Targeting glycolysis: a fragment based approach towards bifunctional inhibitors of *h*LDH-5†‡

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***h*LDH-5 has emerged as a promising target for anti-glycolytic cancer chemotherapy. Here we report a first generation of bifunctional inhibitors, which show promising activity against *h*LDH-5.**

The lactate dehydrogenase (LDH) enzyme catalyses the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. It converts pyruvate, the final product of glycolysis, to lactate when oxygen is absent or in short supply, and it performs the reverse reaction during the Cori cycle in the liver.[1] At high concentrations of lactate, the enzyme exhibits feedback inhibition and the rate of conversion of pyruvate to lactate is decreased.

In humans, *h*LDH exists as five tetrameric isozymes, designated *h*LDH-1 through 5, each formed from a combination of two protein subunits M and H. *h*LDH-5 (M_4) predominates in the liver and skeletal muscle while *h*LDH-1 (H_4) prevails in the cardiac muscle, where it catalyses the reverse reaction using lactate as an aerobic source of energy.[2]

In tumour cells, an over-expression of oncogenes including *Ras*, *Src*, and *HER-2/Neu* through stabilisation of *HIF-1α* protein leads to an up-regulation of most proteins/enzymes involved in the glycolytic pathway, including *h*LDH-5 and the glucose transporters *GLUT-1* and *GLUT-3*.[3] This up-regulation is found in greater than 70% of human cancer cases, suggesting that tumour cells must acquire selective advantages by shifting from normal glucose metabolism (OXPHOS) to lactic acid production, even under normoxic conditions, the so called glycolytic switch[4] or Warburg effect.[5] Recent gene knock-out experiments demonstrated that reduction in the *h*LDH-5 activity of *neu*-initiated mammary tumor cells resulted in stimulation of mitochondrial respiration and a decrease of mitochondrial membrane potential.[3b] It also compromised the ability of these tumour cells to proliferate under hypoxia.

The diminished tumorigenicity of these *h*LDH-5 deficient cells could be reversed by complementation with the human ortholog *h*LDH-A (gene coding for the M unit) and *h*LDH-5 (enzyme), thus demonstrating that *h*LDH-5 plays a key role in tumour maintenance.[3b] Although the hierarchy of the possible genetic alterations, and/or adoption of optimum metabolic pathways, involved in the Warburg effect are still being uncovered,[6] *h*LDH-A and indeed the M_4 isozyme (*h*LDH-5) are primary targets for anti-glycolytic based therapeutic intervention.[7]

We report here a new class of bifunctional ligands which show selectivity towards *h*LDH-5. These novel compounds were prepared using a fragment based 'click' chemistry[8] approach, taking advantage of the high fidelity of the CuAAC fusion reaction.[9]

The structure of *h*LDH-5 cocrystallised with the active site inhibitor oxamate and co-factor NADH has been solved,[2] enabling the rational design of molecular inhibitors. Due to the close proximity between substrate and co-factor binding sites, we envisaged a fragment based approach could be used to unify, through a triazole linkage, the substrate and co-factor mimetics (Fig. 1).

In the design of our inhibitors, we required synthetically viable 'NADH-like' fragments, and were drawn towards bis(indolyl)maleimides (BIM's) as potential candidates. BIM's are known adenosine mimetics and have been shown to be potent inhibitors of NAD^+ dependent sirtuins.[10] Considering their ready synthetic availability,[11,12] opportunities for diversification and validated binding, BIM's were an excellent starting point for our ligand design. Oxamate on the other hand is a known inhibitor of LDH enzymes.[13] Since oxamic acid derivatives have also proven to inhibit *pf*LDH,[14] we hypothesised that *N*-alkylation to allow tethering would not adversely affect binding interactions. Furthermore, we envisaged replacing the oxamic acid functionality with a simple carboxylic acid

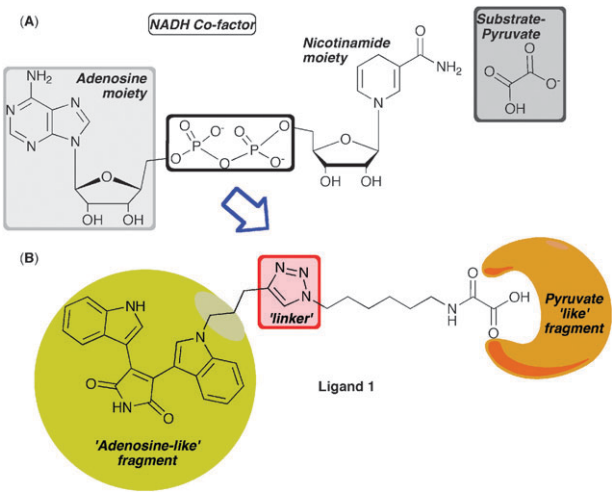

Fig. 1 (A) Structure of the NADH cofactor and substrate, (B) designed bifunctional inhibitor using BIM as adenosine mimetic and oxamate-substrate mimetic tethered through a triazole linker.

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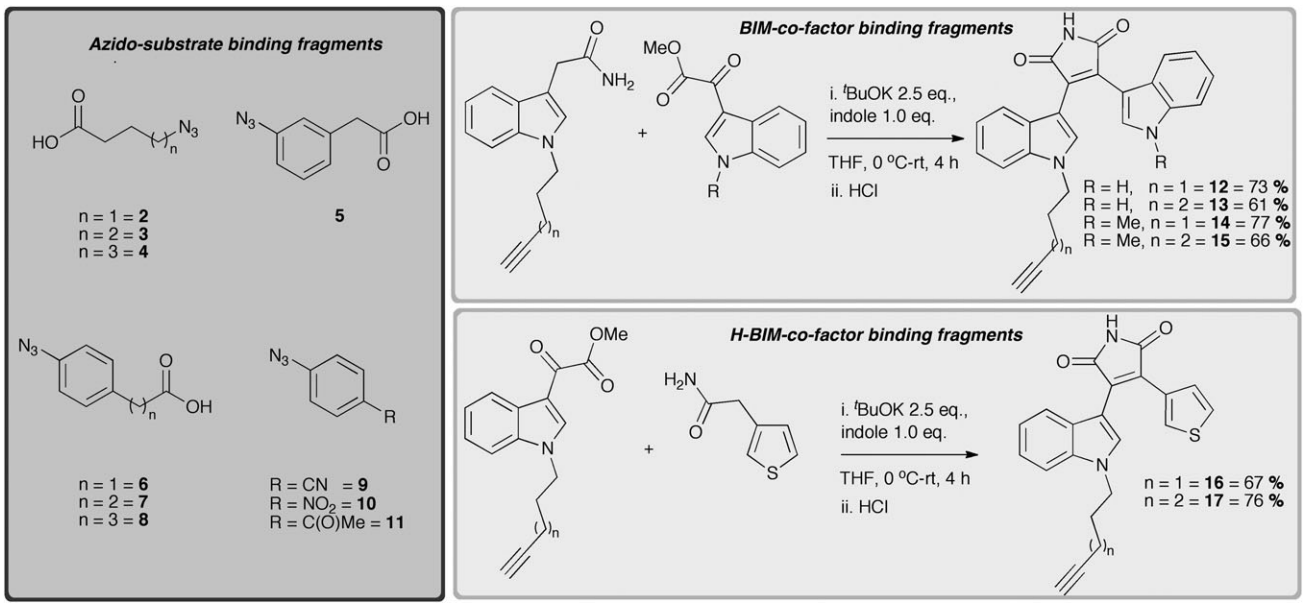

Scheme 1 Syntheses of 'NADH-like' and 'substrate-like' azido and alkyne functionalised fragments.

group. The tether would comprise alkyl chains of varying length, fused through a triazole linker. Modelling studies using the known crystal structure of the human lactate dehydrogenase ternary complex with NADH and oxamate were performed with hypothetical ligand **1**, which appeared to bind appropriately across both the substrate and co-factor binding site (see ESI†, Fig. S1).

Encouraged by these preliminary modelling studies, we began the syntheses of the 'NADH-like' and 'substrate-like' binding moieties, containing complementary azide and alkyne groups ready to be tethered using the CuAAC.[8] Readily available azide functionalised fragments, aliphatic carboxylic acids **2–4**,[15] the aromatic azido carboxylic acids derivatives **5–8**, and aromatic azido compounds **9–11** as potential substrate binding mimetics were prepared (Scheme 1).[16]

A selection of alkyne functionalised BIM's **12–15** were made by condensation of the corresponding indole-3-acetamides and indole-3-glyoxylates[17] in the presence of ^{t}BuOK,[12] as were the indoyl heteroaryl maleimide (H-BIM) substrates **16–17**, using a similar condensation approach (Scheme 1). Uniting the corresponding azido-substrate and alkynyl-co-factor mimetics using microwave enhanced CuAAC[16b] gave a 31-member library of 1,4-triazole fused ligands (**18–48**, see ESI†, Table S1) of varied linker length/structure, in good yields (33–83%).[18] Both the target ligands and fragments were screened for *h*LDH-5 activity at relatively high concentrations (50 and 100 μg mL^{-1}) using a high-throughput 96-well plate UV assay, where decrease in the concentration of NADH was measured at 340 nm.[19] The rate curves ($\Delta A/\Delta t$) at these concentrations were compared with those of a blank, and against the curve of the known inhibitor sodium oxamate (at 50 and 100 μg mL^{-1}) respectively. Compounds demonstrating inhibition more than/or similar to sodium oxamate were identified and their IC_{50} subsequently evaluated. Three compounds (~10%), **24**, **29** and **31**, demonstrated promising activity at the concentration tested and were taken forward for further study (Fig. 2). Interestingly, the oxamate-like fragments did not show any

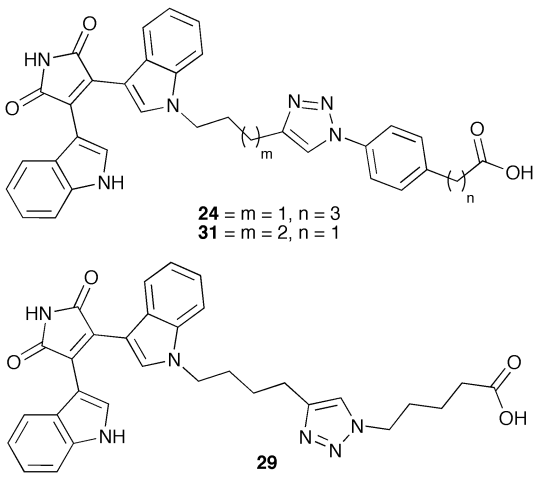

Fig. 2 Lead hit *h*LDH-5 bifunctional ligands **24**, **29** and **31**.

significant activity, whereas the BIM fragments were insoluble in the assay medium and could not be evaluated.

The activities against three enzymes were tested, human *h*LDH-5 (liver), rabbit *r*LDH-5 (muscle) and bovine *b*LDH-1 (heart). The IC_{50}'s of the three lead compounds **24**, **29** and **31** were measured using the four-parameter logistic equation (GraphPad Prism for Windows V5.02). The IC_{50} of sodium oxamate was also recorded for comparative purposes (ESI†, Table S2). Interestingly, and unlike sodium oxamate, none of the bifunctional compounds (**24**, **29** and **31**) demonstrated activity against the isozyme *b*LDH-1, which is comprised exclusively of the LDH-B gene product, the H-domain (H_4). This unprecedented observation was very promising and a step closer towards achieving selective inhibition of *h*LDH-5, the major LDH in carcinoma. Such precise targeting of the LDH-A gene product will be essential for future development as an anti-cancer treatment.

The IC_{50} values revealed that compounds **24** and **29** were particularly promising lead structures, demonstrating notable selectivity for the *h*LDH-5 isozyme over other isozymes tested.

Relatively low micromolar IC_{50}'s were exhibited for *h*LDH-5 (14.8 μM and 35.9 μM, respectively) (ESI†, Table S2), whereas neither compound displayed inhibition of the *b*LDH-1 even at high concentrations. The activity of **24** against *h*LDH-5 was 9-fold better than the standard sodium oxamate, making this candidate a promising target for further development. It is noteworthy that none of the bifunctional ligands bearing an *N*-methyl indole showed any kind of activity against LDH (ESI†, Table S1, **36**, **37**, **38**, **39**, **42**, **43**, **44**, **45**, **46**). This may imply a possible H-bonding interaction of the indole group of **24**, **29** and **31** in the enzyme active site. Unfortunately, bifunctional ligands bearing either a thiophene group (BIM fragment) or a non-carboxylic group (azido-fragment) were insoluble in the buffer medium (ESI†, Table S1, **25–27**, **34**, **35**, **40–42**, **47** and **48**), and no conclusions could be drawn from these entries.

To help us understand the nature of ligand–target interaction, docking experiments on the lead compounds were performed.

Of the three compounds, ligand **24** was the best fit for the protein binding site, and overlayed well with the cofactor from the crystal structure. A favourable interaction between the protein and **24** was also apparent (binding energy = −10 kcal mol^{-1}) with hydrogen bonds formed between the protein and carboxylic and BIM moiety (ESI†, Fig. S2). In line with the observed IC_{50}'s, ligands **29** and **31** were found to interact less favourably (*cf.* **29** = −8.4 kcal mol^{-1} and **31** = −7.5 kcal mol^{-1}).

Using a fragment based CC approach we have synthesised a small library of bifunctional compounds, specifically designed to target *h*LDH-5. We have identified three promising lead structures with impressive selectivity and activity. These ligands represent a new generation of bifunctional inhibitors specifically designed to target the *h*LDH-5 isozyme with a means to interfering with tumour metabolism. Molecular docking experiments corroborated our initial hypothesis and revealed favourable interactions with both the substrate and co-factor binding sites.

Detailed molecular modelling investigations, and further biological testing with a panel of cancer cell-lines are currently underway and will be reported in due course.

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