RESEARCH ARTICLE

[View Article Online](#)
[View Journal](#) | [View Issue](#)

Cite this: *Med. Chem. Commun.*,
2018, 9, 1369

Small molecule cores demonstrate non-competitive inhibition of lactate dehydrogenase†

Brooke A. Andrews and R. Brian Dyer *

Lactate dehydrogenase (LDH) has recently garnered attention as an attractive target for cancer therapies, owing to the enzyme's critical role in cellular metabolism. Current inhibition strategies, employing substrate or cofactor analogues, are insufficiently specific for use as pharmaceutical agents. The possibility of allosteric inhibition of LDH was postulated on the basis of theoretical docking studies of a small molecule inhibitor to LDH. The present study examined structural analogues of this proposed inhibitor to gauge its potency and attempt to elucidate the molecular mechanism of action. These analogues display encouraging *in vitro* inhibition of porcine heart LDH, including micromolar K_i values and a maximum inhibition of up to 50% in the steady state. Furthermore, Michaelis-Menten kinetics and fluorescence data both suggest the simple, acetaminophen derivatives are non-competitive in binding to the enzyme. Kinetic comparisons of a panel of increasingly decorated structural analogues imply that the binding is specific, and the small molecule core provides a privileged scaffold for further pharmaceutical development of a novel, allosteric drug.

Received 21st June 2018,
Accepted 11th July 2018

DOI: 10.1039/c8md00309b

rsc.li/medchemcomm

1. Introduction

Healthy cells rely primarily on mitochondrial oxidative phosphorylation to convert glucose to pyruvate and generate the cellular energy currency ATP; however, cancerous cells largely derive ATP from the anaerobic conversion of pyruvate to lactate.¹ The interconversion of pyruvate and lactate is regulated physiologically by the enzyme lactate dehydrogenase (LDH). In recognition of this role, LDH has become an attractive target for cancer therapies.^{2–4}

Lactate dehydrogenase is a 136 kDa, tetrameric, NADH-dependent enzyme capable of reversibly reducing pyruvate *via* proton and hydride transfer. Five isoforms of the enzyme exist as combinations of muscle, heart, or sperm subunits, which constitute the populations of enzyme found in all parts of the body and tailor the enzyme's catalytic properties. LDH found in the heart (also referred to as LDH-B), for example, is a homotetramer of four heart subunits assembled as a dimer of dimers, while enzyme found in the lungs is a heterotetramer of two heart and two muscle subunits. While slightly different in activity, the muscle homotetramer is structurally very similar to that of the heart, and is referred to as LDH-A.^{5–11}

As a target for competitive inhibition, LDH presents a challenge in that the active site is quite small, restricting the

available space within which an inhibitor may bind. The substrate binding site is also conserved across all isoforms, resulting in poor selectivity of the designed inhibitors.¹² NADH-competitive strategies suffer from the same pitfall of poor selectivity, in addition to interference with unrelated nicotinamide-employing cellular processes.^{13,14} An alternative approach would be to design an allosteric inhibitor, where the drug binds at an isoform-specific, non-competitive site remote from the location of catalysis.

Predicting allosteric binding sites on enzymes is a formidable challenge, in part because allosteric mechanisms are poorly understood.^{15,16} Some enzymes are naturally allosterically regulated, such as bacterial LDHs, which bind fructose-1,6-bisphosphate (FBP) to promote tetramerization from weakly active dimers. While this small-molecule binding site is conserved in eukaryotic LDHs, there is a negligible effect of FBP binding on activity.¹⁷ Both substrates, pyruvate and lactate have effects on the oligomeric state of the enzyme and, in sufficient concentration, can cause inhibition. Malate also stabilizes tetramer formation.^{8,18,19}

In some cases, the binding of an allosteric effector induces a conformational change or maintains a specific conformation of the enzyme,^{17,20–22} reiterating the importance of dynamic processes in biocatalysis. Obstructing a necessary catalytic motion, then, in principle becomes one approach to the design of an allosteric inhibitor.

Dynamic processes generally accepted as relevant to enzymatic turnover include loop motions, which occur on the microsecond to millisecond timescale and often permit binding of a substrate or release of a product.^{7,23–26} However,

Department of Chemistry, Emory University, Atlanta, 30322, Georgia, USA.

E-mail: briandyer@emory.edu

† Electronic supplementary information (ESI) available: Kinetic and fluorescence controls, DLS data, chromatography, analytical ultracentrifugation. See DOI: 10.1039/c8md00309b

dynamics explicitly relevant to the chemical catalysis performed by the enzyme must proceed much faster since crossing the transition state usually occurs on the femtosecond timescale. LDH belongs to a class of enzymes believed to contain such motions, referred to as rate promoting vibrations (RPVs).^{26–28} An axis of residues within each monomer of human heart LDH (hhLDH) has been identified computationally to participate in a low-frequency (80 cm^{-1}), collective compressional motion. This compression facilitates the necessary tunnelling processes of hydride and proton transfer.^{28,29}

The Schwartz group proposed that allosteric inhibition of LDH might be possible by binding of a small molecule that disrupts the RPV. They identified a binding site for a small molecule between two monomers of the human heart LDH dimer, in proximity to the RPV axis.²⁹ After optimizing the structure *via* docking studies, evaluation of the reaction coordinate revealed a perturbed transition state, with an increase in reaction lag time, suggesting the designed allosteric inhibitor had a profound dynamic effect on the enzyme. While the site-specific docking methodology made no consideration for competitive binding sites, covalent reactivity of the small molecule inhibitor, or water solubility, a large panel of molecules were docked, and those amenable to *in vitro* enzymatic assays were selected for the present study.

While this computational examination was performed on the human enzyme, we have crystallized tetrameric porcine heart LDH as a ternary complex with NADH and oxamate (PDB ID: 6CEP) to illustrate the high structural homology responsible for the similar catalytic behavior of the human and pig enzymes. The position of bound NADH and oxamate ligands varies $<0.01\text{ Å}$ between the two species and the pig muscle isoform (LDH-A, PDB ID: 9LDT) at 2.0 Å resolution. The core active site structure, shown in Fig. 1 for the two heart isoforms, is conserved across all three enzymes.

Experimental observations of small molecules similar to the structure tested computationally (including shared core geometry, hydrogen bonding potential, and comparable

docking scores) demonstrate specific, 40–50% inhibition of the highly homologous porcine heart LDH in the steady state and generally non-competitive behaviour. While the preliminary results were consistent with the proposed dynamics-driven allosteric mechanism of action, no change in the rate of chemistry was observed in the single turnover studies. Kinetic differences were observed for one of the inhibitors when tested on dimeric and tetrameric populations of the enzyme, suggesting a possible mechanism of action of dimer dissociation or a binding site at the dimer:dimer interface.

2. Experimental

Materials

Unless otherwise specified, all materials were obtained from Sigma Aldrich and were of the highest purity available. All solutions were prepared fresh daily from solids. Tryptophan was obtained from Acros, 3,5-dihydroxybenzamide was obtained from AstaTech.

phLDH stock solutions

Porcine heart LDH (phLDH) was received as an ammonium sulfate suspension, which was pelleted *via* centrifugation, re-suspended in the appropriate buffer, and buffer exchanged into 100 mM sodium phosphate buffer, pH 7.2 *via* centrifugation in Amicon 0.5 mL centrifuge insert filters (50 kDa cut-off). The concentration of phLDH was quantified spectrophotometrically, using $\epsilon_{280} = 50\,000\text{ N}^{-1}\text{ cm}^{-1}$,⁷ where normality replaces the more standard use of molarity due to the existence of multiple identical active sites per macromolecular unit. For reference, a $1.4\text{ }\mu\text{M}$ phLDH solution is equivalent to approximately $50\text{ }\mu\text{g mL}^{-1}$ enzyme.

phLDH inhibition kinetics

All kinetics data were collected using an Applied Photophysics stopped-flow spectrometer with a path length in the sample cell of 1 cm. The enzyme was assayed as previously reported; briefly, NADH absorbance at 340 nm ($\epsilon_{340} = 6220\text{ M}^{-1}\text{ cm}^{-1}$) was monitored as the cofactor was oxidized to NAD^+ due to the reduction of pyruvate to lactate.⁷ The reaction was initiated by the simultaneous injection of the enzyme:cofactor:inhibitor complex with the pyruvate substrate. All samples were incubated with inhibitor for 2 hours at 4 °C prior to data collection. Final assay conditions were as follows: 5 nM phLDH, $100\text{ }\mu\text{M}$ NADH, $300\text{ }\mu\text{M}$ sodium pyruvate, 100 mM sodium phosphate buffer, pH 7.2, 37 °C . For $3\text{ }\mu\text{M}$ samples, the NADH concentration was maintained at $100\text{ }\mu\text{M}$ and pyruvate concentration was increased to a maximum of 5 mM .

NADH is prone to oxidation and prior to preparation of samples, the A_{260}/A_{340} ratio was determined to be <2.40 . Data points included on all plots are averaged from linear fits to the initial velocities of five consecutive shots with error bars representing the standard deviation. Single turnover results are the average of at least six consecutive shots, with final assay concentrations of $25\text{ }\mu\text{M}/100\text{ }\mu\text{M}$ phLDH, $5\text{ }\mu\text{M}$ NADH, 3

Fig. 1 Conserved active site architecture of porcine and human LDH. Pig heart LDH, bound oxamate and NADH cofactor shown in greens (PDB ID: 6CEP), human heart LDH and ligands (PDB ID: 1IOZ)⁵ shown in blues, hydrogen bonding interactions shown in gray, hydride transfer in red. Image created with PyMol (Shroedinger, Version 1.6.8.0).

mM 3-AP, 5 mM sodium pyruvate, 100 mM sodium phosphate buffer, 20 °C, pH 7.2.³⁰ Experiments were repeated using independent preparations to ensure reproducibility.

All data were fit analytically using Igor Pro, Version 6.37 (Wavemetrics). EC₅₀ values were obtained by fitting the data to eqn (1):

$$f(x) = \min + \frac{(\max - \min)}{\left(1 + \frac{x}{EC_{50}}\right)^{-\text{Hill Coefficient}}} \quad (1)$$

where x is the concentration of inhibitor and $f(x)$ is relative enzyme activity. 'Min' and 'max' refer to the minimum and maximum observed relative enzyme activity. This equation was used as a custom fit function where min, max, EC₅₀, and the Hill coefficient were iteratively refined variable parameters.

Experimental K_i values were determined by titrating pyruvate as a substrate against enzyme pre-incubated with fixed concentrations of the inhibitors. The generated substrate concentration *versus* velocity curves were globally fit to a model of noncompetitive inhibition to estimate the respective inhibition constants. Eqn (3) is substituted into eqn (2):

$$y = \frac{v_{\max \text{ inh}} \cdot x}{(K_m + x)} \quad (2)$$

$$v_{\max \text{ inh}} = \frac{v_{\max}}{\left(1 + \frac{[I]}{K_i}\right)} \quad (3)$$

where y is the velocity of the reaction, $V_{\max}$ is the linked maximum velocity, $[S]$ is the substrate concentration, $[I]$ is the inhibitor concentration, K_m is the Michaelis-Menten constant, and K_i is the inhibition constant.³¹ Visual inspection of the type of inhibition was aided by the creation of double reciprocal Lineweaver-Burk plots.

Dynamic light scattering

Data were collected with a Micromeritics NanoPlus HD. Filtered samples contained 80 μN pHLDH: 4 μM NADH: 2.14 mM inhibitor in 100 mM sodium phosphate buffer, pH 7.2, and experiments were performed at room temperature. Auto-correlation functions and particle size distributions were collected every two minutes for a total of ten minutes.

Fluorescence spectra

Measurements collected with a Horiba Dual FL fluorometer outfitted with a Peltier temperature controller. Measurements were collected at 20 °C, with sample concentrations of 3 μN pHLDH, 6 μM NADH, 5 μM sodium oxamate in 100 mM sodium phosphate buffer, pH 7.2. Samples were excited at 340 nm in a sub-micro (1 cm path length) quartz cuvette, and

emission spectra were collected with a 5 sec integration time. A 2 hour incubation was performed prior to collection consistent with kinetic trials, all samples were also filtered (0.22 μm).

Size exclusion chromatography

Both 5 nN and 3 μN pHLDH samples in 100 mM sodium phosphate, pH 7.2 were analyzed using a size-exclusion Superdex 200 column (GE Healthcare). The wavelength used for detection was 230 nm.

Analytical ultracentrifugation

Velocity sedimentation measurements of both 800 nN and 4 μN pHLDH samples in 100 mM sodium phosphate, pH 7.2 were conducted at 50 000 rpm and 20 °C by measuring protein absorbance at 280 nm in a Beckman XL-I analytical ultracentrifuge. Sedimentation coefficient ($s_{20,w}$) distributions were determined using SEDFIT.³²

3. Results and discussion

Four of the molecules from the Schwartz docking exploration were tested as possible inhibitors against pig heart LDH, including acetanilide, 3-acetamidophenol (3-AP), 3,5-dihydroxybenzamide (DHB), and L-tryptophan. Given the high structural homology of the human and pig enzymes,⁵ the relative docking scores were assumed to be comparable. The EC₅₀ curves for these molecules shown in Fig. 2 were obtained under steady-state kinetic conditions. Docking scores for each molecule are included with the corresponding structure. Three of the four molecules represent a panel of increasing complexity, and likely specificity, from acetanilide to DHB. The aromatic amino acid tryptophan was included to rule out non-specific inhibition, such as cation-π interactions from surface lysines.

DHB demonstrates the strongest inhibition, reducing enzymatic turnover by roughly 50% with a modest EC₅₀ of 712 μM, followed by 40% inhibition by the less decorated 3-AP. The response demonstrates clear selectivity for the structure predicted to be the best fit for the defined docking site, DHB. A control has been included to eliminate the possibility of hydride transfer to the inhibitor DHB (Fig. S1†).

Interestingly, L-tryptophan demonstrates no appreciable inhibition, but rather up to a 24% increase in pHLDH activity by a 1 mM dose. Allosteric, single amino acid activation (and inhibition) has been seen in other enzymes,^{33–35} notably pyruvate kinase,³⁶ and could potentially be operative here. While the binding site(s) remains to be determined for all of the molecules studied, the tryptophan and acetanilide data do exclude a general aromatic inhibitory mechanism, suggesting there may be a specific interaction that hinders the enzyme's operation; however, the dose-dependent response does not reveal any additional insight into the mechanism of action.

Dynamic light scattering (DLS) analysis was employed to ensure the decreased activity was not due to a change in oligomeric state of the enzyme. No appreciable change in the

Fig. 2 EC_{50} curves for molecules included in JPCL 2016 docking study,²⁷ along with their structures and docking scores. Relative activity is measured against uninhibited enzyme activity.

average hydrodynamic radius was observed in the presence of 3-AP, DHB, or acetanilide over the course of 10 minutes (see ESI† Fig. S2), suggesting a specific binding interaction is occurring.

Michaelis–Menten kinetic experiments³⁷ were employed to discern the type of inhibition using substrate and cofactor competition assays, shown in Fig. 3. Fitting Michaelis parameters to substrate competition experiments, the outcome of

Fig. 3 Substrate and cofactor competition assays. (A) and (C), Michaelis–Menten fits and (B) and (D) double reciprocal Lineweaver–Burk plots for substrate competition of 3-AP and DHB, respectively in the presence of 5 nM pHLDH. (E) Cofactor competition of 3-AP vs. NADH in the presence of 5 nM pHLDH. (F) and (G) Michaelis–Menten fits and Lineweaver–Burk plot for 3-AP in the presence of 3 μM pHLDH.

Table 1 Michaelis parameters for substrate and cofactor competition assays

	Substrate competition				Cofactor competition	
	3-AP		DHB		3-AP	
	Apparent K_m pyruvate (μM)	k_{cat} (s^{-1})	Apparent K_m pyruvate (μM)	k_{cat} (s^{-1})	Apparent K_m NADH (μM)	k_{cat} (s^{-1})
No inhibitor	73 ± 10	232 ± 13	93 ± 6	319 ± 8	8 ± 2	221 ± 10
10 μM	98 ± 5	211 ± 5	87 ± 7	281 ± 9		
25 μM	98 ± 5	190 ± 4	90 ± 5	229 ± 5		
50 μM	155 ± 47	175 ± 27	102 ± 6	242 ± 6	7 ± 2	190 ± 8
100 μM	163 ± 36	211 ± 23	116 ± 9	216 ± 7		
300 μM	133 ± 22	132 ± 10	111 ± 14	176 ± 10	7 ± 3	191 ± 12

which are reported in Table 1, demonstrates mixed inhibition, with increasing apparent K_m , but also decreasing k_{cat} . Double reciprocal Lineweaver–Burk plots for 3-AP and DHB (Fig. 3B and D) reveal mostly non-competitive behavior toward the sodium pyruvate substrate. 3-AP also appears entirely non-competitive vs. the NADH cofactor, as the K_m does not change within error (Fig. 2E).

Inhibition parameters are summarized in Table 2. For DHB, the globally extracted K_i was $355 \pm 55 \mu\text{M}$. While the K_i of 3-AP was $498 \pm 156 \mu\text{M}$, the EC_{50} value of approximately $78 \mu\text{M}$ is considerably lower than that of the trisubstituted aromatic DHB. Given that the EC_{50} values are not perfectly equivalent to the extracted K_i values, it is possible that there are multiple mechanisms of inhibition occurring in this system.³¹ Residuals for the global fits are included in the ESI† (see Fig. S3).

Although eukaryotic LDHs are generally thought of as operative tetramers, the dimeric symmetry allows for the dissociation into homodimers, which have been shown to be active.^{8,11} The oligomeric equilibrium appears to be of physiological relevance, as studies have shown the relative populations can be altered by both substrate and enzyme concentrations. By exploiting the enzyme concentration dependence of the dimer–tetramer equilibrium, the kinetics of the dimeric and tetrameric forms can be measured; solutions more concentrated than $1\text{--}2 \mu\text{M}$ exist as $>90\%$ tetramer, while dilution to 5 nM reportedly generates $>90\%$ dimeric pHLDH.⁸ The molecular weight of the higher concentration, tetrameric sample was validated by both size exclusion chromatography and analytical ultracentrifugation (see ESI† Fig. S4 and S5), and non-competitive inhibition was observed (Fig. 3F and G) confirming that this class of inhibitors is active against the predominant form of pHLDH *in vivo*.

Steady state kinetics of pyruvate reduction are rate limited by an enzymatic loop motion, gating product release.^{7,30}

Table 2 K_i values determined from global non-competitive fit, compared to EC_{50} values

[pHLDH]	3-AP	DHB
	5 nM pHLDH	5 nM pHLDH
K_i	$498 \pm 156 \mu\text{M}$	$355 \pm 55 \mu\text{M}$
EC_{50}	78 ± 21	712 ± 257

Single-turnover conditions allow for exposure of the chemical step of the reaction, the hydride transfer. The single turnover kinetics in the presence and absence of the 3-AP inhibitor are shown in Fig. 4, yielding hydride transfer lifetimes of 3.92 ms and 3.73 ms respectively, both reasonable when compared to literature precedent.³⁸ The rate appears unaffected within the error of the measurement, inconsistent with Schwartz's proposed mechanism of RPV disruption.²⁹ However, we cannot definitively rule out inhibitor disruption of the RPV as the rate of the chemical step is difficult to isolate, even at low pH uninhibited by proton availability.³⁰ The measured kinetic isotope effect of the LDH hydride transfer is lower than anticipated for a tunneling reaction³⁹ and rapid mixing microfluidic flow experiments have shown that the rate of loop closure occurs on the same approximate timescale as the pyruvate chemistry.⁴⁰ Any small effect on the rate itself may be unresolved due to the convolution of these additional processes.

With evidence for non-competitive behavior, and uncertainty about the mechanism of RPV interruption, discerning a binding site for the inhibitors on the enzyme or a precise mechanism of action becomes critically important. Further evidence against active site competition by the inhibitor was provided by a fluorescence experiment using the native nicotinamide cofactor. The enzyme–cofactor complex has an innate 450 nm fluorescence when excited at 340 nm . Binding of pyruvate, the substrate, or oxamate, a structural

Fig. 4 Single turnover kinetic traces and hydride transfer rate of pHLDH.

Fig. 5 Fluorescence spectra and relative intensities in presence of oxamate and inhibitors. Sample concentrations 3 μ N pHLDH, 6 μ M NADH, 5 μ M sodium oxamate.

Table 3 Fluorescence emission maxima and relative intensities

	Emission maximum (nm)	Rel. fluorescence intensity (A.U.)
3 μ N pHLDH: 6 μ M NADH	449.8 $\pm$ 0.43	1.007 $\pm$ 0.020
+oxamate	452.9 $\pm$ 0.36	0.775 $\pm$ 0.013
+oxamate, 6 μ M DHB	453.2 $\pm$ 0.25	0.764 $\pm$ 0.014
+oxamate, 35 μ M DHB	453.1 $\pm$ 0.57	0.759 $\pm$ 0.019
+oxamate, 961 μ M DHB	455.2 $\pm$ 0.68	0.773 $\pm$ 0.043
+oxamate, 1000 μ M 3-AP	449.3 $\pm$ 0.89	0.793 $\pm$ 0.071

analogue,^{23,24} has been observed to quench strongly the co-factor fluorescence by a possible photoinduced electron transfer process.^{41,42}

Introduction of an inhibitor that binds in the active site, displacing the bound oxamate, would alleviate that quenching. Fig. 5 demonstrates that no alleviation of NADH fluorescence was observed when the enzyme:cofactor:substrate analogue complex was incubated with 3-AP or DHB. At approximately 1 mM of 3-AP, a minimal blue shift (2 nm) was observed, potential evidence of increased solvent exposure of the cofactor due to inhibitor binding. Emission maxima and relative intensities with error are reported in Table 3. The persistently quenched state of the cofactor fluorescence is supportive of lack of competition for the enzyme active site, consistent with kinetic evidence for non-competitive inhibition. A complete Stern–Volmer analysis is included in the ESI† (Fig. S6) to demonstrate the negligible quenching effects of the studied inhibitors against the protein-cofactor complex.

4. Conclusions

Partial, non-competitive inhibitors for lactate dehydrogenase have been identified, with modest K_i values under 0.5 mM.

Fluorescence data reporting on the enzymatic active site are consistent with non-competitive inhibition identified *via* Michaelis–Menten kinetics, both suggesting the molecular binding is possibly allosteric in nature. As no impact has been observed on the rate of enzymatic hydride transfer, the molecule may inhibit rate-limiting loop motions associated with product release, inducing a maximum 50% reduction in steady state turnover. Assays employing the verified tetrameric form of pHLDH demonstrate inhibition, suggesting that these inhibitors function against the physiological form of the enzyme. Kinetic comparisons of structural analogues imply that the binding is specific, and the small core provides a simple scaffold for facile derivatization and pharmacological development.

Possible future avenues of investigation include crystallographic resolution of the inhibitors in the pig heart or human heart enzyme and site directed mutagenesis at the proposed binding site to elucidate the importance of specific residue contacts. In the absence of suitable crystallographic conditions, hydrogen deuterium exchange (HDX) could be explored as an option to resolve regions of flexibility appearing or disappearing in the presence of the inhibitors.

As a reversible enzyme, LDH can either reduce pyruvate or oxidize lactate by employing a bound nicotinamide cofactor. As both directions involve the inherently distance-dependent hydride transfer, presumably, both directions would be affected by an inhibitor targeting the RPY. Comparing inhibition in both directions could be an additional piece of indirect evidence supporting the idea that these inhibitors allosterically target the hydride transfer. Selectivity for LDH isoforms, not investigated in this work, could also be a critical advantage of an allosteric approach to inhibitor design.

Abbreviations

3-AP	3-Acetamidophenol
ATP	Adenosine triphosphate
DHB	3,5-Dihydroxybenzamide
DLS	Dynamic light scattering
hhLDH	Human heart lactate dehydrogenase
LDH	Lactate dehydrogenase
NADH	Nicotinamide adenine dinucleotide, reduced
NAD ⁺	Nicotinamide adenine dinucleotide, oxidized
pHLDH	Porcine heart lactate dehydrogenase

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This project was supported by the National Institutes of Health grant P01 GM068036 awarded to R. B. Dyer. The authors also thank Dr. Emily Weinert for access to stopped-flow instrumentation, Dr. Eric Hoffer and Dr. Christine Dunham for help with both protein crystallography and size exclusion

chromatography, and John “Pete” Lollar M. D. for analytical ultracentrifugation analysis.

Notes and references

- 1 M. G. C. Vander Heiden, L. C. Cantley and C. B. Thompson, Understanding the Warburg effect: the metabolic requirements of cell proliferation, *Science*, 2009, **324**, 1029–1033.
- 2 S. J. Allison, J. R. Knight, C. Granchi, R. Rani, F. Minutolo, J. Milner and R. M. Phillips, Identification of LDH-A as a therapeutic target for cancer cell killing via (i) p53/NAD(H)-dependent and (ii) p53-independent pathways, *Oncogenesis*, 2014, **3**, e102.
- 3 N. Lu, Z. Weng, Q. Chen, D. Boison, X. Xiao and J. Gao, Evaluation on the inhibition of pyrrol-2-yl ethanone derivatives to lactate dehydrogenase and anticancer activities, *Spectrochim. Acta, Part A*, 2016, **165**, 21–25.
- 4 Y. Shi and B. M. Pinto, Human Lactate Dehydrogenase A Inhibitors: A Molecular Dynamics Investigation, *PLoS One*, 2014, **9**, e86365.
- 5 J. A. Read, V. J. Winter, C. M. Eszes, R. B. Sessions and R. L. Brady, Structural basis for Altered Activity of M- and H-Isozyme Forms of Human Lactate Dehydrogenase, *Proteins*, 2001, **43**, 175–185.
- 6 J. W. Burgner and W. J. Ray, Mechanistic Study of the Addition of Pyruvate to NAD⁺ Catalyzed by Lactate Dehydrogenase, *Biochemistry*, 1978, **17**, 1654–1661.
- 7 L. Qui, M. Gulotta and R. Callender, Lactate Dehydrogenase Undergoes a Substantial Structural Change to Bind its Substrate, *Biophys. J.*, 2007, **93**, 1677–1686.
- 8 S. Yamamoto and K. B. Storey, Dissociation-Association of Lactate Dehydrogenase Isozymes: Influences on the Formation of Tetramers versus Dimers of M4-LDH and H4-LDH, *Int. J. Biochem.*, 1988, **20**, 1261–1265.
- 9 R. E. Vanderlinde, Measurement of Total Lactate Dehydrogenase Activity, *Ann. Clin. Lab. Sci.*, 1985, **15**, 13–31.
- 10 W. Eventoff, M. L. Hackert and M. G. Rossman, A Low-Resolution Crystallographic Study of Porcine Heart Lactate Dehydrogenase, *J. Mol. Biochem.*, 1975, **98**, 249–258.
- 11 S. Yamamoto and N. Eizo, H4-Isozyme of Lactate Dehydrogenase in a Solution of Sodium Chloride- I. The Molecular Weight of H4-Lactate Dehydrogenase in Relation to Sodium Chloride Concentration, Enzyme Concentration, and Temperature, *Int. J. Biochem.*, 1982, **14**, 159–163.
- 12 L. Rodriguez-Paez, M. A. Chena-Taboada, A. Cabrera-Hernandez, J. Cordero-Martinez and C. Wong, Oxamic acid analogues as LDH-C4-specific competitive inhibitors, *J. Enzyme Inhib. Med. Chem.*, 2011, **26**, 579–586.
- 13 A. Merk, A. Bartesaghi, S. Banerjee, V. Falconieri, P. Rao, M. I. Davis, R. Pragani, M. B. Boxer, L. A. Earl, J. L. S. Milne and S. Subramaniam, Breaking Cryo-EM Resolution Barriers to Facilitate Drug Discovery, *Cell*, 2016, **165**, 1698–1707.
- 14 S. Kolappan, D. L. Shen, R. Mosi, J. Sun, E. J. McEachern, D. J. Vocadlo and L. Craig, Structures of lactate dehydrogenase A (LDHA) in apo, ternary and inhibitor-bound forms, *Acta Crystallogr., Sect. D: Biol. Crystallogr.*, 2015, **71**, 185–195.
- 15 G. K. Ackers, Deciphering the Molecular Code of Hemoglobin Allostery, *Adv. Protein Chem.*, 1998, **51**, 185–253.
- 16 A. W. Fenton, *Allostery: Methods and Protocols*, Humana Press, New York City, 2012.
- 17 S. Fushinobu, K. Kamata, S. Iwata, H. Sakai, T. Ohta and H. Matsuzawa, Allosteric Activation of L-Lactate Dehydrogenase Analyzed by Hybrid Enzymes with Effector-sensitive and -insensitive Subunits, *J. Biol. Chem.*, 1996, **271**, 25611–25616.
- 18 R. Stambaugh and D. Post, Substrate and Product Inhibition of Rabbit Muscle Lactic Dehydrogenase Heart (H4) and muscle (M4) Isozymes, *J. Biol. Chem.*, 1966, **241**, 1462–1467.
- 19 C.-S. Wang, Inhibition of Human Erythrocyte Lactate Dehydrogenase by High Concentrations of Pyruvate, *Eur. J. Biochem.*, 1977, **78**, 569–574.
- 20 R. Roskoski, Allosteric MEK1/2 inhibitors including cobimetanib and trametinib in the treatment of cutaneous melanomas, *Pharmacol. Res.*, 2017, **117**, 20–31.
- 21 A. P. Kornev and S. S. Taylor, Dynamics-Driven Allostery in Protein Kinases, *Trends Biochem. Sci.*, 2015, **40**, 628–647.
- 22 J. Zhang, P. L. Yang and N. S. Gray, Targeting cancer with small molecule kinase inhibitors, *Nat. Rev. Cancer*, 2009, **9**, 28–39.
- 23 M. Gulotta, H. Deng, H. Deng, R. B. Dyer and R. H. Callender, Toward an Understanding of the Role of Dynamics on Enzymatic Catalysis in Lactate Dehydrogenase, *Biochemistry*, 2002, **41**, 3353–3363.
- 24 M. J. Reddish, H. Peng, H. Deng, K. S. Panwar, R. Callender and R. B. Dyer, Direct Evidence of Catalytic Heterogeneity in Lactate Dehydrogenase by Temperature Jump Infrared Spectroscopy, *J. Phys. Chem. B*, 2014, **118**, 10854–10862.
- 25 R. Callender and R. B. Dyer, Advances in Time-Resolved Approaches to Characterize the Dynamical Nature of Enzymatic Catalysis, *Chem. Rev.*, 2006, **106**, 3031–3042.
- 26 J. R. Exequiel, T. Pineda, R. Callender and S. D. Schwartz, Ligand Binding and Protein Dynamics, *Biophys. J.*, 2007, **93**, 1474–1483.
- 27 S. Hay and N. S. Scrutton, Good Vibrations in Enzyme-Catalysed Reactions, *Nat. Chem.*, 2012, **4**, 161–168.
- 28 D. Antoniou and S. D. Schwartz, Internal Enzyme Motions as a Source of Catalytic Activity: Rate-Promoting Vibrations and Hydrogen Tunneling, *J. Phys. Chem. B*, 2001, **105**, 5553–5558.
- 29 M. W. Dzierlenga and S. D. Schwartz, Targeting a Rate-Promoting Vibration with an Allosteric Mediator in Lactate Dehydrogenase, *J. Phys. Chem. Lett.*, 2016, **7**, 2591–2596.
- 30 Z. Wang, E. P. Chang and V. L. Schramm, Triple Isotope Effects Support Concerted Hydride and Proton Transfer and Promoting Vibrations in Human Heart Lactate Dehydrogenase, *J. Am. Chem. Soc.*, 2016, **138**, 15004–15010.
- 31 B. Srinivasan, J. V. Rodrigues, S. Tonddast-Navaei, E. Shakhnovich and J. Skolnick, Rational Design of Novel Allosteric Dihydrofolate Reductase Inhibitors Showing Antibacterial Effects on Drug-Resistant Escherichia coli Escape Variants, *ACS Chem. Biol.*, 2017, **12**, 1848–1857.

- 32 P. Schuck, Size-Distribution Analysis of Macromolecules by Sedimentation Velocity Ultracentrifugation and Lamm Equation Modeling, *Biophys. J.*, 2000, **78**, 1606–1619.
- 33 J. Monod, J. Wyman and J. Changeux, On the Nature of Allosteric Transitions: A Plausible Model, *J. Mol. Biol.*, 1965, **12**, 88–118.
- 34 C. A. Stanley, Hyperinsulinism/hyperammonemia syndrome: insights into the regulatory role of glutamate dehydrogenase in ammonia metabolism, *Mol. Genet. Metab.*, 2004, **81**, S45–S51.
- 35 G. N. Cohen, R. Y. Stanier and G. Le Bras, Regulation of the Biosynthesis of Amino Acids of the Aspartate Family in Coliform Bacteria and Pseudomonads, *J. Bacteriol.*, 1969, **99**, 791–801.
- 36 W. J. Israelsen and M. G. Vander Heiden, Pyruvate kinase: Function, regulation and role in cancer, *Semin. Cell Dev. Biol.*, 2015, **43**, 43–51.
- 37 L. Michaelis, M. L. Menten, K. A. Johnson and R. S. Goody, The original Michaelis constant: translation of the 1913 Michaelis-Menten paper, *Biochemistry*, 2011, **50**, 8264–8269.
- 38 M. J. Boland and H. Gutfreund, Pig Heart Lactate Dehydrogenase: Binding of Pyruvate and the Interconversion of Pyruvate-Containing Ternary Complexes, *Biochem. J.*, 1975, **151**, 715–727.
- 39 N. Zhadin, M. Gulotta and R. Callender, Probing the Role of Dynamics in Hydride Transfer Catalyzed by Lactate Dehydrogenase, *Biophys. J.*, 2008, **95**, 1974–1984.
- 40 M. J. Reddish, R. Callender and R. B. Dyer, Resolution of Submillisecond Kinetics of Multiple Reaction Pathways for Lactate Dehydrogenase, *Biophys. J.*, 2017, **112**, 1852–1862.
- 41 P. D. Boyer, *The Enzymes*, 1975, pp. 191–292.
- 42 H. L. Peng and R. Callender, Mechanistic Analysis of Fluorescence Quenching of Reduced Nicotinamide Adenine Dinucleotide by Oxamate in Lactate Dehydrogenase Ternary Complexes, *Photochem. Photobiol.*, 2017, **93**, 1193–1203.