



Discovery of small molecules interacting at lactate dehydrogenases tetrameric interface using a biophysical screening cascade

Léopold Thabault ^{a, b, 2}, Chiara Brustenga ^{a, b, 2}, Perrine Savoyen ^{a, b}, Mégane Van Gysel ^c, Johan Wouters ^c, Pierre Sonveaux ^b, Raphaël Frédérick ^{a, *, 1}, Maxime Liberelle ^{a, b, 1}

^a Medicinal Chemistry Research Lab (CMFA), Louvain Drug Research Institute (LDRI), Université Catholique de Louvain (UCLouvain), B-1200, Brussels, Belgium

^b Pole of Pharmacology and Therapeutics, Institut de Recherche Expérimentale et Clinique (IREC), Université Catholique de Louvain (UCLouvain), B-1200, Brussels, Belgium

^c Laboratoire de Chimie Biologique Structurale, Unité de Chimie Physique Théorique et Structurale, Department of Chemistry, NAMur Research Institute for Life Sciences (NARILIS), NAMur Medicine & Drug Innovation Center (NAMEDIC), University of Namur, B-5000 Namur, Belgium

ARTICLE INFO

Article history:

Received 12 June 2021

Received in revised form

30 December 2021

Accepted 3 January 2022

Available online 12 January 2022

Keywords:

Biophysics

Cancer

Drug screening

Oligomerization

Protein-protein interaction

Nuclear magnetic resonance (NMR)

Lactate dehydrogenases

MicroScale thermophoresis (MST)

Nanoscale differential fluorimetry

(NanoDSF)

ABSTRACT

Lactate dehydrogenases (LDHs) are tetrameric enzymes of therapeutic relevance for cancer therapy due to their important implications in cancer cell metabolism. LDH active site inhibition suffers from different drawbacks due to several features such as high cellular concentration and a shared active site among the dehydrogenase family. Conversely, targeting the LDH oligomeric state is an exciting strategy that could provide a suitable alternative to active-site inhibition. In the present study, we developed a biophysical screening cascade to probe the LDHs tetrameric interface. Using nanoscale differential fluorimetry (nanoDSF) as a primary screening method, we identified a series of hits that destabilize the tetrameric protein. From this primary screening, we validated selected hits using saturation transfer difference nuclear magnetic resonance (STD NMR) and microscale thermophoresis (MST) as a combination of orthogonal biophysical techniques. Finally, we characterized the validated hits and demonstrated that they specifically interact at the tetrameric interface of LDH-1 and LDH-5 and can inhibit the LDH tetramerization process. Overall, this work provides a convenient method for screening ligands at the LDH tetrameric interface and has identified promising hits suitable for further optimization. We believe that this biophysical screening cascade, especially the use of (nano)DSF, could be extended to other homomeric proteins.

© 2022 Elsevier Masson SAS. All rights reserved.

1. Introduction

Lactate dehydrogenases (LDHs) are tetrameric enzymes at the core of lactate metabolism that, in tumors, allow glycolytic cancer cells to sustain their proliferative agenda and oxidative cancer cells to use lactate as a fuel. They catalyze the reversible conversion of pyruvate + NADH + H⁺ in lactate + NAD⁺, with LDH-5 preferentially catalyzing the forward reaction and LDH-1 the reverse reaction [1]. LDHs promote cancer cells proliferation and survival through several mechanisms, including intracellular pH buffering, maintenance of an optimal redox-balance [2], autophagy [3],

promotion of a metabolic symbiosis [4], angiogenesis [5,6] and invasiveness [7,8]. Functional LDHs are constituted of two main subunits, LDH-H and LDH-M, which can assemble in homo- and heterotetramers to constitute five different active tetrameric proteins, LDH-1 (LDH-H₄) to LDH-5 (LDH-M₄) [1]. LDHs constitute appealing targets in anticancer therapy, and intense efforts were devoted to developing LDH active-site inhibitors [9,10]. However, despite promising recent reports [11,12], these drug design campaigns struggled to translate in the development of drug-like compounds displaying lasting and potent LDH inhibition *in vivo*. One of the main reasons for these relative failures can be accounted for by the high intracellular level of LDHs, with concentration reported as high as 10–20 μM in some glycolytic cancer cell lines [13]. Such an elevated target concentration reduces the efficacy of stoichiometric, occupancy-based, active-site inhibitors that need to reach a high cellular concentration, often well above their K_d's, to

* Corresponding author

E-mail address: raphael.frederick@uclouvain.be (R. Frédérick).

¹ R.F. and M.L. are co-senior authors and share the last authorship position.

² L.T. and C.B. share the first position.

Abbreviation used			
NMR	Nuclear magnetic resonance	PAINS	Pan-assay interference
MST	MicroScale Thermophoresis	TRIC	Temperature-related intensity-change
NanoDSF	Nanoscale differential fluorimetry	ΔF norm	Normalized variation in fluorescence
LDHs	Lactate dehydrogenases	SAR	Structure-activity relationships
STD NMR	Saturation transfer difference nuclear magnetic resonance	IPTG	Isopropyl- β -D-1-thiogalactopyranoside
LDH-M	Lactate dehydrogenase muscle isozyme	TB	Terrific broth
LDH-1	Lactate dehydrogenase heart isozyme homotetramer	FID	Free induction decay
LDH-5	Lactate dehydrogenase muscle isozyme homotetramer	SEM	Standard error of the mean
DSF	Differential scanning fluorimetry	λ_{exc}	Wavelength of excitation
		λ_{em}	Wavelength of emission
		BSA	Bovine serum albumin
		EDTA	2,2',2'',2'''-(Ethane-1,2-diylidinitrilo)tetraacetic acid

inhibit LDH efficiently [12,13]. Another drawback lies in the ubiquity of the dinucleotide-binding domain (Rossmann fold) [14,15] found in more than 800 human enzymes (Interpro ID:IPR036291) [16], leading to a potential lack of selectivity and significant cytotoxic side-effects.

Previously, we and others [17–20] suggested an alternative approach to active-site inhibition that consists in targeting LDH self-association. This approach led, on our side, to the development and characterization of a truncated version of LDH-H (LDH-Htr) lacking its tetramerization arm, which we demonstrated behaves as a well-folded dimer incapable of forming tetramers at low concentrations. Using this dimeric protein, we identified two different tetramerization sites essential for LDH stability that we could target with peptidic probes, leading to *in vitro* LDH destabilization and disruption [18,19].

Targeting protein self-association is an emerging and exciting drug design strategy. Different mechanisms can account for inhibition through targeting of protein self-assembly such as (i) a ligand-induced oligomeric disruption where ligand binding to its target induces the direct disruption of the protein complex, (ii) subunit intercalation where ligand insertion between subunits do not directly induce dissociation but is associated with conformational change which translates into a loss of protein function, or for instance (iii) the capture of an inactive monomeric conformation where the inhibitors can indeed block a monomer into an inactive conformation through a specific interaction with this subunit [21].

The strategy of targeting protein self-association can moreover provide several advantages over active-site inhibition [21]. Among the different benefits this approach can offer, compounds interacting at a protein homomeric interface can promote protein destabilization and degradation [22]. Moreover, homomeric interfaces display higher divergence than orthosteric sites, allowing the design of specific compounds that can discriminate even closely related proteins [21]. However, this approach currently lacks methodical ways for identifying compounds that target homomeric interfaces.

Recently, we reviewed several techniques aimed to evaluate compounds interacting at proteins homomeric interfaces [21]. Among these different methods, differential scanning fluorimetry (DSF) appeared to be a promising way to identify ligands that influence their target self-association. Briefly, DSF consists of exposing a protein of interest to a temperature gradient and following its thermal denaturation in the presence or absence of a potential inhibitor [23]. Thermal stability is usually highly sensitive to a protein oligomeric state, with higher-order oligomers being more stable than lower-order ones [21,24]. As a result, compounds interacting at a protein oligomeric interface often destabilize their target, most likely by exposing their hydrophobic core [21,25].

Conversely, we reasoned that such compounds interacting with the same target blocked in a lower-order oligomeric state would instead stabilize it by interacting with this same hydrophobic interface and masking it to the surrounding, thus increasing the protein Gibbs free energy of unfolding (Fig. 1).

Our recent efforts to target the LDH oligomeric state led us to identify and characterize two families of peptides interacting with the LDH tetrameric interface [18,19]. LDH tetrameric interface is highly conserved between the two isozymes (Figure S1), and LDH-H and LDH-M can form heterotetramers in solution [1]. As a result, the peptides we previously developed to target LDH tetrameric interface could interact with both LDH isozymes [18,19]. These two sets of peptides targeted different self-assembly sites of LDH and, according to DSF, destabilized tetrameric LDH to yield dimers. Consistently with our hypothesis, these compounds either stabilized or had a neutral effect on the thermal stability of dimeric LDH-Htr. Therefore, we decided to exploit this complementary effect that such ligands would display between tetrameric LDHs and dimeric LDH-Htr to identify new binders of the LDH tetrameric interface. The present study describes a cascade of biophysical assays, relying on DSF as a primary screening method to identify compounds interacting at the LDH homomeric interface.

2. Results & discussion

2.1. Primary screening using nanoDSF

The first part of this biophysical screening relies on identifying compounds combining destabilizing properties on tetrameric LDH with a stabilizing or neutral effect on dimeric LDH-Htr. We set out to perform the primary screening assay using the nanoDSF technology, which monitors protein stability with low sample consumption. We evaluated a subset of 29 compounds preselected from a focused Prestwick library using nanoDSF against tetrameric LDH-1 (LDH-H₄) and LDH-5 (LDH-M₄) (Fig. 2). As previously mentioned, we consider both LDH-1 and LDH-5 to be interchangeable for the probing of LDH tetrameric interface due to the high conservation of the dimer-dimer interface (Figure S1). However, the use of both LDH in this context adds an internal control to the screening to ensure that we select compounds with homogeneous profiles between both isozymes.

In parallel, we evaluated the same compounds for their stabilizing or neutral effect on dimeric LDH-Htr. This step also served as an internal control by eliminating either promiscuous binders that would stabilize unfolded fractions of the protein and chaotropic compounds that would be intrinsic destabilizers [26]. Of note, we performed this second step in parallel with the first screening on tetrameric LDHs for exemplification purpose. However, to facilitate

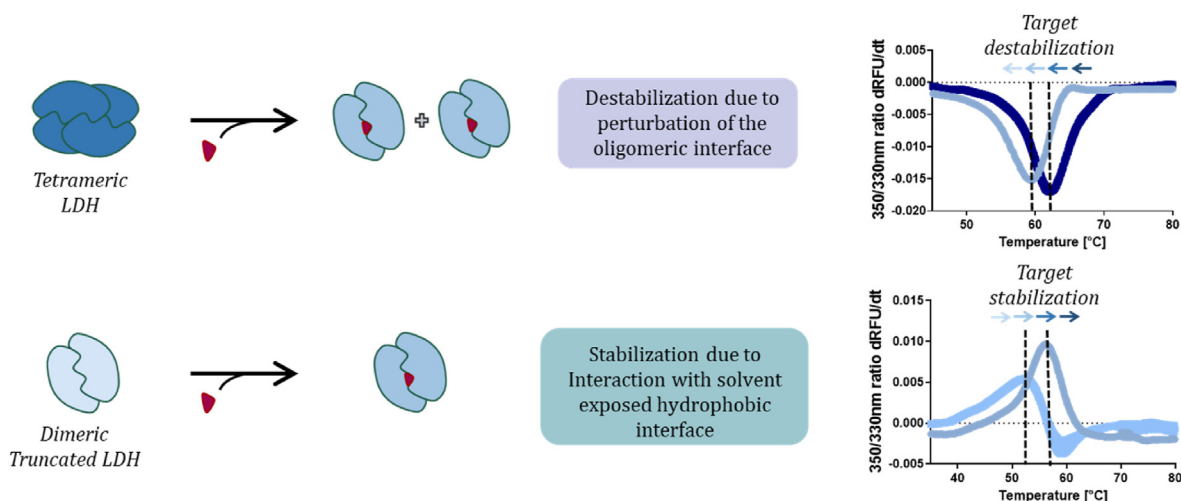


Fig. 1. Compounds interacting at the LDH tetrameric interface destabilize tetrameric LDH while keeping a neutral or stabilizing effect on already dimeric LDH-Htr. The DSF profile displayed in the figure corresponds to compound **F2050** that was identified in this study. RFU: relative fluorescence unit.

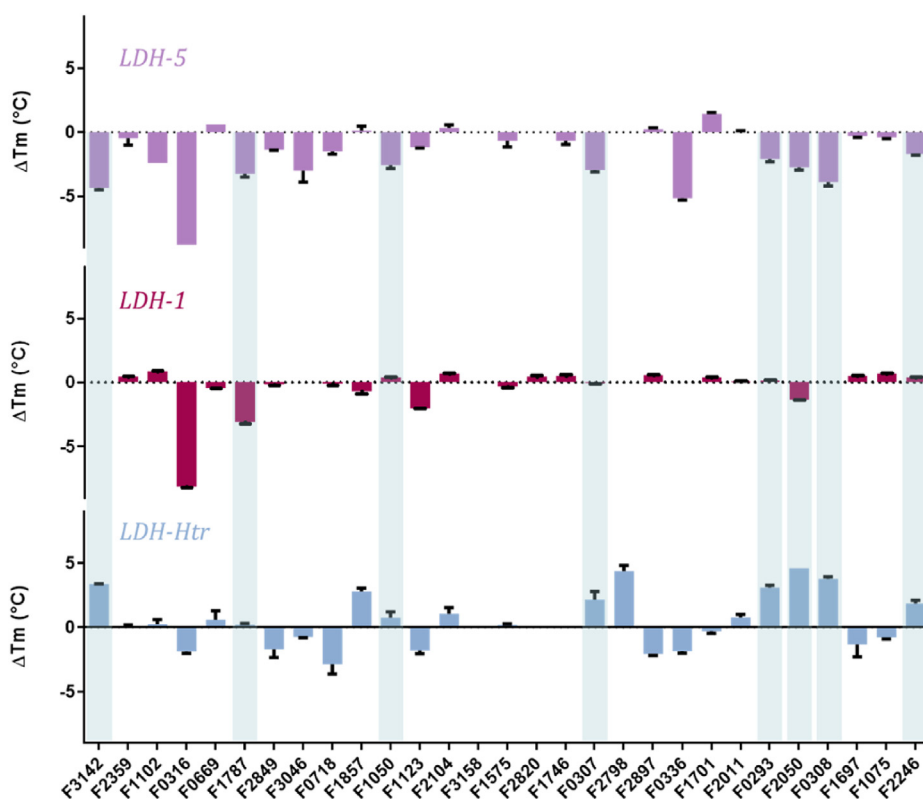


Fig. 2. Primary screening using nanoDSF identifies 8 hits that can destabilize tetrameric LDHs and stabilize dimeric LDH-Htr. Top, thermal shifts of the different compounds screened at 1 mM against tetrameric LDH-5 (25 μ g/ml) ($n = 3$). Middle, thermal shifts of the different compounds screened at 1 mM against tetrameric LDH-1 (25 μ g/ml) ($n = 3$). Bottom, the thermal shift of the different compounds screened at 1 mM against dimeric LDH-Htr (25 μ g/ml) ($n = 3$). The blue vertical lines correspond to the hits that displayed a complementary profile and were selected for further investigation.

the screening procedure, this screening on dimeric LDH-Htr could be run only with “hits” compounds that can destabilize tetrameric LDH.

Following this primary screening on tetrameric LDHs, we identified 14 and 5 compounds that destabilized LDH-5 and LDH-1, respectively (Fig. 2). As previously reported [19], the higher stability of LDH-1 compared to LDH-5 resulted in a lower sensibility to destabilization and, thus, to a lower hit rate. On the other hand, we identified 13 compounds displaying a stabilizing effect on dimeric

LDH-Htr (Fig. 2). By merging these results, we identified 8 compounds that combined a stabilizing or neutral effect on dimeric LDH-Htr with a destabilization of either tetrameric LDH-1, LDH-5 or both proteins. Among these 8 hits, we decided to discard 3 compounds from further evaluation due to their structure: compound **F0308** because of its estrogenic core, and compounds **F3142** and **F0307** due to their pan-assay interference (PAIS) profiles, noticeably because of the catechol moiety (Table S1) [27].

2.2. Hit validation

We next evaluated the ability of the five selected hits to interact with the LDH tetrameric interface using LDH-Htr. We monitored the interaction of each compound with LDH-Htr at 1 mM, using MST binding check experiments. The realization of this step was, in our case, optional due to the low number of hits. However, we set out to perform this first binding assessment to demonstrate our method scalability, as this step would become necessary for more extensive libraries. We evaluated the different hits at both 1.5 s and 20 s of MST on-time to account for ligands that would induce a change in the temperature-related intensity-change in fluorescence (TRIC) and the thermophoretic pattern of LDH-Htr. Interestingly, all five hits displayed an interaction at 1 mM with dimeric LDH-Htr (Fig. 3).

Hits were then validated using saturation transfer difference NMR (STD) as an orthogonal biophysical technique. STD relies on the selective saturation of a target protein in solution. Upon binding, saturation is transferred from the protein to the ligand [28]. In accordance with MST, STD demonstrated a saturation transfer between dimeric LDH-Htr and the five different hits, thus validating these compounds for their interaction with dimeric LDH-Htr (Figures S2-S6).

To further characterize the five compounds, we measured their affinities for dimeric and tetrameric LDH. According to our model, compounds interacting at the LDH tetrameric interface should exhibit a weaker interaction with tetrameric LDH than with dimeric LDH-Htr. We expected this weaker interaction to stem from a competition between the ligands and LDH native subunits for the LDH tetrameric interface. All 5 hits displayed a concentration-dependent interacting profile with dimeric LDH-Htr (Table 1). Strikingly, 3 hits exhibited a K_d in the μM range, with **F1050** interacting in the high micromolar range ($183 \pm 56 \mu\text{M}$) and **F1787** and **F2050** in the mid micromolar range ($79 \pm 37 \mu\text{M}$ and $37 \pm 12 \mu\text{M}$ respectively). Compounds **F0293** and **F2246** interacted in the low millimolar range (Table 1, K_d 's of $1.0 \pm 0.5 \text{ mM}$ and $0.9 \pm 1.2 \text{ mM}$, respectively). All MST binding curves are displayed in Figures S7-S11.

Next, we evaluated these compounds against tetrameric LDHs to look for potential competitions with the native subunits for the tetrameric interface that would result in weaker interactions. We assessed all 5 hits against tetrameric LDH-5 and LDH-1. All five hits that interacted with dimeric LDH-Htr demonstrated a concentration-dependent interaction with either tetrameric LDH-1, LDH-5 or both isozymes. Compounds **F1050**, **F0293** and **F2246**

showed a similar or better interacting profile with LDH-1 [LDH-5] than with LDH-Htr, with K_d 's of $48 \mu\text{M}$ [$324 \mu\text{M}$], $116 \mu\text{M}$ [$343 \mu\text{M}$] and 1.7 mM , respectively, suggesting that these compounds did not compete with LDH subunits for LDH tetrameric interface. Consistently, NMR STD revealed that these hits displayed a similar or higher saturation transfer against tetrameric LDH-1 than with dimeric LDH-Htr (Figures S2-4). Considering the high polarity and the structural homology with previously described ligands [14], we suspected that these compounds could interact with LDH active-site and discarded them from this study. On the other hand, compounds **F1787** and **F2050** showed a 5 to 10-fold decrease in their binding affinity for tetrameric LDHs (Table 1 and Fig. 4a and b), indicating a potential competition with LDH subunits for LDH tetrameric interface. In accordance with MST results, **F2050** showed a 70% decay in saturation transfer to tetrameric LDH-1 in NMR STD (Fig. 4c), while **F1787** demonstrated only a very weak saturation transfer (Fig. 4d). Overall, compounds **F1787** and **F2050** displayed a profile consistent with an interaction with the LDH tetrameric interface. These two compounds were the only hits that could destabilize both LDH-1 and LDH-5 while keeping a neutral or stabilizing effect on dimeric LDH-Htr. This result highlights the advantages of running the screening on both LDH-1 and LDH-5 to eliminate promiscuous destabilizers.

2.3. Hit characterization

We next characterized the ability of **F2050** and **F1787** to destabilize tetrameric LDHs. Interestingly, the two compounds displayed different destabilizing profiles, as assessed by nanoDSF (Fig. 4e and f). Along with its K_d in the mid micromolar range, **F2050** demonstrated a concentration-dependent ability to destabilize tetrameric LDH and to stabilize dimeric LDH-Htr (Fig. 4e). These two events occurred around the same concentration range for LDH-Htr and LDH-5, suggesting that the same process was underlying stabilization and destabilization. However, **F2050** could only destabilize tetrameric LDH-1 at higher concentrations (Figure S12a), consistent with this isozyme higher stability. Conversely, compound **F1787** only destabilized tetrameric LDH at high concentrations (1 mM and above, Fig. 4f and S12b-d), consistent with its weaker interaction with tetrameric LDH. Interestingly, **F1787** induced a destabilizing conformational change that we could follow with nanoDSF. This conformational change corresponds to a shift of LDH fluorescent ratio upon denaturation, from a blue-shift (reduction of the 350/330 nm ratio) to a red-shift (elevation of the 350/330 nm ratio) (Fig. 4f). Such pattern is

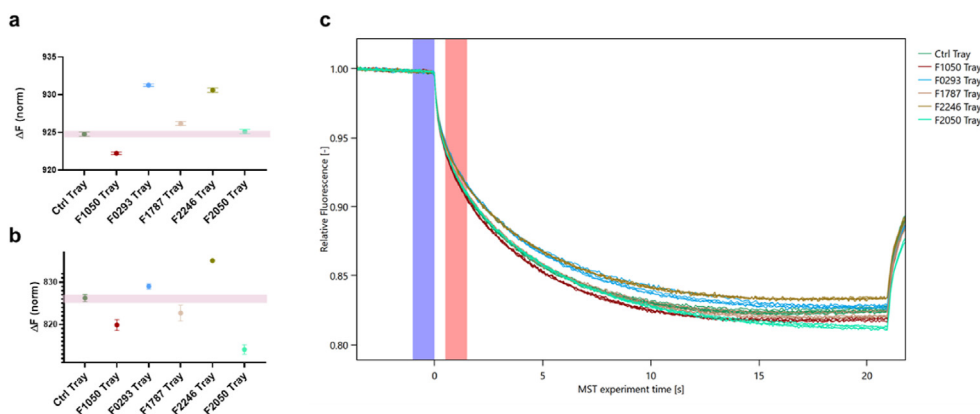
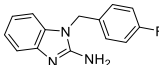
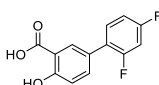
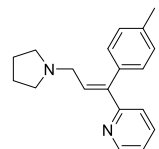
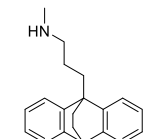
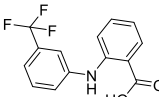


Fig. 3. Binding check experiments using MST reveal that all five hits bind to LDH-Htr. a) Normalized variation in fluorescence (ΔF norm) at 1.5 s of MST time for the five identified hits compared to the control experiment ($n = 4$). b) Normalized variation in fluorescence (ΔF norm) at 20 s of MST time for the five identified hits compared to the control experiment ($n = 4$). c) MST traces of the different hits. The red and blue columns correspond to the MST on and off time used to extract the data to a).

Table 1
Characterization of the different hits identified.

Code	Structure	K _d (LDH-Htr)*	K _d (LDH-5)*	K _d (LDH-1)*	ΔTM LDH-Htr (°C)	ΔTM Tetrameric LDH-5 and LDH-1 (°C)	Binding (MST)*	Binding (STD)**
F1050		183 μM (± 56 μM)	324 μM (± 100 μM)	48 μM (± 11 μM)	0.8 (± 0.9)	-2.6 (± 0.5) 0.4 (± 0.1)	Yes	Yes
F0293		1.0 mM (± 0.5mM)	343 μM (± 100 μM)	116 μM (± 35 μM)	3.1 (± 0.3)	-2.1 (± 0.3) 0.1 (± 0.1)	Yes	Yes
F1787		79 μM (± 37 μM)	760 μM (± 357 μM)	N.B	0.2 (± 0.1)	-3.3 (± 0.4) -3.1 (± 0.2)	Yes	Yes
F2050		37 μM (± 12 μM)	205 μM (± 40 μM)	150 μM (± 48 μM)	4.6 (± 0.0)	-2.7 (± 0.4) -1.4 (± 0.1)	Yes	Yes
F2246		0.9 mM (± 1.2 mM)	N.B	1.7 mM (± 3.2 mM)	1.9 (± 0.5)	-1.7 (± 0.3) 0.4 (± 0.1)	Yes	Yes

N.B: No binding up to 1 mM.

* MST profiles of the different hits are displayed in [Figures S7–S11](#).

** STD spectra are displayed in [Figures S2–S6](#).

coherent with a destabilization of LDH tetramer, as buried tryptophane residues tend to shift to longer wavelengths upon solvent exposure. Previously, we observed a similar destabilization of tetrameric LDH with a macrocyclic peptide (macrocyclic **7**) that competed with LDH N-terminal domain and disrupted tetrameric LDH, suggesting that compound **F1787** could interact in the same fashion [19].

We also studied the ability of the two identified hits to prevent LDH tetrameric association. To do so, we relied on a biochemical assay recently described by Nadal-Bufi et al. on LDH-5 [29]. Briefly, tetrameric LDH is denaturated to partially unfolded monomers in acidic conditions. Following denaturation, neutral pH is restored in the presence or absence of the studied compound, which allows for a slow reassociation of LDH in its native tetrameric structure. The protein then renaturates at room temperature, and its enzymatic activity, corresponding to the recovery of its proper quaternary structure, is followed afterwards. Interestingly, both compounds inhibited LDH activity at high concentrations under these conditions, with an IC₅₀ of 970 μM (CI_{95%}: [830–1277 μM]) for **F2050** and 7.6 mM (CI_{95%}: [4.4–12.0 mM]) for **F1787** (Fig. 4g and h). Conversely, neither **F2050** nor **F1787** could inhibit LDH activity without this pre-dissociative step (Figure S12e). These results are consistent with the destabilizing profiles of both compounds and their putative interaction at the LDH tetrameric interface. These interactions would not lead to any inhibition *a priori*, but would only weakened the strength of the tetrameric association, without significantly disturb the activity *in vitro*. Interestingly, **F1787** and **F2050** inhibited tetrameric LDH-5 at concentrations higher than

their respective K_d assessed by MST. Recent reports of compounds interacting at proteins interfaces demonstrated similar differences between K_d and IC₅₀ [29,30]. We suspect that such a difference could result from cooperativity between LDH subunits that would reduce the efficacy of compounds interacting at the tetrameric interface.

We then set out to perform epitope mapping on **F1787** and **F2050** using NMR STD to guide future hit optimization (Fig. 5). Of note, we apply the term epitope mapping here to describe the characterization of portions of the small molecule ligand, which are in contact with our target of interest (in this case LDH). Briefly, the signal intensity in NMR STD depends on the distance between a ligand proton and the protein. Protons close to the protein, and hence involved in the interaction, would display a high STD signal, while protons farther away would show little to no saturation transfer. This process allows one to identify the protons that make close contact with the protein and are thus important for the interaction. Consistent with the expected hydrophobic nature of the LDH tetrameric interface, epitope mapping analysis revealed a similar interaction pattern between **F1787** and **F2050**. According to NMR STD, the aromatic protons of both hits are the main contributors to the interaction with the LDH tetrameric interface. On the other hand, both **F1787** and **F2050** possess an aliphatic chain with a secondary amine that makes less interaction with LDH-Htr than the aromatic protons.

Finally, we attempted to further characterize the two hits' biophysical profiles to precisely identify their site(s) of interaction and lay the ground for future optimization. To that end, we performed

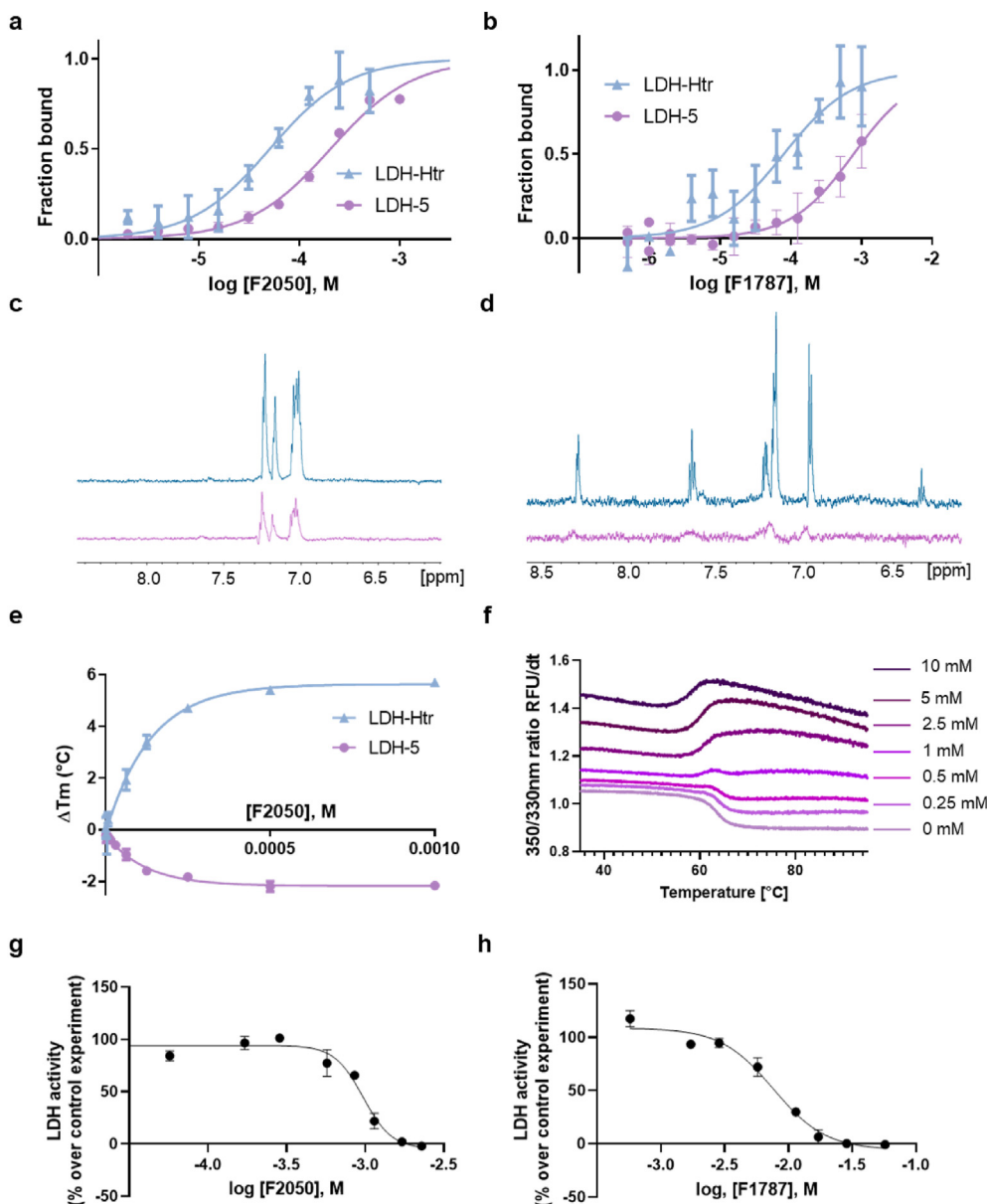


Fig. 4. Hits F1787 and F2050 compete for the LDH tetrameric interface and destabilize the tetrameric proteins. a) MST binding curves of **F2050** with LDH-Htr ($K_d = 37 \mu\text{M} \pm 12 \mu\text{M}$) and LDH-5 ($K_d = 205 \mu\text{M} \pm 40 \mu\text{M}$) ($n = 3$). b) MST binding curves of **F1787** with LDH-Htr ($K_d = 79 \mu\text{M} (\pm 12 \mu\text{M})$) and LDH-5 ($K_d = 760 \mu\text{M} (\pm 40 \mu\text{M})$) ($n = 3$). c) STD spectra of **F2050** (500 μM) with LDH-Htr (up, 15 μM) and LDH-5 (bottom, 15 μM) with 512 scans on and off-resonance. Both spectra have the same scale. d) STD spectra of **F1787** (500 μM) with LDH-Htr (up, 15 μM) and LDH-5 (bottom, 15 μM) with 512 scans on and off-resonance. Both spectra have the same scale. e) ΔT_m ($^{\circ}\text{C}$) of tetrameric LDH-5 (25 $\mu\text{g}/\text{ml}$) and dimeric LDH-Htr (25 $\mu\text{g}/\text{ml}$) as a function of **F2050** concentrations ($n = 3$). f) NanoDSF denaturation of tetrameric LDH-5 (25 $\mu\text{g}/\text{ml}$) with a gradient of **F1787** (from top to bottom: 10 mM, 5 mM, 2.5 mM, 1 mM, 500 μM , 250 μM and 0 μM) ($n = 3$). g) Inhibition of LDH-5 activity after renaturation upon exposure of increasing amount of **F2050** ($\text{IC}_{50} = 970 \mu\text{M}$ [$\text{CI}_{95\%}$: 830–1277 μM]). h) Inhibition of LDH-5 activity after renaturation upon exposure of increasing amount of **F1787** ($\text{IC}_{50} = 7.6 \text{ mM}$ [$\text{CI}_{95\%}$: 4.4–12.0 mM]).

competition experiments with the different peptidic probes that we had previously developed and that are selective for different binding sites at the tetrameric LDH interface [18,19]. A competition with one of the two probes would mean that the compound and the probe share the same interaction site, which would facilitate the understanding of their structure-activity relationships (SARs). Hence, we performed competition experiments using NMR STD with macrocycle **7**, previously identified as competing with LDH N-terminal tetramerization domain [19]; and peptide **LP-22**, previously identified as interacting at a second site, buried at the core of LDH tetrameric interface [18]. Competition experiments with **LP-22** did not result in a diminution of STD intensity signals (Figure S13), meaning that the two hits do not compete with this peptide.

However, when performing the competition experiments with macrocycle **7**, we noticed a substantial decay in the STD signal of **F1787** (~40%, Fig. 6a). This reduction in saturation transfer indicated a competition between the probe and the hit, suggesting that **F1787** shared the same site of interaction as macrocycle **7** and was hence a competitor of the LDH N-terminal domain. Conversely, **F2050** did not demonstrate any competition with either **7** or **F1787**, meaning that the two compounds do not share the same site of action. This result is in line with the different profile that **F2050** and **F1787** showed in both nanoDSF and MST, confirming that these two compounds, despite their structural similarity, do not share the same site of interaction. Based on these competition experiments, and on the epitope mapping, we provided a speculative binding

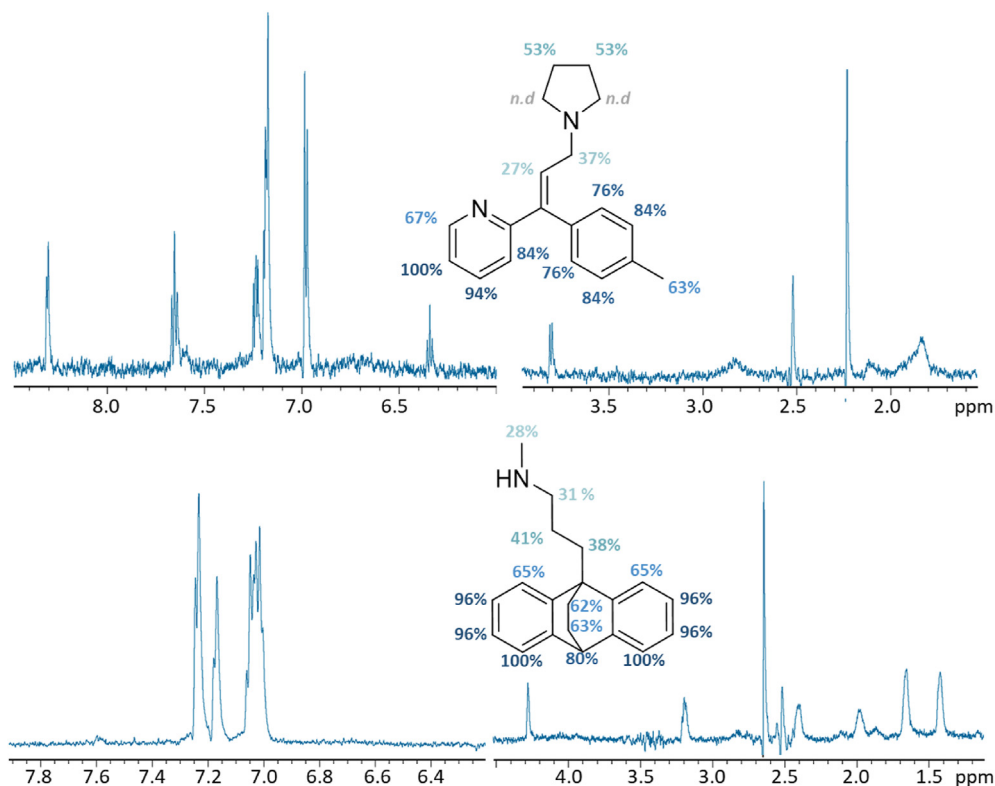


Fig. 5. Epitope mapping reveals that F1787 and F2050 share a similar pattern of interaction with LDH-Htr. Top, NMR STD spectrum of F1787 (500 μM) with LDH-Htr (15 μM). The aromatic region is displayed on the left, while the aliphatic region is on the right. Bottom, NMR STD spectrum of F2050 (500 μM) with LDH-Htr (15 μM). The aromatic region is displayed on the left, while the aliphatic region is on the right. The different percentages correspond to the protons' relative signal intensity, compared to their intensity in ^1H NMR and normalized regarding the most interacting proton. Complete proton assignments were based on 2D NMR (Figure S14 and S15) and previous reports [31,32].

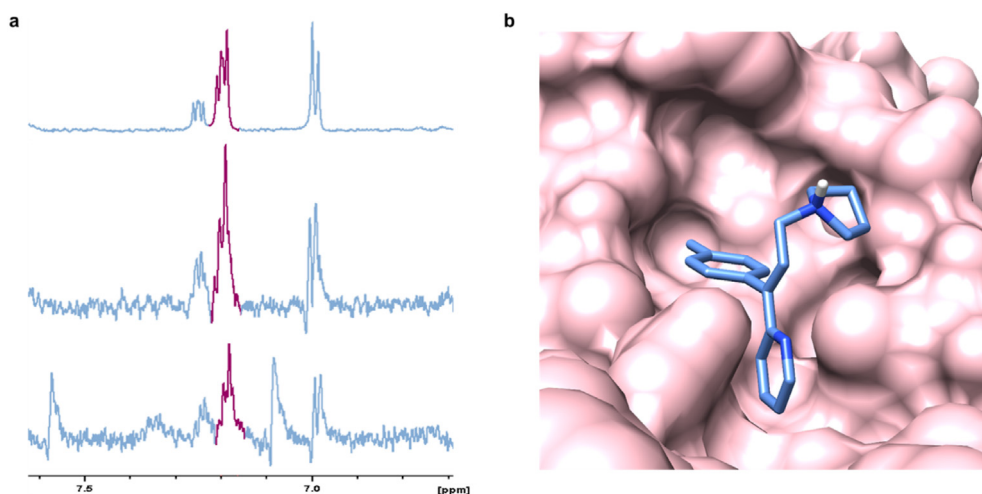


Fig. 6. F1787 competes with macrocycle 7. a) Top, ^1H NMR spectrum of F1787 (100 μM) with LDH-Htr (7.5 μM). Middle, ^1H NMR STD spectrum of F1787 (100 μM) interacting with LDH-Htr (7.5 μM). Bottom, ^1H NMR STD spectrum of F1787 (100 μM) and macrocycle 7 (1 mM) interacting with LDH-Htr (7.5 μM). The signals highlighted in purple correspond to aliphatic protons of F1787 that show a ~40% decay in intensity upon macrocycle 7 addition. b) Proposed binding mode of F1787 with LDH tetramerization site. Best binding pose of F1787 in LDH-H tetramerization site resulting from Autodock Vina docking on the available crystallographic structure (PDB entry: 110Z, chain B, D, pictures made using UCSF Chimera).

mode for compound F1787 at the LDH tetramerization site. Docking of F1787 in the LDH tetramerization pocket showed several docking poses congruent with epitope mapping experiments (Fig. 6b), with the aromatic residues buried within the lipophilic tetramerization site and the aliphatic amine exposed to the surrounding solvent.

Overall, hit characterization revealed that F1787 interacted at the tetramerization site occupied by the LDH N-terminal domain.

However, we could not identify the site of interaction of F2050, as this compound did not compete with any of the peptidic probes. Recent studies reported other allosteric sites at the LDH-1 and LDH-5 tetrameric interface, highlighting the high druggability of this interface [17,33]. We suggest that F2050 might interact in one of these recently disclosed sites or elsewhere at the large and hydrophobic LDH tetrameric interface.

3. Conclusion

Targeting protein self-association is an emerging strategy that can provide different advantages over active site targeting. LDHs constitute attracting targets in cancer therapy that could benefit from alternative targeting approaches, such as the disruption of their oligomeric state. In the present work, we exemplify the concept of probing the LDH oligomeric interface using nanoDSF as a primary screening method, followed by MST and NMR STD for hit validation and characterization. We demonstrated that we could quickly and efficiently identify binders of the LDH tetrameric interface from a small initial subset of compounds. The apparent high hit rate (~6%) can be explained because this screening method probes the entire LDH tetrameric interface, which encloses multiple allosteric binding sites [17–19,33]. The two hits identified in this work specifically target the LDH tetrameric interface and prevent its tetrameric association. They constitute promising starting points for further optimization. Interestingly, both hits were aromatic compounds sharing structural homology with tricyclic antidepressants and antihistaminics. This structural similarity probably highlights the propensity of the hydrophobic LDH tetrameric interface to accommodate large and lipophilic molecules. These scaffolds contrast with LDH small and polar active sites and highlight the potential of this approach to identify more druggable ligands. We anticipate this screening concept to be readily transposable to other obligatory homo-oligomers, pending that these targets can first be stabilized in a lower order oligomeric state.

4. Material and methods

4.1. General

All reagents were purchased from chemical suppliers and used without purification. The 29 compounds evaluated as potential hits were purchased from Prestwick and used without further purification. Structure conformity of **F1787** and **F2050** was confirmed using NMR. Hits **F1787** and **F2050** were repurchased from different suppliers and reevaluated with same results.

4.2. Virtual screening and library selection

A focused library was designed from a Prestwick Drug-Fragment library through docking using the Autodock Vina software [34]. Briefly, we relied on three complementary docking studies on the apoLDH dimer (PDB ID: 1i0z, subunits B-D). A primary docking study was performed on the dimer without area restriction to probe for the entire tetrameric interface. Two other docking studies were done by restricting the binding zone to the two tetramerization sites that we previously described [18,19]. All docking poses were processed together, and poses with Vinascores higher than –6.8, as well as active-site binders, were discarded, allowing a cutoff of two-third of the initial library. Visual selection was performed as the last step, using the docking focused on tetramerization sites as a comparison to eliminate non-reliable binders that did not display homogeneous binding poses between the different docking studies. Unique docking poses on alternative binding pockets at the interface were also considered. From this virtual screening, 29 virtual hits were selected and then processed towards biophysical evaluation.

4.3. Protein production

Production of LDH-1, LDH-5 and LDH-Htr was performed as described elsewhere [19]. Briefly, sequences of *hLDHA*, *hLDHB* and

hLDHBtr were inserted in a pET-28a expression vector using *NdeI* and *Bpu1102I* as restriction sites. Bacteria were grown in Terrific broth (TB) medium supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol at 37 °C until an optical density of 0.8 was reached. Protein expression was induced by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) at 20 °C for 20 h. Then, cells were collected by centrifugation at 5000 rpm, 4 °C for 25 min. Pellets were suspended in a lysis buffer (Tris-HCl 50 mM, pH 8.5, MgCl₂ 10 mM, NaCl 300 mM, imidazole 5 mM, and glycerol 10%) and then disrupted by sonication, followed by centrifugation at 4 °C, 10 000 rpm for 30 min. The insoluble fraction was discarded, and 1 µL of β-mercaptoethanol was added per mL of soluble fraction. The purification of recombinant proteins was performed using 1 mL of His-trap FF-crude columns (GE Healthcare) according to the manufacturer's instructions.

4.4. NanoDSF

Solutions of proteins (LDH-1, LDH-5 or LDH-Htr) were evaluated on a Tycho NT.6 device (NanoTemper Technologies), at 25 µg/ml with and without 1 mM of compounds. Evaluations were run in a 50 mM sodium phosphate and 100 mM NaCl pH 7.6 buffer. According to standard manufacturer's procedures, samples were poured into capillaries and heated up to 95 °C in 3 min while following fluorescence emission at 330 and 350 nm. Melting temperatures were extracted from the derivative of the 350/330 nm fluorescence ratios upon increasing temperature.

4.5. Nuclear magnetic resonance

Human LDH-H (full length and truncated)-6His proteins for 1D NMR were expressed and purified from *E. coli*, as described above. All experiments were performed on a Bruker Ascend Avance III 600 MHz system equipped with a broadband cryoprobe (Bruker).

For STD NMR studies, samples were prepared in PBS containing 2% deuterated DMSO. The concentration of LDHs was ranging from 7.5 to 15 µM of monomer, depending on the experiment. Ligand binding was detected using a STD stddiffesg.3 sequence with a 2 s saturation time. Water signal suppression was achieved using an excitation-sculpting scheme, and a 50 ms spinlock was used to suppress protein background signals. Experiments were performed at 277 K to avoid protein degradation.

For epitope mapping experiments, 512 scans were collected on (0 ppm) and off-resonance (40 ppm) to yield a 32 K point free induction decay (FID). STD spectra correspond to the difference of spectra between on and off-resonance experiments. Signal intensities in STD spectra were compared to reference ¹H spectra in the same conditions to account for signal broadening due to protein addition.

For 2D experiments, **F2050** was dissolved at 5 mM in a 50 mM phosphate buffer, pH 7.6, containing 100 mM NaCl, 90% D₂O and 10% DMSO-*d*₆. **F1787** was dissolved at 50 mM in a 50 mM phosphate buffer, pH 7.6, containing 100 mM NaCl and 100% D₂O. 2 K time-domain points and 256 increments were applied for all the 2D spectra. Experiments were performed at 298 K.

4.6. MST

MST measurements were performed on a Nanotemper Monolith NT.115 instrument (Nano-Temper Technologies) using Red-dye-NHS fluorescent labelling. LDH-5 and LDH-Htr purified to homogeneity were labelled with Monolith Red-dye-NHS second-generation labelling dye (Nanotemper Technologies), according to the supplied protocol. LDH-1, purified to homogeneity, was labelled using first generation labelling dye. Measurements were performed

in 50 mM sodium phosphate, pH 7.6, and 100 mM NaCl containing 0.01% Tween-20 in standard-treated capillaries (Nano- Temper Technologies). The ligands (NADH and hits) were titrated in 1:1 dilutions following manufacturer's recommendations. Experiments were performed in triplicates using 40% LED power, medium MST power, laser on-time 20 s, and laser off time 3 s. Hits were evaluated for their thermophoretic pattern, and K_d 's were extracted from raw data at a 1.5 s–20 s MST on time depending on the hit MST profile.

4.7. Denaturation assay

The experiments were performed with opaque 96-well plates using a Spectramax m2e spectrophotometer (Molecular Devices). The denaturation assay was adapted from a previous report [29]. Active LDH-5 was diluted to 0.9 μ M in 0.01 M phosphate buffer (pH 7.6), 1 mM EDTA, and 0.05% BSA (w/v). 500 μ L of protein solution was incubated with 1 ml of 0.1 M glycine buffer (pH 2.5) and 0.05% BSA (w/v) for 2 min. Afterwards, 30 μ L of the denaturated enzyme was added to 40 μ L of 0.25 M phosphate buffer (pH 7.6), 1 mM EDTA, and 0.05% BSA containing different concentrations of the studied compounds. LDH-5 was then left at room temperature for 2 h to allow tetrameric reassociation. After this incubation, 10 μ L of LDH-5 + inhibitor sample were transferred into a 96-well microplate containing 90 μ L of 0.25 M phosphate buffer (pH 7.6), 1 mM EDTA, and 0.05% BSA (w/v) per well. Finally, 20 μ L of substrate buffer containing 18 mM pyruvate and 1.8 mM NADH was then added to the mix to initiate the enzymatic reaction. NADH consumption was followed over time by measuring the fluorescence emission intensity ($\lambda_{\text{excitation}} = 340$ nm, $\lambda_{\text{emission}} = 460$ nm).

Notes

The authors declare the following competing financial interest(s): P.S., R.F. and L.T. are inventors of International Patent Application WO 2020/221899.A1, Lactate dehydrogenase inhibitor polypeptides for use in the treatment of cancer. P.S., R.F., L.T. and M.L. are inventors of European Patent Application EP21154636.1, Polypeptides inhibitors of lactate dehydrogenase activity for use in cancer therapy. Authors declare no other conflicts of interest.

Author contributions

M.L. and R.F. are co-senior authors. L.T., P.S., R.F. and M.L. designed the research. L.T., C.B., and P.S. performed the research experiments. L.T., C.B. and M.L. analyzed the data. L.T. set up the experimental methods, and L.T., P.S., R.F. and M.L. wrote the paper.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors declare the following competing financial interest(s): P.S., R.F. and L.T. are inventors of International Patent Application WO 2020/221899.A1, Lactate dehydrogenase inhibitor polypeptides for use in the treatment of cancer. P.S., R.F., L.T. and M.L. are inventors of European Patent Application EP21154636.1, Polypeptides inhibitors of lactate dehydrogenase activity for use in cancer therapy. Authors declare no other conflicts of interest.

Acknowledgements

This work was supported by ERC independent researcher Starting Grant 243188, TUMETABO, the Belgian Science Policy Office (Belspo; Interuniversity Attraction Pole Grant P7/03), the French Community of Belgium (ARC 14/19–058 and ARC 21/

26–115), the Belgian *Fonds National de la Recherche Scientifique* (F.R.S.-FNRS; Grants 3.4575.11, 2.5025.12, and 31288018), the Belgian *Fondation contre le Cancer* (Fundamental Research Grant FAF-F/2018/1282), and the Télévie (Grants 7.4507.14, 7.8504.18, and 7.4647.20). P.S. is a Research Director of the F.R.S.-FNRS. L.T. was a Ph.D. Fellow of the F.R.S.-FNRS then a Postdoctoral Researcher of the Belgian *Fondation contre le Cancer*. Funding sources had no role in study design, data collection and interpretation, or the decision to submit the work for publication. The authors thank the Nuclear and Electron Spin Technology (NEST) platform for providing access to the Bruker Ascend Avance III 600 MHz system.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ejmech.2022.114102>.

References

- [1] L. Fiume, M. Manerba, M. Vetrano, G. Di Stefano, Inhibition of lactate dehydrogenase activity as an approach to cancer therapy, *Future Med. Chem.* 6 (2014) 429–445.
- [2] A. Chiarugi, C. Dölle, R. Felici, M. Ziegler, The NAD metabolome - a key determinant of cancer cell biology, *Nat. Rev. Cancer* 12 (2012) 741–752.
- [3] L. Brisson, P. Bański, M. Sboarina, C. Dethier, P. Danhier, M.J. Fontenille, V.F. Van Hée, T. Vazeille, M. Tardy, J. Falces, C. Bouzin, P.E. Porporato, R. Frédérick, C. Michiels, T. Copetti, P. Sonveaux, Lactate dehydrogenase B controls lysosome activity and autophagy in cancer, *Cancer Cell* 30 (3) (2016) 418–431.
- [4] P. Sonveaux, F. Végran, T. Schroeder, M.C. Wergin, J. Verrax, Z.N. Rabbani, C.J. De Saedeleer, K.M. Kennedy, C. Diepart, B.F. Jordan, M.J. Kelley, B. Gallez, M.L. Wahl, O. Feron, M.W. Dewhirst, Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice, *J. Clin. Invest.* 118 (12) (2008) 3930–3942.
- [5] C.J. de Saedeleer, T. Copetti, P.E. Porporato, J. Verrax, O. Feron, P. Sonveaux, Lactate activates HIF-1 in oxidative but not in warburg-phenotype human tumor cells, *PLoS One* 7 (10) (2012), e46571.
- [6] F. Végran, R. Boidot, C. Michiels, P. Sonveaux, O. Feron, Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-KB/IL-8 pathway that drives tumor angiogenesis, *Cancer Res.* 71 (7) (2011) 2550–2560.
- [7] H. Izumi, M. Takahashi, H. Uramoto, Y. Nakayama, T. Oyama, K.Y. Wang, Y. Sasaguri, S. Nishizawa, K. Kohno, Monocarboxylate transporters 1 and 4 are involved in the invasion activity of human lung cancer cells, *Cancer Sci.* 102 (5) (2011) 1007–1013.
- [8] C.B. Colen, Y. Shen, F. Ghoddoussi, P. Yu, T.B. Francis, B.J. Koch, M.D. Monterey, M.P. Galloway, A.E. Sloan, S.P. Mathupala, Metabolic targeting of lactate efflux by malignant glioma inhibits invasiveness and induces necrosis: an in vivo study, *Neoplasia* 13 (7) (2011) 620–632.
- [9] R. Rani, V. Kumar, Recent update on human lactate dehydrogenase enzyme 5 (HLDH5) inhibitors: a promising approach for cancer chemotherapy, *J. Med. Chem.* 59 (2016) 487–496.
- [10] S.L. Zhang, Y. He, K.Y. Tam, Targeting cancer metabolism to develop human lactate dehydrogenase (HLDH)5 inhibitors, *Drug Discov. Today* 23 (7) (2018) 1407–1415.
- [11] G. Rai, D.J. Urban, B.T. Mott, X. Hu, S.M. Yang, G.A. Benavides, M.S. Johnson, G.L. Squadrito, K.R. Brimacombe, T.D. Lee, D.M. Cheff, H. Zhu, M.J. Henderson, K. Pohida, G.A. Sulikowski, D.M. Dranow, M. Kabir, P. Shah, E. Padilha, D. Tao, Y. Fang, P.P. Christov, K. Kim, S. Jana, P. Muttli, T. Anderson, N.K. Kunda, H.J. Hathaway, D.F. Kusewitt, N. Oshima, M. Cherukuri, D.R. Davies, J.P. Norenberg, L.A. Sklar, W.J. Moore, C.V. Dang, G.M. Stott, L. Neckers, A.J. Flint, V.M. Darley-Usmar, A. Simeonov, A.G. Waterson, A. Jadhav, M.D. Hall, D.J. Maloney, Pyrazole-based lactate dehydrogenase inhibitors with optimized cell activity and pharmacokinetic properties, *J. Med. Chem.* 63 (19) (2020) 10984–11011.
- [12] N. Oshima, R. Ishida, S. Kishimoto, K. Beebe, J. Brender, K. Yamamoto, D. Urban, G. Rai, M.S. Johnson, G. Benavides, G. Squadrito, D. Crooks, J. Jackson, A. Joshi, B.T. Mott, J. Shrimp, M.A. Moses, M.-J. Lee, A. Yuno, T. Lee, X. Hu, T. Anderson, D. Kusewitt, H.H. Hathaway, A. Jadhav, D. Picard, J. Trepel, J.B. Mitchell, G. Stott, W. Moore, A. Simeonov, L. Sklar, J. Norenberg, D.J. Maloney, C.V. Dang, A. Waterson, M. Hall, V. Darley-Usmar, M.C. Krishna, L. Neckers, Dynamic imaging of LDH inhibition in tumors reveals rapid in vivo metabolic rewiring and vulnerability to combination therapy, *Cell Rep.* 30 (6) (2020) 1798–1810.
- [13] J. Billiard, J.B. Dennison, J. Briand, R.S. Annan, D. Chai, M. Colón, C.S. Dodson, S.A. Gilbert, J. Greshock, J. Jing, H. Lu, J.E. McSurdy-Freed, L.A. Orband-Miller, G.B. Mills, C.J. Quinn, J.L. Schneek, G.F. Scott, A.N. Shaw, G.M. Waitt, R.F. Wooster, K.J. Duffy, Quinoline 3-sulfonamides inhibit lactate dehydrogenase A and reverse aerobic glycolysis in cancer cells, *Cancer Metabol.* 1 (1)

- (2013) 19.
- [14] R.A. Ward, C. Brassington, A.L. Breeze, A. Caputo, S. Critchlow, G. Davies, L. Goodwin, G. Hassall, R. Greenwood, G.A. Holdgate, M. Mrosek, R.A. Norman, S. Pearson, J. Tart, J.A. Tucker, M. Vogtherr, D. Whittaker, J. Wingfield, J. Winter, K. Hudson, Design and synthesis of novel lactate dehydrogenase A inhibitors by fragment-based lead generation, *J. Med. Chem.* 55 (7) (2012) 3285–3306.
 - [15] A. Kohlmann, S.G. Zech, F. Li, T. Zhou, R.M. Squillace, L. Commodore, M.T. Greenfield, X. Lu, D.P. Miller, W.S. Huang, J. Qi, R.M. Thomas, Y. Wang, S. Zhang, R. Dodd, S. Liu, R. Xu, Y. Xu, J.J. Miret, V. Rivera, T. Clackson, W.C. Shakespeare, X. Zhu, D.C. Dalgarno, Fragment growing and linking lead to novel nanomolar lactate dehydrogenase inhibitors, *J. Med. Chem.* 56 (3) (2013) 1023–1040.
 - [16] S. Hunter, R. Apweiler, T.K. Attwood, A. Bairoch, A. Bateman, D. Binns, P. Bork, U. Das, L. Daugherty, L. Duquenne, R.D. Finn, J. Gough, D. Haft, N. Hulo, D. Kahn, E. Kelly, A. Laugraud, I. Letunic, D. Lonsdale, R. Lopez, M. Madera, J. Maslen, C. Mcanulla, J. McDowall, J. Mistry, A. Mitchell, N. Mulder, D. Natale, C. Orengo, A.F. Quinn, J.D. Selengut, C.J.A. Sigrist, M. Thimma, P.D. Thomas, F. Valentin, D. Wilson, C.H. Wu, C. Yeats, InterPro: the integrative protein signature database, *Nucleic Acids Res.* 37 (2009) 211–215.
 - [17] A. Friberg, H. Rehwinkel, D. Nguyen, M. Quanz, I. Heisler, G. Langer, Structural evidence for isoform-selective allosteric inhibition of lactate dehydrogenase A, *ACS Omega* 5 (22) (2020) 13034–13041.
 - [18] L. Thabault, M. Liberelle, K. Koruza, E. Yildiz, N. Joudiou, J. Messens, L. Brisson, J. Wouters, P. Sonveaux, R. Frédérick, Discovery of a novel lactate dehydrogenase tetramerization domain using epitope mapping and peptides, *J. Biol. Chem.* 296 (2021), 100422.
 - [19] L. Thabault, L. Brisson, C. Brustenga, S.A.M. Gache, A. Kozlova, Q. Spillier, M. Liberelle, Z. Benyahia, J. Messens, T. Copetti, P. Sonveaux, R. Frédérick, Interrogating the lactate dehydrogenase tetramerization site using (stapled) peptides, *J. Med. Chem.* 63 (9) (2020) 4628–4643.
 - [20] F. Jafary, M.R. Ganjalikhany, A. Moradi, M. Hemati, S. Jafari, Novel peptide inhibitors for lactate dehydrogenase A (LDHA): a survey to inhibit LDHA activity via disruption of protein-protein interaction, *Sci. Rep.* 9 (2019) 4686.
 - [21] L. Thabault, M. Liberelle, R. Frédérick, Targeting protein self-association in drug design, *Drug Discov. Today* 26 (5) (2021) 1148–1163.
 - [22] A.Y. Gunderwala, A.A. Nimbavikar, N.J. Cope, Z. Li, Z. Wang, Development of allosteric BRAF peptide inhibitors targeting the dimer interface of BRAF, *ACS Chem. Biol.* 14 (7) (2019) 1471–1480.
 - [23] F.H. Niesen, H. Berglund, M. Vedadi, The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability, *Nat. Protoc.* 2 (9) (2007) 2212–2221.
 - [24] N.J. Marianayagam, M. Sunde, J.M. Matthews, The power of two: protein dimerization in biology, *Trends Biochem. Sci.* 29 (11) (2004) 618–625.
 - [25] W.G. Seetoh, C. Abell, Disrupting the constitutive, homodimeric protein-protein interface in CK2 β using a biophysical fragment-based approach, *J. Am. Chem. Soc.* 138 (43) (2016) 14303–14311.
 - [26] P. Cimmerman, L. Baranauskiene, S. Jachimovičiute, J. Jachno, J. Torresan, V. Michailoviene, J. Matuliene, J. Sereikaite, V. Bumelis, D. Matulis, A quantitative model of thermal stabilization and destabilization of proteins by ligands, *Biophys. J.* 95 (7) (2008) 3222–3231.
 - [27] J.L. Dahlin, J.W.M. Nissink, J.M. Strasser, S. Francis, L. Higgins, H. Zhou, Z. Zhang, M.A. Walters, PAINS in the assay: chemical mechanisms of assay interference and promiscuous enzymatic inhibition observed during a sulfhydryl-scavenging HTS, *J. Med. Chem.* 58 (5) (2015) 2091–2113.
 - [28] M. Mayer, B. Meyer, Characterization of ligand binding by saturation transfer difference NMR spectroscopy, *Angew. Chem. Int. Ed.* 38 (12) (1999) 1784–1788.
 - [29] F. Nadal-Bufi, J.M. Mason, L.Y. Chan, D.J. Craik, Q. Kaas, S. Troeira Henriques, Designed β - hairpins inhibit LDH5 oligomerization and enzymatic activity, *J. Med. Chem.* 64 (7) (2020) 3767–3779.
 - [30] K. Gao, S. Lunev, M.P.M. Berg, B. van den, Z.M. Al-Dahmani, S. Evans, D.A.L.J. Mertens, H. Meurs, R. Gossens, M.R. Groves, A synthetic peptide as an allosteric inhibitor of human arginase I and II, *Mol. Biol. Rep.* 48 (2021) 1959–1966.
 - [31] R.C. Reddy, G. Venkateswara Rao, B. Narayana Swamy, P. Hareesh Kumar, A simple and convenient synthesis of triprolidine, *Org. Prep. Proced. Int.* 41 (2) (2009) 168–171.
 - [32] T. Winkler, W. Dieterle, P. Hug, H.-P. Kriemler, The structure of diastereomeric oxaprotiline metabolites. An NMR study, *Helv. Chim. Acta* 67 (8) (1984) 2082–2086.
 - [33] Sachio Shibata, S. Sogabe, M. Miwa, T. Fujimoto, N. Takakura, A. Naotsuka, S. Kitamura, T. Kawamoto, T. Soga, Identification of the first highly selective inhibitor of human lactate dehydrogenase B, *Sci. Rep.* (2021) 21353.
 - [34] Oleg Trott, J. Olson Arthur, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multi-threading, *J. Comput. Chem.* 31 (2) (2010) 455–461.