Optimization of 3-Hydroxy-5-(2,6-dichlorophenyl)-2-mercaptocyclohex-2-enones as Potent Inhibitors of Human Lactate Dehydrogenase

Sharada Labadie^a, Peter S. Dragovich^a, Jinhua Chen^b, Benjamin P. Fauber^a, Jason Boggs^a, Laura B. Corson^a, Charles Z. Ding^b, Charles Eigenbrot^a, HongXiu Ge^b, Qunh Ho^b, Kwong Wah Lai^b, Shuguang Ma^a, Shiva Malek^a, David Peterson^a, Hans E. Purkey^a, Kirk Robarge^a, Laurent Salphati^a, Steven Sideris^a, Mark Ultsch^a, Erica VanderPorten^a, BinQing Wei^a, Qing Xu^b, Ivana Yen^a, Qin Yue^a, Huihui Zhang^b, Xuying Zhang^b, Aihe Zhou^a

^aGenentech, Inc., 1 DNA Way, South San Francisco, California 94080, USA

^bWuXi AppTec Co., Ltd. 288 Fute Zhong Road, Waigaoqiao Free Trade Zone, Shanghai 200131, P. R. China.

Tumor cells exhibit altered glucose metabolism compared to normal cells.¹ They often rely on glycolysis for energy generation in contrast to more efficient mitochondrial tricarboxylic acid (TCA) cycle utilized by healthy cells.² This dependency on glycolysis occurs even in the presence of normal oxygen concentrations.³ Lactate dehydrogenase A (LDHA) plays a critical role by catalyzing the conversion of pyruvate to lactate in the last step of the process.⁴ Over-expression of LDHA is detected in many types of cancer cells⁵ and shRNA-mediated LDHA knock-down results in significant inhibition of tumor growth in glycolytically dependent cancer cell lines.⁶ Moreover, humans deficient in LDHA show only mild phenotype suggesting that inhibition of the enzyme will not lead to unwanted toxicities.^{4,7} Collectively, these facts suggest that the selective inhibition of LDHA would provide new therapeutic potential in cancer treatment.

A number of LDHA inhibitors have recently been disclosed (Figure 1, **1-3**).⁸⁻¹² Optimization of high throughput screening hits (Figure 1, **4-6**) by our group have also been reported.¹³⁻¹⁵ To date, low enzyme potency and poor physiochemical properties have resulted in poor cellular potency.¹⁵⁻¹⁷ In the case of 3-hydroxycyclohex-2-enone series **6**, containing two lipophilic phenyl rings and an acidic 1,3-dicarbonyl moiety, the lack of cell potency¹⁵ was ascribed to a high cell-shift associated with the lipophilic acidic template.¹⁸ To overcome the cell shift associated with these templates without altering the acidity of the molecule, further improvement in biochemical potency was required relative to previously disclosed¹⁵ inhibitors **6a** and **6b**. Herein, we describe the optimization and evaluation of 3-hydroxycyclohex-2-enone series **6** in biochemical and related cellular assays.

Distinct binding pockets observed in the crystal structure of 3-hydroxy-2-(2-chlorophenylthio)-5-(2,6-dichlorophenyl)cyclohex-2-enone, **6b**, bound to LDHA¹⁵ provided possible functionalization strategies to further enhance the biochemical potency (Figure 2). First, the pocket near the nicotinamide adenine dinucleotide (NADH) binding site contained unoccupied space encompassed by the flexible 100's loop. Favorable interactions with the polar side-chains of the residues R98, Q99, Q100 and E103 of the 100's loop might therefore be realized by functionalization (Figure 1) of the phenyl ring A at C-3 and C-4. The substituents at C-3 might also point inward toward the NADH and make interactions with the functionalities as observed earlier.¹⁴ Secondly, the C-5' position of the phenyl ring B was in close proximity to the acidic side-chain and backbone NH of the D194 residue of LDHA. Previous SAR exploration from our group showed that the substitution at this position was tolerated¹⁵ and additional favorable interactions with the protein could be obtained.¹³ This indicated that incorporation of H-bond acceptor/donor moieties at C-5' of the ring B might afford more favorable interactions with LDHA. These structural features collectively suggested that functionalization of C-3 and C-4 of the phenyl ring A and C-5' of the phenyl ring B present in molecule, such as **6b**, were reasonable strategies to enhance biochemical potency.

Accordingly, several **6b** analogs containing various H-bond donor/acceptor functionalities were evaluated for their LDHA enzyme potencies (Table 1). In comparison to **6b**, the C-3 N-linked morpholine analog (**7**) showed a modest four-fold gain in enzyme potency. However, a reduction in potency was observed for the compound with N-linked morpholine at C-4 (**8**). We then directed our focus towards

further exploration of C-3 modifications of the phenyl ring A. N-Linked piperazine analogs (**9-12**) displayed biochemical potencies comparable to **7**. Compounds **13** and **14**, substituted with 4-aminotetrahydropyran and isopropoxy moieties, respectively, showed 15- and 10-fold improvement relative to **6b**. We then explored a few C-linked analogs. Compounds containing phenyl (**15**), 2-chlorophenyl (**16**) and heteroaryls (**17-19**) in this position of the molecule displayed similar potencies except for the pyrimidine analog **19**.

In order to understand the gain in potency from this exploration, a co-crystal structure of a representative molecule, **13**, bound to LDHA (Figure 3a) was obtained.¹⁹ As expected, the inhibitor was bound in the substrate binding site and the neighboring side-chains of residues R168, H192 and N137 were within the H-bond distance from the 1,3-dicarbonyl portion of the ligand. The 2, 6-dichlorophenyl ring A was positioned in the equatorial orientation as observed in the crystal structure of **6b**.¹⁵ Nicotinamide adenine dinucleotide (NADH) co-factor was also present in the complex. The 4-aminotetrahydropyranyl group on the ligand appeared solvent exposed and was not pointed inward toward NADH.¹⁴ Some residues of the 100's loop were disordered due to crystal packing contacts. Overlay of the X-ray crystal structures of **6b** and **13** (Figure 3b) revealed that a portion of the 100's loop was considerably closer to the phenyl ring A in **13** compared to the phenyl ring A in the crystal structure of **6b**. However, the polar moieties on the side-chains of the 100's loop residues were not in close proximity to make favorable interactions with the 4-aminotetrahydropyranyl group of the ligand **13**. The disorderliness and mobility of the 100's loop made it difficult to ascertain

interactions that may be responsible for potency enhancement for this molecule. We then directed our efforts towards functionalization of C-5' of the aryl ring B.

To obtain interactions with the acid side-chain and backbone NH of D194, as discussed earlier, a new set of compounds with hydrogen-bond donor/acceptor moieties at C-5' of the phenyl ring B were synthesized and evaluated in biochemical assay (Table 2, **20-32**). Compounds with the hydroxyl, methoxy, amino and methyamino groups at C-5 (**20-23**) exhibited a modest potency gain relative to **6b**. Compound containing carboxylic acid²⁰ (**24**) and amides (**25-27**) were weaker than the corresponding carboxylic acid esters²⁰ (**28-32**). Among the esters, the (*R*)-2-phenylethyl ester **31** was the most potent analog with a 145-fold improvement in biochemical potency over **6b**. This dramatic improvement in potency for **31** was possibly due to the H-bond interaction between the ester moiety and the backbone NH of D194 and additional favorable interaction between the phenyl ring and the side-chain of the residue encompassing the binding pocket (see below).

Encouraged by these results, we envisioned that incorporation of functionalities on both rings A and B might produce synergistic effects and provide an inhibitor with greatly improved biochemical potency. Therefore, compounds with substituents at both the C-3 of ring A and the C-5' of ring B were investigated (Table 3, **33-39**). The isopropoxy substituents at C-3 of ring A was selected to test this hypothesis because of the ease of synthesis and the potency improvement with this was comparable to the other groups tested (Table 1). This moiety was combined with hydroxyl and methoxy on C-5' of ring B. These combinations improved enzyme potency only 3-fold over **14** (Table 1). A slight improvement in enzyme potency was observed when the C-2' Cl was

replaced by CN providing inhibitor **35**. Other combinations, 4-pyridyl at C-3 and methoxy at C-5 (**37**), phenyl at C-3 and ethyl acetic acid ester at C-5 (**38**) and 4-aminotetrahydropyranyl group at C-3 and methoxy at C-5 (**39**) showed only modest potency improvement relative to the mono-substituted analogs. Generally, compounds with substituents both at C-3 of the phenyl ring A and the C-5 of the phenyl ring B were more potent than the compounds with substituent on only one of the rings. However, the dramatic synergistic effect was not realized.

A subset of the potent analogs was profiled in a panel of in vitro DMPK assays and against structurally related dehydrogenases. As shown in Table 4, the tested compounds displayed good solubility in pH 7.4 buffered aqueous solutions and low to moderate MDCK permeability. The inhibitors exhibited very high protein binding in plasma from humans. All tested compounds displayed low stability in human liver microsomes because of high cLogPs.²¹ The most potent analog **31** was stable in human plasma and unstable in mouse plasma (72% of parent remained after 1hr in mouse plasma at 37 °C). The inhibitors were, typically, 4-10 fold selective for LDHA over LDHB isoform except for the analog **31** which displayed similar potency for both LDHA and LDHB. The inhibitors were inactive ($IC_{50} = >10 \mu M$) against related malate dehydrogenase 1 (MDH1) and malate dehydrogenase 2 (MDH2) enzymes.

A few potent analogs were tested in MIA PaCa2 cells to assess their ability to inhibit lactate production.²² All tested compounds were inactive under normal serum conditions (10% fetal bovine serum) at inhibitor concentrations of up to 10 μM . The reasons for lack of cell potency for these inhibitors are unknown but it could be

potentially due to high protein binding and the high cell-shift associated with the lipophilic acidic templates¹⁸ and high intracellular LDHA concentration.¹⁷

A co-crystal structure of the most potent analog **31** bound to LDHA was obtained to understand the considerable improvement in enzyme potency over **6b** (Figure 4). The inhibitor was bound in the substrate binding pocket similar to that observed for compounds **6b** and **13**. The usually mobile and disordered R105 was well ordered in this complex and the guanidinium moiety of the side-chain was within the H-bond distance (2.6 Å) from the acid side-chain of D194.^{19,23} This interaction positioned the guanidinium moiety in close proximity to the phenyl ring of the ester at C-5' of **31** enabling favorable partial π -cation interaction.²⁴ In addition, the carbonyl of the ester moiety was within the H-bond distance (2.8 Å) from the backbone NH of D194. We believe, these two additional favorable interactions with the protein were responsible for the observed gain in potency for the inhibitor **31**.

Syntheses²⁵ of 3-hydroxy-2-(2-chlorophenylthio)-5-(2,6-dichlorophenyl)cyclohex-2-enone analogs with substitutions on ring A are described in Scheme 1a-c. Deprotonation of 1-bromo-2,4-dichlorobenzene with LDA followed by DMF quench provided the intermediate aldehyde **40**. The aldehyde was converted to **41** as previously described.¹⁵ Treatment of **41** with the 1,2-bis(2-chlorophenyl)disulfane in the presence of potassium carbonate formed **42**. Coupling of **42** with various secondary amines in the presence of Pd₂(dba)₃, Xanthphos and cesium carbonate in toluene afforded the amine substituted analogs **7**, **9-11** and **13**. Deacetylation of **11** under basic conditions yielded **12**. Compound **8** was prepared, in a similar fashion to **7**, starting from 3,5-dichlorobromobenzene. Alkylation of 2,4-dichlorophenol with 2-iodopropane produced

43. Deprotonation of **43** with n-BuLi in hexanes at -78 °C and subsequent DMF quench produced the aldehyde **44** which was converted to **14** via **45** as previously described.¹⁵ Compounds **15-19** were obtained by Suzuki couplings between **42** and boronic acids in the presence of a palladium catalyst.

The compounds with substitutions on the ring B were synthesized as described in Scheme 2. 5-(2,6-Dichlorophenyl)cyclohexane-1,3-dione **46**¹⁵ was treated with various thiols, **47a-d** and **47f**^{26,27} in the presence of potassium carbonate in acetonitrile to generate compounds **20-24**. The carboxylic acid **24** was converted to esters and amides (**25-32**) using either Mitsunobu or HATU coupling conditions.

Scheme 3 describes the synthesis of compounds with substitutions on both rings A and B. Treatment of the intermediate **45** with the thiols **47a,b,g** and **h** provided **33-36**. Suzuki coupling on the intermediate **41** and subsequent treatment of the product **48** with thiols **47a** and **47e** provided compounds **37** and **38**, respectively. Intermediate **41** was converted to **39** via the intermediate **49**.

In summary, structure based design strategies were applied to obtain analogs with considerable improvement in biochemical potency relative to **6b**. These inhibitors were substrate competitive and were typically selective for LDHA over LDHB and other structurally related malate dehydrogenases, MDH1 and MDH2. A co-crystal structure of the most potent inhibitor **31** revealed that the carbonyl and the phenyl group of the ester moiety in **31** were in close proximity to allow H-bond and partial π -cation interactions, respectively, with the protein. Because of the flexibility of the 100's loop, it was difficult to efficiently fill the binding pocket. In addition, the 100's loop was very disordered near

the inhibitor binding site, perhaps arising from the crystal packing interactions. Therefore, in some cases, interactions that may be responsible for affinity enhancement could not be observed in the crystal structure. Additional efforts to identify inhibitors, in the related scaffold, with improved ability to inhibit LDHA in cell-culture experiments were undertaken and will be reported in due course.

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19. Most procedures to produce and crystallize LDHA/inhibitor complex crystals were as described in ref. 15. LDHA crystals in space group P2(1) were grown by vapor diffusion at 18 °C using a 50:50 mixture of protein stock (10 mg/mL) and reservoir (20% (w/v) PEG 3350, 0.2M sodium malonate pH 7.0, 2mM NADH). Inhibitors were incorporated by placing crystals in a solution lacking malonate ion (30% (w/v)PEG 3350, 0.1M HEPES pH 7.2, 1mM NADH) made 5 mM in inhibitor using a 50 mM DMSO stock solution and waiting 5 - 7 days. See *Supplementary Material* and deposited pdb file for additional structural details.

20. The benzoic acid (R = COOH) and the corresponding methyl ester (R = COOMe) analogs were inactive against LDHA ($IC_{50} = >4 \mu M$, data not shown). The required thiol was prepared according to Godt, H. C.; Wann, R. E. *J. Org. Chem.*; **1961**, 26, 4047.

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22. See supplementary material for experimental details.

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Table 1. Structure-activity relationship of compounds **7-19**

Compd. No.	R ¹	R ²	LDHA IC ₅₀ (nM)	Compd. No.	R ¹	R ²	LDHA IC ₅₀ (nM)
6b	H	H	870	13		H	60
7		H	240	14		H	90
8	H		2,600	15		H	100
9		H	230	16		H	60
10		H	370	17		H	108
11		H	100	18		H	85
12		H	170	19		H	1,100

See ref.15 for experimental details associated with each assessment. LDHA biochemical inhibition results are reported as the average of 2 separate runs for compounds with LDHA IC₅₀ <100 nM. For others, the result is from a single run.

Table 2. Structure-activity relationship of compounds **20-32**

Compd. No.	R	LDHA IC ₅₀ (nM)	Compd. No.	R	LDHA IC ₅₀ (nM)
6b	H	870	26	CH ₂ CONMe ₂	10,000
20	OH	120	27	CH ₂ CONHCH ₂ CH ₂ OH	300
21	OMe	370	28	CH ₂ COOMe	40
22	NH ₂	640	29	CH ₂ COOEt	130
23	NHMe	170	30		120
24	CH ₂ COOH	3,500	31		6
25	CH ₂ CONHMe	1,800	32		90

See ref.15 for experimental details associated with each assessment. Biochemical results as the arithmetic mean of at least 2 separate runs for compounds with LDHA IC₅₀ <100 nM. Data for others is the result of a single run.

Table 3. Structure-activity relationship of compounds **33-39**

Compd No.	R ¹	R ²	R ³	LDHA IC ₅₀ (nM) ^a	LDHB IC ₅₀ (nM) ^b
33		OH	Cl	30	180
34		OMe	Cl	25	410
35		OH	CN	15	140
36		OMe	CN	460	2,400
37		OMe	Cl	35	230
38	Ph	CH ₂ COOEt	Cl	30	120
39		OMe	Cl	30	490

^aLDHA biochemical inhibition results are reported as the arithmetic mean of 2 separate runs. ^bLDHB inhibition data are from a single run.

Table 4. In vitro properties for representative examples

Compd. No.	HLM_Cl _{hep} (ml/min/kg)	Human PPB (%)	MDCK P _{app} .A : B (x 10 ⁻⁶ cm/s)	Kinetic Solubility (μM) ^a	cLogP	LLE ^b
31	18	ND ^c	10.6	61	7.5	0.7
35	19	>99.9	1.1	50	5.0	2.8
39	20	>99.9	14	49	6.1	1.3

^aKinetic solubility was measured at pH 7.4 in PBS buffer. ^bSee ref. 28. ^cPPB could not be determined as mass peak (M+H) was not detected.

Figure(s)

Figure 1. Previously disclosed LDHA inhibitors.

Figure 2

Figure 3a

Figure 3b

Figure 4

Figure 2. Depiction of the optimization strategy from the crystal structure of **6b** (cyan) bound to LDHA (grey). Accessible space in the binding pocket is shown with blue ellipses. Red arrows indicate possible positions on ring A and B for functionalization.

Figure 3a. Co-crystal structure (3.2 Å, PDB code = 4R69) of the inhibitor **13** (cyan) in complex with LDHA (grey). Hydrogen bonds are depicted as dashed red lines. **Figure 3b.** Overlay of co-crystal structures of inhibitor **13** (cyan) in complex with LDHA (grey) and inhibitor **6b** (green) in complex with LDHA (green).

Figure 4. Co-crystal structure (2.11 Å, PDB code = 4R68) of inhibitor **31** (cyan) in complex with LDHA (grey). Hydrogen bonds are depicted as dashed red lines.

Scheme 1. Synthesis of compounds **7-19**

Reagents and reaction conditions: See Table 1 for R¹ substituents **Scheme 1a:** (i) LDA 2 M in THF/heptane/diethylbenzene, -78 °C, 1 h; (ii) DMF, -78 °C, 2 h; (iii) (2-ClPhS)₂, K₂CO₃, AcCN, 80 °C, 2h; (iv) amine, Cs₂CO₃, Pd₂(dba)₃, Xanthphos, toluene, 110 °C, 16 h; (v) NaOH, water, 60 °C.

Scheme 1b: (i), (ii) and (iii) are the same as in Scheme 1a; (vi) 2-Bromopropane, NaOH, MeCN, 80 °C, 12 h.

Scheme 1c: (vii) R¹B(OH)₂, Pd(PPh₃)₄, K₂CO₃, 1,4-dioxane, 6 h.

Scheme 2. Synthesis of compounds **20-32**

Reagents and reaction conditions: (i) K₂CO₃, acetonitrile, 80 °C, 14 h; (ii) amine, HATU, DIPEA, DMF, 16h; (iii) alcohol, PPh₃, DEAD, PhI(AcO)₂, THF, RT, 16 h.

Scheme 3. Synthesis of compounds **33-39**

Reagents and reaction conditions: See Table 3 for R^1 and R^2 and R^3 substituents: (i) K_2CO_3 , acetonitrile, 80 °C, 14 h (ii) $R^1B(OH)_2$, $Pd(dppf)Cl_2$, Cs_2CO_3 , DMF, 1 h, 110 °C microwave; (iii) 4-aminotetrahydropyran, $NaOt-Bu$, $Pd_2(dba)_3$, Brettphos, toluene, 110 °C, 12 h.