Mapping Aldehyde Dehydrogenase 1A1 Activity using an \[^{18}F\]Substrate-Based Approach

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Abstract: Aldehyde dehydrogenases (ALDHs) catalyze the oxidation of aldehydes to carboxylic acids. Elevated ALDH expression in human cancers is linked to metastases and poor overall survival. Despite ALDH being a poor prognostic factor, the non-invasive assessment of ALDH activity in vivo has not been possible due to a lack of sensitive and translational imaging agents. Presented in this report are the synthesis and biological evaluation of ALDH1A1-selective chemical probes composed of an aromatic aldehyde derived from \(N,N\)-diethylamino benzaldehyde (DEAB) linked to a fluorinated pyridine ring either via an amide or amine linkage. Of the focused library of compounds evaluated, \(N\)-ethyl-6-(fluoro)-\(N\)-(4-formylbenzyl)nicotinamide 4b was found to have excellent affinity and isozyme selectivity for ALDH1A1 in vitro. Following \(^{18}\)F-fluorination, \(^{18}\)F]4b was taken up by colorectal tumor cells and trapped through the conversion to its \(^{18}\)F-labeled carboxylate product under the action of ALDH. In vivo positron emission tomography revealed high uptake of \(^{18}\)F]4b in the lungs and liver, with radioactivity cleared through the urinary tract. Oxidation of \(^{18}\)F]4b, however, was observed in vivo, which may limit the tissue penetration of this first-in-class radiotracer.

Introduction

Aldehyde dehydrogenases (ALDHs) are a family of enzymes that catalyze the NAD(P)\(^+\)-dependent oxidation of a wide variety of aldehydes to their corresponding carboxylic acids.\(^{[1]}\)

There are currently 20 known functional human ALDHs\(^{[2]}\) that mediate the metabolism of aldehydes generated during oxidative stress,\(^{[3]}\) amino acid and biogenic amine metabolism,\(^{[4]}\) retinoic acid biosynthesis,\(^{[5]}\) and ethanol metabolism.\(^{[6]}\) In addition, ALDHs control the detoxification of exogenous reactive aldehydes and therapeutic drugs such as cyclophosphamide.\(^{[7]}\)

A aberrant expression of ALDH is associated with many diseases, including cancer, with increased expression and activity of ALDH shown to be a predictor of metastatic potential and poor overall survival.\(^{[8]}\) In particular, the ALDH1A1 isozyme is a well-characterized marker of cancer stem cells, which are known for their tumor-initiating properties and resistance to conventional therapy.\(^{[9]}\) Studies have shown resistance to chemotherapy and poor prognosis is associated with high ALDH1A1 activity in breast,\(^{[10]}\) ovarian,\(^{[11]}\) prostate,\(^{[12]}\) colon\(^{[13]}\) and lung\(^{[14]}\) cancer. As a consequence, ALDH1A1 has been selected as a target for anti-cancer therapy, with ALDH inhibitors shown to reverse chemoresistance in a range of preclinical tumor models.\(^{[15]}\)

Given the causal link between ALDH expression and cancer drug resistance, the non-invasive identification of ALDH-expressing tumors is of great clinical importance. The measurement of chemoresistance through ALDH imaging could potentially enable the clinician to select the most suitable therapeutic intervention for the individual patient (e.g. chemotherapy versus immunotherapy) with the possibility to improve outcomes and reduce unnecessary treatment. Currently, the in vitro assessment of ALDH activity has been restricted to fluorescence-based assays.\(^{[16]}\) Despite these commercially available imaging agents being widely-adopted for the isolation of...
ALDH-positive cells in cell culture, the poor tissue penetration of the fluorescent signal currently limits their use in vivo. In order to circumvent these inherent limitations, we propose the use of positron emission tomography (PET) as an alternative to fluorescence-based assays.\textsuperscript{[16]} Previous attempts to develop ALDH1A1-specific radiotracers have so far failed due to the poor cellular retention of the carboxylate product, presumed to be a consequence of its high hydrophobicity.\textsuperscript{[17]} Here, we report the synthesis and biological evaluation of \textsuperscript{18}F-fluorinated aldehyde-based probes for the non-invasive detection of ALDH1A1 activity in tumor cell models.

Results and Discussion

ALDH1A1 chemical probes were designed to have a) an aldehyde that can serve as a substrate for ALDH1A1; b) contain a (radio)fluorine atom that would allow for detection via gamma counting/PET imaging; c) a suitable hydrophobic-hydrophilic balance which would allow for passive diffusion in and out of cells, and importantly; d) subsequent trapping of the in situ generated carboxylic acid product within the cytosol as a result of the acquired negative charge (Figure 1A). We took a substrate-based approach for the imaging of ALDH1A1 to provide a functional readout of enzymatic activity. Substrate-based radiotracers provide an advantage over radiolabeled inhibitors which only report on enzyme expression. Moreover, multiple substrate molecules can be turned over by a single enzyme, thereby increasing the sensitivity of detection when compared to radiolabeled inhibitors.

The starting point for our small molecule probe development was \textit{N,N-}diethylaminobenzaldehyde (DEAB) 1, which is a well-known inhibitor of ALDH1A1 (K\textsubscript{i} values of 10–40 nM).\textsuperscript{[18]} Given the advantages of substrate-based radiotracers, our initial goal was to convert 1 in to a substrate whilst maintaining suitable ALDH1A1 selectivity over other commonly expressed ALDH isozymes. 1 is thought to form a stalled acyl-enzyme intermediate as a result of the delocalization of the electron lone pair of the \textit{para}-substituted amine into the aromatic ring.\textsuperscript{[18a,c]} To convert 1 from an inhibitor to a substrate, we uncoupled the amine nitrogen from the aromatic \textit{r}-system through the introduction of a methylene linker to give 2 (Figure 1B), which was rapidly converted to the carboxylate product under the action of ALDH1A1 (Figure S1). In order to assess the enzyme kinetics of the compounds in this study, we examined the effect of substrate concentration on the initial enzyme velocity using recombinant human ALDH1A1, ALDH2 and ALDH3A1 enzymes, which are commonly expressed in human cancer. ALDH2 is expressed in the mitochondrial matrix and plays a critical role in alcohol metabolism,\textsuperscript{[19]} whereas ALDH3A1 is localized in both the nucleus and cytosol and functions to detoxify aldehydes formed during UV-induced lipid peroxidation.\textsuperscript{[20]} Furthermore, these three isoforms exhibit three different rate-limiting steps: ALDH1A1’s being the cofactor dissociation,\textsuperscript{[21]} the deacylation-step for ALDH2,\textsuperscript{[22]} and hydride transfer for ALDH3A1.\textsuperscript{[23]}

We made a single-point modification to 2, with the addition of a fluorine to the aromatic ring—an essential requirement for \textsuperscript{18}F-radiofluorine-based radiotracers—to give compounds 3\textsuperscript{a} and 3\textsuperscript{b}. The lithium/bromine exchange on 11 followed by quenching with DMF afforded aldehyde 12. Stiring 12 with NaBH\textsubscript{(OAc)}\textsubscript{3} and diethylamine in DCE afforded the crude reductive amination product which was directly treated with aqueous hydrochloric acid in THF to furnish 3\textsuperscript{a} (Scheme 1A). Reduction of nitrile 13 with diisobutylaluminium hydride cleanly produced aldehyde 14 which was thereby stirred with excess diethylamine in THF to yield aldehyde 3\textsuperscript{b} (Scheme 1B).

Amide 16 was prepared by reacting the acid chloride of 5-fluoronicotinic acid 15 with amine 3\textsuperscript{b} (Scheme 1C). The acid-catalyzed cleavage of the acetal furnished compound 4\textsuperscript{a}. LiAlH\textsubscript{4} reduction of the amide bond in 16 followed by the acid-catalyzed acetal cleavage afforded amine 5 (Scheme 1C). In a similar manner, the acid chloride of 6-fluoronicotinic acid 16 was re-acted with amine 3\textsuperscript{a} to yield amide 18 which following an acetyl-catalyzed acetal cleavage furnished aldehyde 4\textsuperscript{b}. The sulfonamide 6 was accessed by reacting the commercially available 5-fluoropyridine-3-sulfonyl chloride 19 with amine 3\textsuperscript{a} following an acid-catalyzed acetal cleavage (Scheme 1E).

The benzylic amine 2 showed a higher affinity (lower K\textsubscript{i}) and catalytic efficiency (V\textsubscript{max}/K\textsubscript{m}) for ALDH1A1 over both ALDH2 and ALDH3A1 (Table 1, entry 1 and Figure 2B, respectively), indicating that the isozyme selectivity of DEAB was maintained. Interestingly, 3\textsuperscript{a} and 3\textsuperscript{b} exhibited lower affinity for ALDH1A1 than the non-fluorinated analogue 2, with a K\textsubscript{i} of 0.28±0.12 mM, 0.26±0.08 mM and 0.16±0.03 mM, respectively (Table 1, entries 1–3).

Given that fluorination proximal to the aldehyde moiety decreased affinity for ALDH1A1, we next explored compounds with fluorine atoms that were remote from the aldehyde. Compounds 4\textsuperscript{a} and 4\textsuperscript{b} exhibited a five-fold increase in ALDH1A1 affinity over 2 (Table 1, entries 1, 4 and 5). Furthermore, the enzyme efficiency for 4\textsuperscript{a} was 7-fold higher for ALDH1A1 than ALDH2, with the enzyme efficiency for ALDH1A1 over 20-fold higher with respect to ALDH3A1; that is, the linking of the pyridine via an amide bond resulted in improved selectivity for ALDH1A1 (Figure 2B; Table 1, entry 4). The position of the fluorine on the pyridine ring crucially did not play a key role in substrate kinetics, as seen with amide 4\textsuperscript{b} which exhibited analogous behavior to 4\textsuperscript{a}, albeit with marginally decreased

![Figure 1. A) Schematic illustrating ALDH-mediated trapping of \textsuperscript{18}F-labeled aldehydes by conversion to their corresponding acid. B) Chemical structures of DEAB (1), an ALDH1A1 inhibitor, and 2, an ALDH1A1 substrate.](image-url)
ALDH1A1 enzyme selectivity (Table 1, entry 5). In the absence of the amide linkage, the tertiary amine 5, whilst exhibiting a similar ALDH1A1 binding profile as compounds 4a and 4b, was readily oxidized by ALDH2 when compared to the other compounds tested (Figure 2B; Table 1, entry 6). Replacing the amide of compound 4a with a sulfonamide gave us compound 6 with diminished ALDH1A1 enzyme efficiency when compared to 4a (Figure 2B; Table 1, entries 5 and 7). In summary, we have shown 4a, 4b and 5 to be excellent substrates for ALDH1A1, with 4a and 4b to have good ALDH1A1 selectivity over the other isozymes tested (Figure 2B).

To understand why compounds 4a and 4b exhibited enhanced selectivity for ALDH1A1 over the other isozymes tested, we carried out an in silico docking study wherein compounds 2–6, and the natural ALDH1A1 substrates 9-cis-retinal, 13-cis-retinal, and all-trans-retinal, were docked into protein structures for ALDH1A1, ALDH2, and ALDH3A1 (Protein data bank ID: 4WB9, 1O01 and 4L20 respectively). In comparison to...
ALDH2 and ALDH3A1, ALDH1A1 has the largest access tunnel to the active site residue (Figure S3), which suggests that bulkier and more rigid substrates would be preferentially turned over by ALDH1A1. Tunnel topography may also explain why reducing the amide in compound 4a to a flexible tertiary amine in compound 5, resulted in increased affinity for ALDH2 and ALDH3A1. Compounds 4a and 4b displayed similar binding modes as 2 to ALDH1A1 in our in silico model, as shown in Figure 3. The benzaldehyde portions of compounds 4a, 4b, 5, and 6 occupied a similar site to that of compound 2; however, the pyridyl-ring was predicted to make additional contacts, presumably via π-stacking between the pyridine and the tyrosine-Y296 of ALDH1A1 (Figure 3). This putative π-stacking interaction may explain the increase in binding affinity for compounds 4a, 4b, 5, and 6 when compared to compounds that lack the pyridine functional group. The low binding affinity of compounds 4a, 4b, 5, and 6 for ALDH2 (Figure 2 and Table 1) might be ascribed to the equivalent phenylalanine residue within the active site, which being less electron-rich than tyrosine, results in a lower π-stacking efficiency. In ALDH3A1 the active site tyrosine is replaced by methionine, which is incapable of π-stacking. Consequently, no pattern in binding affinity for ALDH3A1 was observed for substrates with the pyridyl substituent.

Compound 4b was further considered as a potential radiolabeling probe to assess cellular ALDH activity as it not only had excellent enzyme efficiencies and selectivity for ALDH1A1, but also potential radiolabeling accessibility. Radiolabeling via nucleophilic substitution with [18F]fluoride allows for substitution at the 2-position on a pyridine ring, whilst the 3-position 4a exhibits poor reactivity. Consequently, the [18F]-radiolabeled analogue of 4b, N-ethyl-6-(fluoro-18F)-N-(4-formylbenzyl)-nicotinamide was chosen as our lead candidate radiotracer. To access [18F]4b, a nucleophilic aromatic substitution (SnAr) was performed on the 6-chloronicotinamide precursor 7, which was prepared from the acid chloride of 6-chloronicotinic acid 20 and amine A (Scheme 1F). Stirring 7 with [18F]KF/K222 in DMSO at 150°C for 25 min followed by an acid-catalyzed acetal cleavage step furnished [18F]4b in 35 ± 1% (n = 3) decay-corrected radiochemical yield (RCY) after reverse phase semi-preparative HPLC purification following manual radiolabeling (Scheme 2). Automation of this procedure improved the RCY to 43 ± 1% (decay-corrected; n = 3). Starting the synthesis with 1.0 GBq of [18F]fluoride, the radiotracer was obtained with a radiochemical purity of 99% (see Supporting Information) and a molar activity of up to 4.4 GBq μmol⁻¹ (manual) and up to 7.2 GBq μmol⁻¹ (automated).

Furthermore, reference compounds for cellular metabolite analysis, carboxylic acid 8 and alcohol 9, were prepared by the

### Table 1. Kinetic properties of human ALDH1A1, ALDH2 and ALDH3A1 towards oxidation of aldehydes in this study.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>ALDH1A1</th>
<th>ALDH2</th>
<th>ALDH3A1</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Vmax</td>
<td>Km</td>
<td>Vmax</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(nmol min⁻¹ μg⁻¹)</td>
<td>(μM)</td>
<td>(nmol min⁻¹ μg⁻¹)</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>1.22 ± 0.09</td>
<td>0.16 ± 0.03</td>
<td>7.9</td>
</tr>
<tr>
<td>2</td>
<td>3a</td>
<td>1.10 ± 0.18</td>
<td>0.28 ± 0.12</td>
<td>3.9</td>
</tr>
<tr>
<td>3</td>
<td>3b</td>
<td>1.42 ± 0.17</td>
<td>0.26 ± 0.08</td>
<td>5.5</td>
</tr>
<tr>
<td>4</td>
<td>4a</td>
<td>1.08 ± 0.07</td>
<td>0.03 ± 0.01</td>
<td>33.9</td>
</tr>
<tr>
<td>5</td>
<td>4b</td>
<td>0.98 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>28.7</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>1.36 ± 0.08</td>
<td>0.04 ± 0.01</td>
<td>33.3</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>1.5 ± 2.68</td>
<td>0.10 ± 0.24</td>
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</table>
KMN0 mediated oxidation of 4b, and the NaBH4 mediated reduction of aldehyde 4b, respectively (Scheme 1G). With a tracer candidate in hand we assessed whether 18F]4b could provide a readout of ALDH activity in tumor cells grown in culture. We used the HCT116 KrasG12D- mutant (HCT116 mut) human colorectal cancer cell line as a model of aggressive, therapy-resistant cancer. HCT116 mut lines displayed high ALDH activity, which was specifically inhibited through the incubation of cells with the ALDH inhibitor DEAB (30 μM; Figure 4A). Incubation of HCT116 mut cells with 18F]4b resulted in rapid cell uptake and intracellular retention of radioactivity, reaching 5.7±0.2% radiotracer dose mg⁻¹ protein at 20 min (n = 3). Treatment of cells with DEAB resulted in an 83% reduction in cell-associated radioactivity to 1.0±0.1% radiotracer dose mg⁻¹ protein (n = 3; P < 0.0001), indicating ALDH-specific intracellular trapping of either 18F]4b or its products (Figure 4B). To confirm the identity of the intracellular radioactive species present, we performed radio-HPLC analysis of the resulting cell lysates. 20 min after the addition of the radiotracer, near-complete conversion of the 18F]4b aldehyde to the corresponding carboxylic acid 18F]8 was observed (Figure 4C; see Scheme 1G for structures of 8 and 9), confirmed through coinjection with non-radioactive 18F]8 (See Supporting Information for details). Incubation of cells with DEAB resulted in a substantial reduction in the production of 18F]8, with >90% of radioactivity present as the parent compound (Figure 4C), suggesting that in the absence of ALDH1A1 activity the aldehyde does not undergo intracellular oxidation and therefore is not retained.

Whilst the levels of 18F]4b in DEAB-treated cells did not significantly change over the 60 min time course of the experiment (P > 0.05; Figure 4B, red circles), the amount of intracellular radioactivity decreased in a time-dependent fashion in vehicle (DMSO)-treated cells following addition of 18F]4b (Figure 4B, blue circles). Despite washout of radioactivity from vehicle-treated cells, cell-associated radioactivity at 60 minutes remained 2.4-fold higher than cells treated with DEAB, at 2.8±0.3% radiotracer dose mg⁻¹ and 1.2±0.1% radiotracer dose mg⁻¹ protein, respectively (P = 0.0005; n = 3). Analysis of media samples by radio-HPLC following cell incubation showed a progressive increase in the levels of 18F]8 and the subsequent appearance of the alcohol 18F]9 (see Scheme 1G for structures), indicating imperfect intracellular trapping of 18F]8 following its production by ALDH (Figure 4D). Appearance of 18F]8 in the media therefore accounts for the time-dependent reduction in cell-associated radioactivity observed in vehicle-treated cells following incubation with 18F]4b, potentially as the result of efflux pump-mediated excretion of the carboxylate. Media incubation of 18F]4b at 37 °C for 60 min in the absence of cells did not result in the production of 18F]8 (Supporting Information; chromatogram S3), confirming that the conversion to the carboxylate was cell-mediated. Together, these data show 18F]4b to be specific and sensitive marker of ALDH activity in tumor cells.

Given that 18F]4b can measure ALDH activity in HCT116 mut cells with high sensitivity and specificity, we next explored 18F]4b’s in vivo biodistribution. Dynamic microPET imaging following intravenous injection of 18F]4b into healthy balb/c mice (Figure 5) revealed rapid and extensive uptake in the lungs, known to express high levels of ALDH1A1. Liver uptake peaked at 5 min post injection (p.i.) at 20.0±2.6% injected dose (ID) g⁻¹ tissue (n = 3 mice). 18F]4b clearance was initially via the kidneys and bladder to afford high contrast images with low uptake in background tissue (2.2±0.3%ID g⁻¹ in the muscle at 5 min p.i.; n = 3 mice). However, hepatobiliary excretion was evident by 30 min p.i., as shown by radiotracer.
Figure 5. PET/CT maximum intensity projections of $^{18}$F$\text{4b}$ in a healthy balb/c mouse. Representative time course images are shown to illustrate $^{18}$F$\text{4b}$ pharmacokinetics. Gray scale is CT image, and color scale is PET image. Uptake in key organs are illustrated. GI, gastrointestinal tract. Images are representative of three separate mice.

 uptake in the gallbladder and gastrointestinal (GI) tract, indicating possible metabolism of the parent compound at this time point. $^{18}$F$\text{4b}$ was further cleared from all other organs other than gallbladder and GI over the remainder of the imaging time course. For time activity curves for organs with high $^{18}$F$\text{4b}$ uptake, see Figure S4.

The combined renal and hepatobiliary excretion observed at 30 min p.i., prompted us to assess the in vivo stability of $^{18}$F$\text{4b}$. Radio-HPLC analysis of plasma taken by terminal exsanguination from anesthetized mice following tail vein intra- venous (i.v.) injection of $^{18}$F$\text{4b}$ revealed complete conversion to $^{18}$F$\text{8}$ by 2 min (Supporting Information; chromatogram S7). The appearance of a second, unknown peak of similar retention time to $^{18}$F$\text{8}$ (Supporting Information chromatogram S7) was evident by 5 min, increasing to $\approx 40\%$ total radioactivity in the blood by 60 min, which may account for the mixed routes of excretion observed at later imaging time points. Importantly, the rapid metabolism of the free aldehyde may limit the in vivo tissue penetration of $^{18}$F$\text{4b}$.

Conclusions
In conclusion, we have developed a focused library of ALDH substrates based on the well-known inhibitor DEAB. The addition of a fluoronicotinamide dramatically increased ALDH1A1 isoform specificity, thought to result from $\pi$-stacking interactions with a tyrosine residue proximal to the active site. N-Ethyl-6-(fluoro)-N-(4-formylbenzyl)nicotinamide $\text{4b}$ was taken forward for radiolabeling and evaluation in both tumor cells and in mice. $^{18}$F$\text{4b}$ was rapidly taken up by ALDH-expressing colorectal cancer cells in culture and intracellularly trapped through ALDH-specific conversion to the corresponding carboxylic acid. In vivo, high radiotracer uptake in the lung and liver were observed, combined with rapid clearance from background tissues and excretion via the urinary tract. Rapid oxidation of this lead compound in vivo however highlights a potential limitation of aldehyde-based radiotracers for ALDH imaging. Future strategies will focus on the development of second generation ALDH1A1 radiotracers with improved in vivo stability to image drug resistance in animal models of cancer.

Experimental Section
See Supporting Information for detailed synthetic, radiochemical methods, enzyme assays, cellular and studies in mice.

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Conflict of interest
The authors declare no conflict of interest.

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