

Research paper

Resorufin-derived esters as fluorescent probes to test for the esterase activity and active-site topology of aldehyde dehydrogenase

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ABSTRACT

Aldehyde dehydrogenase (ALDH) isoforms are crucial for cellular detoxification and metabolic regulation, yet their enzymatic properties with non-canonical substrates remain underexplored. ALDH1A isoforms are overexpressed in several cancer types and contribute to tumor aggressiveness and chemoresistance. In this study, we characterize the esterase activity of ALDH1A isoforms using novel resorufin-derived fluorogenic substrates. Steady-state kinetic analysis reveals distinct catalytic efficiencies (k_{cat}/K_m) among isoforms, emphasizing differences in substrate hydrolysis rates and binding affinities. Structural modeling and fluorescence-based assays indicate that NAD^+ binding induces conformational rearrangements that modulate esterase activity. Resorufin-derived esters clearly outperformed 4-nitrophenyl acetate and standard aldehyde substrates of ALDH. These findings provide mechanistic insights into ALDH1A isoform selectivity and expand the application of resorufin-based probes for ALDH functional assays. Our study establishes a foundation for developing targeted ALDH assays and potential applications in cancer drug screening and biomarker discovery.

1. Introduction

Aldehyde dehydrogenase (ALDH) is an oxidoreductase that catalyzes the irreversible $NAD(P)^+$ -dependent oxidation of aldehydes to their corresponding carboxylic acids. ALDH is a ubiquitous enzyme found in all living domains in nature, with diverse functions in hormone, amino acid, neurotransmitter and energy metabolism, and antioxidant defense. The catalytic mechanism of ALDH proceeds through the nucleophilic attack by the sulfur atom of a strictly conserved active-site cysteine residue (Cys302, ALDH2 nomenclature) to the carbon atom of the aldehyde carbonyl group, and the formation of a covalent thiohemiacetal intermediate, followed by deacylation and carboxylic acid release [1]. The cofactor (NAD^+ or $NADP^+$) is needed for substrate oxidation to uptake the hydride anion from the carbon atom to the C4

atom of the nicotinamide ring. Active-site residues Asn169 and Glu268, and water molecules also participate in the acid-base catalysis. The active-site Cys residue sits at the intersection between two channels located in opposite sites: the substrate- and the cofactor-binding pockets.

In humans, several ALDH isoforms with distinct substrate specificity have been described. ALDH1A1, ALDH1A2 and ALDH1A3 isoforms are cytosolic, homotetrameric enzymes, with 55-kDa subunits. They are involved in retinoic acid synthesis from retinaldehyde, although they show wide substrate specificity [2]. These ALDH isoforms, mostly ALDH1A1 and ALDH1A3, are cancer stem cell biomarkers and have been associated with cancer chemoresistance, metastasis and recurrence [3–7]. ALDH inhibitors are emerging as good candidates as pharmacological drugs in adjuvant anticancer therapies and have been the subject of active research [8–12].

Abbreviations: ABD0171, S-methyl 4-[dimethyl(2-(2,4-dimethylphenoxy)ethyl)amino]-4-methylpent-2-ynethioate; ALDH, aldehyde dehydrogenase; ALDH1A1, aldehyde dehydrogenase family 1 member A1; CES2, carboxylesterase 2; MONAL-62, 6-methoxy-2-naphthaldehyde; MONAL-71, 7-methoxy-1-naphthaldehyde; RMSD, root-mean-square deviation.

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Interestingly, in addition to the aldehyde-oxidizing activity, ALDH also catalyzes cofactor-independent ester hydrolysis, yielding the corresponding carboxylic acid and alcohol as reaction products. The esterase activity is shared with thiol proteases and serine hydrolases, such as proteases and carboxylesterases [13–15]. Ester molecules bind to the same active site as aldehydes in ALDH and the hydrolytic activity uses the same active-site Cys residue for the nucleophilic attack to the carbon atom of the carbonyl ester [1]. At this point, it is unknown whether the esterase activity of ALDH is simply a side reaction of aldehyde oxidation using the same catalytic mechanism or it has any physiological significance. In this regard, during the bioactivation of glyceryl trinitrate (nitroglycerin) to release vasodilator nitric oxide, the active-site Cys residue of ALDH2 performs a nucleophilic attack on the nitrate ester bond of nitroglycerin without NAD^+ requirement, mimicking the esterase activity [16].

Classically, ALDH esterase activity was detected *in vitro* using 4-nitrophenyl acetate (4-NPA) as a substrate [17–19]. 4-NPA hydrolysis was typically measured by the absorbance increase at 400 nm, due to 4-nitrophenol production. The esterase activity did not require the participation of a redox cofactor, although it was enhanced 2–4 fold by NAD^+ in ALDH1 and ALDH2, but inhibited in the ALDH3 isoform [20]. Recently, we reported that ALDH1A1, ALDH1A2 and ALDH1A3 isoforms showed esterase activity with 4-NPA in the presence of NAD^+ [2], while ATP inhibited esterase activity of ALDH1A3 in a concentration-dependent manner [21]. Other nitrophenyl derivatives, such as 4-nitrophenyl esters of acyl groups from three to six carbon atoms, 4-nitrophenyl pivalate, methyl 4-nitrophenyl carbonate, 4-nitrophenyl dimethylcarbamate and 4-nitrophenyl guanidinobenzoate, were used as substrates of ALDH. Some of them did not show any activation or even inhibition by cofactor binding [22–25]. A different rate-limiting step or cofactor conformation was proposed to explain this effect [20, 25].

The esterase activity of ALDH was also measured fluorometrically by using resorufin acetate (**1**) as a substrate. When compared to 4-NPA, resorufin acetate (**1**) afforded a much more sensitive method and its hydrolysis rate resulted dramatically accelerated (more than 20 fold) by cofactor binding [26,27]. In addition, a variety of resorufin derivatives, such as resorufin bromoacetate, were used as covalent ALDH inhibitors [28]. Resorufin is a phenoxazine, a planar tricyclic heterocycle composed of a central 1,4-oxazine ring fused to two benzene rings. Resorufin-derived compounds have been used as probes in the fluorescent-based detection of hydrolases and ligases [29,30] and non-hydrolyzable analogs in crystallographic studies of enzyme-substrate complexes [31]. Other fluorescent compounds along with resorufin which have been used to detect esterase activity are listed in Table S1.

Recently, we reported the use of fluorogenic naphthalene-derived substrates to assess the dehydrogenase activity of ALDH1A isoforms in cell extracts [32]. Following this line of research, here we describe the chemical synthesis and kinetic properties of five different resorufin-derived compounds (**2–6**) that were tested as substrates of ALDH1A1, ALDH1A2 and ALDH1A3, in the presence and absence of NAD^+ . Commercially available resorufin acetate (**1**) was also included in the study for comparison. Molecular docking analyses revealed steric hindrances depending on the various active-site topologies of ALDH isoforms. The experimental results were rationalized in terms of the structure-based and *in silico* predictions. Resorufin-derived esters clearly outperformed 4-NPA and standard aldehyde substrates of ALDH. Thus, these compounds could be applied to the fluorescent high-throughput screening of ALDH inhibitors and to the detection of ALDH in low-activity cell extracts. The usefulness of resorufin acetate was tested to detect esterase activity of ALDH isoforms in two different cancer cell lines.

2. Materials and methods

2.1. ALDH substrates and inhibitors

Resorufin-derived compounds (**2–6**) were synthesized in-house by Advanced Biodesign (France) and served as the fluorescent substrates for measuring ALDH activity *in vitro*. Resorufin acetate (**1**) was purchased from Santa Cruz Biotechnology. Hexanal was obtained from Sigma-Merck and utilized as a standard aldehyde substrate for comparative assays. MONAL-62 and MONAL-71, fluorescent naphthalene derivatives, were purchased from CymitQuimica. Inhibitors: ABD0171 was provided by Advanced BioDesign (France) and orlistat was from Merck.

2.2. Chemistry

Commercial reagents and solvents were used without purification, except for dichloromethane distilled under an inert atmosphere on CaH_2 . The reactions were monitored by thin layer chromatography on aluminum sheets coated with Macherey-Nagel ALUGRAM SIL G/UV 25 4 silica gel (thickness 0.2 mm), the plates being observed under ultraviolet lamp at 254 and 312 nm. The chromatography was carried out on Macherey-Nagel 60 M silica gel (40–63 μm) under air pressure. Melting points were measured with a Tottoli Büchi SMP-20 device and were not corrected. The ^1H NMR spectra were recorded with Bruker ALS300 or DRX300 devices at 300 MHz. The ^{13}C NMR spectra were obtained on Bruker DRX300 devices at 75 MHz. The chemical shifts δ are expressed in parts per million (ppm), the residual peak of the solvent having been taken as an internal reference. The coupling constants J are expressed in Hz. Mass spectra were recorded in positive mode on a hybrid time-of-flight mass spectrometer (MicroTOFQ-II, Bruker Daltonics, Bremen) with an electrospray source (ESI). The spray gas flow was 0.4 bar and the capillary voltage at 3500 V. The solutions were perfused at 10 $\mu\text{L}/\text{min}$ in a mixture of solvents (methanol/dichloromethane/water 45/40/15) with 1% formic acid. The mass range of the analysis was 50–1000 m/z and the calibration was carried out with sodium formate.

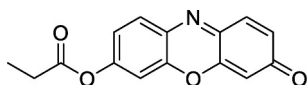
The compounds were prepared from resorufin (sodium salt) (Sigma) using the acid chloride (2 equivalents) corresponding in dichloromethane (DCM, 0.05 M) in the presence of diisopropylethylamine (DIPEA, 2 equivalents). Purified resorufin derivatives were dissolved in DMSO and stored at -20°C .

2.2.1. Resorufin propionate (**2**)

For propionate, a suspension of sodium salt of resorufin (235.2 mg, 1.0 mmol) in 20 mL of anhydrous DCM were added DIPEA (2 equivalents) at room temperature then propionyl chloride (2 equivalents) dropwise at 0°C . After 5 min at 0°C , the reaction medium was brought to ambient temperature and stirred overnight. Thirty mL of water was then added and an extraction with 3×30 mL of DCM was carried out. The combined organic phases were washed with 30 mL of a saturated NaHCO_3 solution and then 30 mL of a saturated NaCl solution. After drying over Na_2SO_4 , filtration and evaporation of the solvent, the residue was taken up in EtOH. Sonication was carried out and the solid obtained was filtered through a frit and washed with EtOH (2 times). After drying under vacuum, 218 mg (81%) of an orange solid was obtained.

Mp $176\text{--}177^\circ\text{C}$ (EtOH); 177°C [33]; ^1H NMR (300 MHz, DMSO) δ 7.89 (d, $J = 8.7$ Hz, 1 H), 7.57 (d, $J = 9.8$ Hz, 1 H), 7.40 (d, $J = 2.3$ Hz, 1H), 7.25 (dd, $J = 8.7, 2.4$ Hz, 1H), 6.84 (dd, $J = 9.8, 2.0$ Hz, 1H), 6.31 (d, $J = 2.0$ Hz, 1H), 2.66 (q, $J = 7.4$ Hz, 2H), 1.15 (t, $J = 7.5$ Hz, 3H); ESI-MS m/z 270.1 $[\text{M} + \text{H}]^+$. Weight: 269.2 g mol^{-1} .

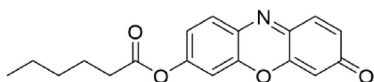
2.2.1.1. Formula: $\text{C}_{15}\text{H}_{11}\text{NO}_4$



2.2.2. Resorufin hexanoate (3)

For hexanoate, the procedure is identical to that of propionate. Hexanoyl chloride (scale: 1.0 mmol) was used. The compound obtained was isolated by precipitation in EtOH, followed by 2 washes with EtOH, yield 65%. Mp 130-132°C; ^1H NMR (300 MHz, DMSO) δ 7.89 (d, J = 8.7 Hz, 1H), 7.57 (d, J = 9.8 Hz, 1H), 7.39 (d, J = 2.3 Hz, 1H), 7.24 (dd, J = 8.7, 2.4 Hz, 1H), 6.84 (dd, J = 9.8, 2.0 Hz, 1H), 6.30 (d, J = 2.0 Hz, 1H), 2.63 (t, J = 7.4 Hz, 2H), 1.75-1.57 (m, 2H), 1.43-1.25 (m, 4H), 0.90 (t, J = 7.0 Hz, 3H); ESI-MS m/z 312.1 $[\text{M} + \text{H}]^+$. Weight: 311.3 g mol $^{-1}$.

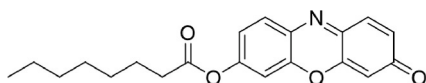
2.2.2.1. Formula: $\text{C}_{18}\text{H}_{17}\text{NO}_4$



2.2.3. Resorufin octanoate (4)

For octanoate, the procedure is identical to that of propionate. Octanoyl chloride (scale: 1.0 mmol) was used. The compound obtained was isolated by precipitation in EtOH, followed by 2 washes with EtOH, yield 81%. Mp 127-129°C; ^1H NMR (300 MHz, DMSO) δ 7.89 (d, J = 8.7 Hz, 1H), 7.57 (d, J = 9.8 Hz, 1H), 7.39 (d, J = 2.4 Hz, 1H), 7.23 (dd, J = 8.7, 2.4 Hz, 1H), 6.84 (dd, J = 9.8, 2.0 Hz, 1H), 6.30 (d, J = 2.1 Hz, 1H), 2.63 (t, J = 7.4 Hz, 2H), 1.72-1.57 (m, 2H), 1.44-1.19 (m, 8H), 0.87 (t, J = 6.8 Hz, 3H); ESI-MS m/z 340.2 $[\text{M} + \text{H}]^+$. Weight: 339.4 g mol $^{-1}$.

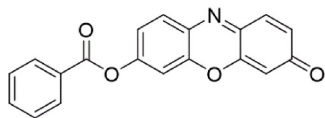
2.2.3.1. Formula: $\text{C}_{20}\text{H}_{21}\text{NO}_4$



2.2.4. Resorufin benzoate (5)

For benzoate, the procedure is identical to that of propionate. Benzoyl chloride was used (scale: 1.0 mmol). The isolated solid (166 mg) was purified by chromatography on silica gel (MeOH/DCM: 1/99) to give 156 mg (49%) of pure benzoate. Mp > 210°C: 203°C [33]; ^1H NMR (300 MHz, DMSO) δ 8.21 - 8.14 (m, 2H), 7.95 (d, J = 8.7 Hz, 1H), 7.84 - 7.75 (m, 1H), 7.62 (ddd, J = 11.7, 10.5, 8.1 Hz, 4H), 7.44 (dd, J = 8.7, 2.4 Hz, 1H), 6.86 (dd, J = 9.8, 2.1 Hz, 1H), 6.33 (d, J = 2.1 Hz, 1H); ESI-MS m/z 318.1 $[\text{M} + \text{H}]^+$. Weight: 317.3 g mol $^{-1}$.

2.2.4.1. Formula: $\text{C}_{19}\text{H}_{11}\text{NO}_4$

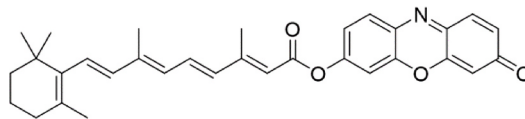


2.2.5. Resorufin retinoate (6)

For resorufin retinoate, the compound was prepared in the same manner as the previous ester using commercial retinoic acid (md 40%). The crude product (orange-red solid) obtained was washed with MeOH and purified by chromatography on silica gel (MeOH/DCM: 1/99). Additional washing with EtOH then MeOH gave 100 mg (40%) of pure product. Mp 150-160°C (decomposition); ^1H NMR (300 MHz, CDCl $_3$) δ

7.82 (d, J = 8.6 Hz, 1 H), 7.46 (d, J = 9.8 Hz, 1H), 7.25 - 7.10 (m, 3H), 6.89 (dd, J = 9.8, 2.0 Hz, 1H), 6.46 - 6.31 (m, 3H), 6.26 - 6.14 (m, 2H), 6.00 (s, 1H), 2.45 (d, J = 0.9 Hz, 3H), 2.10-2.00 (m, 5H), 1.75 (d, J = 0.6 Hz, 3H), 1.70-1.58 (m, 2H), 1.54-1.46 (m, 2H), 1.06 (s, 6H); ESI-MS m/z 496.2 $[\text{M} + \text{H}]^+$. Weight: 495.6 g mol $^{-1}$.

2.2.5.1. Formula: $\text{C}_{31}\text{H}_{33}\text{NO}_4$



2.3. Protein expression and purification

Recombinant human ALDH1A1, ALDH1A2 and ALDH1A3 containing an N-terminal (His) $_6$ tag were prepared following previously described protocols [2,7]. Purification involved nickel-charged agarose affinity chromatography, followed by storage at -20°C in 20 mM Tris/HCl, 500 mM NaCl, 5 mM DTT, pH 8.0.

2.4. Fluorescence assay for the determination of the kinetic constants

To determine the kinetic parameters of ALDH1A1, ALDH1A2 and ALDH1A3 using resorufin-derived substrates, the enzymatic activity was determined utilizing a Cary Eclipse Varian fluorimeter. The assays were performed in 1-mL quartz cuvettes, containing 50 mM HEPES, pH 7.5. All the resorufin-derived compounds were prepared in DMSO and assayed in a final concentration of 1% (v/v) DMSO and were characterized in the absence and in the presence of 500 μM NAD $^+$ cofactor at 25°C. The reaction was initiated by the addition of the corresponding substrate and the fluorescence of resorufin product was monitored at 583 nm with excitation at 560 nm, using 2.5 and 5 nm for the excitation and emission spectral bandwidths, respectively. Furthermore, 5 μM resorufin was added as an internal standard to obtain the absolute reaction rate, which was calculated as described previously [34]. A control sample without enzyme was included to account for spontaneous hydrolysis. The enzymatic kinetic data were analyzed using GraFit 5.0 (Erithacus Software) and all data were fitted to the standard Michaelis-Menten equation. Experimental values were represented as the mean $\pm$ standard deviation from three independent measurements, while kinetic parameters were the mean $\pm$ standard error.

2.5. Molecular docking analysis

The 3D structures of ALDH1A1 (PDB code 4WB9), ALDH1A2 (PDB code 6ALJ), and ALDH1A3 (PDB codes 7QK7 and 7QK8) were used. The co-crystallized ligands, ions, and water molecules were removed from the X-ray structures and polar hydrogens were added to ensure the simulation of a biological environment by Autodock Tools [35]. The Gasteiger charges were assigned for a precise modeling of the electrostatic interactions. Then, the rotatable bonds in the resorufin series of compounds were determined and given some flexibility to improve interaction with the target. The NAD $^+$ cofactor was adapted from ALDH1A3 structure to ALDH1A1. The processed ligands were used in a PDBQT file format. All docking calculations were carried out on the X-ray structures of human ALDH1A1 and ALDH1A3 in complex with resorufin substrates, both in the presence and absence of NAD $^+$ cofactor. The molecular docking analysis was performed using PyRx - Python Prescription virtual screening software [36], which incorporates tools mainly combining AutoDock [35] and AutoDock Vina [37]. The protein and ligands were selected in the environment associated with Vina and the binding site was defined as an inclusion region with the focus located in the catalytic cysteine residue, which entirely encompassed all the binding-pocket conformations of the trajectory. The binding site was

defined by a box along the x, y, and z axes centered on the catalytic cysteine (ALDH1A1: x = 21.5 Å; y = 21.0 Å; z = 22.0 Å; ALDH1A2: x = 22.0 Å; y = 19.5 Å; z = 21.0 Å; ALDH1A3: x = 21.0 Å; y = 21.0 Å; z = 21.0 Å). Once the grid was defined, a total of 8 models ordered according to free binding energy (ΔG) criteria and the expected pose of the ligand were generated and the top-ranked docking pose was considered as the final pose. The reliability of the docking program was assessed by calculating the root-mean-square deviation (RMSD), both for lower bound (RMSD lb) and upper bound (RMSD ub).

2.6. Cell culture and ALDH activity and inhibition assays in cell extracts

Non-small cell lung cancer (A549) and glioblastoma (A172) and cell lines were obtained from ATCC and cultured in DMEM culture medium, supplemented with 10% FBS without antibiotics. A172 knockout (KO) cells had been described previously [38]. Cells were maintained at 37°C

with 5% CO₂ until reaching the exponential growth phase. Cell lysates were prepared using mammalian protein extraction reagent (M-PER, Thermo Scientific). Cell pellets were resuspended in 200 µL M-PER, lysed with two cycles of ultrasound at 110–120 V, 50–60 Hz for 5 min, and centrifuged at 16,100 xg for 10 min at 4°C. The supernatant was collected for protein quantification. Enzymatic activity assays were conducted using hexanal (under ALDH1A1 and ALDH1A3 conditions), MONAL-62 and MONAL-71, and resorufin acetate as substrates. Working stocks of hexanal were prepared in 50 mM HEPES, 0.5 mM EDTA, pH 7.2 using 30 µM hexanal for ALDH1A1 assay; and 50 mM HEPES, 50 mM MgCl₂, pH 7.2 using 250 µM hexanal for ALDH1A3 assay, as described previously [34]; MONAL series of compounds were prepared in water: acetonitrile (60:40, v/v) and fresh working stocks were prepared daily in 50 mM pyrophosphate-NaOH, 0.5 mM EDTA, pH 8.1 using 5 µM MONAL-62 and 15 µM MONAL-71 in enzymatic assays; 15 µM resorufin acetate was assayed in a final concentration of 1% (v/v) DMSO in 50 mM

Table 1

Kinetic constants of ALDH1A1, ALDH1A2 and ALDH1A3 with resorufin-derived substrates.

Substrate and parameter	ALDH1A1	ALDH1A2	ALDH1A3
Resorufin acetate (1)			
K_m (µM)	0.16 ± 0.0003	0.35 ± 0.05	0.20 ± 0.06
k_{cat} (min ⁻¹)	9.24 ± 0.06	2,246 ± 53	59.7 ± 3.74
k_{cat}/K_m (mM ⁻¹ min ⁻¹)	56,900 ± 2400	6,500,000 ± 925,000	298,000 ± 86,000
Resorufin acetate (1) + NAD⁺			
K_m (µM)	4.27 ± 0.93	3.89 ± 0.78	2.14 ± 0.83
k_{cat} (min ⁻¹)	410 ± 24.95	8,857 ± 434	310 ± 45
k_{cat}/K_m (mM ⁻¹ min ⁻¹)	96,000 ± 21,700	2,300,000 ± 467,000	145,000 ± 60,200
Resorufin propionate (2)			
K_m (µM)	0.74 ± 0.20	1.59 ± 0.54	0.43 ± 0.06
k_{cat} (min ⁻¹)	49.4 ± 3.26	485 ± 43.73	23.9 ± 0.76
k_{cat}/K_m (mM ⁻¹ min ⁻¹)	66,800 ± 18,100	304,900 ± 107,400	54,900 ± 7,200
Resorufin propionate (2) + NAD⁺			
K_m (µM)	2.87 ± 0.32	8.77 ± 1.58	2.91 ± 0.31
k_{cat} (min ⁻¹)	369 ± 12.36	1981 ± 130	112 ± 3.49
k_{cat}/K_m (mM ⁻¹ min ⁻¹)	128,500 ± 15,000	225,900 ± 43,300	38,500 ± 4,300
Resorufin hexanoate (3)			
K_m (µM)	1.53 ± 0.24	2.54 ± 0.52	0.07 ± 0.01
k_{cat} (min ⁻¹)	21.70 ± 0.70	532 ± 31.28	20.9 ± 0.27
k_{cat}/K_m (mM ⁻¹ min ⁻¹)	14,200 ± 2,300	209,800 ± 44,900	298,300 ± 23,300
Resorufin hexanoate (3) + NAD⁺			
K_m (µM)	2.66 ± 0.65	2.43 ± 0.52	5.05 ± 0.45
k_{cat} (min ⁻¹)	27.91 ± 1.54	28.50 ± 1.04	6.45 ± 0.09
k_{cat}/K_m (mM ⁻¹ min ⁻¹)	10,500 ± 2,600	11,700 ± 2,500	1300 ± 115
Resorufin octanoate (4)			
K_m (µM)	0.35 ± 0.06	0.89 ± 0.23	0.16 ± 0.04
k_{cat} (min ⁻¹)	23.1 ± 0.77	382 ± 26.65	16.5 ± 0.94
k_{cat}/K_m (mM ⁻¹ min ⁻¹)	66,800 ± 11,900	428,100 ± 115,900	104,200 ± 29,400
Resorufin octanoate (4) + NAD⁺			
K_m (µM)	0.36 ± 0.09	1.01 ± 0.25	NA
k_{cat} (min ⁻¹)	11.2 ± 0.51	9.55 ± 0.54	NA
k_{cat}/K_m (mM ⁻¹ min ⁻¹)	31,400 ± 7,600	9,500 ± 2,450	NA
Resorufin benzoate (5)			
K_m (µM)	1.52 ± 0.50	0.51 ± 0.20	0.47 ± 0.10
k_{cat} (min ⁻¹)	1.34 ± 0.09	4.89 ± 0.32	1.32 ± 0.07
k_{cat}/K_m (mM ⁻¹ min ⁻¹)	880 ± 300	9,550 ± 3,800	2,800 ± 600
Resorufin benzoate (5) + NAD⁺			
K_m (µM)	7.79 ± 1.19	NA	NA
k_{cat} (min ⁻¹)	2.97 ± 0.09	NA	NA
k_{cat}/K_m (mM ⁻¹ min ⁻¹)	380 ± 59	NA	NA
Resorufin retinoate (6)			
K_m (µM)	52.9 ± 5.96	97.3 ± 15.41	34 ± 11
k_{cat} (min ⁻¹)	0.35 ± 0.02	2.7 ± 0.23	1.99 ± 0.20
k_{cat}/K_m (mM ⁻¹ min ⁻¹)	6.7 ± 1	27.7 ± 5	58.7 ± 20
Resorufin retinoate (6) + NAD⁺			
K_m (µM)	54 ± 9.20	NA	NA
k_{cat} (min ⁻¹)	0.46 ± 0.02	NA	NA
k_{cat}/K_m (mM ⁻¹ min ⁻¹)	8.6 ± 2	NA	NA

Enzymatic activity was measured fluorometrically in 50 mM HEPES, pH 7.5, at 25°C in the presence of 500 µM NAD⁺. Fluorescence of resorufin was followed at 583 nm with excitation at 560 nm. To calculate k_{cat} values, the molecular weight used for ALDH1A1 was 220,000 g/mol, for ALDH1A2 was 226,800 g/mol and for ALDH1A3 was 224,000 g/mol. NA, no activity was detected. Data are the mean ± standard deviation of triplicates (n = 3).

HEPES, pH 7.5. The assays were performed in a 1-mL quartz cuvette at 37°C, adding 500 μM NAD^+ when hexanal and resorufin compounds were assayed and 150 μM NAD^+ when MONAL series of compounds were analyzed. All substrate concentrations were chosen to be at or near saturation for each substrate and isoform, according to previous reports [32,34] and this work (Table 1). The reaction was initiated by the addition of the corresponding substrate and the fluorescence of NADH was monitored at 460 nm with excitation at 340 nm, using 10 and 10 nm for the excitation and emission spectral bandwidths, respectively, for hexanal assays; MONOIC-62 product was monitored at 360 nm with excitation at 288 nm, using 5 and 10 nm for the excitation and emission spectral bandwidths, respectively; the fluorescence of MONOIC-71 product was monitored at 395 nm with excitation at 331 nm, using 10 and 10 nm for the excitation and emission spectral bandwidths, respectively; and the fluorescence of resorufin was monitored at 583 nm with excitation at 560 nm, using 2.5 and 5 nm for the excitation and emission spectral bandwidths, respectively, for esterase activity. Furthermore, 5 μM NADH, 1 μM MONOIC-62 or MONOIC-71 and 5 μM resorufin were added as an internal standard to obtain the absolute reaction rates, which were calculated as described previously [34]. Inhibition of ALDH activity was performed with 100 μM ABD0171 in 50 mM HEPES, pH 7.5, at 37°C. Cell extracts were pre-incubated for 20 min with inhibitor in the absence of NAD^+ . Inhibition of carboxylesterase 2 (CES2) activity was performed under the same conditions with 200 μM orlistat. Then 500 μM NAD^+ and 15 μM resorufin acetate were added.

3. Results and discussion

3.1. Kinetic characterization of ALDH1A isoforms using resorufin derivatives as substrates

All active isoforms displayed Michaelis-Menten kinetics with the resorufin ester derivatives tested. The kinetic parameters (K_m , k_{cat} and k_{cat}/K_m) of ALDH1A1, ALDH1A2 and ALDH1A3 were determined, revealing distinct patterns depending on the molecular volume of the carboxylate moiety, in the absence and presence of NAD^+ (Table 1). The K_m values were all grouped in the low micromolar range, 0.07–2.54 μM substrate in the absence of NAD^+ , and 2.14–8.77 μM in the presence of NAD^+ , except for the retinoate compound (6) that showed much higher values (34–97.3 μM). The k_{cat} values were much more variable, being those of ALDH1A2 the highest ($k_{cat} = 2,246 \pm 53 \text{ min}^{-1}$), resulting in a catalytic efficiency of $6,500,000 \pm 925,000 \text{ mM}^{-1} \text{ min}^{-1}$ for resorufin acetate (1), the highest of any ALDH1A isoform and for any substrate reported so far. The higher turnover rates of ALDH1A2 are a characteristic feature among ALDH1A isoforms, which was previously observed with other substrates such as alkanals, alkenals, 6-methoxy-2-naphthaldehyde and BODIPY-aminoacetaldehyde, including 4-nitrophenyl acetate ester [2,32]. An interesting feature of ALDH1A2 is the presence of a disordered loop at the entrance of the substrate channel. It has been proposed that upon substrate binding a disorder to order transition may occur. In fact, it was reported that two point mutations in the disordered segment resulted in a reduced apparent V_{max} in rodent ALDH1A2 [39].

The presence of cofactor in the esterase reaction resulted in a wide array of catalytic effects, from increasing to decreasing enzymatic activity, depending on the molecular volume of the carboxylate moiety of the resorufin derivative (Table 1). With resorufin acetate, the smallest substrate, the inducing effect of adding 500 μM NAD^+ was greatest since it increased the k_{cat} value for all isoforms between 4- and 40-fold. As the length of the aliphatic chain increased from acetate (1) to octanoate (4), the k_{cat} values of resorufin derivatives decreased, suggesting a less catalytically productive interaction within the enzyme active site. The isoform least sensitive to substrate bulkiness was ALDH1A1, followed by ALDH1A2. The extreme case was ALDH1A3 which rapidly lost enzymatic activity since it did not show any detectable activity with resorufin octanoate (4) (Table 1 and Fig. S1).

When comparing the effect of adding cofactor for each substrate, resorufin acetate (1) and resorufin propionate (2) kept the activity enhancement in all isoforms. Both resorufin derivatives were much better substrates of ALDH1A isoforms as compared to 4-NPA [2]. Only ALDH1A1 tolerated well the cofactor presence when resorufin hexanoate (3) was used as a substrate. Resorufin octanoate (4) showed activity inhibition in all isoforms upon cofactor binding. Again, as the number of carbon atoms increased the cofactor enhancing effect was diminished to different extents among the various isoforms, being ALDH1A1 the most resilient and ALDH1A3 the least.

Interestingly, with resorufin benzoate (5) and resorufin retinoate (6), the k_{cat} values of all isoforms were very low, likely reflecting the unfavorable binding configuration when linked to resorufin. With resorufin retinoate, ALDH1A3 demonstrated the highest catalytic efficiency, followed by ALDH1A2 and ALDH1A1. This parallels the fact that ALDH1A3 also exhibited the highest efficiency with the physiological substrate, all-*trans*-retinaldehyde, followed by ALDH1A2 and ALDH1A1 [2]. In contrast, when the cofactor was present, only ALDH1A1 kept the esterase activity.

The results obtained reveal profound differences in catalytic performance depending on the nature of the carboxyl moiety of the resorufin derivatives. Bulkiness, hydrophobicity and the presence of π electrons in aromatic and conjugated systems, and their adaptation to the active site topology of each ALDH isoform are likely determinants.

3.2. Molecular docking of resorufin esters into ALDH1A active sites

In order to rationalize the observed experimental results, molecular docking analysis of resorufin esters, in the absence and presence of NAD^+ , was performed for ALDH1A1, ALDH1A2 and ALDH1A3, revealing distinct patterns in substrate binding, orientation, and catalysis.

3.2.1. Resorufin hexanoate

In the ALDH1A3 active site, in the absence of NAD^+ , resorufin hexanoate (3) adopts an extended conformation, allowing the ester carbonyl carbon to locate at 3.5 Å from Cys314, in a catalytically productive configuration, making possible the nucleophilic attack (Fig. 1A). Additionally, multiple interactions with the benzene ring of resorufin hexanoate are observed, including an offset-stacked (RING OT) interaction with Trp189, a sulfur- π interaction with Cys313, and edge-to-face (RING EF) aromatic interactions involving Phe182 and Phe477. These contacts highlight the involvement of key residues such as Phe182, Trp189, Cys313, and Phe477, in stabilizing the ligand within the binding site. Conversely, in the presence of NAD^+ , the hydrocarbon chain of resorufin hexanoate experiences steric hindrance with the cofactor, preventing optimal positioning near the catalytic residue Cys314 (Fig. 1B). Instead, the resorufin aromatic rings engage in carbon- π interactions with residues Ile132 and Leu471, stabilizing the substrate but limiting its access to the active site for nucleophilic attack. These findings align well with the lower ΔG and RMSD values for the complex in the absence of NAD^+ .

The kinetic data (Table 1) strongly support the molecular docking findings since, in the absence of NAD^+ , the K_m value is lower ($K_m = 0.07 \mu\text{M}$ vs 5 μM), catalytic turnover is higher (21 min^{-1} vs 6.5 min^{-1}), and overall enzyme efficiency is significantly enhanced ($298,300 \text{ mM}^{-1} \text{ min}^{-1}$ vs $1,300 \text{ mM}^{-1} \text{ min}^{-1}$). These results indicate that ALDH1A3 functions optimally without NAD^+ for its esterase activity with resorufin hexanoate.

In the ALDH1A1 active site, resorufin hexanoate bound in the presence of NAD^+ reveals a distinct binding mode compared to that of ALDH1A3. This is likely related to the higher accessible volume of the ALDH1A1 pocket compared to that of ALDH1A3 [2]. The hydrocarbon chain of the substrate successfully accommodates within the ALDH1A1 active site lined by Trp178 and Val460 without inducing steric clashes with the NAD^+ cofactor (Fig. 2). Besides, offset face-to-face (RING OF)

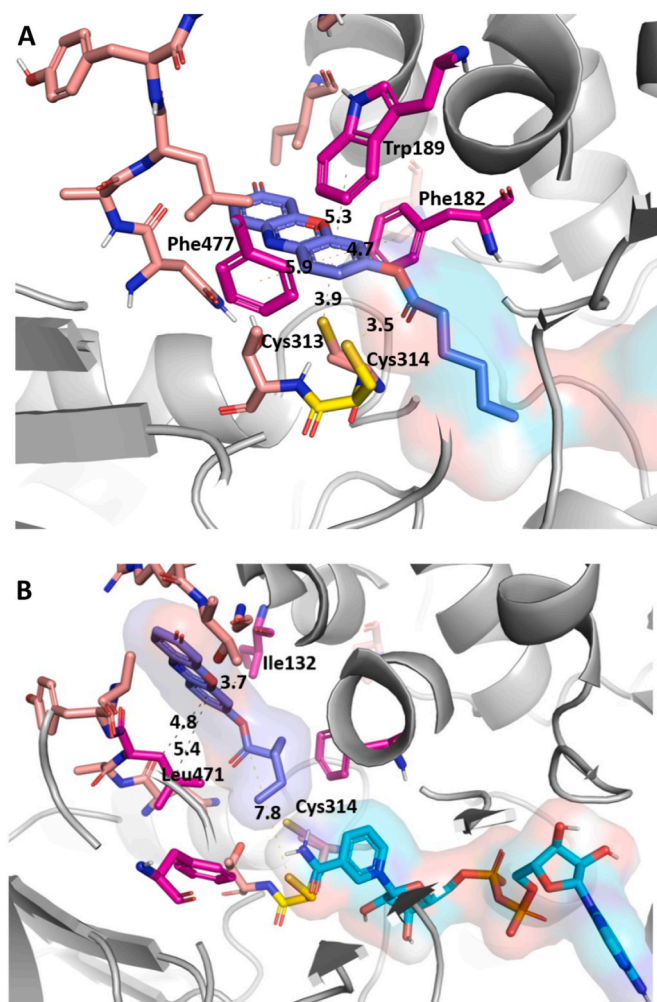


Fig. 1. Molecular docking analysis of resorufin hexanoate into the active site of the ALDH1A3 isoform. A) Docking of resorufin hexanoate with ALDH1A3 in the absence of NAD^+ ($\Delta G = -8.8$ kcal/mol, RMSD lb = 1.26 Å, RMSD ub = 1.93 Å). B) Docking of resorufin hexanoate to ALDH1A3 in the presence of NAD^+ ($\Delta G = -7.9$ kcal/mol, RMSD lb = 3.58 Å, RMSD ub = 3.34 Å). Multiple non-covalent interactions involving aromatic π -systems (carbon- π , sulfur- π , RING OT and RING EF type) contribute to the stabilization of resorufin hexanoate, positioning the carbonyl carbon atom at 3.5 Å and 7.8 Å from Cys314, in the absence and presence of NAD^+ , respectively.

interactions of Phe171 and Tyr297 with the benzene ring of resorufin hexanoate contribute to the favorable stabilization of the substrate within the active site of ALDH1A1. The lower RMSD values observed in ALDH1A1 relative to ALDH1A3 align well with the corresponding decreased K_m value (Table 1), suggesting that ligands adopting non-constrained conformations tend to exhibit improved binding affinities. Thus, these findings highlight the interplay between structural flexibility, active site dynamics, and catalytic efficiency in ALDH1A3, reinforcing the inhibitory role of NAD^+ in restricting optimal substrate alignment.

3.2.2. Resorufin octanoate

Binding of resorufin octanoate, in the presence of NAD^+ , was compared between ALDH1A2 and ALDH1A3. In ALDH1A2, resorufin octanoate adopted a catalytically productive orientation characterized by a favorable binding energy (-8.4 kcal/mol), moderate RMSD values (2.33 Å lb; 3.19 Å ub), and a short distance (4.3 Å) between the ester carbonyl carbon and the catalytic Cys320 (Fig. 3A). The bulky hydrocarbon chain of resorufin octanoate could still be accommodated within ALDH1A2 through conformational folding of the aliphatic tail, thereby

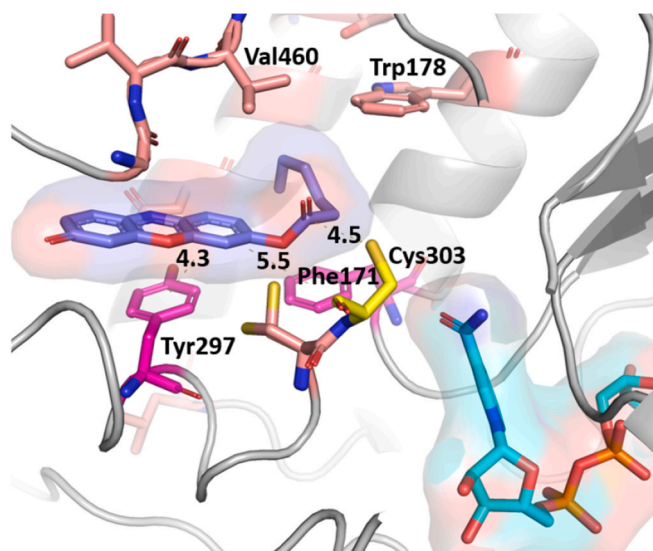


Fig. 2. Molecular docking analysis of resorufin hexanoate into the active site of the ALDH1A1 isoform. Docking of resorufin hexanoate to ALDH1A1 in the presence of NAD^+ ($\Delta G = -7.5$ kcal/mol, RMSD lb = 2.24 Å, RMSD ub = 3.01 Å). The hydrocarbon chain of resorufin hexanoate is effectively accommodated within the active site, delineated by Trp178 and Val460 and the RING OF interactions of Tyr297 and Phe171 with the benzene ring of resorufin hexanoate, which contribute to the stabilization of the substrate, positioning the carbonyl carbon atom at 4.5 Å from nucleophilic Cys303.

minimizing steric clashes with NAD^+ and allowing the ester carbonyl to remain oriented toward the catalytic cysteine residue. In contrast, docking resorufin octanoate into ALDH1A3 produced less favorable structural parameters, with substantially higher RMSD values (3.37 Å lb; 5.33 Å ub), and, most importantly, a larger distance (8.6 Å) between the ester carbonyl and the catalytic Cys314, which is incompatible with an efficient catalytic attack (Fig. 3B). The reduced pocket volume and the presence of NAD^+ generate significant steric hindrance that limits substrate penetration into the catalytic site. These results therefore suggest that, although substrate binding may still occur in ALDH1A3, the orientation adopted is not compatible with catalysis. The kinetic results support the molecular docking findings: activity is low but detectable in ALDH1A2 and is completely lost in ALDH1A3 (Table 1).

3.2.3. Resorufin benzoate

An additional intriguing scenario emerges when the interaction between resorufin benzoate (5) and the ALDH1A3 active site was analyzed. In this case, the benzene ring occupies a significant space within the active site and, in the absence of NAD^+ (Fig. 4A), it is attracted towards Cys314 through several interactions (sulfur- π , donor- π , and carbon- π), being the sulfur- π interaction the most significant. At this point, the thiol group of the catalytic Cys establishes a non-covalent π -type bond at a distance of 2.5 Å with the benzene ring. As a result, the thiol group is no longer able to make direct contact with the ester group of resorufin benzoate, effectively preventing the enzymatic activity. This situation could hypothetically enable some catalytic function to adjacent Cys313, which may still exhibit partial or full nucleophilic activity due to the electron-donating capacity of the thiol group. However, the steric or electrostatic interaction with Cys314 could limit the nucleophilic potential of Cys313. The observed distance between the sulfur atom of Cys313 and the carbonyl carbon of the ester (3.2 Å) suggests potential for transient covalent bonding, supporting the idea of a nucleophilic reaction. On the other hand, in the presence of NAD^+ (Fig. 4B), the substrate cannot approach the catalytic Cys314, as it would cause a steric clash. Additionally, the high electron density of the benzene ring prevents it from advancing through the catalytic channel. Instead, it establishes aromatic interactions with nearby residues,

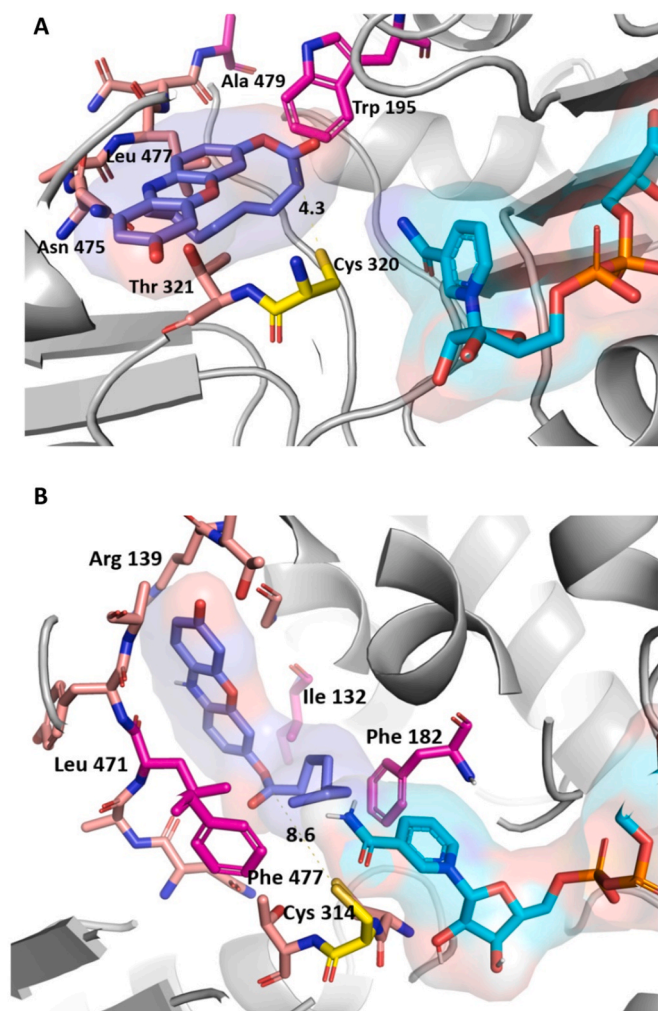


Fig. 3. Molecular docking analysis of resorufin octanoate, in the presence of NAD^+ , into the active site of the ALDH1A2 and ALDH1A3 isoforms. A) Docking of resorufin octanoate with ALDH1A2 in the presence of NAD^+ ($\Delta G = -8.4$ kcal/mol, RMSD lb = 2.33 Å, RMSD ub = 3.19 Å). The carbonyl carbon atom is positioned at 4.3 Å from Cys320. B) Docking of resorufin octanoate to ALDH1A3 in the presence of NAD^+ ($\Delta G = -7.8$ kcal/mol, RMSD lb = 3.37 Å, RMSD ub = 5.23 Å). The carbonyl carbon atom is positioned at 8.6 Å from Cys314.

including a RING FF interaction with Phe182, a RING OT interaction with Trp189, and a sulfur- π interaction with Cys313, collectively contributing to hinder enzymatic activity when NAD^+ is present. The kinetic results support the molecular docking findings: activity is low in the absence of NAD^+ and is completely lost in the presence of NAD^+ (Table 1).

3.2.4. Resorufin retinoate

In the ALDH1A3 active site, for resorufin retinoate (**6**), molecular docking in the absence of NAD^+ revealed exceptionally high RMSD values (Fig. 5A). This unstable binding mode is consistent with the highest K_m value (34 ± 11 μM) among all tested compounds for ALDH1A3 (Table 1). However, the substrate carbonyl group is located at 3.5 Å from the sulfur atom of Cys314, suggesting a potential nucleophilic reaction, further supported by carbon- π interactions between Cys314 and Thr256 with the resorufin aromatic ring, and a sulfur- π interaction with Met186.

In fact, the retinoic acid moiety of resorufin retinoate mimics the binding pose and conformation of all-*trans*-retinoic acid in the 5FHZ structure [40], particularly in the substrate tunnel and around the

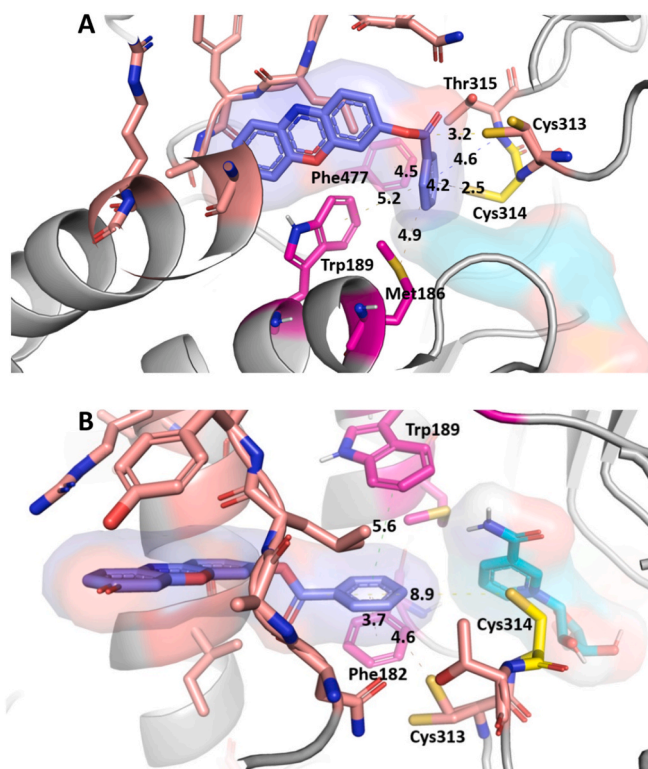


Fig. 4. Molecular docking analysis of resorufin benzoate into the active site of the ALDH1A3 isoform. A) Docking of resorufin benzoate to ALDH1A3 in the absence of NAD^+ ($\Delta G = -9.5$ kcal/mol, RMSD lb = 1.753 Å, RMSD ub = 1.899 Å). The benzoate ring is oriented towards Cys314 through sulfur- π (2.5 Å) interactions with Met186 and Cys314, donor- π interactions with Thr315, carbon- π interactions with C314 (4.2 Å), RING OF type with Trp189 and RING OT type with Phe477. B) Docking of resorufin benzoate to ALDH1A3 in the presence of NAD^+ ($\Delta G = -7.6$ kcal/mol, RMSD lb = 1.833 Å, RMSD ub = 2.755 Å). RING FF and RING OT interactions of resorufin benzoate with aromatic residues such as Phe182 and Trp189, respectively, and a sulfur- π interaction with Cys313, avoid enzymatic activity.

catalytic cysteine. The accommodation of the resorufin group, which may interact with additional residues at the NAD^+ binding site, does not disrupt the canonical binding mode of the retinoic acid moiety. The polyene chain and β -ionone ring of the retinoic acid moiety form hydrophobic and van der Waals contacts with Ile132, Gly136, Arg139, Thr140, Trp189, Leu471 and Ala473, stabilizing the ligand at the entrance of the substrate-binding pocket, as observed with retinoic acid in the 5FHZ structure. In contrast, in the presence of NAD^+ , severe steric clashes between resorufin retinoate and ALDH1A3 precluded any productive interaction of the substrate in the active site (Fig. 5B), which is consistent with the lack of enzymatic activity (Table 1).

In the ALDH1A1 active site, molecular docking analysis of resorufin retinoate reveals a binding mode analogous to that observed with resorufin hexanoate, where the substrate adopts a conformation confined to the catalytic pocket rather than extending into the NAD^+ binding site, both in the absence and in the presence of cofactor. This configuration results in the accommodation of the polyene chain within the region defined by residues Phe171 and Ile304, in the absence of NAD^+ , and Gly458 and Ala462 in the presence of NAD^+ .

In the absence of NAD^+ cofactor, resorufin retinoate establishes RING EF interactions with Trp178 and Phe171, and carbon- π interactions with Val174 (Fig. 6A). On the other hand, in the presence of cofactor, the substrate is also stabilized by π -type interactions between the phenoxazine aromatic ring of resorufin substrate and surrounding aromatic residues, including RING OF type with Phe171 and face-to-face (RING FF) interactions with Tyr297 (Fig. 6B). However, unlike

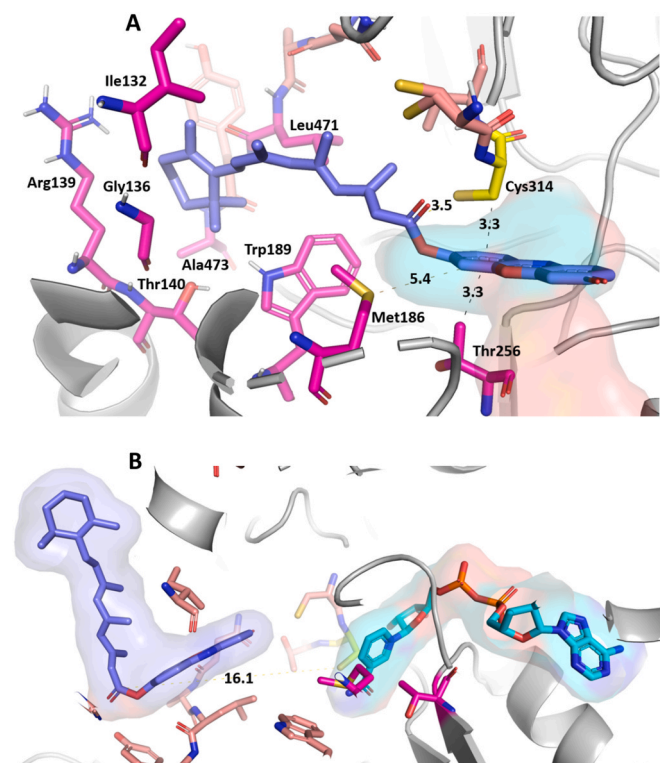


Fig. 5. Molecular docking analysis of resorufin retinoate into the active site of the ALDH1A3 isoform. A) Docking of resorufin retinoate to ALDH1A3 in the absence of NAD^+ ($\Delta G = -9.7$ kcal/mol, RMSD lb = 2.548 Å, RMSD ub = 4.548 Å). Carbon- π interactions between Cys314 and Thr256 with the resorufin aromatic ring, and a sulfur- π interaction with Met186 are shown, positioning the carbonyl carbon atom at 3.5 Å from Cys314. Hydrophobic and van der Waals contacts with Ile132, Gly136, Arg139, Thr140, Trp189, Leu471 and Ala473 (shown in magenta) mimic the residue-by-residue binding pattern of retinoic acid in the 5FHZ structure [40]. B) Docking of resorufin retinoate to ALDH1A3 in the presence of NAD^+ ($\Delta G = -6.9$ kcal/mol, RMSD lb = 10.134 Å, RMSD ub = 10.976 Å). No detectable interactions within the active site were observed in the docking of resorufin retinoate to ALDH1A3 in the presence of NAD^+ .

ALDH1A3, where resorufin retinoate binds in a more linear conformation that extends into the NAD^+ binding pocket, potentially enhancing catalytic alignment, its positioning in ALDH1A1 reflects a constrained geometry. This alternative binding mode may contribute to the comparatively poorer kinetic parameters observed for ALDH1A1 (Table 1), primarily due to the increased distance between the catalytic cysteine and the ester bond of resorufin retinoate (4.6 Å and 4.8 Å, in the absence and in the presence of NAD^+ , respectively), which may limit the efficiency of the nucleophilic attack. In contrast, the smaller distance observed in ALDH1A3 (3.5 Å) suggests a more favorable geometry, which may contribute to the more efficient catalytic activity exhibited by ALDH1A3 towards resorufin retinoate in the absence of NAD^+ . These findings suggest that while ALDH1A1 accommodates bulky aromatic substrates through favorable local interactions, its inability to support the extended binding configuration seen in ALDH1A3 may limit catalytic efficiency.

In general, RMSD lb and ub values were higher for resorufin derivatives docked in the presence of NAD^+ than without NAD^+ , especially for resorufin retinoate bound to ALDH1A3 (Fig. S2). RMSD values for resorufin hexanoate and benzoate in the presence of NAD^+ remained between 1.8 and 3.6 Å, while for resorufin retinoate reached above 10 Å within the ALDH1A3 active site. In this latter case, when comparing ALDH1A1 and ALDH1A3, RMSD values in ALDH1A3 were higher than in ALDH1A1, reflecting steric clashes within the narrower site pocket of

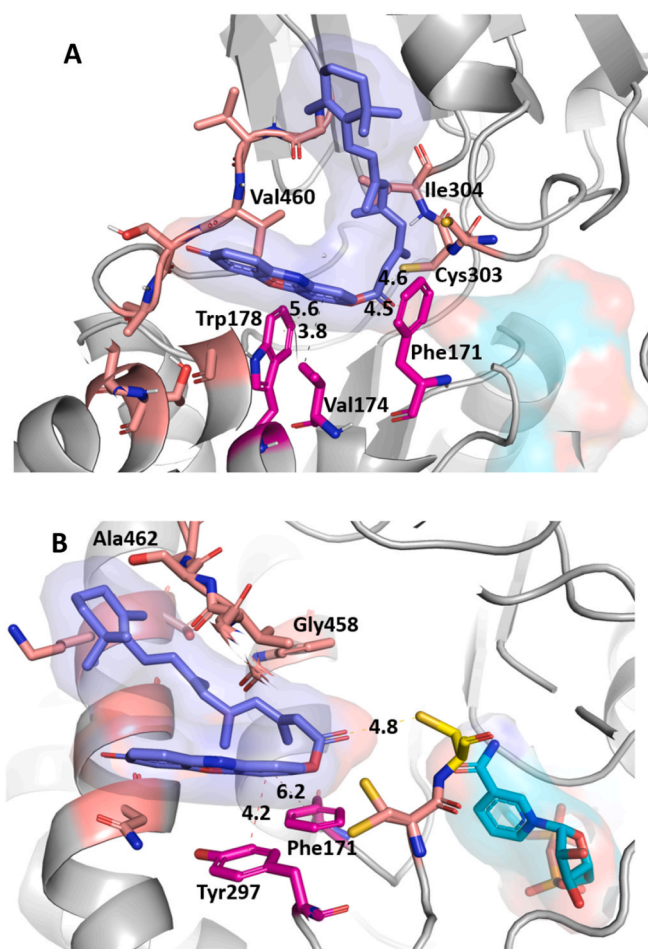


Fig. 6. Molecular docking analysis of resorufin retinoate into the active site of the ALDH1A1 isoform. A) Docking of resorufin retinoate to ALDH1A1 in the absence of NAD^+ ($\Delta G = -11.1$ kcal/mol, RMSD lb = 2.954 Å, RMSD ub = 6.784 Å). Additional RING EF interactions with Trp178 and Phe171, as well as carbon- π interactions with Val174, further stabilize the binding pose in the absence of NAD^+ . Under both conditions, the substrate adopts a constrained conformation confined to the catalytic pocket, avoiding the NAD^+ cofactor binding site. B) Docking of resorufin retinoate to ALDH1A1 in the presence of NAD^+ ($\Delta G = -9.0$ kcal/mol, RMSD lb = 2.456 Å, RMSD ub = 4.876 Å). π -type interactions stabilize the substrate via RING OF and RING FF interactions with Phe171 and Tyr297, respectively.

ALDH1A3. The observed differences in substrate binding and catalytic efficiency between ALDH1A1, ALDH1A2 and ALDH1A3 can be attributed, in part, to the distinct structural characteristics of their active sites, particularly the size of the substrate-binding pocket. ALDH1A1 features a larger active site pocket (534 Å³) compared to ALDH1A2 (387 Å³) and ALDH1A3 (357 Å³) [2], which directly impacts the ability of the enzyme to accommodate larger substrates such as resorufin octanoate, resorufin benzoate, or resorufin retinoate in the presence of NAD^+ . The increased volume of the ALDH1A1 active site provides greater topological flexibility, allowing substrates to bind more easily and adopt conformations that promote effective nucleophilic attack and catalysis even when the cofactor is bound. In contrast, the smaller active site of ALDH1A3 results in more limited space, which can induce steric clashes or hinder proper substrate orientation, especially when larger molecules like resorufin retinoate are involved. This structural limitation is further exacerbated by the presence of NAD^+ , which induces conformational changes that reduce the capacity of enzymes to accommodate substrates efficiently. Thus, the size of the substrate-binding pocket is a critical factor influencing the differential catalytic behavior of ALDH1A1 and ALDH1A3.

3.3. Comparative analysis of ALDH activity in lung cancer A549 and glioblastoma A172 cell extracts

To assess the potential use of resorufin ester derivatives in ALDH activity measurements in cell extracts, two different human cell lines, A549 and A172, were analyzed by using both dehydrogenase (with aldehyde substrates) and esterase (with resorufin acetate) activities. A549 cells, from lung adenocarcinoma, typically express high levels of several ALDH isoforms and have been widely used as a model to study lung cancer and the development of drug therapies [38,41]. On the other hand, A172 glioblastoma cells also express multiple ALDH isoforms but at much lower levels than A549 cells [42].

Fig. 7 displays the specific activity of ALDH isoforms towards different substrates in A549 and A172 cells. A549 cell extracts showed high dehydrogenase activity (15–70 mU/mg) compatible with the known expression of several ALDH isoforms, i.e. ALDH1A1, ALDH1A3, ALDH2, ALDH3A1, and ALDH7A1 at mRNA, protein and enzymatic activity levels [38,41,43–49]. Comparatively, A172 cells were much less active (0.5–2.5 mU/mg) with lower expression of ALDH1A1 and ALDH3A1, as assessed from their null activity with MONAL-62 or MONAL-71. This fact aligns well with the presence of ALDH1A3 in glioblastoma cell lines [42], which typically exhibited the lowest catalytic efficiency for aromatic or bulky aldehydes, such as MONAL-62 (more specific for ALDH1A1 and ALDH3A1) [32]. When resorufin acetate was used as a substrate, both A549 and A172 exhibited the highest activity (220 and 35 mU/mg, respectively). This elevated specific activity indicates that the ALDH isoforms present in A549 and A172 cell lines efficiently hydrolyze this substrate.

3.4. Contribution of ALDH to esterase activity in glioblastoma A172 cell extracts

To show whether the esterase activity in cell extracts was due to ALDH, an ALDH-specific inhibitor and ALDH-knockout cells were used (Fig. 8). ABD0171 is a selective inhibitor of ALDH1A isoforms which had been found to be successful in inhibiting the dehydrogenase activity in glioblastoma and breast cancer cell lines [7,32,42]. Here, when ABD0171 was added, the esterase activity was inhibited by 53% in glioblastoma A172 cell extracts. This result is consistent with the

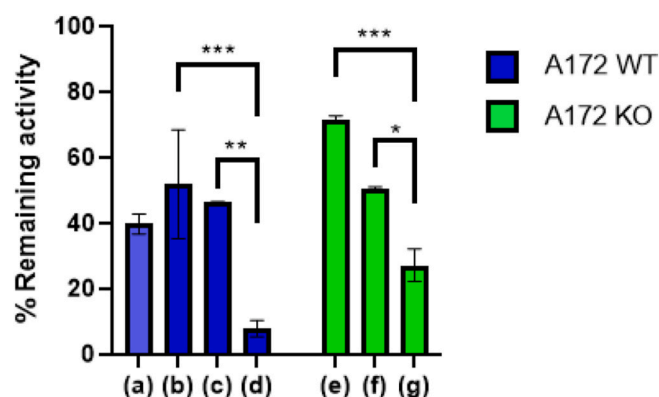


Fig. 8. Effect of ABD0171 and orlistat on the enzymatic activity in A172 WT and KO cells. Bar diagram showing the remaining activity (%) of A172 wild-type (WT, blue) and knockout (KO, green) cells after treatment using ALDH (ABD0171) and carboxylesterase (orlistat) inhibitors. Dehydrogenase activity in (a) was measured using 250 μM hexanal in 50 mM HEPES, 50 mM MgCl₂, pH 7.2, at 37°C, whereas esterase activity in (b–h) was determined using 15 μM resorufin acetate and 500 μM NAD⁺, in 50 mM HEPES, pH 7.5, at 37°C. Incubation time with the inhibitors was 20 min: (a) 15 μM ABD0171 (data taken from Ref. [38]); (b) 100 μM ABD0171; (c) 200 μM orlistat; (d) 100 μM ABD0171 and 200 μM orlistat; (e) 100 μM ABD0171; (f) 200 μM orlistat; (g) 100 μM ABD0171 and 200 μM orlistat. Data are expressed as the mean ± S.D. Statistical analysis was performed using one-way ANOVA followed by Sidak's multiple comparisons test. Statistical significance is indicated as **p* < 0.05, ***p* < 0.01, ****p* < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

inhibitory potency (60%) of ABD0171 against the dehydrogenase activity in A172 cell extracts [42].

A172 KO cells had been characterized previously [42] and were virtually devoid of any ALDH1A dehydrogenase activity. Thus, they were also used to assess the contribution of ALDH to the esterase activity. ABD0171 inhibition decreased from 53% in A172 WT cells to 30% in A172 KO cells, suggesting that ALDH partially contributes to esterase activity. Thus, it is conceivable that any other remaining activity could be due to non-ALDH1A isoforms or other esterases. In fact, it has been reported that A172 cells show detectable levels of CES2 activity [50]. To

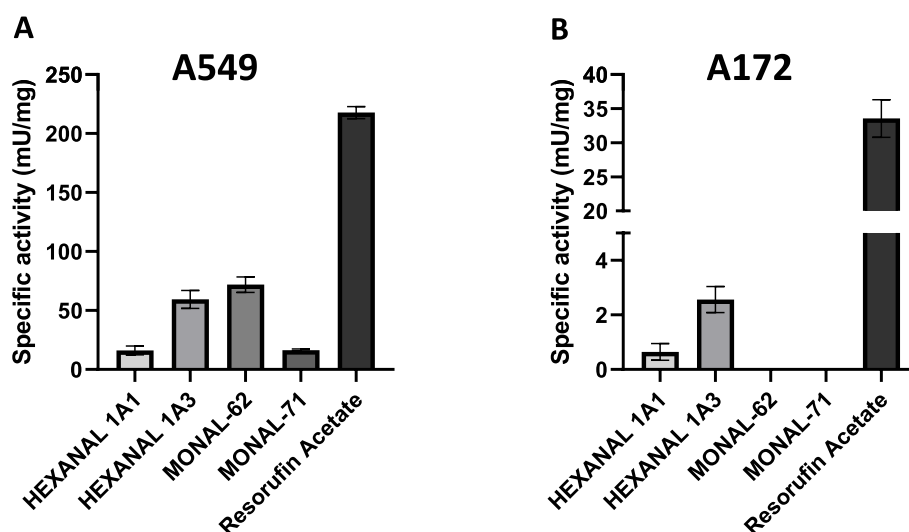


Fig. 7. Dehydrogenase and esterase activities of ALDH in tumor cell extracts. Specific activity was measured in mU/mg protein. (A) A549 cells. (B) A172 cells. Activity assays were performed in 50 mM HEPES, 0.5 mM EDTA, pH 7.2 using 30 μM hexanal for ALDH1A1 assay; 50 mM HEPES, 50 mM MgCl₂, pH 7.2, using 250 μM hexanal for ALDH1A3 assay; 50 mM pyrophosphate-NaOH, 0.5 mM EDTA, pH 8.1 using 5 μM MONAL-62 and 15 μM MONAL-71, respectively. The esterase activity was monitored in 50 mM HEPES, pH 7.5, using 15 μM resorufin acetate. As a cofactor, 500 μM NAD⁺ was added when hexanal or resorufin acetate was used as a substrate, and 150 μM NAD⁺ when MONAL series of compounds were analyzed. In both cases, these corresponded to saturating concentrations of cofactor. All activities were measured at 37°C. Data are the mean ± standard deviation of triplicates (n = 3).

sort this point out, orlistat, a potent inhibitor of CES2 [51,52], was used alone or in combination with ABD0171. As expected, 200 μ M orlistat had a similar effect on A172 WT and A172 KO cells, the remaining activity being 46% and 51%, respectively. In contrast, when 200 μ M orlistat was combined with 100 μ M ABD0171, the remaining activity in A172 WT cells was only 8%, much lower than that in A172 KO cells (27%). Importantly, 200 μ M orlistat did not inhibit any of the ALDH1A isoforms *in vitro*. Overall, it could be estimated that ALDH1A isoforms account for approximately 50% of the esterase activity in A172 cells, while CES2 contributes the rest of the activity.

4. Conclusions

In summary, we examined the performance of resorufin-derived ester compounds, with acyl moieties of different chemical nature and atomic volume, as substrates of the esterase activity of three ALDH1A isoforms: ALDH1A1, ALDH1A2 and ALDH1A3. All isoforms were active with the resorufin ester derivatives tested. The best compound in terms of substrate specificity (k_{cat}/K_m) was resorufin acetate with ALDH1A2, while the poorest substrate was resorufin retinoate with ALDH1A1. When adding cofactor, the catalytic rate of resorufin derivatives decreased, as the length of the aliphatic chain increased from acetate (1) to octanoate (4). The isoform less sensitive to substrate bulkiness was ALDH1A1, followed by ALDH1A2 and ALDH1A3, in good agreement with their active-pocket accessible volumes. ALDH1A3 did not show any detectable activity with resorufin octanoate (4), benzoate (5) and retinoate (6) in the presence of NAD⁺. The results with resorufin derivatives were reminiscent of the previous observation made using 4-nitrophenyl derivatives, where the enhancement of esterase activity by cofactor was limited to esters of short acyl chain length, while with longer acyl chain length or increased bulkiness no effect or even inhibition was observed [25]. Molecular docking simulations, backed by free energy and RMSD calculations, supported the hypothesis that bulky acyl moieties overlapped with the cofactor-binding channel of ALDH, resulting in a less catalytically productive interaction of the resorufin ester, especially in those ALDH isoforms with a narrower active site.

Resorufin acetate was used as an alternative substrate to detect ALDH activity in cancer cell extracts and compared favorably with standard dehydrogenase substrates, such as hexanal and even fluorescent naphthaldehyde derivatives. The potential use of other resorufin derivatives, such as resorufin octanoate or benzoate, in the presence or absence of NAD⁺, and the combination with specific inhibitor compounds could afford additional selectivity to identify the contribution of distinct ALDH isoforms.

Finally, resorufin-derived esters, with acyl moieties of different atomic volumes, and particularly resorufin acetate, will be suitable probes to test compounds as inhibitors of ALDH. In fact, by using 4-NPA, esterase activity had already been employed successfully in high-throughput assays to screen for ALDH1A1 and ALDH1A3 inhibitors [53–55]. We recently used a similar approach, based on fluorescent umbelliferone-derived esters, to screen for ALDH inhibitors (Covaleda et al., unpublished results). Thus, the ALDH esterase activity using a fluorescence-based assay will be a convenient inhibitor screening assay instead of aldehyde oxidation since it might exclude compounds that compete with structurally conserved elements of the cofactor-binding site and minimize spectral overlap with the absorbance characteristic of compounds in chemical libraries.

CRedit authorship contribution statement

Raquel Pequerul: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. **Daniela Covaleda:** Investigation, Methodology, Software, Visualization. **Andrés S. Sánchez-Vaca:** Investigation, Methodology, Software, Visualization. **Tarik Benali:** Investigation, Methodology. **Ismail Ceylan:** Conceptualization,

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jaume Farrés reports financial support, article publishing charges, and equipment, drugs, or supplies were provided by Spanish Ministerio de Ciencia, Innovación y Universidades. Raquel Pequerul reports financial support was provided by Advanced BioDesign. Ismail Ceylan, Guillaume Martin, Guy Fournet, and Mileidys Perez-Alea are inventors on patent #US11,519,014B2 issued to Advanced BioDesign. Ismail Ceylan, Guillaume Martin and Mileidys Perez-Alea are shareholders in Advanced BioDesign. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix. A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cbi.2026.112209>.

Data availability

Data will be made available on request.

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