Discovery, development and SAR of aminothiazoles as LIMK inhibitors with cellular anti-invasive properties

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ABSTRACT: As part of a program to develop a small molecule inhibitor of LIMK, a series of aminothiazole inhibitors were discovered by high throughput screening. Scaffold hopping and subsequent SAR directed development led to a series of low nanomolar inhibitors of LIMK1 and LIMK2 that also inhibited the direct biomarker p-cofilin in cells and inhibited the invasion of MDA MB-231-luc cells in a matrigel inverse invasion assay.

Tumour cell invasion and metastasis are the primary causes of mortality in cancer patients. During progression of tumour cells to a metastatic phenotype, they undergo a series of changes that begin with loss of contact inhibition and increased motility, allowing them to migrate from the primary tumour site, invade distant organs and induce neovascularization resulting in metastasis.¹ Despite numerous developments, cancer cell invasion and metastasis is still a poorly studied process. Most strategies to treat cancer do not rely on inhibiting invasion and metastasis as the primary phenotype due to the requirement for lengthy and complicated clinical trials. However a detailed understanding of the drivers of cancer cell invasion and migration is essential to develop new treatments for cancer patients.

The LIM kinases (LIMK1 and LIMK2; collectively LIMK) are TKL kinases that act downstream of Rho GTPases. LIM kinases phosphorylate and inactivate the filamentous-actin severing protein cofilin. Cycles of cofilin inactivation and activation enable dynamic actin rearrangements that are required for cell motility (Figure 1). Once phosphorylated at Ser3 by the LIM kinases cofilin can no longer bind to actin leading to the accumulation of actin polymers. LIM kinases are therefore centrally positioned regulators of actin cytoskeletal dynamics and also play important roles in microtubule organization.²,³

LIMK1 has been reported to play a key regulatory role in tumour cell invasion and the level of LIMK1 is increased in invasive breast,⁴ prostate,⁵ and pancreatic⁶ cancer cell lines in comparison with less invasive cells. Overexpression of LIMK1 in MCF-7 and in MDA MB-231 human breast cancer cell lines increased their motility, while inhibition of LIMK1 activity in MDA MB-231 cells by expression of a dominant negative LIMK1 resulted in decreased motility and formation of osteolytic bone lesions in an animal model of tumour invasion.⁷ As such, the LIM kinases have been proposed to be attractive drug targets to block tumour cell invasion and metastasis.

A number of groups have previously reported inhibitors of the LIM kinases⁸,¹³ as treatments for cancer and for their ability to lower intraocular pressure for glaucoma. Herein we describe the discovery and development of a series of LIMK inhibitors that demonstrate inhibition of p-cofilin and inhibit invasion of cancer cells in a 3D inverse invasion assay.

We used a commercially available kinase Glo²⁵ kit measuring ATP depletion using full length LIMK and cofilin to screen 60,000 compounds.¹² From this we identified two lead series, a series of pyrimidines that our partners CTx developed¹⁵,¹⁶ and a

Figure 1 LIMK’s are key regulators of the actin cytoskeleton, through their modulation of cofilin function.
series of aminothiazoles exemplified by 1 LIMK1 IC<sub>50</sub>=4 μM (Figure 2) as modestly potent inhibitors of LIMK1. Although these compounds could be improved in terms of their isolated enzyme potency, no evidence of activity in the cellular assay was observed. They also contained numerous undesirable functionalitites such as an alkene, a ketone and a potentially unstable methylene dioxy group. In removing the amino functionality, we reasoned that we could improve the cellular potency by reducing the H bond count. We also removed the undesirable ketone functionality, replacing it with a variety of groups. This led to pyridine 2 a compound that was similarly potent to the original hit 1. Bristol Myers Squibb reported that substituted pyrazoles with an aryl di-ortho chloro group were potent LIMK inhibitors. 13 Similarly we found that pyridines substituted with aryl rings adjacent to the thiazole ring provided substantial improvements in potency as in 3a which had a LIMK1 potency of 15 nM. Even more importantly compound 3a was active in the p-cofilin cellular assay with an IC<sub>50</sub>=3 μM.

Figure 2 Evolution from original HTS lead
In order to better understand the binding mode, we created a homology model of the kinase domain of hLIMK1 based on c-Src (PDB code 1Y57.pdb) as a template. hLIMK shares 33.5% sequence identity and 50.6% sequence similarity with the c-Src template. Whilst there are some sequence inserts in the activation loop region (LIMK1 compared with c-Src) the rest of the sequences align well with no major inserts or deletions.

The improvements in potency were explained by docking 3a into the homology model (Figure 3). The amino thiadiazole in 1, 2 and 3a can form the key donor-acceptor interaction to the Ile428, Tyr 427 hinge residues. The pyridyl nitrogen present in 2 and 3a is well placed to interact with the salt bridge Lys380 residue. This agreed well with the SAR because the other pyridine isomers 4, 5 and 6 were much less potent than 3a (Figure 4). The chlorine atom was postulated to be making a halogen bond interaction to the Val 378 residue.

Figure 3 Compound 3a docked into a LIMK1 homology model based on c-Src (PDB code 1Y57.pdb) with surfaces coloured by lipophilicity, with purple indicating hydrophilic surfaces, green indicating lipophilic surfaces and neutral areas of the surface in white. Dotted lines indicate the key bonding interactions between the ligand and protein.

Reagents and conditions: (i) NaBH₄, MeOH, rt (ii) SOCl₂, DCM, DMF, reflux (iii) MeCN, 10a, 10b or 10c, Et₃N, reflux (iv) Boc₂O, DMAP, DCM, rt (v) (PPh₃)₂PdCl₂, R²ArB(OH)₂, K₂CO₃, 1,4-dioxane, water, 150 °C (vi) TFA (vii) 3 equivs Boc₂O, DMAP, THF (viii) (PPh₃)₂PdCl₂, 2-chlorophenylboronic acid, K₂CO₃, 1,4-dioxane, water, 150 °C then TFA (ix) NaN₂, HCl (x) NHMePr, MeOH, 150 °C

Figure 4 Pyridine isomer SAR
To understand the SAR and key binding interactions we set out to synthesize analogues of the cell active lead 3a. A variety of synthetic strategies were employed to allow late stage modifications of either the aryl ring (Scheme 1) or the amine side chain attached to the aminothiazole (Schemes 2 and 3).

Starting from the commercially available 3-bromopyridine-4-carbaldehyde 7 and reducing to the alcohol 8 followed by chlorination with thionyl chloride to the benzyl chloride 9 proceeded smoothly. The thiazole ring could then be constructed by heating in acetonitrile with triethylamine and the appropriate arylamines 10a-10c to provide the thiazoles 11 a-c. Protection of 11a with a Boc group allowed the introduction of a range of aryl groups via a Suzuki coupling to furnish starting materials 12 followed by Suzuki coupling to afford 14a.
that could then be converted to the chloride 15 by diazotization in hydrochloric acid. Displacement with N-propylmethylamine gave the final target 3i.

Initial SAR modifications focused mainly on the aryl ring (Table 1) and demonstrated that the presence of an ortho substituent was required to provide activity, in particular where the group was ortho chloro as shown in entry 3a. The ortho chloro group could be replaced with methyl or trifluoro methyl although larger groups such as iso-propyl resulted in large losses in LIMK1 potency. Removal of the propyl side chain as in entry 14 or methylation of the NH as in entry 3i resulted in large losses in potency with the latter result being consistent with the proposed binding mode where the NH forms a key donor interaction to the hinge region of the kinase (Figure 3).

The amine side chain could be modified (Scheme 2) by di Boc protection of 11b to give 13 followed by a Suzuki coupling gave the Boc protected and the unprotected compounds 14a and 16 respectively. The mono Boc protected aminothiazole 14a could be derivatised using a Mitsunobu coupling providing a convenient method to deliver benzylamines 3i and 3m. Alternatively 11b could be mono Boc protected to give 17, the amine side chain could be introduced via a Mitsunobu coupling to give 18a and 18b. Suzuki couplings provided access to the targets 3j and 3k. Using an analogous strategy that was employed in (Scheme 1) N-aryl analogues could be introduced by Boc protection of 11c to afford 19 and subsequent Suzuki coupling to yield 3n.

Table 1 Initial aryl ring SAR investigations

<table>
<thead>
<tr>
<th>Entry</th>
<th>R^1</th>
<th>R^2</th>
<th>LIMK1 IC_50 μM</th>
<th>Cell p-cofilin* IC_50 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>-NPr</td>
<td>o-Cl</td>
<td>0.015</td>
<td>3.8</td>
</tr>
<tr>
<td>3b</td>
<td>-NPr</td>
<td>m-Cl</td>
<td>0.50</td>
<td>ND</td>
</tr>
<tr>
<td>3c</td>
<td>-NPr</td>
<td>p-Cl</td>
<td>0.62</td>
<td>ND</td>
</tr>
<tr>
<td>3d</td>
<td>-NPr</td>
<td>H</td>
<td>0.85</td>
<td>ND</td>
</tr>
<tr>
<td>3e</td>
<td>-NPr</td>
<td>o-Me</td>
<td>0.082</td>
<td>11.5</td>
</tr>
<tr>
<td>3f</td>
<td>-NPr</td>
<td>o-i-Pr</td>
<td>3.3</td>
<td>ND</td>
</tr>
<tr>
<td>3g</td>
<td>-NPr</td>
<td>o-CF_3</td>
<td>0.037</td>
<td>2.2</td>
</tr>
<tr>
<td>3h</td>
<td>-NPr</td>
<td>o-CF_3, p-Ome</td>
<td>0.013</td>
<td>2.5</td>
</tr>
<tr>
<td>14a</td>
<td>-NH_2</td>
<td>o-Cl</td>
<td>0.27</td>
<td>&gt;30</td>
</tr>
<tr>
<td>3i</td>
<td>-NMePr</td>
<td>o-Cl</td>
<td>3.1</td>
<td>ND</td>
</tr>
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</table>

*measured in ZR75-1 cells using a Cellomics array scan instrument. ND=not determined.

To allow further diversification of the aminothiazole side chain at the last step the aryl rings could be introduced on the alcohol intermediates 20a and 20b (Scheme 3), followed by chlorination to provide 21a and 21b, thiazole formation with 10b or 10d provided final compound 3o and intermediates 14b-c. This then allowed for a series of amides to be prepared 22a-d.

The N-aryl analogue 3n in particular was very potent providing a compound that was sub micromolar in the p-cofilin cellular assay. Smaller alkyl groups such as ethyl 3j were less potent compared to iso-butyl 3k and benzylamines 3i and 3m. Amides 22a-22d were also well tolerated particularly 22b which was 0.8 μM in the p-cofilin cellular assay.

Scheme 2 Synthetic strategy to access amine side chain analogues

Reagents and conditions: (i) 3 equivs Boc_2O, DMAP, THF (ii) (PPh_3)_2PdCl_2, 2-chlorophenylboronic acid, K_2CO_3, 1,4-dioxane, water, 150 °C (iii) R^3OH, DIAD, PPh_3, THF (iv) TFA, DCM (v) Boc_2O, DMAP, DCM (vi) Boc_2O, DMAP, DCM

Scheme 3 Synthetic strategy to access amide side chain analogues

Reagents and conditions: (i) (PPh_3)_2PdCl_3, R^2ArB(OH)_2, K_2CO_3, 1,4-dioxane, water, 150 °C (ii) SOCl_2, DCM, DMF, reflux (iii) MeCN, 10b or 10d, Et_3N, reflux (iv) R^3COCl, Et_3N, DCM, rt
Table 2. SAR of amine side chain modifications

<table>
<thead>
<tr>
<th>Entry</th>
<th>R²</th>
<th>R³</th>
<th>LIMK1 IC₅₀ μM</th>
<th>Cell p- combination IC₅₀ μM</th>
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<tbody>
<tr>
<td>3j</td>
<td>-NHeT</td>
<td>o-Cl</td>
<td>0.12</td>
<td>14</td>
</tr>
<tr>
<td>3k</td>
<td>-NH-i-Bu</td>
<td>o-Cl</td>
<td>0.005</td>
<td>1.5</td>
</tr>
<tr>
<td>3l</td>
<td>-NHCHMe(S)Ph</td>
<td>o-Cl</td>
<td>0.04</td>
<td>9.2</td>
</tr>
<tr>
<td>3m</td>
<td>-NHCHMe(R)Ph</td>
<td>o-Cl</td>
<td>0.004</td>
<td>2.1</td>
</tr>
<tr>
<td>3n</td>
<td>-NHeT</td>
<td>o-Cl</td>
<td>0.003</td>
<td>0.6</td>
</tr>
<tr>
<td>3o</td>
<td>-NH4-PhOH</td>
<td>o-Cl, p- Me</td>
<td>0.003</td>
<td>6.6</td>
</tr>
<tr>
<td>22a</td>
<td>-NHeT</td>
<td>o-Cl</td>
<td>0.02</td>
<td>1.4</td>
</tr>
<tr>
<td>22b</td>
<td>-NHCO-i-Pr</td>
<td>o-Cl</td>
<td>0.003</td>
<td>0.8</td>
</tr>
<tr>
<td>22c</td>
<td>-NHCO-i-Pr</td>
<td>o-Cl, p-Me</td>
<td>0.001</td>
<td>ND</td>
</tr>
<tr>
<td>22d</td>
<td>-NHCO-i-Pr</td>
<td>o-CF₃, p-OMe</td>
<td>0.008</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*measured in ZR75-1 cells using a Cellomics array scan instrument. ND=not determined.

The ADME properties of the compounds required optimisation, in particular the alkyl substituted analogues were liable to high metabolic turnover in vitro. However the propyl group could be replaced with N-aryl analogues and amide groups. The amide analogue 22d in particular had lower in vitro microsomal turnover (Table 3).

Table 3. ADME properties and isoform selectivity of selected analogues

<table>
<thead>
<tr>
<th>Entry</th>
<th>22c</th>
<th>22d</th>
<th>3o</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIMK1 IC₅₀/nM</td>
<td>1</td>
<td>8</td>
<td>0.3</td>
</tr>
<tr>
<td>LIMK2 IC₅₀/nM</td>
<td>3</td>
<td>48</td>
<td>1</td>
</tr>
<tr>
<td>cLogP</td>
<td>4.3</td>
<td>4.3</td>
<td>4.8</td>
</tr>
<tr>
<td>Microsomes (m/h) mL/min/g liver</td>
<td>19.4/8.3</td>
<td>5.2/3.7</td>
<td>4.5/0.9</td>
</tr>
<tr>
<td>PB (h)%</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

In order to understand the functional effects of the compounds, we employed a 3D inverse invasion assay to measure the inhibition of invasion of cells into a matrigel matrix. In order to purely understand the metastatic phenotype it was necessary to select non-toxic compounds, so cell viability at 10 μM was measured. We used an MTT cytotoxicity assay to select four compounds for further investigation based on their lack of cytotoxic effects (Table 4). To measure the invasive phenotype, MDA MB-231-luc cells were plated on the underside of a transwell filter plate. The ability of cells to invade was measured by confocal microscopy of sections through a matrigel plug. The percentage inhibition of invasion was measured as a function of the proportion of cells that invaded more than 60 µm into the matrigel plug compared to cells that invaded less than 60 µm, relative to the control. Compounds 3k, 22c, 22d and 3o all inhibited the invasion of the cells through matrigel at a concentration of 3 μM, whilst not significantly affecting cell viability even at the higher compound concentration of 10 μM. Compound 3o was found to be the most effective at inhibiting invasion. However the more selective compound 22d also significantly inhibited the invasion of the cells by 52%. In addition, two other compounds 3k and 22c also inhibited invasion without having a marked effect on cell viability.

Table 4. Functional effects of selected LIMK inhibitors in an inverse invasion assay

<table>
<thead>
<tr>
<th>Entry</th>
<th>MTT % viable cells@ 10 μM inhibitor</th>
<th>% inhibition of invasion@ 3 μM in MDA MB-231-luc</th>
</tr>
</thead>
<tbody>
<tr>
<td>3k</td>
<td>88%</td>
<td>34 (n=5)</td>
</tr>
<tr>
<td>22c</td>
<td>131%</td>
<td>48 (n=2)</td>
</tr>
<tr>
<td>22d</td>
<td>106%</td>
<td>52 (n=3)</td>
</tr>
<tr>
<td>3o</td>
<td>70%</td>
<td>96 (n=3)</td>
</tr>
</tbody>
</table>

To measure selectivity against the LIMK2 isoform we developed an assay using a lanthanabrid format. The compounds were similarly potent Vs LIMK2 as LIMK1 (Table 3). The lead compounds were also profiled for their selectivity against a broader panel of 442 kinases using KINOMEscan® at Millipore (now DisoverRx). The selectivity of 22d, 3o and 22c is represented in the kinome phylogenetic trees from screening at 10 μM inhibitor concentration (Figure 5). Selectivity scores were calculated as the number of non mutant kinases with % activity relative to control X35/number of non mutant kinases tested. Compounds 22d and 22c were the particularly selective with selectivity S(35) scores of 0.083 and 0.132 respectively.

Figure 5 Kinase selectivity Vs 442 kinases for compounds 22d, 3o and 22c

In summary we have developed a series of novel LIMK inhibitors that are effective in inhibiting cellular invasion through a 3D matrix. An additional publication by Olson et al.¹⁸ will detail further biological data using compounds 22d and 3o (also known as CRT0105446 and CRT0105950 respectively) to investigate the effects of LIMK inhibition on microtubule organisation. The lead compounds are available externally for further profiling and investigation to determine their pharmacological applicability in conditions where LIMK plays a role.

ASSOCIATED CONTENT

Supporting Information. Detailed synthetic methods, analytical data, detailed selectivity data, procedures for the enzymatic and cellular assays and information on the computational approaches used can be found in the supplementary information. This material is available free of charge via the Internet at http://pubs.acs.org.
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Inter LIM kinase 1 increases tumor metastasis of human breast cancer cells
(4) nases are required for in
Machesky, 284 semination to organ
(1) 53 LIM kinases: function, regulation and
tyrosine kinase like, GTP: Guanosine-5'-triphosphate, ATP: Adenosine tri-
phosphate; HTS: High throughput screen; SAR: Structure activity relationship;
PDB: Protein data bank; MOE: Molecular operating environment; ND: Not determined; m: mouse; h: human.

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