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Development of a potent and selective cell penetrant Legumain inhibitor

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ABSTRACT

This Letter describes the continued SAR exploration of small molecule Legumain inhibitors with the aim of developing a potent and selective in vitro tool compound. Work continued in this Letter explores the use of alternative P2–P3 linker units and the P3 group SAR which led to the identification of **10t**, a potent, selective and cellularly active Legumain inhibitor. We also demonstrate that **10t** has activity in both cancer cell viability and colony formation assays.

Legumain, or Asparaginyl endopeptidase (AEP), is a member of the CD clan of proteases, which includes caspases, separase and the gingipains.[1,2] Mammalian legumain is expressed within the lysosome and plays an important role in MHC class II-mediated antigen presentation and regulation of the activity of the papain family cathepsins, specifically B, L and H.[3–5] In addition, legumain has also been reported to play a major role in the conversion and activation of pro-MMP2 into the active enzyme.[6]

Recently, several publications have highlighted the use of legumain as a marker of prognosis in many human cancers including breast, ovarian, glioma, pancreatic and prostate.[7–11] These studies demonstrate that within the cancer setting higher levels of legumain expression directly correlate with poor patient outcome and aggressive disease.[7–11] Furthermore, localisation of active legumain determined by vesicular staining is correlated with lower survival and more aggressive disease in prostate cancer patients.[11] A transgenic murine model of prostate cancer also revealed an association of increased legumain expression with disease progression.[12]

Our group identified the transcription factor TBX2 to be a potent regulator of legumain activity in breast cancer, via suppression of CST6, the endogenous legumain inhibitor.[13] CST6 loss leads to increased cancer cell proliferation and invasion in breast cancer cells.[13] Re-expression of CST6 in prostate cancer cells significantly impairs tumour growth and metastasis when xenografted.[14] In addition, suppression of TBX2 or legumain, and re-introduction of CST6, were performed and found to have a potent cytotoxic effect against a panel of breast cancer cells but not the corresponding normal cell lines[13] demonstrating the dependence of tumorigeneic cells on legumain signalling.

We recently identified compound **1** as a novel legumain inhibitor (Fig. 1).[15] Whilst use of the cyano warhead in our initial inhibitor design was beneficial further development was required to achieve an optimal in vivo tool compound. As the SAR appeared to be shallow in both the P1 and P2 we focused on exploring the SAR of the P2–P3 linker group. Holding both P1 and P2 constant based upon compound **1** the benzamide **2** and benzylamine **3** were both found to be poor replacements for the carbamate group in terms of potency (Fig. 1). The use of the sulphonamide group (**4**) however afforded a sub-μM inhibitor. The identification of a potent alternative to the carbamate linker warranted further exploration.

Proline **5** was converted to the methyl ester **6** with thionyl chloride in methanol under refluxing conditions (Scheme 1). The resulting amino methyl ester was converted to the corresponding

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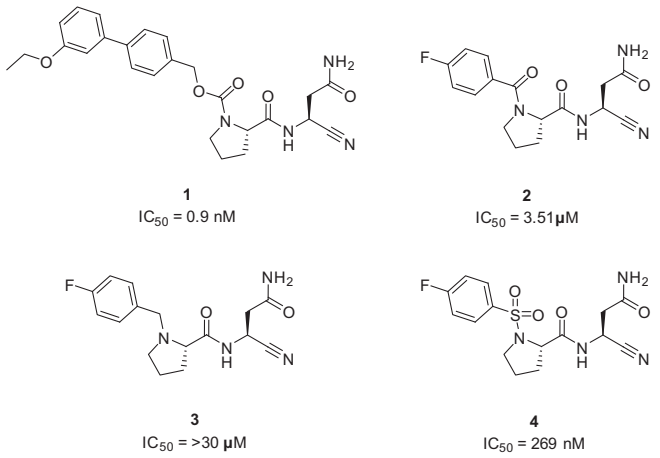


Figure 1. Preceding Legumain inhibitor and P2–P3 linker scan results.

sulphonamide (**7a**–**h**) in good yield. The ester was cleaved under standard conditions and the corresponding acid was coupled to H-Asp(OBzl)-$CONH_2$. The dipeptides (**8a**–**h**) were subsequently dehydrated with cyanuric chloride to install the cyano warhead followed by cleavage of the benzyl ester and coupling with ammonium chloride to afford desired analogues (**9a**–**h**) in moderate to good yield.

Based upon results from compounds that were synthesized for screening the SAR appeared limited. Increasing the size of the halogen from fluoro (**4**) to chloro (**9b**) had a positive effect upon the potency with a 3.7-fold increase observed (Table 1). This increase in potency was lost when moving to the *para*-bromo analogue (**9d**). Incorporation of di-halo substitution patterns was poorly tolerated with a 8-fold loss in activity observed with the 2,4-DiCl analogue (**9a**) and the 3,4-DiCl compound (**9e**) inactive. Additional functionalities were attempted, however these led to significant drop in potency with the exception of the *para*-phenyl analogue **9h**. This biphenyl compound, **9h**, was highly potent against recombinant enzyme and out of this collection of compounds it was the only analogue to display intracellular activity. After an incubation period of 2 h compound **9h** significantly suppressed intracellular legumain activity by ~50% (Table 1). Additional time points revealed that this activity was lost after 24 h with no effect upon cancer cell.

Table 1
Potency values of progenitor compound **4** and analogues

Compound	X group	Legumain IC_{50}[a] (μM)	Cell Inh @30 μM[b] (%)
4	4-F	0.269	0
9a	2,4-DiCl	0.593	0
9b	4-Cl	0.073	0
9c	4-OMe	>10	ND
9d	4-Br	0.282	0
9e	3,4-DiCl	>10	ND
9f	4-CF_3	0.332	0
9g	4-*t*Bu	>10	ND
9h	4-Ph	0.012	47

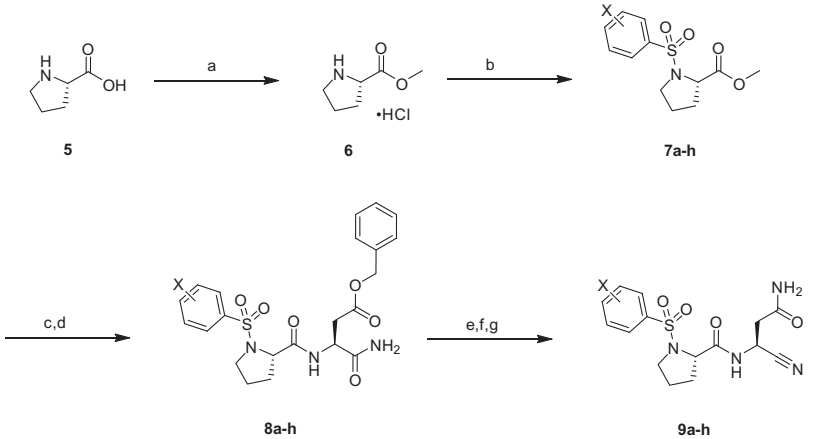
[a] IC_{50} performed in triplicate against recombinant human Legumain via an 8 point dose response curve. Z-Ala-Ala-Asn-AMC fluorogenic substrate (R&D Systems) was used.
[b] Compound was incubated with MCF7 cells @37 °C for 24 h, assay media removed, cells washed with assay buffer and lysed. Inhibition was determined with fluorogenic substrate and carried out in triplicate.

To explore the SAR of the bi-aryl sulfonamides the 4-bromophenylsulphonamide analogue (**9d**) was used as a chemical handle for a series of Suzuki cross coupling reactions to afford **10a**–**t** (Scheme 2 and Table 2).[16]

The SAR of the distal phenyl ring (**10a**–**t**) was robust when compared to that of the mono-phenyl sulfonamides (**9a**–**h**) with a number a functionalities and substitution patterns displaying low nanomolar activity (Table 2). All active compounds were tested to determine the intracellular activity at a single drug concentration of 30 μM in MCF7 cells 24 h post treatment. With a few exceptions (**10f**, **10p** and **10q**) all of the analogues displayed varying levels of intracellular legumain inhibition (Table 2).

Compounds that displayed ⩾70% intracellular legumain inhibition were assayed for effects on cellular viability against a panel of breast (MCF7 and T47D) and prostate cancer cells (PC3 and DU145). Each of the cell lines were treated with 10 and 30 μM of each compound for 5 days. From this study only one compound, **10t**, reduced cell survival with >50% cell viability at 30 μM across all cell lines (Fig. 2a). To further understand this finding we assessed the ability of each of the selected compounds to retain intracellular activity across the timeline of the cellular experiment (5 days). This study revealed that only **10t** inhibited legumain activity post 48 h drug treatment suggesting that this compound has a slower off rate than the other analogues tested.

Legumain has been reported to play a significant number of roles in cancer biology, including cellular invasion and formation



Scheme 1. Reagents and conditions; (a) thionyl chloride (2 equiv), MeOH, reflux, 2 h, 99%; (b) X-benzenesulphonyl chloride, NMM, DCM, 4 h, 72–98%; (c) LiOH (6 equiv), H_2O/MeOH/THF (1:1:9), 1–3 h; (d) H-Asp(OBzl)-$CONH_2$, HBTU, NMM, DMF, 18 h (86–95%); (e) cyanuric chloride, DMF, 3 h (85–97%); (f) LiOH (4 equiv), H_2O/THF (1:9), 1–2 h; (g) NH_4Cl, HBTU, NMM, DMF, 18 h (32–67%).