Structure-based design, synthesis and biological evaluation of a novel series of isoquinolone and pyrazolo[4,3-c]pyridine inhibitors of fascin 1 as potential anti-metastatic agents

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ARTICLE INFO

Keywords:
Virtual screening
Fragments
Drug discovery
Medicinal chemistry
Cancer

ABSTRACT

Fascin is an actin binding and bundling protein that is not expressed in normal epithelial tissues but over-expressed in a variety of invasive epithelial tumors. It has a critical role in cancer cell metastasis by promoting cell migration and invasion. Here we report the crystal structures of fascin in complex with a series of novel and potent inhibitors. Structure-based elaboration of these compounds enabled the development of a series with nanomolar affinities for fascin, good physicochemical properties and the ability to inhibit fascin-mediated bundling of filamentous actin. These compounds provide promising starting points for fascin-targeted anti-metastatic therapies.

Introduction

Fascin 1 (hereafter termed fascin) is an evolutionarily conserved actin binding protein that cross-links filamentous actin (F-actin) into tightly packed parallel bundles driving the formation of various cell surface protrusions, such as filopodia and invadopodia, that promote cell migration and invasion.3 It’s expression is highly restricted in adult human tissues.2 Fascin is either absent or shows low expression in normal epithelial tissues but is overexpressed in a number of cancers, with expression levels correlating with overall cancer aggressiveness and predicting poor clinical outcome.3,4 Conversely, genetic knockdown of fascin decreases tumor cell invasion both in vitro and in vivo,5,6 suggesting that fascin may be a viable target for anticancer/antimetastatic drugs. Huang et al. have disclosed compounds which bind to fascin and inhibit actin bundling.7–9 However, in our hands a number of these compounds bound with modest (10–100µM) affinity and suffered from poor solubility.

In order to identify novel fascin compounds, with favourable properties, we undertook a surface plasmon resonance (SPR) screen using our collection of 1050 fragments and identified 53 hits (hit rate 5%). Follow-up protein crystallography yielded a range of fragments binding to multiple sites on the protein. The N-phenylacetamide 1 (Fig. 1A) binds efficiently (SPR \( K_d = 92\mu M \); ligand efficiency 0.43) in a buried pocket between the first and second \( \beta \)-trefoil domain (Fig. 1B) and was therefore selected for further development. Binding of compound 1 induces a hydrophobic pocket in domain 1, mainly by displacing Trp101. In addition, domain 1 pivots by ≈15°, reorienting residues on either side of the domain interface and creating a narrow channel to the protein surface (Fig. 1C and D). The dichlorophenyl moiety of 1 fills the hydrophobic pocket, while the acetamide engages in hydrophilic interactions with domain 2, with its carbonyl accepting a hydrogen bond from the backbone of Leu214 (Fig. 1E). While the completely enclosed binding site makes compound 1 a highly efficient ligand, it hampered attempts to develop this series.

The importance of this fragment binding site was highlighted when we determined the crystal structure of compound 2 (Fig. 2A).
previously identified by Huang et al. to inhibit fascin-mediated actin bundling.\textsuperscript{1,2} To accommodate the larger compound 2, domain 1 undergoes a more dramatic conformational change, rotating 35° around an axis almost parallel to a line connecting the first two β-trefoil domains (Fig. 2B). For the compound binding site (Fig. 2C) this almost pure rotation results in a 6 Å ‘drop’ of domain 2 relative to domain 1, so that the Phe216 side chain, which in the fascin-compound 1 complex lines the channel, now extends the hydrophobic pocket (cf. Figs. 1D and 2C) where the compound’s dichlorobenzyl moiety binds. More importantly, the conformational change opens up the channel so that it can accomodate the rest of the compound. The pyrazolopyrimidinone core rests on a relatively flat surface created by the salt-bridged side chains of Glu215 and Arg217. Additionally, compound 2 accepts a hydrogen bond from Phe216 and makes a relatively weak water-bridged interaction with the side chain of Glu215 (Fig. 2D). The induced conformational changes provide 2 with access to the protein surface creating opportunities for structure-driven compound elaboration. Notably, the recently disclosed structure of fascin in complex with an unrelated small molecule bound in the same site\textsuperscript{9} shows fascin adopting a similar conformation (RMSD < 1.3 Å for 483 Cz atoms).

To identify compounds which could potentially fill these additional areas a hybrid virtual screen was carried out. The first method was structure-based: 809 commercial analogues of compound 2 and ≈ 2.7 million lead-like molecules from our in-house virtual library were docked\textsuperscript{13} in the enclosed site. To prioritize the docked compounds, a multi-parameter optimization was performed using Pareto efficiency\textsuperscript{14} as the ranking technique with calculated free energy of binding (from docking), QED\textsuperscript{15} and interaction pattern similarity (based on compound 2 interaction fingerprints)\textsuperscript{16} as the parameters to optimize. The second method was ligand-based using shape-similarity\textsuperscript{17} to find analogues of compound 2 in our in-house library. Combining the results, 110 commercial compounds were tested by SPR at a screening concentration of 100 µM. The resulting binders were validated by co-crystalization and SPR dose-response experiments. Eighteen compounds were confirmed to bind in the same site as 2, and three yielded $K_d$ values < 100 µM. Of these three, we decided to progress the isoquinolinone 3 (SPR $K_d = 29.3 ± 5.8$ µM; Fig. 2A and E), which maintains affinity similar to 2 despite substituent truncations from both the benzyl and the core ring system. The pyrazolamide moiety forms an edge-to-face aromatic interaction with the Trp101 side chain. It also moves the bridging water seen in the fascin-compound 2 complex structure deeper into the binding site. This water now hydrogen-bonds to the Thr213 side chain, the Leu214 backbone carbonyl and the ligand amide in addition to the Ghul215 side chain, which ‘follows’ it by adopting a different rotamer (Fig. 2D and E). Analysis of this water with MOE 3D-RISM\textsuperscript{18} suggests it is relatively tightly bound ($\Delta$G = $-3.9$ kJ/mol) and that displacing it would likely be energetically unfavorable.

In order to measure the effect of these compounds on fascin activity we developed a robust and highly reproducible in vitro F-actin bundling assay, based on a previously published method.\textsuperscript{19} Unlike compound 1, both 2 and 3 inhibit fascin in this bundling assay. Compound 3 inhibits actin bundling with an IC$_{50}$ of 67.9 ± 3.5 µM, giving an approximately two-fold fall-off from SPR binding affinity. A similar fall-off is seen for other compounds in this series.

Considering that both 1 and 2 place a substituted phenyl into the hydrophobic pocket, it seemed plausible that the plain benzyl of 3 is not optimal for filling the available space. Additionally, given that this induced pocket is likely to show some plasticity, it may be possible to open it up further by extending the compound. To investigate this, we synthesized a series of benzyl derivatives of 3 (Table 1). Introduction of a para-halo substituent (compounds 4 and 5) increased binding affinity.
≈ 14-fold compared to 3. The crystal structure of 5 bound to fascin shows a binding mode without substantial changes in either ligand or protein conformation compared to the fascin-3 complex (Fig. S1A). A single meta-fluoro substituent (6) is equipotent while m-chloro substitution (7) improved affinity, though less so than 4 or 5. This may be partly explained by the slight differences in binding mode for meta substituted compounds compared to 3, in which the benzyl moiety is rotated to line up the m-substituent with a pocket indentation (Fig. S1B). In light of this conformational divergence, it is not surprising that m,p-dihalo compounds exhibit sub-additive affinity gains, though both 8 and 9 showed improved binding over mono- or unsubstituted analogues with SPR Kd values of 1.2 and 1.5 μM respectively. The structure of the fascin-9 complex shows the compound adopts a conformation with the phenyl rotated to an intermediate position compared to the meta and para analogues (Fig. S1C). Larger meta or para substituents (e.g. 10 and 11; Fig. S1D) are tolerated by expanding the hydrophobic pocket. However, the associated conformational penalty consistently
makes these compounds less potent. Introduction of an ortho substituent decreases potency both in combination with a para substituent (12) or as a single substituent (data not shown). Taken together the SAR suggests that m,p-dichloro substitution (9) is close to optimal in this series.

We next investigated altering the bicyclic scaffold of 3 (Table 2), taking care to retain the pyridinone moiety, which we found to be essential for binding. Complete deletion of the outer ring (13) led to a significant loss of affinity, which can be partly recovered with the addition of a 3-amino substituent (14), which donates an additional hydrogen bond to the Phe216 backbone carbonyl. The naphthyridinones 16 and 17 maintain binding affinities similar to the parent isoquinolinone, while placement of the naphthyridinone nitrogen next to

| Compound | $K_d \pm SD^a (\mu M)$ | $IC_{50} \pm SD^b (\mu M)$ |
|----------|------------------------|--------------------------|
| 9        | X                      | 1.5 ± 1                   | 1.3 ± 0.2 |
| 13       | Y                      | >100                      | nd        |
| 14       | Y                      | 21 ± 4.2                  | >100      |
| 15       | X                      | >100                      | nd        |
| 16       | X                      | 1.6 ± 0.1                 | 5.3 ± 0.8 |
| 17       | X                      | 1.3 ± 0.1                 | 3.8 ± 0.3 |
| 18       | X                      | >100                      | nd        |
| 19       | X                      | 10.0 ± 1.4                | 10.4 ± 0.2 |

$x$: R = Me; $\gamma$: R = 3-pyrolidine;

$^a$ $K_d$: SPR binding affinity, values are mean ± standard deviation from $n$ = 2–10 separate experiments;

$^b$ $IC_{50}$: compound concentration that reduces actin bundling by 50%, values are mean ± standard deviation from $n$ = 2–4 separate experiments; nd: not determined.
sterically restricted from adopting an optimal core binding mode. In light of this we chose to revisit the pyrazolo series, where we had previously observed that the equally bulky cyclic sulphone substituent of 2 was compatible with the ‘standard’ core orientation (Fig. S2B). The truncated compound 25 showed diminished binding (Table 4), revealing that addition of either the pyrazolamide in the 7-position (to obtain 19) or the cyclic sulphone at N1 (to obtain 2) to this compound improves fascin affinity > 50-fold and 23-fold, respectively. Combining these key features into one compound gave 26, with an SPR $K_d$ of 0.6 μM, suggesting near-additive gains in binding affinity compared to 24 and almost closing the potency gap to our best naphthyridinones.

We next investigated changes to the heteroaromatic amide. In line with the predicted importance of the bridging water discussed above, any changes to the amide (N-alkylation, carbonyl deletion) abolish fascin binding (not shown). On the other hand, the methylpyrazole interacts with the protein through stacking with the indole of Trp101. A number of compounds with alternative heterocycles were made to probe this interaction, focusing on five- and six-membered nitrogen-containing heterocycles. Of these the pyridin-4-yl 27 yielded the largest, albeit still modest, improvement over 26 in terms of both affinity and inhibition. QM calculations (Fig. S4) suggest that at least for (hetero)aryl derivatives improved stacking with Trp101 may be a major factor in this increase in potency.

Given the success of 24 and to alleviate concerns about chemical stability of the cyclic sulphone substituent, we opted to replace it with a 1-piperidin-4-yl.

The crystal structure of fascin in complex with BDP-13176 shows the compound adopting the expected overall binding mode (Fig. 4), but with a slight core rotation in the opposite direction from that observed for 24 (Fig. S5). The compound’s in vitro physicochemical and DMPK properties (Table S1) are within acceptable limits with low lipophilicity ($LogD_{7.4} = 1.8$) and reasonable kinetic solubility (midpoint = 65 μM). The compound was also moderately stable in both human and mouse

### Table 4
Further substitutions of the pyrazolopyridinone scaffold.

| Compound | $R^1$-group | $R^2$-group | $K_d \pm SDa$ (µM) | $IC_{50} \pm SDb$ (µM) |
|----------|-------------|-------------|---------------------|------------------------|
| 25       | N           | H           | > 500               | nd                     |
| 2        | N           | SO$_2$      | 29.5 ± 8.3          | 67.6 ± 2.4             |
| 26       |             |             | 0.60 ± 0.05         | 0.63 ± 0.15            |
| 27       |             |             | 0.27 ± 0.04         | 0.33 ± 0.06            |
| 28       |             |             | 0.09 ± 0.02         | 0.24 ± 0.01            |

*a* $K_d$: SPR binding affinity, values are mean ± standard deviation from n = 2–10 separate experiments.

*b* $IC_{50}$: compound concentration that reduces actin bundling by 50%, values are mean ± standard deviation from n = 2–4 separate experiments; nd: not determined.

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**Scheme 1.** Reagents and conditions: (a) $N,N$-dimethylformamide dimethyl acetal, ethanol r.t. (75%); (b) 4 tert-butyl 4-hydrazinopiperidine-1-carboxylate, ethanol, reflux, (27%); (c-e) Bredereck’s, toluene reflux; 3,4-dichlorophenyl) methanamine, acetic acid, toluene; sodium ethoxide, ethanol (3 steps 85%); (f) Sodium hydroxide, ethanol r.t. (84%); (g) HATU, $N,N$-disopropylethylamine, DMF, r.t., (83%); (h) TFA, DCM r.t. (52%).
liver microsomes (Human Cl\textsubscript{int} = 6.4, Mouse Cl\textsubscript{int} = 11.8, µL/min/mg protein). However, the compound has low Caco-2 permeability (A-B P\textsubscript{app} = 0.15x10\textsuperscript{-6} cm/s) and high efflux (efflux ratio = 37.8).

In conclusion, fragment screening coupled with crystallography and computational approaches allowed identification of a cryptic pocket within fascin. Using compound 2 (K\textsubscript{d} = 29.5 ± 8.3 µM)\textsuperscript{12} as a starting point combined with virtual screening and structure-based design allowed us to develop potent and functionally active fascin binders. Crystal structures reveal the compounds bind with a hydrophobic ‘hook’ in an induced pocket between the first two β-trefoil domains of fascin and from there extend towards the protein surface, effecting a substantial conformational change in domain 1. While the mechanism by which our compounds inhibit bundling remains unclear, it is worth noting that fascin’s two major proposed actin binding regions involve domain 1 and cross a domain boundary. The ligand-induced conformational change would deform both these regions and thereby disrupt actin binding (Fig. S6). We anticipate these compounds to be a useful foundation to further probe fascin’s potential role in tumor invasion and metastasis.
Experimental section

Experimental details can be found within the supplementary data file provided.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.01.035.

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