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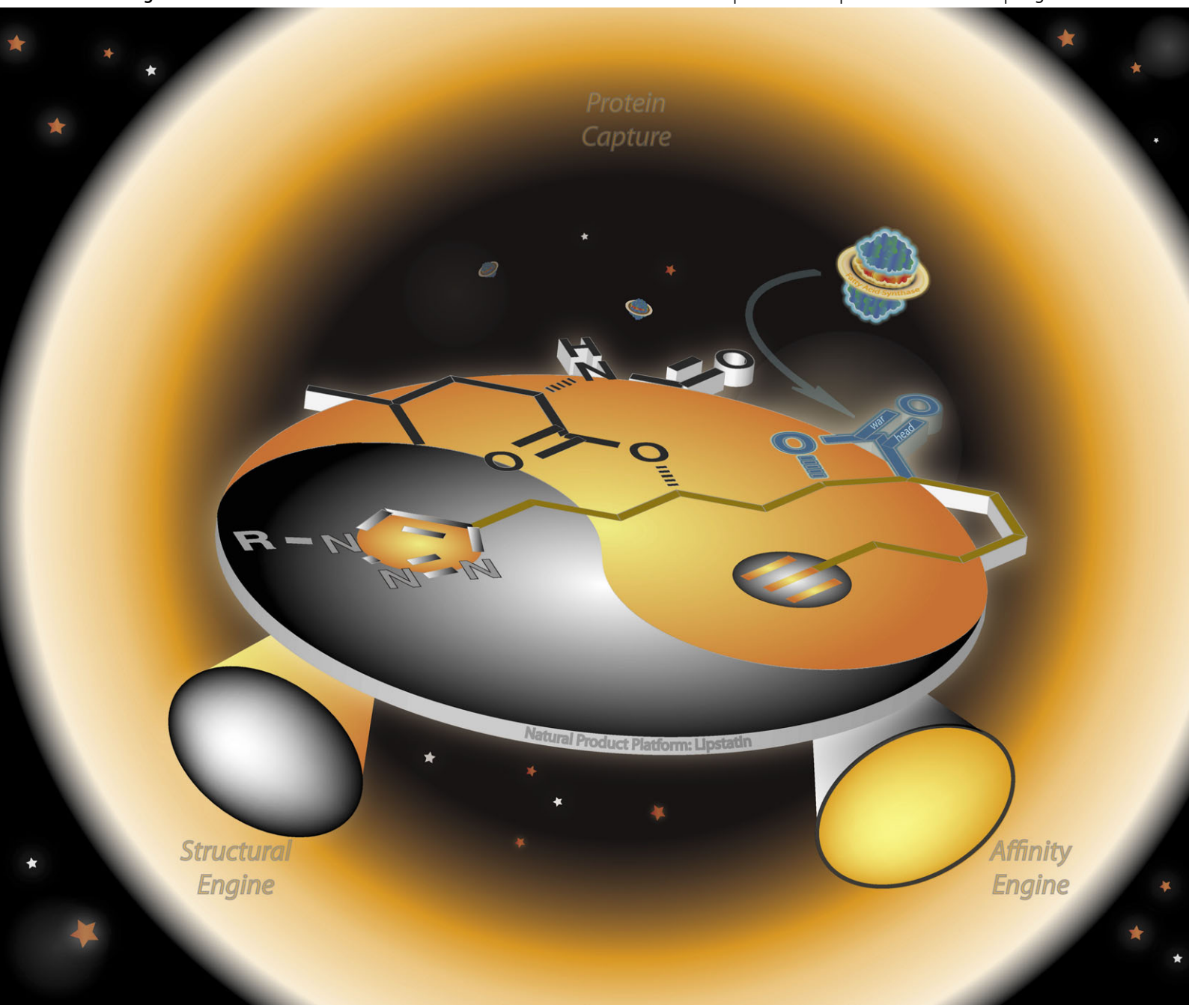

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Click-based synthesis and proteomic profiling of lipstatin analogues

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Click-based synthesis and proteomic profiling of lipstatin analogues†

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Using click chemistry to enable both structural diversity and proteome profiling within a natural product derived library, two out of nineteen lipstatin analogues showed similar activity to Orlistat against fatty acid synthase (FAS), but with an improved ability to induce tumour cell death.

Tetrahydrolipstatin (THL, **1**; Fig. 1) is an FDA-approved antiobesity drug called Orlistat, which works primarily on pancreatic and gastric lipases within the gastrointestinal (GI) tract.[1] Among other lipstatins, THL (**1**) has recently shown promising activity against off-targets such as human fatty acid synthase (FAS), an enzyme essential for the survival of tumour cells.[2] There are, however, a number of challenges that prevent **1** from being considered as a potential antitumour drug; it has poor solubility and bioavailability,[3] and, most importantly, lacks sufficient potency and specificity.[4] Recognising the need to address these impending issues, we decided to search for better lipstatin analogues that might possess similar (or improved) potency against FAS, and at the same time affect fewer cellular off-targets.[4] Herein, we describe the identification of two such compounds, made possible by the combination of a rapid synthesis of THL-like compounds (using click chemistry) and a cell-based chemical proteomic approach to enable the direct activity-based protein profiling (ABPP) of potential cellular targets (on and off) of THL, **1** (Fig. 1).[5]

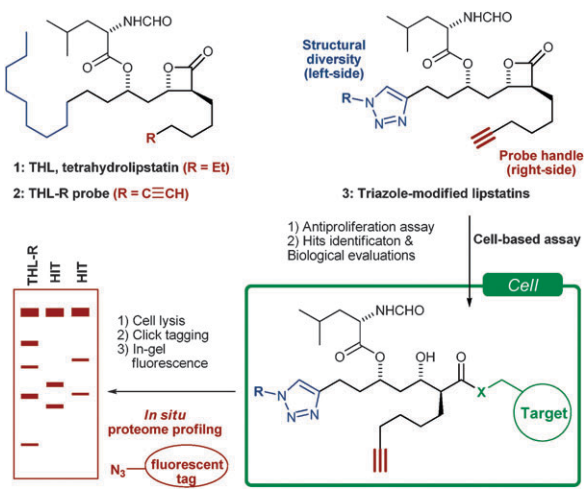

Fig. 1 Click-based design of lipstatin analogues **3**.

The design of our lipstatin-derived library is based on the general structure of THL (**1**) and the success of our THL-R alkyne-probe **2** (Fig. 1).[4] The β-lactone pharmacophore and amino ester would thus be preserved (to maintain sufficient binding/activity to FAS) and a terminal alkyne handle introduced to the right aliphatic side chain (for identification of cellular targets *via* the downstream conjugation to reporter tags).[6,7] A left alkyne-terminated aliphatic side chain would then be diversified through a triazole ring (*cf.* **3**), *via* click chemistry, with a variety of aliphatic/aromatic azides.[8] Here, we anticipated the known functional group compatibility of click conditions would tolerate a relatively reactive β-lactone component.[6] We were, however, uncertain whether the triazole moiety in **3** would engage in favourable or unfavourable interactions with FAS, thereby influencing the potency and specificity.

Our synthesis parallels the elegant work of Romo, Smith and co-workers, who adopted olefin metathesis to diversify an alkene lipstatin analogue.[3] Herein, we targeted a di-alkyne lipstatin analogue (**9**) as our chemical progenitor (Scheme 1). Starting from 4-pentynal **4**, Keck allylation[9] with allyltributyltin (in the presence of a titanium-(*S*)-BINOL complex) afforded the chiral homoallylic alcohol (*R*)-**5** in an excellent *ee* of >97% (*cf.* modified Mosher esters analysis; Scheme S1 and Fig. S1†). The alcohol **5** was then TBS-protected and the olefin oxidatively cleaved with potassium osmate and sodium periodate to afford the aldehyde **6**.[10] The key $ZnCl_2$-mediated, tandem Mukaiyama aldol-lactonisation (TMAL)[3] between the β-hydroxy aldehyde **6** and the thiopyridyl ketene acetal **7** (prepared in four steps from 6-bromohexanoic acid; Scheme S2†) afforded the *trans*-β-lactone **8** as separable 9 : 1 *anti*/*syn* diastereomers in 39% yield after desilylation.

^{1}H NMR analysis of the β-lactone ring protons showed a characteristic coupling constant of 3.8 Hz for a *trans*-β-lactone ($^3J_{cis}$ = 6.5 Hz and $^3J_{trans}$ = 4–4.5 Hz).[11] Formation of the β-lactone was further confirmed by the presence of a strong IR absorption band at 1818 cm^{-1}. Subsequent Mitsunobu reaction of the readily separable, major *anti*-hydroxy-β-lactone **8** with *N*-formyl-L-leucine afforded the desired di-alkyne β-lactone **9** in 66% yield. Next, several click chemistry conditions were examined;[12] it was found that triazole formation was highly sensitive to the catalyst, additives and solvents used (Supporting Information†). Upon optimisation, we found the use of CuI (0.4 equiv.) and ^{i}Pr$_2$NEt (0.8 equiv.) in DCE/H_2O (1 : 1) gave the desired triazole-adduct with complete

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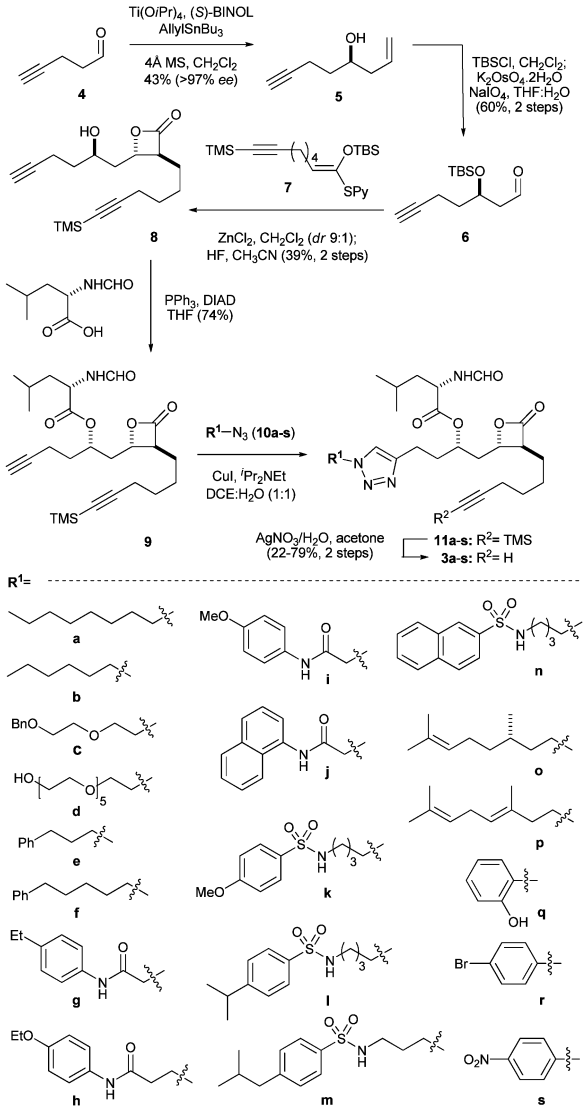

Scheme 1 Synthesis of β-lactone **9** and lipstatin analogues **3**.

consumption of the alkyne in high purity and within 24 h. A total of 19 aromatic and aliphatic azides (**10a–s**) with different electronic properties were used (*cf.* R^1 below Scheme 1) to give the TMS-protected click compounds, **11a–s**. Subsequent C-desilylation of the crude click adducts with $AgNO_3/H_2O$ gave the desired THL analogue library, **3a–s**.[13]

With nineteen THL analogues (**3a–s**) in hand, we next studied their biological activities (Fig. 2). On the basis of established lipstatin biology,[3,4] three cellular assays were evaluated for cell proliferation, eIf2α phosphorylation and caspase-8 activation. For the proliferation assay, a human hepatocellular liver carcinoma cell line (HepG2) was selected. As shown in Fig. 2A, the 19 THL compounds, as well as the wildtype THL (**1**), THL-R (**2**) and library progenitor **9**, showed differential degrees of activity. The azides **10** alone did not show any cellular activity even up to 50 μM (see **10k** in Fig. 2A as a representative example). Four compounds from the library (Fig. 2B), derived from two sulfonamide-containing azides (**3k** and **3m**) and two aromatic azides (**3r** and **3s**), respectively, displayed the highest activity in blocking the proliferation of HepG2 cells (highlighted in blue), comparable to that seen with THL (**1**) and THL-R (**2**) (highlighted in red). In contrast, other library members (*i.e.* those derived from aliphatic, PEG, and carbonyl azides), including the intermediate **9**, showed a significantly reduced anti-proliferative activity. We next carried out comparative analysis with two of the four hits (**3k** and **3s**) by measuring their ability to induce phosphorylation of eIf2α in prostate cancer PC-3 cells (Fig. 2C); PC-3 cells treated with different amounts of each compound showed similarly elevated eIf2α phosphorylation. Lastly, we tested the compounds against the invasive human breast cancer MCF-7 cells (Fig. 2D); similar degrees of caspase-8 activation were observed (at either 5 or 25 μM). Collectively, all these lines of evidence indicate that both **3k** and **3s** are at least as potent as THL (**1**) and THL-R (**2**) as potential antitumour agents.

We next compared the *in situ* proteome reactivity profiles of the hits against THL-R (**2**; a close mimic of Orlistat) to directly reveal the potential range of cellular protein targets. Ideally, for example, a hit compound should predominantly (*i.e.* specifically) target FAS and no other protein. Live HepG2 cells were thus directly treated with each of the four THL analogues (**3k**, **3m**, **3r** and **3s**; 20 μM final concentration), either alone or in the presence of the THL competitor **1** (over a concentration range of 50–200 μM).[3] The cells were subsequently washed (to remove excessive probes), homogenised, incubated with rhodamine-azide under click-chemistry conditions,[4] separated by SDS-PAGE gel, and analysed by in-gel fluorescence scanning (Fig. 2E; left gel).

All hit compounds (except **3s**, in which the FAS band was significantly weakened) labeled FAS (the 265 kDa band) as efficiently as THL-R (**2**), indicating they likely possess similar potency as THL (**1**) in targeting FAS. The proteins involved were indicated to be Orlistat-specific since competition studies with THL (**1**) (center gel in Fig. 2E) or hydroxylamine displayed diminished band-labeling levels (see Supporting Information†). Similar proteome labeling profiles, albeit with significantly higher background levels, were also obtained when cell lysates were used (right gel in Fig. 2E). This underscores the importance of *in situ* labeling as a prerequisite for accurate and specific identification of true cellular targets.

In summary, a small natural product-based library of lipstatin analogues (*i.e.* **3a–s**) was synthesised and tested for cellular activity and protein specificity. The synthesis of the library showcases the chemical compatibility of click conditions to tether various aliphatic/aromatic azides with a relatively labile β-lactone lipstatin core (**9**). It is noted that, though click chemistry has previously been used in many other biological applications,[7] our current report is one of the few examples[7c] where it has been successfully implemented to facilitate the rapid lead identification of natural product-like compounds. The introduction of a triazole ring into the aliphatic side chain of the analogues **3** appeared to be tolerated in many cases by FAS (a validated THL enzyme target). Our current approach highlights the dual use of click chemistry to not only diversify a compound library in an expedient manner, but also allow the rapid activity-based protein profiling (ABPP) and identification of cellular targets of hit compounds. From this study, we have tentatively identified four candidates (**3k/m/r/s**) to possess similar anti-proliferative activity against HepG2 cells as compared to Orlistat (THL, **1**).

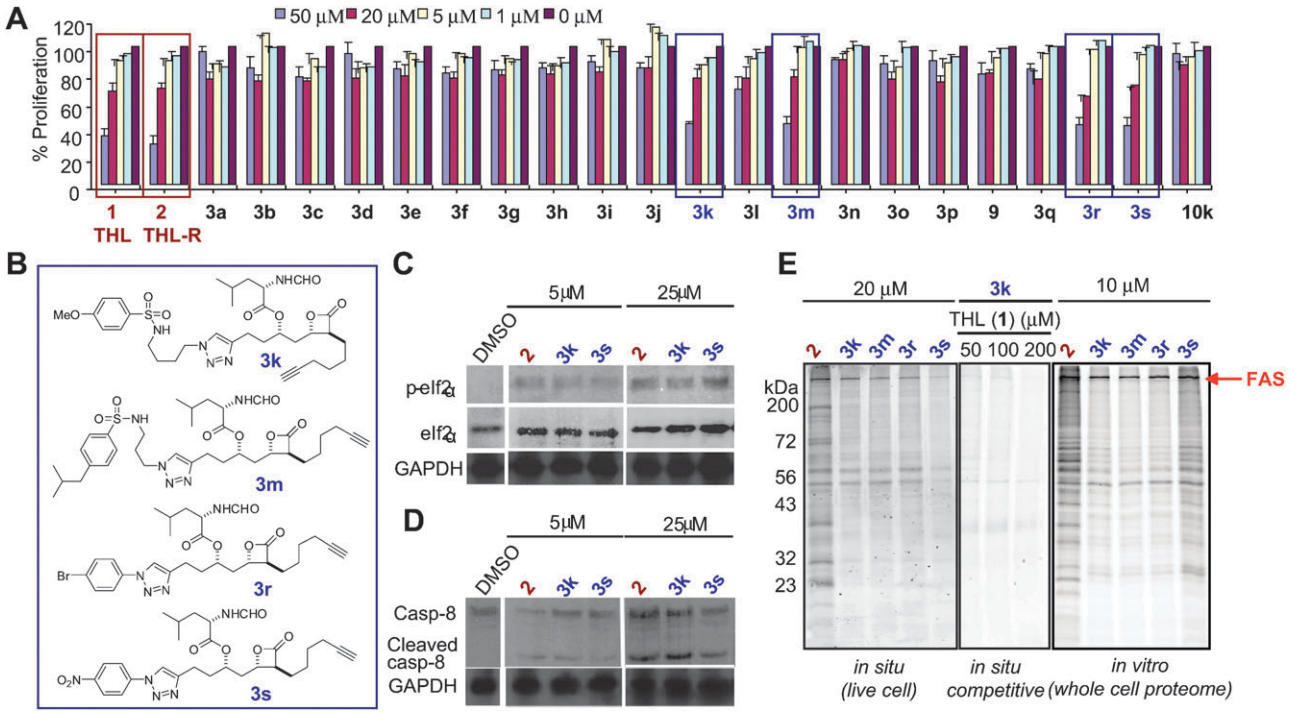

Fig. 2 (A) Dose-dependent inhibition of HepG2 cell proliferation by THL (**1**), THL-R (**2**), and triazole analogues **3a–s** using XTT assay.[4] Data represent the average s.d. for three trials. (B) Hits identified from screening. (C) Western blot analysis of eIf2α phosphorylation in PC-3 cells upon treatment with **2** and **3k/s**. GAPDH was used as a loading control. (D) Activation of caspase-8 in MCF-7 cells treated with the indicated concentrations of **2** and **3k/s**. (E) Proteome profiling of HepG2 cells using **1**, **2** and **3k/m/r/s**. Both *in situ* (live cell) and *in vitro* (whole-cell) lysate labelings were carried out. *In situ* proteome labeling of HepG2 cells, with or without Orlistat (over a concentration range of 50–200 μM), followed by click chemistry with rhodamine-azide, SDS-PAGE analysis, and in-gel fluorescence scanning (for *in situ* dose-dependent profile and in-gel hydroxylamine treatment profiles, see Fig. S2).

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