Discovery of NCT-501, a Potent and Selective Theophylline-Based Inhibitor of Aldehyde Dehydrogenase 1A1 (ALDH1A1)

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Supporting Information

ABSTRACT: Aldehyde dehydrogenases (ALDHs) metabolize reactive aldehydes and possess important physiological and toxicological functions in areas such as CNS, metabolic disorders, and cancers. Increased ALDH (e.g., ALDH1A1) gene expression and catalytic activity are vital biomarkers in a number of malignancies and cancer stem cells, highlighting the need for the identification and development of small molecule ALDH inhibitors. A new series of theophylline-based analogs as potent ALDH1A1 inhibitors is described. The optimization of hits identified from a quantitative high throughput screening (qHTS) campaign led to analogs with improved potency and early ADME properties. This chemotype exhibits highly selective inhibition against ALDH1A1 over ALDH3A1, ALDH1B1, and ALDH2 isozymes as well as other dehydrogenases such as HPGD and HSD17β. Moreover, the pharmacokinetic evaluation of selected analog 64 (NCT-501) is also highlighted.

INTRODUCTION
The human genome encodes 19 aldehyde dehydrogenase (ALDH) enzymes that metabolize reactive aldehydes to their corresponding carboxylic acid derivatives.1 Unbalanced biological activity of ALDHs and specific contribution to their metabolism pathway have been associated in a variety of disease states, including alcoholic liver disease, Sjögren–Larsson syndrome (SLS), type 2 hyperprolinemia, hyperammonemia, Parkinson’s disease, and cancers.2–5 It is well established that overexpression of certain ALDHs, especially ALDH1A1, in a number of malignancies and cancer stem cells (CSCs) correlates with poor prognosis and tumor aggressiveness and that ALDH1A1 is linked to drug resistance in traditional cancer chemotherapy.6–8 Although the majority of the research community has considered ALDH1A1 as a marker of cancer stem cells and a predictor of the prognosis, this enzyme also plays an important role in the biology of tumors and cancer stem cells.9–10 Initial evidence toward this end has been established using nonspecific ALDH inhibitors and siRNA silencing techniques.9 More recently, ALDH1A1 has also been implicated in obesity11,12 and inflammation,13 suggesting that inhibition of this enzyme may offer new therapeutic options for obese patients and patients with Crohn’s disease.13 These data suggest that discovery of small molecule ALDH inhibitors is a prudent approach for identifying potential cancer and/or CSC-directed therapeutics as well as a better understanding of the physiological and pathophysiological actions of the ALDHs.14,15

Among reported ALDH inhibitors,16 CVT-1021 (1) represents an attractive small molecule that effectively inhibits both ALDH1A1 (1.3 μM) and ALDH2 (0.029 μM) (Figure 1).17 This reversible inhibitor was designed and optimized based on the interaction of daidzin (2) with human ALDH2.18,19 As a result of the potent ALDH2 inhibition, 1 has been developed for the treatment of alcoholism and has also demonstrated activity in the reduction of cocaine-seeking behavior.20 More recently, indolinedione-based analogs (e.g., 3)21 and substituted tricyclic pyrimidinone 422 reported by

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Hurley and co-workers exhibit significant hALDH1A1 inhibitory activity (0.02 and 4.6 μM for 3 and 4, respectively) and were reported to be selective inhibitors against other ALDH isozymes such as ADLH2 and ALDH3A1. The 3-carbonyl functionality of these indolinedione analogs appears to form reversible covalent adducts with catalytic cysteine residue, particularly with ALDH3A1, and additional mechanism of action studies suggest that these molecules are substrate competitive inhibitors.

Furthermore, the inhibition of ALDH1A1 activity by compound 4 resulted in disruption of ovarian cancer spheroid formation and cell viability. Despite the favorable activity of these compounds, to the best of our knowledge a systematic and thorough medicinal chemistry effort had not been reported on ALDH1A1 inhibitors. As such, our efforts toward identifying novel, more druglike small molecule of ALDH1A1 inhibitors for cancer and CSCs research began with a qHTS campaign (PubChem assay identifier 1030: http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=1030, last modified March 15, 2010) that ultimately led to identification of the theophylline-based chemotype, exemplified as hits 5 (0.084 μM) and 6 (0.17 μM), along with other active analogs (Figure 1).

Furthermore, we aimed to fill the need for optimized druglike ALDH1A1 inhibitors with improved ADME/PK properties required for evaluating the fundamental biological roles (e.g., cancer and CSCs) of ALDH1A1 in vivo. These theophylline-based hits (e.g., 5 and 6) are structurally distinct from existing ALDH inhibitors and appear quite specific with minimal activity across 570 assays screened in PubChem, which suggests the theophylline core is a suitable scaffold for further investigation.

Herein, we report the systematic medicinal chemistry optimization and structure–activity relationships (SARs) of theophylline-based analogs as potent and selective ALDH1A1 inhibitors.

## CHEMISTRY

The synthetic routes that allow access to the majority of designed molecules with modification of two arms (N7 and C8) of the theophylline core are described in Scheme 1. Alkylation of commercially available 8-chlorotheophylline 7 with representative bromides provided intermediates 8–10 in which the Cl functionality is poised for derivatization of the C8 substitution. For instance, analog 11 was readily prepared by utilizing a substitution protocol with ethoxycarbonylpiperazine under microwave irradiation. Analogs 12–13 were synthesized similarly by Cl displacement with methyl 4-hydroxybenzoate followed by hydrolysis and methyl amide formation. The key intermediates 14–18 with diverse C8 substitutions that focused on the variation of ring size (four-membered vs six-membered rings) and linkers (O or NH) were generated in a similar manner after Boc deprotection.

With these intermediates in hand, the piperidine moiety was further reacted with halosubstituted heteroarenes to give 19 and 20, acylated or sulfonated with substituted carbonyl chloride or sulfonyl chloride, respectively, to furnish analogs 21–28 (see Table 1). These transformations allowed for efficient SAR investigations around ethyl carbamate portion of qHTS hits. Further utilizing intermediate 16 by acylation with cyclopropanecarbonyl chloride followed by removal of the benzyl group under palladium-catalyzed hydrogenation conditions gave intermediate 22. This is then poised for derivatization of the N7 position by alkylation or a Mitsunobu reaction with the requisite halides and linkers (O or NH) to furnish analogs 25–30 (see Table 1).

The piperazine-type analogs, including resynthesized hits 5 and 6, were prepared in a different manner as highlighted in Scheme 2. The hydroxyl intermediate 24 was generated via a two-step sequence involving acylation of 23 with hydroxysacetic acid followed by cyclization under basic conditions. Further alkylation with various alkyl and benzyl bromides followed by oxidation with Dess–Martin periodinane gave aldehyde 25. Subsequent reductive amination with corresponding piperazine derivatives using sodium triacetoxyborohydride as the reducing agent afforded qHTS hits 5 and 6 and desired analogs 31–36 (see Table 3).

## RESULTS AND DISCUSSION

### Structure–Activity Relationships (SARs)

The inhibitory activity of these analogs was tested using human ALDH1A1
Scheme 1. Synthetic Routes Assessing Theophylline-Based ALDH1A1 Inhibitors

Reagents and conditions: (a) R1-Br, K$_2$CO$_3$, DMF, rt to 60 °C, 1–24 h, 8 (98%), 9 (95%), 10 (99%); (b) 1-ethoxycarbonylpiperazine, DMSO, microwave, 160 °C, 30 min, 11 (58%); (c) methyl 4-hydroxybenzoate, K$_2$CO$_3$, DMF, microwave, 160 °C, 1 h, 66–83%; (d) 1.5 N LiOH$_{aq}$/THF/MeOH, 50 °C, 2–3 h, 48–70%; (e) MeNH$_2$ (2 M in THF), HATU, DMF, (i-Pr)$_2$NEt, rt, 2 h, 46–59%; (f) N-Boc-4-hydroxypiperidine or N-Boc-4-hydroxyazetidine (for 14–16 and 18), NaH, DMF, rt, 0.5–2 h, 81–96%; or N-Boc-4-aminoazetidine (for 17), DMSO, microwave, 160 °C, 45 min, 66%; (g) 4 M HCl in dioxane, rt, 3–24 h, 90–99%; (h) 2-chloro-4-methylpyrimidine or 2-bromo-5-methylthiazole, K$_2$CO$_3$, DMF, microwave, 160 °C, 1 h; (i) corresponding substituted carbonyl chloride, Et$_3$N, CH$_2$Cl$_2$, rt, 0.5–1 h, for 26–27, 29–34, and 36–38; or ethyl isocyanate, Et$_3$N, CH$_2$Cl$_2$, rt, 0.5 h, for 28; or cyclopropanesulfonyl chloride, Et$_3$N, CH$_2$Cl$_2$, rt, 0.5 h, for 35; (j) 16, cyclopropanecarbonyl chloride, Et$_3$N, CH$_2$Cl$_2$, rt, 0.5 h, 21 (96%); (k) Pd(OH)$_2$ (20 mol %), EtOAc/EtOH, 70 °C, 24 h, 22 (75%); (l) corresponding alkyl or benzyl bromide or chloride, K$_2$CO$_3$, DMF, 50–60 °C, 3–24 h, for 39–52; or corresponding hydroxyl compounds, Bu$_2$O$_2$CN=NCO$_2$Bu, PPH$_3$, THF, rt or 60 °C, 1–3 h, for 53–56; (hALDH1A1). Importantly, the resynthesized material of 5 and 6 had comparable potency to that obtained in the primary HTS assay (Table 1). As part of our medicinal chemistry optimization campaign, we also characterized all synthesized compounds for early ADME (eADME) data such as microsomal stability, PAMPA permeability, and aqueous solubility (pH 7.4) to concurrently develop structure–property relationships. Thus, we immediately realized that our hit compounds possessed poor rat liver microsomal (RLM) stability (5, $t_{1/2} = 5$ min) which would need to be improved along with retaining the desired potency and selectivity profile. Initial structural modifications were primarily focused on the R$_2$ portion as shown in Table 1. With the 3-methylbenzyl R$_1$ substitution held constant, direct attachment of the piperazine substituent (11) or rearranging the nitrogen atom from piperazine to amino-piperidine substituent (26) resulted in ~14-fold loss of potency. While the hydroxypiperidine analog exhibited reasonable inhibitory activity (27, 213 nM), removal of the ethyl carbamate functionality significantly dropped the potency to the micromolar range (14, 13.7 μM), indicating further investigation of this position was required. Subsequently, changing the ethyl carbamate to ethyleneurea (28) or short chain amides (29 and 30) minimally affected the potency. However, increasing the size of side chain, such as 31 and 32, resulted in a loss of potency. In comparison with linear amides such as 31, the branched amides (e.g., 33 and 34), particularly cyclopropylamide, showed significant improvement in potency (34, 84 nM). Switching the cyclopropylamide to sulfonamide (35) caused a 3-fold potency loss. Other attempts to structurally mimic ethyl carbamate with simple methylheteroarenes, such as methylpyrimidine (19) and methylthiazole (20), were unsuccessful and led to inhibitory activities in the micromolar range (3.8–19.2 μM). The isopentyl-substituted analogs again demonstrated that the cyclopropylamide contributed significantly to the potency (37, 87 nM vs 36, 138 nM). Furthermore, reducing the ring size of piperidine to four-membered azetidine also caused a 6-fold potency drop (37 vs 38). Finally, the phenoxy moiety was found to be a suitable replacement of 4-hydroxyperipiderine and improved the inhibition dramatically (12 and 13, 57–89 nM). In comparison to the lead analogs (such as 5, 6, 36, and 37) with aqueous solubility greater than 62 μg/mL, the phenoxy analogs exhibited poor solubility (<1 and 6.1 μg/mL) for analogs 12 and 13, respectively. As such, despite the potent inhibitory
potency, these compounds were deprioritized for further development. Beyond the demonstrated potency increase by cyclopropylamide modification, no further improvement in RLM stability was observed. Other key SAR findings taken from Table 1 include the following: (1) the 3-methylbenzyl...
group seemed responsible for RLM instability as suspected (e.g., 5 vs 6; 12 vs 13; 34 vs 37), and (2) the methyl piperazine analogs are slightly more potent than 4-hydroxypiperidine analogs, e.g., 5 (40 nM) and 6 (77 nM) vs 27 (213 nM) and 36 (138 nM), respectively.

Table 3. Combination of Favored R<sup>1</sup> Substitution and Piperazine-Type R<sup>2</sup> Substitution

<table>
<thead>
<tr>
<th>Cpd</th>
<th>R&lt;sup&gt;1&lt;/sup&gt;</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ± SD (nM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RLM&lt;sup&gt;b&lt;/sup&gt; (t&lt;sub&gt;1/2&lt;/sub&gt;, min)</th>
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<tbody>
<tr>
<td>57</td>
<td></td>
<td></td>
<td>33 ± 5</td>
<td>6</td>
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<td>58</td>
<td></td>
<td></td>
<td>65 ± 14</td>
<td>26</td>
</tr>
<tr>
<td>59</td>
<td></td>
<td></td>
<td>256 ± 48</td>
<td>&gt; 30</td>
</tr>
<tr>
<td>60</td>
<td>F</td>
<td></td>
<td>390 ± 73</td>
<td>20</td>
</tr>
<tr>
<td>61</td>
<td>Cl</td>
<td></td>
<td>69 ± 8</td>
<td>13</td>
</tr>
<tr>
<td>62</td>
<td>3-F</td>
<td></td>
<td>86 ± 26</td>
<td>20</td>
</tr>
<tr>
<td>63</td>
<td>4-F</td>
<td></td>
<td>35 ± 10</td>
<td>5</td>
</tr>
<tr>
<td>64</td>
<td>(NCT-501)</td>
<td></td>
<td>40 ± 23</td>
<td>&gt; 30</td>
</tr>
<tr>
<td>65</td>
<td></td>
<td></td>
<td>92 ± 35</td>
<td>&gt; 30</td>
</tr>
<tr>
<td>66</td>
<td></td>
<td></td>
<td>69 ± 31</td>
<td>&gt; 30</td>
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<td>67</td>
<td></td>
<td></td>
<td>320 ± 111</td>
<td>5</td>
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</tbody>
</table>

<sup>a</sup>Values with standard deviation (SD) represent the average of three to six runs from one to two experiments. The IC<sub>50</sub> values are shown in nM unless otherwise specified. <sup>b</sup>RLM represents rat liver microsomal stability conducted at NCATS in the presence of NADPH.

Given that the cyclopropylamide moiety improved inhibition of ALDH1A1, we maintained this motif on the second set of analogs aimed at addressing the relationships between R<sup>1</sup> substitution and RLM instability from newly identified leads 34 and 37. Analogs with various substituted aryl and alkyl R<sup>1</sup> substitutions were synthesized and screened (Table 2). Removal of R<sup>1</sup> group (22) led to a significant loss in activity, while benzyl (21) or phenylethyl (39) only slightly diminished potency in a comparison with 34. Importantly, these analogs exhibit improved RLM stability, leading us to suspect the 3-Me group as key factor in the observed RLM instability. This was further supported by the finding that the RLM stability is greatly influenced by substitution pattern of phenyl ring. For instance, electron-withdrawing groups, such as CF<sub>3</sub> (41), CI (42), or F (45), are preferred, giving marked improvement in RLM stability (t<sub>1/2</sub> ≥ 20 min), though the IC<sub>50</sub> values are decreased compared to the Me substitution (34). The electron-donating group, exemplified by OMe (40), is undesirable in both potency and RLM stability. Interestingly, moving these substitutions to 2-position, such as 43 and 46 vs 42 and 45, respectively, gained potency back while improving RLM stability. However, the 2-F substitution did not overcome the undesired RLM issue caused by 3-Me substitution, as analog 47 had a RLM stability of only 4 min. Replacing the phenyl ring with pyridine produced analogs with improved RLM stability but with significantly lower potency (48, 12.5 μM). Finally, the bicyclic naphthylene substituent also produced potent analog 49 (91 nM) but again resulted in a RLM stability issue.

In parallel, investigation of alkyl type R<sup>1</sup> substitution was conducted (Table 2). Compared to lead compound 37, the shorter alkyl chain (50) increased RLM stability, albeit with significant loss of potency to 3.9 μM, while the opposite results were obtained with longer alkyl chain analog 51 (81 nM; RLM, 3 min), leading us to focus on modifying the isopentyl portion. Changing to a more hydrophobic dimethylbutyl side chain (52) showed comparable potency (76 nM) and RLM stability (12 min) to 37. Further tethering the dimethyl groups of the isopentyl moiety led to analogs with either improved RLM stability (example, 53, RLM, 25 min) or retained potency (54, 30 nM) but not both. Surprisingly, the oxetane analog (56) completely lost inhibitory activity. The data obtained for R<sup>1</sup> modification suggest that hydrophobic interactions with enzyme in this region are preferred. This is consistent with

Scheme 2. Synthesis of Piperazine-Substituted, Theophylline-Based ALDH1A1 Inhibitors<sup>4</sup>

<sup>4</sup>Reagents and conditions: (a) 2-hydroxyacetic acid, neat, 100 °C, 3 h, 49%; (b) NaOH (aq), 100 °C, 2 h, 24 (82%); (c) R<sup>1</sup>-Br, K<sub>2</sub>CO<sub>3</sub>, DMF, rt to 60 °C, 3–24 h; (d) Dess–Martin periodinane, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C to rt, 1–2 h; (e) piperazine or homopiperazine derivatives (or its HCl salt), NaBH(OAc)<sub>3</sub>, Et<sub>3</sub>N, rt, 4 h.

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Table 4. Selectivity against Other ALDH Isozymes and Dehydrogenases

<table>
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<tr>
<th>Compd</th>
<th>hALDH1A1</th>
<th>hALDH1B1</th>
<th>hALDH3A1</th>
<th>hALDH2</th>
<th>HPGD</th>
<th>HSD17/4</th>
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<td>5</td>
<td>0.040</td>
<td>&gt;57</td>
<td>24.3</td>
<td>2.4</td>
<td>&gt;57</td>
<td>&gt;57</td>
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<tr>
<td>6</td>
<td>0.077</td>
<td>&gt;57</td>
<td>&gt;57</td>
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<td>&gt;57</td>
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<tr>
<td>12</td>
<td>0.057</td>
<td>&gt;57</td>
<td>24.3</td>
<td>6.1</td>
<td>&gt;57</td>
<td>&gt;57</td>
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<tr>
<td>13</td>
<td>0.089</td>
<td>&gt;57</td>
<td>1.5</td>
<td>2.4</td>
<td>&gt;57</td>
<td>&gt;57</td>
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<tr>
<td>27</td>
<td>0.213</td>
<td>&gt;57</td>
<td>4.9</td>
<td>3.9</td>
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<td>&gt;57</td>
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<tr>
<td>34</td>
<td>0.084</td>
<td>&gt;57</td>
<td>&gt;57</td>
<td>&gt;57</td>
<td>&gt;57</td>
<td>&gt;57</td>
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<tr>
<td>36</td>
<td>0.138</td>
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<td>&gt;57</td>
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<tr>
<td>37</td>
<td>0.087</td>
<td>&gt;57</td>
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<tr>
<td>57</td>
<td>0.033</td>
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<td>&gt;57</td>
<td>&gt;57</td>
<td>&gt;57</td>
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<td>64</td>
<td>0.040</td>
<td>&gt;57</td>
<td>&gt;57</td>
<td>&gt;57</td>
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<td>&gt;57</td>
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Compounds noted as >57 μM represent a very weak or no inhibition.  

ALDH3A1 and ALDH2 inhibitions (12 and 13). Finally, removal of 3-methyl group resulted in enhanced selectivity (e.g., 58 vs 57). These data, along with the above-mentioned SAR and structure–property relationship (SPR) explorations, suggested that compound 64 should be selected for further evaluation and characterization. Accordingly, compound 64 was profiled for off-target kinase activity using DiscoveRx KINOMEscan screening platform. The results indicated 64 is a clean inhibitor with no significant hits (i.e., inhibition of ≥55% at 10 μM) observed against more than 450 human kinases (Supporting Information Figure S1). To further evaluate any potential off-target activity of 64, we profiled it against the gpcrMAX panel which contains 168 GPCRs covering 60 receptor families and is run in both agonist and antagonist mode. As with the kinase profile, compound 64 demonstrated very little activity in either agonist or antagonist mode (Supporting Information Figure S2).  

Mechanism Studies. During the preparation of this manuscript, Hurley and co-workers published an X-ray crystal structure of a similar theophylline-based analog with ALDH1A1. This structure along with kinetic studies indicated that these analogs are substrate noncompetitive inhibitors, which bind near the solvent exposed exit of substrate-binding site. Independent kinetic studies in our laboratory corroborated these findings (data not shown). This mode differs from the previously reported indolinedione and tricyclic pyrimidinone type of inhibitors (e.g., 3 and 4) which both demonstrated substrate competitive inhibition. To further investigate the mechanism of action, a rapid dilution experiment using 64 was conducted to determine the reversibility of the inhibition. The reaction time courses were collected after incubation with 64 at an IC50 concentration (red triangle, Figure 2) and rapid dilution of enzymatic reaction. The diluted sample rapidly recovered enzymatic activity (orange box), similar to the low-compound incubated control (blue triangle) or in the absence (green circle) of inhibitor, indicating that compound 64 demonstrates reversible inhibition of ALDH1A1.  

Pharmacokinetics and in Vivo ADME Profiles. After demonstrating desirable potency and selectivity with a better understanding of the mechanism of action, compound 64, which possessed favorable kinetic aqueous solubility (>60 μg/mL) and RLM stability (>30 min), was further evaluated for its pharmacokinetics (PK) in CD1 mice. The compound was dosed at 2 mg/kg, 10 mg/kg, and 30 mg/kg for intravenous (iv), oral (po), and intraperitoneal (ip) administration, respectively. Brain and liver tissues are also collected and
low oral bioavailability seems to be attributed to the rapid clearance via first pass metabolism as mentioned above.

### CONCLUSION

The overexpression of specific ALDH isozymes in certain CSCs suggests ALDHs as potential targets for cancer and CSC-directed therapeutics. In addition, it has been shown that selective targeting of ALDH isozymes prevents spheroids formation in vitro and the size of xenograft tumors formed in vivo.33 In this study, we performed a systematic medicinal chemistry optimization of theophylline-based qHTS hits that ultimately led to potent ALDH1A1 inhibitors with improved eADME properties. This chemotype demonstrated a high degree of selectivity over other ALDH isozymes (ALDH1B1, ALDH3A1, and ALDH2) and other dehydrogenases (HPGD and HSD17β4). A rapid dilution experiment suggested the selected analog 64 is a reversible inhibitor. The PK study demonstrated that analog 64 had reasonable drug exposure via ip administration and should be suitable for in vivo proof of concept animal studies. However, further improvement of the half-life and oral bioavailability would be beneficial if this compound is to be dosed po. Current studies are focused on evaluating this class of molecules in cell-based studies (e.g., patient-derived CSCs) and other disease-relevant models, which could benefit from selective pharmacological ALDH1A1 inhibition. As the combined activity of two or more ALDH isoforms is likely to be associated with a specific cancer, the feasibility of using a combination of isoform selective inhibitors or a dual inhibitor as a clinical prognostic concept should also be considered.15

### EXPERIMENTAL SECTION

#### General Methods for Chemistry

All air or moisture sensitive reactions were performed under positive pressure of nitrogen with oven-dried glassware. Chemical reagents and anhydrous solvents were obtained from commercial sources and used as is. Preparative purification was performed on a Waters semipreparative HPLC instrument. The column used was a Phenomenex Luna C18 (5 μm, 30 mm × 75 mm) at a flow rate of 45 mL/min. The mobile phase consisted of acetonitrile and water (each containing 0.1% trifluoroacetic acid). A gradient from 10% to 50% acetonitrile over 8 min was used during the purification. Fraction collection was triggered by UV detection (220 nm). Analytical analysis for purity was determined by two different methods denoted as final QC methods 1 and 2.

#### Method 1

Analysis was performed on an Agilent 1290 Infinity series HPLC instrument. UHPLC long gradient equivalent from 4% to 100% acetonitrile (0.05% trifluoroacetic acid) in water over 3 min run time of 4.5 min with a flow rate of 0.8 mL/min. A Phenomenex Luna C18 column (3 μm, 3 mm × 75 mm) was used at a temperature of 50 °C.

#### Method 2

Analysis was performed on an Agilent 1260 with a 7 min gradient from 4% to 100% acetonitrile (containing 0.025% trifluoroacetic acid) in water (containing 0.05% trifluoroacetic acid) over 8 min run time at a flow rate of 1 mL/min. A Phenomenex Luna C18 column

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Table 5. Pharmacokinetic Results of 64 in CD1 Mice

<table>
<thead>
<tr>
<th>admin (dosage)</th>
<th>sample</th>
<th>C_{max} (ng/mL)</th>
<th>T_{1/2} (h)</th>
<th>AUC_{24h} (b·ng/mL)</th>
<th>V_{ss} (L/kg)</th>
<th>CL (mL·min^{-1}·kg^{-1})</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iv (2 mg/kg)^a</td>
<td>plasma</td>
<td>2070^b</td>
<td>0.5</td>
<td>332</td>
<td>1.8</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>ip (30 mg/kg)^a</td>
<td>plasma</td>
<td>8080</td>
<td>0.7</td>
<td>5670</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>po (10 mg/kg)^a</td>
<td>plasma</td>
<td>696</td>
<td>0.4</td>
<td>484</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>po (10 mg/kg)^a</td>
<td>brain</td>
<td>1130</td>
<td>0.4</td>
<td>261</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>po (10 mg/kg)^a</td>
<td>liver</td>
<td>2690</td>
<td>0.4</td>
<td>1880^d</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a n = 3. The compound (64) was formulated as solution in 30% HP/CD in saline. ^b C_{max} = C_{0} (t = 0) for iv administration. ^c Brain/plasma (B/P) ratio of 0.54. ^d Liver/plasma (L/P) ratio of 3.88.

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Figure 2. Rapid dilution assay. The experiment was conducted using 64 as the inhibitor in the presence of ALDH1A1 (20 nM), substrate (propionaldehyde, 80 μM), and cofactor (NAD+, 1 mM) in 1536-well format.
(3 μm, 3 mm × 75 mm) was used at a temperature of 50 °C. Purity determination was performed using an Agilent diode array detector for both method 1 and method 2. Mass determination was performed using an Agilent 6130 mass spectrometer with electrospray ionization in the positive mode. All of the analogs for assay have purity greater than 95% based on both analytical methods. 1H NMR spectra were recorded on Varian 400 MHz spectrometers. High resolution mass spectrometry results were recorded on Agilent 6210 time-of-flight LC/MS system.

Representative Synthetic Procedures. Preparation of 8-Chloro-1,3-dimethyl-7-(3-methylbenzyl)-1H-purine-2,6-(3H,7H)-dione (8). To a mixture of 8-chloro-1,3-dimethyl-1H-purine-2,6(3H,7H)-dione (7, 21.5 g, 10 mmol) and K2CO3 (207 mg, 1.5 mmol). Then, DMF (3 mL) was added. The mixture was stirred at rt for 5 min, and then diluted with EtOAc (5 mL). The mixture was stirred at rt for 30 min and then heated at 60 °C for 2 h. The mixture was then poured into vigorously stirred H2O (250 mL). The resulting solid was filtered, washed with H2O (30 mL × 2), hexane (5 mL × 2), and then dried to give 8-chloro-1,3-dimethyl-7-(3-methylbenzyl)-1H-purine-2,6(3H,7H)-dione (8, 3,12 g, 9.79 mmol, 98% yield). 1H NMR (400 MHz, chloroform-d) δ 7.23 (dd, J = 8.2, 7.4 Hz, 1H), 7.17−7.09 (m, 3H), 5.51 (s, 2H), 3.55 (s, 3H), 3.40 (s, 3H), 2.33 (s, 3H). LC−MS (method 1): tR = 3.40 min, m/z (M + H)+ = 319.

Preparation of Ethyl 4-((1,3-Dimethyl-7-(3-methylbenzyl)-1,3-dimethyl-1H-purin-8-yl)oxy)benzoate (9). In a microwave tube was placed 8-chloro-1,3-dimethyl-7-(3-methylbenzyl)-1H-purine-2,6(3H,7H)-dione (8, 159 mg, 0.5 mmol) and ethyl piperazine-1-carboxylate (237 mg, 1.5 mmol), and then the mixture was added DMDO (1 mL). The mixture was sealed and heated at 160 °C under microwave irradiation for 30 min. The crude mixture was filtered through a silica gel chromatography using 20−60% EtOAc/hexane as the eluent to give tert-butyl 4-((1,3-dimethyl-7-(3-methylbenzyl)-1,3-dimethyl-1H-purin-8-yl)oxycarbonyl)-1-carbonyl (36 mg, 0.78 mmol, 58% yield).

1H NMR (400 MHz, DMSO-d6) δ 7.19 (d, J = 7.6 Hz, 1H, 7.11−6.98 (m, 2H), 6.93 (d, J = 7.6 Hz, 1H), 5.34 (s, 2H), 4.02 (q, J = 7.1 Hz, 2H), 3.39 (m, 3H), 3.17 (m, 3H), 3.31−3.01 (m, 4H), 2.24 (s, 3H), 1.16 (t, J = 7.1 Hz, 3H). LC−MS (method 2): tR = 5.44 min, m/z (M + H)+ = 441. HRMS calculated for C20H24N5O4 (M + H)+: 441.2245. Found: 441.2262.

Preparation of 4-((1,3-Dimethyl-7-(3-methylbenzyl)-2,6-dioxo-2,3,5,6,7,8,9,10-tetrahydro-1H-purin-8-yl)-N-methylbenzamide (10). In a microwaved tube was placed 8-chloro-1,3-dimethyl-7-(3-methylbenzyl)-1H-purine-2,6(3H,7H)-dione (8, 319 mg, 1 mmol), methyl 4-hydroxybenzoate (183 mg, 1.2 mmol), and K2CO3 (207 mg, 1.5 mmol). After 5 min stirring, 8-chloro-1,3-dimethyl-7-(3-methylbenzyl)-1H-purine-2,6(3H,7H)-dione (8, 683 mg, 2 mmol) was added. The mixture was stirred at rt for 30 min and heated at 60 °C for 2 h. The mixture was then filtered through a vigorously stirred H2O (250 mL). The resulting solid was filtered, washed with H2O (30 mL × 2), hexane (5 mL × 2), and then dried to give 4-((1,3-dimethyl-7-(3-methylbenzyl)-1,3-dimethyl-1H-purin-8-yl)oxy)benzoic acid (42 mg, 0.1 mmol) and HCl (14, 796 mg, 1 mmol, 97% yield). The material was used without further purification. Some material was submitted for purification by semipreparative HPLC to give tert-butyl 4-((1,3-dimethyl-7-(3-methylbenzyl)-1,3-dimethyl-1H-purin-8-yl)oxycarbonyl)-1-carboxylic acid (945 mg, 1.95 mmol) in 1,4-dioxane (2 mL) was added HCl (4 mL, 4.2 mmol, 5% HCl in water). The mixture was stirred at rt for overnight, and the mixture was concentrated to remove all the solvent. Then the product was diluted in vacuo to give 1,3-dimethyl-7-(3-methylbenzyl)-8-((1-(4-methylpyrimidin-2-yl)-piperidin-4-yl)-oxy)-1,3-dimethyl-1H-purine-2,6(3H,7H)-dione, HCl (19, 741 mg, 1 mmol, 97% yield).

Preparation of 1,3-Dimethyl-7-(3-methylbenzyl)-8-((1-(4-methylpyrimidin-2-yl)piperidin-4-yl)oxy)-1H-purine-2,6-(3H,7H)-dione (19). In a microwave tube was placed 1,3-dimethyl-7-(3-methylbenzyl)-8-((piperidin-4-yl)-oxy)-1H-purine-2,6-(3H,7H)-dione (18, 413 mg, 0.61 g, 1.5 mmol) in CH2Cl2 (5 mL). The mixture was sealed and heated at 160 °C under microwave irradiation for 1 h. The mixture was cooled to RT, and the mixture was filtered and submitted for purification by semipreparative HPLC to give 1,3-dimethyl-7-(3-methylbenzyl)-8-((1-(4-methylpyrimidin-2-yl)-piperidin-4-yl)-oxy)-1H-purine-2,6(3H,7H)-dione, TFA (19). The titration of 1,3-dimethyl-7-(3-methylbenzyl)-8-((1-(4-methylpyrimidin-2-yl)-piperidin-4-yl)-oxy)-1H-purine-2,6-(3H,7H)-dione to be dissolved in CHCl3 and then titrated with a 5% HCl in water solution. The titration of 1,3-dimethyl-7-(3-methylbenzyl)-8-((1-(4-methylpyrimidin-2-yl)-piperidin-4-yl)-oxy)-1H-purine-2,6(3H,7H)-dione, TFA (19).
propanecarboxyl chloride (0.3 g, 3 mmol) dropwise. The mixture was stirred at rt for 30 min and then poured into EtOAc/H2O/ Na2CO3(aq) (50 mL/25 mL/25 mL). The organic layer was washed with H2O (50 mL), dried (Na2SO4), and filtered. After removal of solvent, the product was dried to give 7-benzyl-8-((1-cyclopentanecarboxyl)piperidin-4-yloxy)-1,3-dimethyl-1H-purine-2,6(3H,7H)-dione (21, 630 mg, 96%). This material was used for the next step without further purification. Small amount of product was submitted for purification by semi-preparative HPLC to give TFA salt of 21 for screening.

1H NMR (TFA salt, 400 MHz, DMSO-d6) δ 7.46−7.11 (m, 3H), 5.26 (s, 2H), 5.20 (dt, J = 7.0, 3.4 Hz, 1H), 3.67 (s, 2H), 3.48 (s, 2H), 3.36 (s, 3H), 3.20 (s, 3H), 1.68 (m, 2H), 0.76−0.60 (m, 4H). LC−MS (method 1): tR = 3.24 min, m/z (M + H)+ = 438. HRMS calculated for C25H27N5O4Na (M + Na)+: 474.2112. Found: 474.2123.

Preparation of 8-((1-cyclopentanecarboxyl)piperidin-4-yloxy)-1,3-dimethyl-1H-purine-2,6(3H,7H)-dione, TFA (39). To a mixture of 8-((1-cyclopentanecarboxyl)piperidin-4-yloxy)-1,3-dimethyl-1H-purine-2,6(3H,7H)-dione (22, 20.8 mg, 0.06 mmol) and K2CO3 (16.6 mg, 0.12 mmol) was added N,N-dimethylformamide (1 mL). The mixture was stirred at rt for 5 min, and (2-bromoethyl)benzene (33.3 mg, 0.18 mmol) was added. The mixture was heated at 50 °C for 3 h. The mixture was filtered through a filter and then submitted for purification by semi-preparative HPLC to give 8-((1-cyclopentanecarboxyl)piperidin-4-yloxy)-1,3-dimethyl-1H-purine-2,6(3H,7H)-dione, TFA (39, 8.9 mg, 0.016 mmol, 26% yield). 1H NMR (400 MHz, DMSO-d6) δ 7.27−7.13 (m, 3H), 7.09−7.03 (m, 2H), 4.96 (dt, J = 7.1, 3.5 Hz, 1H), 4.22 (t, J = 6.7 Hz, 2H), 3.43 (br s, 4H), 3.35 (s, 3H), 2.33 (s, 3H), 2.99 (t, J = 6.7 Hz, 2H), 2.04−1.78 (m, 5H), 1.78−1.70 (m, 7H), 1.60−1.50 (m, 4H). LC−MS (method 2): tR = 5.08 min, m/z (M + H)+ = 452. HRMS calculated for C25H27N5O4Na (M + Na)+: 492.2292. Found: 452.2275.

Preparation of 7-(2-Cyclohexylethyl)-8-((1-cyclopentanecarboxyl)piperidin-4-yloxy)-1,3-dimethyl-1H-purine-2,6(3H,7H)-dione, TFA (54). In a microwave tube were placed 8-((1-cyclopentanecarboxyl)piperidin-4-yloxy)-1,3-dimethyl-1H-purine-2,6(3H,7H)-dione (22, 20.84 mg, 0.06 mmol) and 2-cyclohexylethanol (23.08 mg, 0.180 mmol). The tube was sealed, and the air was removed and then refilled with N2. Then, (E)-di-tert-butyl diazene-1,2-dicarboxylate (41.4 mg, 0.180 mmol) and Ph3P (47.2 mg, 0.180 mmol) in THF (1 mL) was added, and then the mixture was stirred at rt for 3 h. The mixture was concentrated, redissolved in DMF, filtered through a filter, and then submitted for purification by semi-preparative HPLC to give 7-(2-cyclohexylethyl)-8-((1-cyclopentanecarboxyl)piperidin-4-yloxy)-1,3-dimethyl-1H-purine-2,6(3H,7H)-dione, TFA (54, 14.7 mg, 0.026 mmol, 43% yield). 1H NMR (400 MHz, DMSO-d6) δ 14.70 (br s, J = 7.4 Hz, 1H), 4.94 (t, J = 7.0 Hz, 2H), 3.95−3.39 (m, 4H), 3.36 (s, 3H), 3.19 (s, 3H), 2.19−1.45 (m, 12H), 1.25−1.02 (m, 4H), 0.89 (m, 2H), 0.76−0.63 (m, 4H). LC−MS (method 2): tR = 5.99 min, m/z (M + H)+ = 458. HRMS calculated for C24H30N5O4Na (M + Na)+: 458.2762. Found: 458.2772.

Preparation of Ethyl 4-((1,3-Dimethyl-7-(3-methylbenzyl)-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)methyl)piperazine-1-carboxylate (5). To 1,3-dimethyl-7-(3-methylbenzyl)-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-carboxylic acid (25a, 62.5 mg, 0.22 mmol) was added a solution of ethyl piperazine-1-carboxylate (95 mg, 0.600 mmol) in CH2Cl2 (2 mL) at rt. The mixture was stirred for 3−5 min, and sodium triacetoxorhodanydride (212 mg, 1.000 mmol) was added. The mixture was stirred at rt for 1.5 h and was poured into CH2Cl2/Na2CO3 (aq) (3 mL/3 mL). The aqueous layer was extracted with CH2Cl2 (3 mL×2). The combined organic layer was dried (Na2SO4) and filtered. After removal of solvent, the crude product was purified by silica gel chromatography using 50−80% EtOAc/hexane as the eluent to giveethyl 4-((1,3-dimethyl-7-(3-methylbenzyl)-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-yl)methyl)piperazine-1-carboxylate (87 mg, 0.191 mmol, 96% yield). 1H NMR (400 MHz, chloroform-d) δ 7.24−7.15 (m, 1H), 7.12−7.04 (m, 1H), 6.92 (ddd, J = 7.6, 1.9, 0.9 Hz, 2H), 5.73 (s, 2H), 4.13 (q, J = 7.1 Hz, 2H), 3.59 (s, 3H), 3.54 (s, 2H), 3.41 (s, 7H), 2.39 (t, J = 6.2 Hz, 4H), 2.31 (s, 3H), 1.25 (s, J = 7.1 Hz, 3H). LC−MS (method 1): tR = 2.80 min, m/z (M + H)+ = 455.

Preparation of 8-((4-Cyclopentanecarboxyl)piperazin-1-yl)methyl-7-isopentyl-1,3-dimethyl-1H-purine-2,6(3H,7H)-dione, HCl (64). To a mixture of 7-isopentyl-1,3-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-8-carboxylde (25g, 195 mg, 0.7 mmol) and cyclopropyl(piperazin-1-yl)methanone, HCl (267 mg, 1.400 mol)
were added CHCl₃ (6 mL) and then Et₂N (0.293 mL, 2.100 mmol) at rt. The mixture was stirred for 3–5 min, and sodium triacetoxoborohydride (297 mg, 1.400 mmol) was added. The mixture was stirred at rt for 4 h and was poured into CH₂Cl₂/Na₂CO₃(aq) (5 mL/5 mL). The aqueous layer was extracted with CHCl₃ (5 mL × 2). The combined organic layer was dried (Na₂SO₄) and filtered. After removal of solvent, the crude product was purified by silica gel chromatography using 0–5% MeOH/Et₂O as the eluent to give 8-(4-(cyclopropylcarboxyamidinyl)piperazin-1-yl)methyl)-7-isopentyl-1,3-di- methyl-1H-purine-2,6(3H,7H)-dione (251 mg, 0.603 mmol, 86% yield).¹ H NMR (400 MHz, DMSO-d₆) δ 4.36 (dd, J = 9.7, 5.6 Hz, 2H), 3.70 (s, 2H), 3.63 (s, 2H), 3.43 (s, 3H), 3.39 (s, 3H), 3.21 (s, 2.41 (m, 4H), 1.93 (m, 1H), 1.68 (m, 3H), 0.94 (d, J = 5.9 Hz, 6H), 0.68 (dt, J = 7.9, 2.9 Hz, 4H), 1.3C NMR (100 MHz, chloroform-d) δ 171.1, 154.78, 151.50, 148.47, 147.53, 107.79, 54.48, 53.22, 52.89, 45.14, 44.50, 41.80, 39.73, 29.67, 27.86, 26.15, 22.39, 10.80, 7.40 (four peaks shown on piperazine ring). LC–MS (method 1): tᵣ = 2.82 min, m/z (M + Na)⁺ = 417. HRMS calculated for C₆H₇N₂O₄N₃ (M + Na⁺): 439.2428. Found: 439.2443. This material was converted to its HCl salt for PK study. To a solution of 8-(4-(cyclopropylcarboxyamidinyl)piperazin-1-yl)methyl)-7-isopentyl-1,3-dimethyl-1H-purine-2,6-(3H,7H)-dione (250 mg, 0.600 mmol) in 1,4-dioxane (6 mL) at rt was added HCl solution (4 M in 1,4-dioxane, 0.3 mL, 1.2 mmol, 2 equiv.). The mixture was stirred at rt for 5 min, and hexane (15 mL) was added and stirred for 15 min. The solid was filtered and washed with hexane (5 mL × 3) and dried in vacuo to give 8-(4-(cyclopropylcarboxyamidinyl)piperazin-1-yl)methyl)-7-isopentyl-1,3-dimethyl-1H-purine-2,6-(3H,7H)-dione (250 mg, 0.596 mmol, 99% yield) as a white solid. Mp 187–189 °C; ¹H NMR (400 MHz, DMSO-d₆) δ 4.36 (d, J = 7.6 Hz, 2H), 3.55 (s, 2H), 3.43 (s, 3H), 3.32 (s, 3H), 2.07–1.89 (m, 1H), 1.64 (m, 3H), 0.93 (d, J = 6.2 Hz, 6H), 0.73 (d, J = 7.7 Hz, 4H) (The protons of piperazine ring (8 H) are very broad between 4.6 and 2.8 ppm).

Biological Methods. Protein Expression and Activity Measurement. Human ALDH1A1, ALDH1B1, and ALDH3A1 were expressed and purified as described elsewhere.15–16 Human ALDH2 was purchased from Abcam (Cambridge, MA).

General Protocol for ALDH Enzymatic Assays. Briefly, an amount of 3 μL of enzyme (final concentration 20, 50, 20, and 5 μM for ALDH1A1, ALDH1B1, ALDH2, and ALDH3A1, respectively) or assay buffer (100 mM HEPES, pH 7.5, with 0.01% Tween 20) was dispensed into a 1356-well solid-bottom black plate (Greiner Bio One, Monroe, NC) followed by pin-tool transfer (23 nL) of candidate test articles was determined in a HTS format with the single time point concentration range 968 pM to 57.2 nM and propranolol (for short half-life), loperamide and diclofenac (for long half-life). The assay incubation system consisted of 0.5 μM microsomal protein, 1.0 μM drug concentration, and NADPH regeneration system (containing 0.650 mM NADP⁺, 1.65 mM glucose 6-phosphate, 1.65 mM MgCl₂, and 0.2 unit/mL Glucose 6-phosphate dehydrogenase) in 100 mM phosphate buffer at pH 7.4. The incubation was carried out at 37 °C for 15 min. The reaction was quenched by adding 50 μL of acetonitrile (~1:2 ratio) containing 0.28 μM abendazole (internal standard). After a 20 min centrifugation at 3000 rpm, 30 μL of the supernatant was transferred to an analysis plate and was diluted 5-fold using 1:2 v/v acetonitrile/water before the samples were analyzed by LC/MS–MS.

ASSOCIATED CONTENT

3 Supporting Information
Additional supplemental figures, tables, and detailed experimental procedures and spectroscopic data (¹H NMR, LC–MS, HRMS) for screening compounds; a.xlsx file containing molecular formula strings. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00577.

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Notes
The authors declare no competing financial interest.

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ABBREVIATIONS USED
ADME, absorption, distribution, metabolism, and excretion; ALDH, aldehyde dehydrogenase; CSC, cancer stem cell; ADME, absorption, distribution, metabolism, and excretion; HPGD, 15-hydroxyprostaglandin dehydrogenase; HSD17b4, type 4 hydroxysteroid dehydrogenase; qHTS, quantitative high throughput screening; RLM, rat liver microsomes; SAR, structure–activity relationship

REFERENCES


(19) A library of 220 402 compounds was screened at 7 concentrations against hALDH1A1. Inhibition of ALDH1A1 activity was screened by utilizing propionaldehyde as substrate and NAD+ as cofactor. An increase in the fluorescence intensity due to conversion of NAD+ to NADH was used to measure the enzyme activity. The screening and follow-up results were deposited in PubChem in January, 2008 (PubChem bioassay identifier AID 1030 and AID 493210; compound identifiers CID 607135 for S and CID 596188 for 6).

(20) Notably, indolinedione appeared to be one of active chemotypes in our qHTS screening.

(21) During the preparation of this manuscript, Hurley reported a high-throughput screening that indicated the theophylline core is one of the active chemical series inhibiting ALDH1A1; see the following: Morgan, C. A.; Hurley, T. D. Development of a high-throughput in vitro assay to identify selective inhibitors for human ALDH1A1. *Chem.-Biol. Interact.* 2015, 234, 29–37.


(23) Detailed synthetic procedures for all analogs are provided in Supporting Information.


(26) The multipoint rat liver microsomal stability and human liver microsomal stability (HLM) of 64 are 84 min and >120 min, respectively. Additionally, the HLM of selective analogs 58, 59, 61, 62, 65, and 66 is 94, 57, 47, 57, and 66 min, respectively.


