11) was performed. Of the 97 kinases and three bromodomains included in this panel only MAP2K4 (MKK4) showed a significant ΔT_m > 3 °C (S6K2 not tested). Given the even distribution of the kinases in the panel across all major kinase families, a favourable selectivity of compound **2** can be assumed.

Table 1. Results of the biochemical kinase assays[a]

	S6K2 IC_{50} [nM]	S6K1 IC_{50} [nM]	MAPKAPK2 IC_{50} [nM]	MAPKAPK3 IC_{50} [nM]	FGFR4 IC_{50} [nM]	TTK IC_{50} [nM]
2	22±1.6[b]	>5000	>5000	>5000	216	>5000
21	>5000	ND	ND	ND	ND	ND

[a]Shown IC_{50} values were commercially determined by five-point singlicate measurements with exception of compound **2** for S6K2 (five-point triplicate; see the supporting information) at ReactionBiology Corp. using the Hotspot™ platform.[20] [b]± standard deviation. ND: not determined.

To test whether the biological activity of the title compound **2** stemmed from a potential non-specific reactivity, we evaluated it in a HPLC-based glutathione (GSH) reactivity assay[21] in comparison to the FDA approved covalent kinase inhibitor Afatinib featuring a common acrylamide-derived warhead (see supporting information Figure S7 and S8). Inhibitor **2** showed a >10-fold longer half-life than Afatinib at physiological pH and a 20-fold excess of GSH (5 mM), suggesting a favourable reactivity range for specific covalent targeting. Notably, a major benefit of the S_NAr-based warhead lies in the highly tuneable reactivity of such electrophilic aromatic systems, which can be modulated by adjusting the substitution pattern, ring size, number of annellated systems, and heteroatom content.[22] Compound **2** also showed moderate stability in mouse liver microsomes with no signs of the nitro group being a metabolic hotspot (see supporting information Figure S10). Observed main metabolites had molecular weights differing by -2 Da and +16 Da, which might be attributed to iminium formation via oxidation at the piperazine ring and subsequent ring-opening hydrolysis.

Conclusions

In summary we have designed the first isoform-selective inhibitor of S6K2 (**2**) by merging the structures of S6K1 inhibitor **3** and covalent FGFR4 inhibitor **1**. Incorporation of an electrophilic aromatic system known to bind covalently to a cysteine in the hinge region of FGFR4, which is also present in S6K2, was the key to success. A synthesis route was developed to efficiently deliver key compound **2**, which will also allow for future structure-activity relationship (SAR) exploration. Our inhibitor **2** showed high biochemical potency and selectivity for S6K2. Selectivity over the closely related isoform S6K1 can be rationalized by the putative formation of a covalent bond with Cys_{150} of S6K2, which is a tyrosine in S6K1. This assumption is in accordance with the poor S6K2 inhibitory activity of the unreactive control compound **21**, suggesting that the potency observed for **2** is mainly driven by efficient covalent inactivation rather than by strong reversible binding. Nevertheless, intrinsic reactivity of inhibitor **2** was low and we found no indications of the reactive chloronitropyridine warhead being a hotspot of hepatic metabolism. Future studies will focus on the characterization of the suggested covalent mode-of-action and improvement of reversible binding affinity along with further exploration of warhead chemistry. With the clean thermal shift kinome profile and FGFR4 being the only off-target among the kinases with an equivalent cysteine (MAPKAPK2, MAPKAPK3, FGFR4 and TTK), we expect inhibitor **2** to serve as an

excellent starting point for the generation of highly potent and specific chemical probes for studying S6K2 function *in vitro* and *in vivo*.

Supporting Information

Synthetic methods, spectra and characterization of key compounds, graphs for the GSH assays and microsomal stability, IC_{50} curves, kinome thermal shift scan data and kinase distribution in the human kinome.

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Notes

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Abbreviations

S6K1/S6K2, S6 Kinase beta 1/2: AGC, family of kinases derived from PKA, PKG and PKC; Akt, protein kinase B; mTOR, mechanistic target of rapamycin; RAS, rat sarcoma; RAF, rapidly accelerated fibrosarcoma; MKK4/MAP2K4, mitogen-activated protein kinase 4; ERK, extracellular-signal regulated kinases; ER, estrogen receptor; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; FGF2, basic fibroblast growth factor; PKCε, protein kinase C epsilon type; B-RAF, B-RAF proto-oncogene; ATP, adenosine triphosphate, GLI, glioma-associated transcription factor; MAPKAPK2, mitogen-activated protein kinase-activated protein kinase 2; MAPKAPK3 mitogen-activated protein kinase-activated protein kinase 3; FGFR4, fibroblast growth factor receptor 4; TTK, dual specificity kinase TTK (also known as MPS1); SAR, structure-activity relationship; S_NAr, nucleophilic aromatic substitution; LiHMDS, lithium bis(trimethylsilyl)amide; RuPhos Pd G4, 2-dicyclohexylphosphino-2′,6′-diisopropoxybiphenyl Buchwald 4th generation palladacycle catalyst; MOMBr, bromomethyl methyl ether; MOM, methoxymethyl protecting group; XantPhos Pd G 4, 4,5-bis(diphenylphosphino)-9,9-dimethylxanthene Buchwald 4th generation palladacycle catalyst; DIPEA, *N,N*-diisopropylethylamine; Boc, *tert*-butyloxycarbonyl protecting group; THF, tetrahydrofuran; Boc_2O, di-*tert*-butyl dicarbonate; GSH, glutathione.

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