Generation of Oxamic Acid Libraries: Antimalarials and Inhibitors of *Plasmodium falciparum* Lactate Dehydrogenase

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Received August 3, 2006

Lactate dehydrogenase (LDH) is a key enzyme in the glycolytic pathway of *Plasmodium falciparum* (*pf*) and has several unique amino acids, related to other LDHs, at the active site, making it an attractive target for antimalarial agents. Oxamate, a competitive inhibitor, shows high substrate affinity for *pf*LDH. This class of compounds has been viewed as potential antimalarial agents. Thus, we have developed an effective automated synthetic strategy for the rapid synthesis of oxamic acid and ester libraries to screen for potential lead inhibitors. One hundred sixty-seven oxamic acids were synthesized using a "catch and release" method with overall yields of 20−70%. Most of the compounds synthesized had some inhibitory effects, but compounds **5** and **6** were the most active against both chloroquine- and mefloquine-resistant strains with IC_{50} values of 15.4 and 9.41 μM and 20.4 and 8.40 μM, respectively. Some oxamic acids showed activities against *pf*LDH and *mammalian* LDH (mLDH) at the micromolar range. These oxamic acids selectively inhibited *pf*LDH 2−5 fold over mLDH. Oxamic acid **21** was the most active against *pf*LDH at $IC_{50} = 14$ and mLDH at $IC_{50} = 25$ μM, suggesting that oxamic acid derivatives are potential inhibitors of *pf*LDH and that further study is required to develop selective inhibitors of *pf*LDH over mLDH.

Introduction

Despite tremendous worldwide efforts, malaria remains one of the key parasitic diseases present today with more than 300 million acute illnesses and at least one million deaths annually throughout the tropical and subtropical regions of the world.[1] The prevalence of malaria and the emergence of multidrug-resistant (MDR) strains of the *Plasmodium* sp. require the development of new classes of antimalarial drugs and a renewed effort to eradicate this debilitating disease. Although MDR-reversing agents have shown potential efficacy on drug-resistant malaria, the presence of side effects limit their utility. Moreover, parasites resistant to this line of therapy are also rapidly emerging.[2,3] For example, a sulfadoxine−pyrimethamine combination to treat *Plasmodium falciparum*, a main causative agent of the most lethal form of malaria, is an effective treatment against MDR malaria but has been rendered useless in Southeast Asia by overuse and misuse.[4]

Lactate dehydrogenase (*pf*LDH) is a key enzyme in the glycolytic pathway of *P. falciparum*, reducing pyruvate to lactate with the aid of NADH. *P. falciparum* has been shown to possess the necessary enzymes needed for a functional tricarboxylic acid (TCA) cycle, although it remains unclear as to whether this pathway is used for energy production or for the production of biochemical intermediates for other pathways.[5] Thus energy production is thought to be solely dependent upon anaerobic glycolysis in the cytoplasm, leading to a 30−50-fold higher glucose-consumption rate than their host cells.[6−9] Therefore, *pf*LDH may prove to be an effective target for chemotherapy, eliminating the production of ATP, resulting in death of the *P. falciparum* cell.[10,11]

Lactate dehydrogenase (LDH) is a principal enzyme involved in energy generation found in many eukaryotic organisms, including humans.[12,13] Therefore, to be effective, a drug candidate would need to be selective for *pf*LDH over human LDH (hLDH). The differences found within the active site of parasite and human LDHs suggest that selectivity may be possible. In all LDHs, catalytic residues Arg171, Arg109, Asp168, and His195 are conserved. Asp168 and His196 act as hydrogen donors, while the side chain of Arg171 interacts with the carboxylate of pyruvate. The side chain of Arg109 interacts with the ketone oxygen of pyruvate leading to polarization of the ketone carbonyl and hydride attack from NADH. In addition, *pf*LDH has several unique amino acid residues when compared to hLDHs. The active site of hLDH contains Thr246 and Ile250. These are replaced by proline in *pf*LDH. Likewise, Asp197, Gln102, and Ser163 are replaced by Asn197, Lys102, and Leu163, respectively, in *pf*LDH. Furthermore, the substrate specificity loop in *pf*LDH is 5 amino acids longer than in other LDHs.[14] These active site variations suggest the possibility of developing highly selective inhibitors of *pf*LDH.

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10.1021/cc060110n CCC: $37.00
Published on Web 02/23/2007

To this end, research has focused on the isolation of natural products, synthesis of natural product derivatives, and design of selective drugs using computational chemistry.[15−17] Gossypol, a natural product isolated from cotton seeds, is a known inhibitor of LDHs from both malarial parasites and humans, but it shows high toxicity, possibly because of the presence of two aldehyde groups and catechol functional groups.[18] Gossypol exhibits competitive binding with cofactor NADH in both *pf*LDH and hLDH, and a few derivatives have shown good selectivity for *pf*LDH over hLDH resulting from a variation in the residues in the active site of the LDHs.[10,19] Unfortunately, the vast majority of gossypol derivatives failed to show improved selectivity or activity.[14,16,20]

Oxamate, an isosteric and isoelectric analogue of pyruvate, shows high affinity for *pf*LDH.[21] Oxamate is a competitive inhibitor of the binding of pyruvate to LDH, and several oxamic acid derivatives have been developed as lead compounds for selective *pf*LDH inhibitors. Specifically, Vander Jagt has reported *N*-substituted oxamic acids that have micromolar activities.[17] These studies prompted us to pursue this family of compounds as a potential lead class for the development of selective parasite LDH inhibitors. Thus, *pf*LDH is an attractive target for antimalarial chemotherapy because (1) it controls the production of plasmodial ATP, (2) it has unique amino acids at the active site compared to other LDHs, including human LDH, and (3) many crystal structures of *pf*LDH complexed with various substrates (Protein Data Bank codes 1LDG, 1T26, 1T2C, 1T2D, 1T24, 1U4S, 1U4O, 1U5C, 1U5A, 1CEQ, 1XIV, 2A94, and 1T25) are known, providing a solid drug target around which to design inhibitors.

Although an oxamic acid library has recently been built using solid-phase synthesis by Player,[22] our primary efforts have been focused on the development of an automated synthetic strategy to quickly and efficiently produce oxamic acid and ester libraries for screening potential antimalarial compounds and to find a lead compound that displays selective activity against *pf*LDH over hLDHs.

Results and Discussion

The purpose of any medicinal chemistry program whose primary objective is drug design and discovery is to identify a lead compound as rapidly as possible so that synthesis of suitable derivatives can begin, with the ultimate goal of finding candidates with potent activity. One method of achieving this is through the use of automated parallel synthesis and purification. In this paper, we have developed a short route to an oxamic acid library, using full automation, in our search for a viable drug lead for the dual inhibition of LDH and MDH (malate dehydrogenase) from *P. falciparum*. First, we used a synthetic strategy that could be applied to fully automated systems for the synthesis of the library. To achieve our goal, we designed a synthesis protocol based on a series of automation platforms available in our laboratories. Each step, such as solvent addition, reagent addition, and evaporation, was considered in terms of an automated synthesis because each step represents total success or failure in the preparation of libraries. Reaction

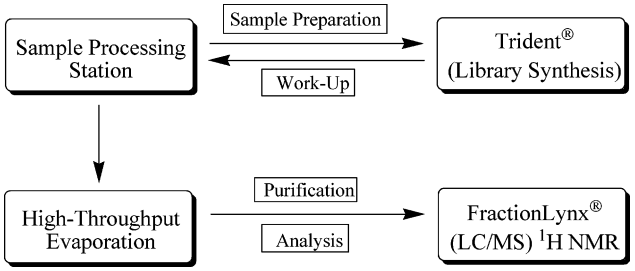
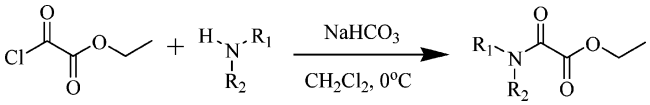


Figure 1. Automated synthesis protocol.

Scheme 1. Synthesis of Various Oxamic Esters



preparation, workup, and format changes were carried out on a Trident sample processing station (SPS, Argonaut Technologies, Inc.). Synthetic steps were performed on a Trident combinatorial synthesizer (Argonaut Technologies, Inc.). LC/MS analysis was accomplished using a FractionLynx HPLC/MS system (Waters, Inc.). High-throughput sample evaporations were conducted using a GeneVac HT-12 evaporator (GeneVac, Inc.) (Figure 1).

The synthesis of oxamic acid derivatives began with the reaction of diverse primary or secondary amines (0.5 M in DCM, 0.75 mmol) and ethyl chlorooxoacetate (1 M solution in DCM, 0.5 mmol) at 0 °C in the presence of sodium bicarbonate (0.75 mmol in 0.2 mL DCM) on the Argonaut Trident combinatorial synthesizer.[23] After 10 h, the heterogeneous mixture was filtered into vials using the Argonaut sample processing station (SPS), transferred to Varian Hydromatrix cartridges, and preconditioned with 1 N HCl to remove unreacted amine and ethyl chlorooxoacetate (Scheme 1).

A key aspect in automated library synthesis is a purification of the final compounds that can be done on large numbers in a streamlined fashion. We accomplished this goal using the catch and release strategy for purification. Thus, the next reaction sequence was conducted by initial dissolution of the esters in 90:10 EtOH/water, followed by the addition of MP-carbonate resin (2.0 equiv, 3 mmol/g loading capacity) using the Argonaut SPS. By using this highly crosslinked resin, which contains ionically immobilized carbonate, we determined that the final hydrolysis step could be catalyzed by the base and the subsequent ionized product could then be scavenged by the free quaternary ammonium moiety of the resin. After reaction incubation at 65 °C for 24 h on the Trident, the mixture was filtered, and the remaining resin was washed with ethanol and methylene chloride to remove any unreacted starting material using the Argonaut SPS. Subsequent treatment with 1 N HCl allowed for the release of the oxamic acid products, which were subjected to parallel liquid−liquid (CH_2Cl_2/H_2O) extraction (Scheme 2).

Overall, library analysis was accomplished with a randomly selected sample of 20 of the 167 compounds.[24] This sample group was evaluated using ^{1}H NMR, LC/MS, and HRMS (Table 1). Product purity was determined by LC-MS screening (UV detection, $\lambda_{max} = 210$ nm; ESI− mode,

Scheme 2. Hydrolysis of Oxamic Esters Using the Catch and Release Method

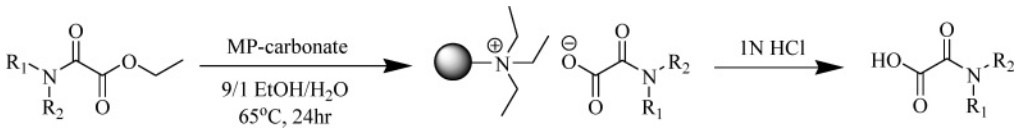


Table 1. MS Analysis (ES^-) of 20 Randomly Selected Oxamic Acids

entry	oxamic acids	MW	MS [M − 1][a]	yield (%)[b]
1	{[3-(trifluoromethyl)benzyl]amino}-oxoacetic acid	247.1	246.0	49
2	{[3-(trifluoromethyl)phenyl]amino}-oxoacetic acid	233.0	231.9	35
3	(1,3-benzodioxol-5-ylamino)-oxoacetic acid	209.0	208.0	45
4	[(cyclohexylmethyl)amino]-oxoacetic acid	185.1	184.1	66
5	[(4-phenoxyphenyl)amino]-oxoacetic acid	257.1	256.1	52
6	[(3-chlorobenzyl)amino]-oxoacetic acid	213.0	212.0	58
7	[(2,6-dibromophenyl)amino]-oxoacetic acid	320.9	319.9	33
8	[(4-chloro-2-methylphenyl)amino]-oxoacetic acid	213.0	212.0	44
9	[(4-bromo−2,3,5,6-tetrafluorophenyl)amino]-oxoacetic acid	314.9	313.9	20
10	[(1-methyl-3-phenylpropyl)amino]-oxoacetic acid	221.1	220.1	68
11	[(3-phenoxyphenyl)amino]-oxoacetic acid	257.1	256.1	49
12	[(2-phenylethyl)amino]-oxoacetic acid	193.1	192.1	68
13	[4-(pentyloxy)phenylamino]-oxoacetic acid	251.1	250.1	62
14	[(2-chloro-4,6-dimethylphenyl)amino]-oxoacetic acid	227.0	226.0	33
15	[(2-*sec*-butylphenyl)amino]-oxoacetic acid	221.1	220.1	43
16	[(4-chloro-2-fluorophenyl)amino]-oxoacetic acid	217.0	216.0	25
17	[(4-pentylphenyl)amino]-oxoacetic acid	235.1	234.1	53
18	[(2,5-dichlorophenyl)amino]-oxoacetic acid	233.0	232.0	38
19	[(4-methoxyphenethyl)amino]-oxoacetic acid	223.0	222.1	70
20	[(2-cyanophenyl)amino)]-oxoacetic acid	190.0	191.0[c]	49

[a] LC/MS(ESI-) spectrum [M − 1]. [b] Isolated percentage yield based on ethyl chlorooxoacetate. [c] LC/MS(ESI+) spectrum [M + 1].

m/*z* 150−400). Analysis revealed that 90% of the products sampled exhibited >95% purity. The only disappointing aspect of this synthetic strategy was the low overall yields. The average yield in the sampled set was 20−70% overall. This can be attributed to the low nucleophilicity of many of the diverse amines used as building blocks, as well as inefficient capture following ester hydrolysis. Even with these yields, however, we still were able to synthesize sufficient material for use in several biological assays.

Antimalarial Activity. These 167 oxamate derivatives were tested using two whole-cell parasite cell lines, W2 (chloroquine resistant) and D6 (mefloquine resistant) strains, and two purified enzymes, *pf*LDH and mLDH (mammalian LDH). Table 2 contains the biological activities (IC_{50}) of 38 compounds from the library.[24] Overall, in antimalarial testing, activities were rather low, with the most active compounds displaying IC_{50} values in the mediocre micromolar range. Most of derivatives showed a little better activity against W2 than against D6. While 46 oxamic acid derivatives showed inhibitory effects with IC_{50} values of less than 50 μM against both D6 and W2 strains, oxamic acids **5** and **6** were the most active compounds tested with IC_{50} values of 15.4 and 20.4 μM against the D6 clone, respectively, and 9.41 and 8.40 μM against W2 clone, respectively. These two acids may be used as potential lead structures from which to develop selective antimalarial drugs in the future. In our series, the *N*,*N*-disubstituted oxamic acids showed relatively higher activities than the monosubstituted acids against both D6 and W2 (Table 2, oxamic acids **7**, **8**, **9**, **11**, and **13**), whereas the oxamic acids substituted with a small moiety, such as simple alkyl groups, showed activities displaying IC_{50} values ranging from 21.6 to 50 μM against both strains (Table 2, oxamic acids **10**, **12**, **21**, **23**, **26**, and **28**) with the exceptions of oxamic acids **25**, **31**, **34**, and **40** which have oxygen atoms or branched side chains. Therefore, it may be possible to design and explore novel oxamic acid derivatives to develop antimalarials based on N,N-disubstituted or monosubstituted compounds.

All bioactive or inactive oxamic acids shown in Table 2 have hydrophobic groups, such as a long alkyl chain or phenyl group, attached to the oxamate group, reflecting that the increased hydrophobic surface area of oxamic acids tend to penetrate the membrane of the cells. However, despite the structural similarity, some of the oxamic acids did not have any activity, such as compounds **35** and **39** which are similar to compounds **15** and **16**, compounds **22** and **32** which are similar to compounds **7** and **8**, and compound **17** which is similar to compound **38**. The inactivity of these compounds may be caused by their poor absorption into the red blood cell and the presence of a carboxylic acid group. Therefore, we have randomly selected and tested 86 ethyl oxamates whose acids are inactive or active via an in vitro malaria screening test to determine if ethyl oxamates may be used as potential prodrugs. Unexpectedly, most of esters are less active than their acids against W2 and D6. Only one ethyl oxamate shows a better activity with an IC_{50} value of 20.5 μM against D6 and a value of 2.79 μM against W2 (Table 2, compound **37**). In addition, one ethyl oxamate shows a little better activity than its acid does in the in vitro test against the W2 strain (Table 2, compound **14**). Four esters show lower or similar activities compared to their acids with micromolar levels (Table 2, compounds **7**, **8**, **13**, and **23**). Interestingly, ethyl 2-(3-(methylthio)phenylamino)-oxoacetate (Table 2, **37**) shows promising activity against W2, whereas its acid does not have inhibitory activities against two strains. All esters, except these six esters, do

Table 2. Biological Activities of Oxamic Acids and Esters against Drug Resistant Strains, D6 and W2, and Purified Enzymes, *pf*LDH and mLDH

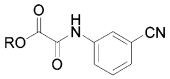
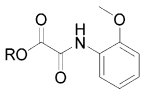
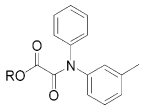
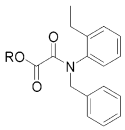
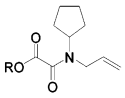
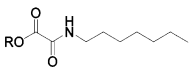
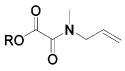
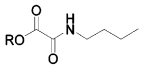
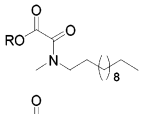
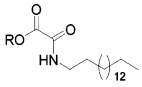
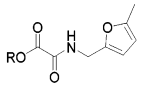
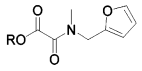
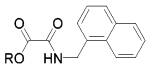
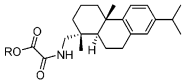
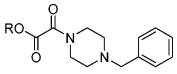
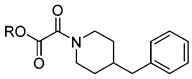
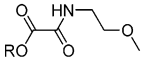
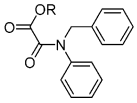
	compound	whole cell assay[a] R = Et IC_{50} (μM)		whole cell assay[a] R = H IC_{50} (μM)		enzyme assay[b] R = H IC_{50} (μM)	
		D6	W2	D6	W2	*pf*LDH	*m*LDH
1	Mefloquine	0.004	0.002	0.003	0.001	N/T[c]	N/T
2	Chloroquine	0.005	0.180	0.008	0.120	N/T	N/T
3	oxamate	N/T	N/T	N/T	N/T	94.4	116
4	gossypol	N/T	N/T	N/T	N/T	3.97	6.9
5		>200	>200	15.4	9.41	N/A[d]	N/A
6		>170	>170	20.4	8.40	>200	>200
7		30.1	23.8	16.7	17.5	97.9	107
8		42.4	95.9	22.0	18.3	>200	158
9		N/T	N/T	26.3	24.9	N/A	N/A
10		>164	>164	26.7	26.7	160	>200
11		N/T	N/T	19.7	21.7	>200	>200
12		N/T	N/T	21.6	28.3	>200[d]	>200
13		25.8	47.9	29.0	19.2	N/A	N/A
14		>77.1	17.4	>83.5	69.4	>200	150
15		>372	>372	26.2	23.3	>200	>200
16		>228	>228	50.1	50.1	>200	186
17		>106	>106	20.5	15.2	43	>200
18		N/T	N/T	25.2	13.4	59	20
19		N/T	N/T	>52.0	>52.0	169	46
20		N/T	N/T	>49.4	28.3	101	59
21		N/T	N/T	>50.1	>50.1	14	25
22		N/T	N/T	>49.8	27.5	>200	>200

Table 2. (Continued)

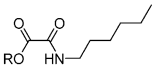
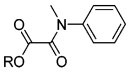
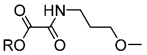
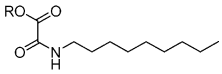
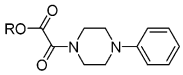
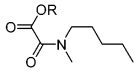
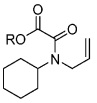
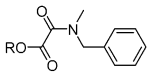
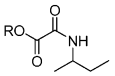
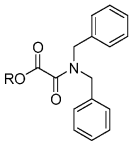
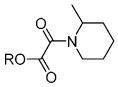
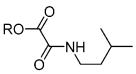
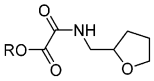
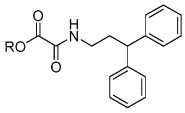
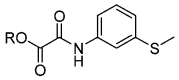
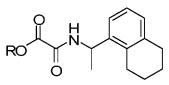
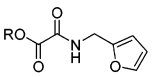
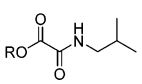
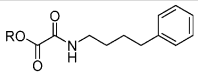
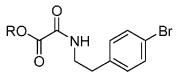
	compound	whole cell assay[a] R = Et IC_{50} (μM) D6	whole cell assay[a] R = Et IC_{50} (μM) W2	whole cell assay[a] R = H IC_{50} (μM) D6	whole cell assay[a] R = H IC_{50} (μM) W2	enzyme assay[b] R = H IC_{50} (μM) *pf*LDH	enzyme assay[b] R = H IC_{50} (μM) *m*LDH
23		>35	>35	28.9	>49.3	47	133
24		N/T	N/T	>50.2	>50.2	>200	>200
25		N/T	N/T	N/A	N/A	43	31
26		N/T	N/T	>50.2	>50.2	88	>200
27		N/T	N/T	>50.0	>50.0	>200	32
28		N/T	N/T	>50.1	>50.1	>200	>200
29		N/T	N/T	>51.2	>51.2	>200	>200
30		N/A	N/A	>129	>129	>200	168
31		N/A	N/A	>172	>172	>200	>200
32		N/A	N/A	>92.9	>92.9	>200	>200
33		N/A	N/A	>146	>146	>200	43
34		N/A	N/A	>157	>157	157	>200
35		N/A	N/A	>144	>144	188	>200
36		N/A	N/A	>88.3	>88.3	35	90
37		20.5	2.79	>118	>118	>200	>200
38		N/A	N/A	>102	>102	146	>200
39		N/A	N/A	>147	>147	>200	>200
40		N/A	N/A	>172	>172	>200	>200

Table 2. (Continued)

	compound	whole cell assay[a] R = Et IC$_{50}$ (μM) D6	W2	whole cell assay[a] R = H IC$_{50}$ (μM) D6	W2	enzyme assay[b] R = H IC$_{50}$ (μM) *pf*LDH	*m*LDH
41		N/T	N/T	24.4	>48.8	41	88
42		N/T	N/T	40.4	25.1	51	>200

[a] R^2= 0.998. [b] Average IC$_{50}$ value of duplicate, mean ± SD was given in Supporting Information. [c] N/T = not tested. [d] N/A = inactive.

not show activity even though their acids have good inhibitory effect, including the two most active oxamic acids (Table 2, oxamic acids **5** and **6**) which inhibit both W2 and D6 strains at a lower micromolar level. The prodrug has been widely used to improve desirable drug properties such as oral bioavailability and solubility. One of strategies for prodrug design is to increase lipophilicity of bioactive drugs to penetrate cell membrane. Esterification of carboxylic acid is one method to improve membrane permeability because polar and charged drugs cannot penetrate cell membrane unless they use membrane transporters. As shown in Table 2, it is unlikely that improved lipophilicity of oxamic acids increases inhibitory activity. These ethyl oxamates may not be hydrolyzed to acids after crossing membrane. Another plausible explanation is that these oxamic acids use a specific transporter to cross membrane.

Activity of Inhibition by pfLDH and mLDH. We also tested our oxamic acid library against the target enzymes *pf*LDH and mLDH to see if these acids have direct and selective inhibitory effects both lactate dehydrogenses. *pf*LDH is the most active enzyme in the glycolytic pathway for energy production, producing abundant lactate in the blood stages of the parasite life cycle. Its unique residues and kinetic properties compared to other LDHs, expecially human isoforms, make it a potential target for development of antimalarial drugs along with other enzymes involved in the glycolytic pathway.[8,25] However, *pf*LDH inhibitors with low inhibitory activities have been reported despite tremendous efforts to develope these inhibitors, such as oxamate derivatives[17] and gossypol-like compounds.[15,16,26−28] Recently, azole-based compounds have been reported to show promising inhibitory activities with selectivity over hLDH along with a crystal structure complexed with OXD1.[29] Because of the high concentration of *pf*LDH in the trophoxoit stage, inhibitors with high inhibitory activity are required to kill *pf*LDH.

We used mLDH instead of hLDH because mLDH is structurally and functionally very similar to hLDH. Of 167 acids, 75 oxamic acids showed various activities against *pf*LDH, mLDH, or both, ranging from millimolar to micromolar.[24] As we expected, most oxamic acids were more active against *pf*LDH than mLDH with a few exceptions. Compound **21** (R = H) was the most active oxamic acid against *pf*LDH with an IC$_{50}$ value of 14 μM, while compound **18** (R = H) was the most active acid against mLDH with an IC$_{50}$ value of 20 μM. Compounds **18**, **21**, and **25** (R = H) were most active oxamic acids against both dehydrogenases. A few oxamic acids were active against only mLDH with IC$_{50}$ values ranging from 32 to 59 μM (Table 2, compounds **19**, **20**, **27**, and **33**). With the exception of oxamic acid **25**, active compounds against *pf*LDH showed 2−5 fold selectivity over mLDH (Table 2, compounds **17**, **21**, **23**, and **36**); **17** showed the best selectivity over mLDH (5 fold), suggesting that these oxamic acids may be used for drug design to develop selective *pf*LDH inhibitors. Interestingly, N,N-disubstitued oxamic acids did not demonstrate significant activities on both dehydrogenases probably because of their steric bulkiness for fitting in the substrate (pyruvate) site of *pf*LDH and mLDH (Table 2, compounds **7**, **8**, **9**, **11**, **13**, **16**, **22**, **24**, **28**, **29**, **30**, and **32**). The most active acids against both dehydrogenases have simple ethyl or propyl methyl ether groups attached to *N*-oxamate (Table 2, compounds **21** and **25**). However, acids that have almost the same groups in terms of chain length and size represent no or less activity on both hydrogenases, indicating that hydrogen bonding of the N-substituted chain may play a critical role in the active sites of enzymes (Table 2, compounds **10**, **12**, **14**, **23**, **26**, **31**, and **34**).

Surprisingly, we did not find any relationship between the whole cell assays and enzyme testing. The most active acids (Table 2, acids **5** and **6**) in the malarial test did not show inhibitory effects against both *pf*LDH and mLDH. In addition, ethyl oxamate **37** showed good antimalarial activity, but its acid did not inhibit both enzymes and was inactive against W2 and D6. It may be that the methythio moiety of ethyl oxamate **37** is oxidized to a sulfoxide or sulfone group which may play a significant role in binding to the enzyme.[30]

Modeling. The molecular docking study was carried out to determine structure−activity relationship and binding modes in the active site of *pf*LDH. We assumed that all oxamic acids are placed in the substrate (pyruvate) binding pocket where the conserved residues in all LDHs are located since oxamic acid is a competitive inhibitor of pyruvate. The crystal structure of *pf*LDH complexed with oxamate and NADH (1LDG) was used for molecular docking.[14] The crystal structure was refined using InsightII 2000.[31] The crystal structure was soaked with water after the addition of the hydrogen atoms. Minimization of the soaked structure was performed with the DISCOVER module (InsightII), using the steepest descents, followed by conjugate gradients, with a force field CVFF94. The refined *pf*LDH structure was applied to molecular docking with the default setting using GOLD 3.01.[32] Constraint was used for the hydrogen bonds between oxamic acids and Arg171, which anchors the

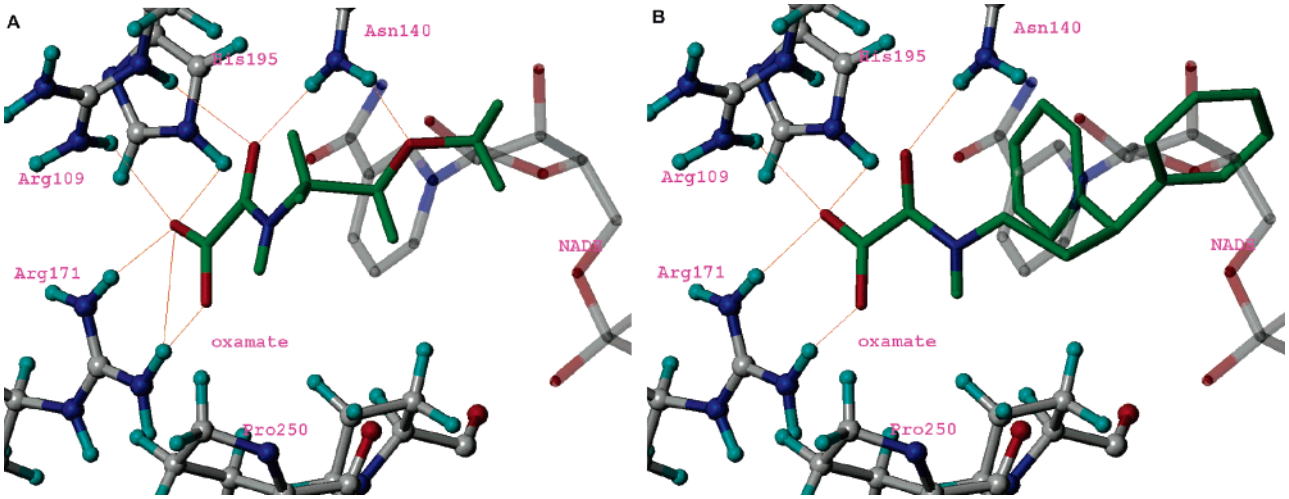


Figure 2. Molecular dockings of oxamic acids **21** and **36** (green) into the active site of *pf*LDH (NADH overlaid on the active site was not involved in docking). (A) Arg71 anchors the acid **21** by forming hydrogen bonds. Arg109 forms hydrogen bonds with two carboxylate oxygen and ketone oxygen atoms of oxamate moiety. The oxygen of the alkyl chain found near the binding site of ribosyl hydroxyl group of NADH (gray) shows hydrogen bonding with Asn140. (B) Acid **36** is anchored by Arg171 via hydrogen bonds. The carboxylate oxygen forms hydrogen bonds with Arg109 and His195. The ketone oxygen of oxamate moiety also shows a hydrogen bond with Asn140. Two phenyl groups are placed closely to the binding sites of the nicotinamide and ribosyl rings.

substrate (oxamate) in the crystal structure of *pf*LDH. The docking of active oxamic acids **21** and **36** may provide insights for the binding mode of the acids, as well as for the design of new inhibitors.

The conserved residues were involved in binding of both oxamic acids, as with oxamate in the crystal structure, but they interact with the conserved residues with a few differences because of the N-substituted groups. Both N-substituted groups occupied part of NADH binding site. In the docking of the oxamic acid **21**, the *N*-methoxyethyl group (green) is found close to the binding site of the ribosyl hydroxyl moiety of NADH, forming hydrogen bonds with side chain of Asn140 (Figure 2A). This docking study explains that oxamic acid **21** is more active than the acid **25**, even though they only differ in the CH_2 chain. Acid **25** is unable to form a hydrogen bond with Asn140. In the docking of oxamic acid **36**, the two phenyl groups (green) were located near where the ribosyl ring and the nicotinamide ring bind in the active site, partly overlapping both ring binding sites (Figure 2B). The residue at 163 (leucine in *pf*LDH, serine in hLDH) is considered to play a critical role in the mechanism of substrate inhibition in *pf*LDH and hLDH.[33] The hydroxyl group of Ser163 in hLDH forms a hydrogen bond with the amide of the nicotinamide via a water molecule, while the amide oxygen of Leu163 participates in hydrogen bonding with the amide of the nicotinamide because of steric hindrance, suggesting that introduction of a hydrogen bonding donor on the phenyl group of oxamic acid **36** or on side chain of oxamic **21** would improve the activity and selectivity for the future design of *pf*LDH inhibitors.[14,25]

Conclusion

Oxamic acid is a competitive inhibitor of pyruvate in the active site of *pf*LDH and other LDHs. A fully automated synthetic strategy was performed to synthesize oxamic acid and ester derivatives. The catch and release method was used to obtain the final acid compounds with high purity after ester compounds were synthesized. Randomly selected oxamic acids (20) were analyzed and showed acceptable high purities (>95%) despite low overall yields (20−70%). Of 167 compounds, 87 showed various micromolar activities against *P. falciparum* (W2, D6). We have found that two oxamic acids (Table 2, acids **5** and **6**) may be potential lead compounds for drug-resistant malaria. It was shown that ethyl oxamates did not increase inhibitory activity as prodrugs probably because of hydrolysis by cells or an inability to penetrate cell membranes. Seventy-five compounds from the oxamic acid library show various activities against *pf*LDH, mLDH, or both. Even though we did not find a relationship between the whole cell assays (W2 and D6 strains) and enzyme bioassays, we did find that oxamic acids are prospective compounds that selectively inhibit *pf*LDH over mLDH. Bulky *N*,*N*-disubstituted oxamic acids did not have inhibitory activities, while some of oxamic acids containing a phenyl moiety and a hydrophobic group, such as alkyl chains, show good activities, indicating that oxamic acids can be potential inhibitors. Oxamic acids **17**, **21**, **37**, **23**, **41**, and **42** may be used as lead structures for designing novel inhibitors of *pf*LDH.

Experimental Methods

All solvents were anhydrous unless otherwise noted. All NMR spectra were recorded in $CDCl_3$, using a Bruker Advance DPX 400 and an internal TMS standard. ^{1}H NMR spectra were recorded at 400 MHz, and shifts are reported as parts per million relative to TMS. Analytical LC/MS was carried out on a Waters FractionLynx LC/MS system using a Micromass ZQ quadrapole mass spectrometer and negative electrospray ionization with an Xterra C_{18} column. Automated synthesis was performed using an argonaut Trident Synthesizer and an argonaut Trident Sample Processing Station. Evaporation of solvents was done using a GeneVac HT-12 evaporation system.

For biological activities, two parasite strains were used. The W2 strain is an Indochina *P. falciparum* that is resistant to chloroquine, pyrimethamine, and sulfadoxine. The D6 strain is an African *P. falciparum* that is resistant to mefloquine.

Synthesis of Ethyl Oxamates. Solid sodium bicarbonate was added to 167 Trident reaction vessels using the Trident sample processing station's (SPS) solid handling system. The reaction vessels were then placed in the Trident combinatorial synthesizer. After a preset reaction time (10 h) at 0 °C, the solutions were collected from the cassettes and transferred to scintillation vials. The reaction mixtures were then transferred to the SPS for automated SPE column (hydromatrix packing) workup of each reaction. After the workup, the solvents were evaporated using a GeneVac H-12. The resulting mixtures were evaporated and prepared for the next reaction step.

Synthesis of Oxamic Acids. Esters were transferred to the reaction cassettes. Resin was then added to each vessel using SPS. The reaction cassettes were transferred to the Trident, and solvent was added, followed by reaction incubation at 65 °C for 16 h. After the reaction time, the cassette wells were drained and washed with solvent, removing any unreacted material; 1 N HCl was subsequently added to each vessel, and they were incubated for 0.5 h to furnish free acids. The solution was collected to scintillation vials and then transferred to the SPS for a liquid−liquid extraction (methylene chloride/water). The organic layer of each vessel was evaporated by GeneVac to give pure oxamic acids.

Bioassay. The assay was performed using red blood cells infected with *P. falciparum*. Compounds were dissolved in DMSO at a starting concentration of 25 μg/mL. They were then subsequently serially diluted 5 times to give a range of concentrations from 1.6 ng/mL to 25 μg/mL. The assay itself was based on the uptake of hypoxanthine as an indication of parasite proliferation. The parasite was incubated with the drug candidate for 42 h, followed by collection of the cells and [H^3]-hypoxanthine uptake measurement by scintillation. Two strains were used, chloroquine resistant (W2) and mefloquine resistant (D6).

The recombinant *pf*LDH used for the target enzyme assay is described in details in the published article.[34] The mLDH (bovine heart) was purchased from Calzyme Laboratories, San Luis Obispo, California. The assay was performed in a 96-well plate, which can be easily adapted to a HTS mode. Initial rapid screening of the compounds was performed at concentrations of 50 and 100 μg/mL. Briefly, the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4) and pyruvate (200 μM). The reaction was initiated with the addition of NADH (250 μM), and the decrease in absorbance at 340 nm was monitored for 5 min at 25 °C. The hit compounds showing inhibition of more than 50% at the 50 μg/mL concentration were retested, and their IC_{50} values were determined.

Oxo{[3-(trifluoromethyl)benzyl]amino}acetic Acid (1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.7 (s, 1H), 7.5 (s, NH), 7.4 (d, J = 7.5 Hz, 1H), 7.35 (d, J = 7.6 Hz, 1H), 7.3 (t, 1H), 4.6 (d, J = 6.0 Hz, 2H).

Oxo{[3-(trifluoromethyl)phenyl]amino}acetic Acid (2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.1 (s, 1H), 8.2 (s, 1H), 7.7 (d, J = 7.7 Hz, 1H), 7.6 (d, J = 7.7 Hz,1H), 7.3 (t, 1H).

(1,3-Benzodioxol-5-ylamino)(oxo)acetic Acid (3). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.6 (br s, NH), 7.2 (d, J = 8.8 Hz, 1H), 6.8 (d, J = 7.8 Hz, 1H), 6.7 (s, 1H), 3.7 (s, 2H)

[(Cyclohexylmethyl)amino](oxo)acetic Acid (4). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.4 (br s, 1H), 7.5 (s, NH), 3.1 (t, 2H), 1.6 (t, 4H), 1.2 (m, 4H), 1.0 (m, 2H).

Oxo[(4-phenoxyphenyl)amino]acetic Acid (5). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.9 (s, H), 7.6 (d, J = 8.8 Hz, 2H), 7.4 (d, J = 8.2 Hz, 2H), 6.9 (m, 5H).

[(3-Chlorobenzyl)amino](oxo)acetic Acid (6). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.6 (br s, 1H), 7.3 (d, J = 4.4 Hz, 1H), 7.25 (s, 1H), 7.15 (d, J = 3.9 Hz, H), 4.5 (d, J = 6.2 Hz, 2H).

[(2,6-Dibromophenyl)amino](oxo)acetic Acid (7). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.8 (s, H), 8.0 (br s, H), 7.7 (d, J = 8.0 Hz, 2H), 7.1 (t, 1H).

[(4-Chloro-2-methylphenyl)amino](oxo)acetic Acid (8). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.8 (s, H), 8.0 (s, H), 7.3 (d, J = 5.6 Hz, 2H), 2.3 (s, 3H).

[(4-Bromo-2,3,5,6-tetrafluorophenyl)amino](oxo)acetic Acid (9). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.7 (s, 1H).

[(1-Methyl-3-phenylpropyl)amino](oxo)acetic Acid (10). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.3 (m, 5H), 2.6 (t, 2H), 2.0 (m, 2H), 1.4 (m, 1H), 1.3 (d, J = 6.6 Hz, 3H).

Oxo[(3-phenoxyphenyl)amino]acetic Acid (11). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.8 (s, 1H), 7.4 (m, 5H), 7.2 (d, J = 7.4 Hz, 1H), 7.1 (t, 1H), 6.8 (d, J = 7.5 Hz, 1H).

Oxo[(2-phenylethyl)amino]acetic Acid (12). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.3 (m, 5H), 3.8 (t, 2H), 2.9 (t, 2H).

Oxo{[4-(pentyloxy)phenyl]amino}acetic Acid (13). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.8 (s, H), 7.6 (d, J = 8.9 Hz, 2H), 6.9 (d, J = 8.9 Hz, 2H), 4.0 (t, 2H), 1.8 (t, 2H), 1.6 (m, 6H), 0.9 (t, 3H).

[(2-Chloro-4,6-dimethylphenyl)amino](oxo)acetic Acid (14). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.6 (s, 1H), 7.1 (s, 1H), 7.0 (s, 1H), 2.3 (s, 3H), 2.2 (s, 3H).

[(2-*sec*-Butylphenyl)amino](oxo)acetic Acid (15). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.9 (s, 1H), 9.1 (s, 1H), 7.9 (d, J = 7.0 Hz, 1H), 7.3 (m, 3H), 2.8 (m, 1H), 1.8 (m, 2H), 1.3 (d, J = 8.3 Hz, 3H), 0.9 (t, 3H).

[(4-Chloro-2-fluorophenyl)amino](oxo)acetic Acid (16). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.4 (br s, 1H), 8.3 (d, J = 9.1 Hz, 1H), 7.25 (d, J = 7.9 Hz, 1H), 7.0 (s, 1H).

Oxo[(4-pentylphenyl)amino]acetic Acid (17). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.0 (s, H), 7.6 (d, J = 8.35 Hz, 2H), 7.2 (d, J = 8.3 Hz, 2H), 2.5 (t, 2H), 1.7 (m, 2H), 1.5 (m, 4H), 1.0 (t, 3H).

[(2,5-Dichlorophenyl)amino](oxo)acetic Acid (18). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.5 (br s, 1H), 8.3 (s, J = 8.0 Hz, 1H), 7.4 (d, J = 8.6 Hz, 1H), 7.2 (d, J = 8.5 Hz, 1H).

[(4-Methoxyphenethyl)amino]-oxoacetic Acid (19). ^{1}H NMR (400 MHz, DMSO): δ 8.5 (br, NH), 7.10 (d, 2H), 6.83 (d, J = 8.0, 2H), 3.69 (s, 3H), 3.25 (t, 2H), 2.67 (t, 2H).

[(2-Cyanophenyl)amino)-oxoacetic Acid (20). ^{1}H NMR (400 MHz, DMSO): δ 10.9 (s, NH), 8.3 (s, 1H), 8.1 (d, J = 6.4 Hz, 1H), 7.5 (m, 2H).

Acknowledgment. We would like to thank Walter Reed Army Institute for in vitro testing and the CDC for providing financial support.

Supporting Information Available. ^{1}H NMR, HPLC, LC/MS, and HRMS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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CC060110N