ALDH1 Bio-activates Nifuroxazide to Eradicate ALDH$^{\text{High}}$ Melanoma-Initiating Cells

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In Brief
A major challenge for cancer treatment is that tumors are comprised of subpopulations with differing growth potential and drug sensitivity. Here, Sarvi and colleagues reveal that the clinically approved antibiotic, nifuroxazide, selectively eliminates ALDH$^{\text{High}}$ melanoma-initiating cell subpopulations. This conceptual advance opens up new avenues in drug repurposing and melanoma therapy.

Highlights
- ALDH1 bio-activates nifuroxazide leading to ALDH1 inactivation and cytotoxicity
- Nifuroxazide selectively eradicates ALDH$^{\text{High}}$ melanoma tumor-initiating cells
- Targeted therapy increases ALDH1 in some patient melanomas and cell line models
- Targeting ALDH$^{\text{High}}$ cells with nifuroxazide is an orthogonal therapeutic strategy
ALDH1 Bio-activates Nifuroxazide to Eradicate ALDH\textsuperscript{High} Melanoma-Initiating Cells

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SUMMARY

5-Nitrofurans are antibiotic pro-drugs that have potential as cancer therapeutics. Here, we show that 5-nitrofurans can be bio-activated by aldehyde dehydrogenase (ALDH) 1A1/1A3 enzymes that are highly expressed in a subpopulation of cancer-initiating (stem) cells. We discover that the 5-nitrofuran, nifuroxazide, is selective for bio-activation by ALDH1 isoforms over ALDH2, whereby it both oxidizes ALDH1 and is converted to cytotoxic metabolites in a two-hit pro-drug mechanism. We show that ALDH1\textsuperscript{High} melanoma cells are sensitive to nifuroxazide, while ALDH1A3 loss-of-function mutations confer drug resistance. In tumors, nifuroxazide targets ALDH1\textsuperscript{High} melanoma subpopulations with the subsequent loss of melanoma-initiating cell potential. BRAF and MEK inhibitor therapy increases ALDH1 expression in patient melanomas, and effectively combines with nifuroxazide in melanoma cell models. The selective eradication of ALDH1\textsuperscript{High} cells by nifuroxazide-ALDH1 activation goes beyond current strategies based on inhibiting ALDH1 and provides a rational basis for the nifuroxazide mechanism of action in cancer.

INTRODUCTION

5-Nitrofurans are pro-drugs (i.e., they require bio-activation for activity) and have been widely used in human and veterinary medicine for over 40 years to treat bacterial and trypanosome infections (Clayton, 2010). Recently, 5-nitrofuran antibiotics, such as nifurtimox and nifuroxazide, have been found to have anti-cancer activity in cancers, including neuroblastoma, melanoma, multiple myeloma, colon, and breast cancer (Nelson et al., 2008; Saulnier Sholler et al., 2006, 2011; Yang et al., 2015; Ye et al., 2017; Zhou et al., 2012; Zhu et al., 2016). Notably, nifurtimox reduced neuroblastoma tumor burden in a child with Chagas disease caused by trypanosome infection (Saulnier Sholler et al., 2006), and is now in a phase II clinical trial to treat children with relapsed or refractory neuroblastoma or medulloblastoma (NCT00601003) (Saulnier Sholler et al., 2011). When used to treat infectious disease, the relative selectivity of the 5-nitrofurans is mediated by bio-activation by bacterial- or parasite-specific nitroreductases (Clayton, 2010). However, the mechanism by which 5-nitrofurans confer anti-cancer activity is not well understood, limiting efforts for 5-nitrofuran drug development, patient stratification, and the development of treatment strategies.

We previously discovered that aldehyde dehydrogenase 2 (ALDH2) is a direct target for nifurtimox and NFN1 (a tool compound featuring a 5-nitrofuran moiety) by a small-molecule phenotypic screen in zebrafish (Zhou et al., 2012). ALDH enzymes are a large family of enzymes that metabolize toxic aldehydes (Koppaka et al., 2012; Ma and Allan, 2011). ALDH\textsuperscript{High} activity marks somatic stem cells, and, in many cancers, cells with ALDH\textsuperscript{High} enzymatic activity have enhanced tumorigenic potential and mark so-called cancer stem cell subpopulations (Marcato et al., 2011; Tomita et al., 2016). Cancer cell heterogeneity, including cancer stem cell populations, is of clinical importance because it can make treatment difficult, drive tumor progression, and promote drug resistance (Kreso and Dick, 2011).
2014). Genetic and phenotypic cancer cell heterogeneity is especially important in melanoma, where a majority of patients with metastatic melanoma succumb to the disease, and even those who initially respond to targeted or immune treatment may eventually die due to melanoma recurrence and drug resistance (Luke et al., 2017).

Despite the prevalence of the ALDH
\textsuperscript{H} subpopulations in multiple cancer types, there are no clinically available drugs that specifically target such subpopulations. Clinically active ALDH inhibitors, such as disulfiram or daidzin, are strong inhibitors for ALDH2, the principal ALDH enzyme responsible for alcohol metabolism, as well as other possible targets (Kona et al., 2011; Kopppaka et al., 2012), whereas ALDH
\textsuperscript{H} activity in melanoma is mediated by ALDH1A1 and ALDH1A3 (Luo et al., 2012; Yue et al., 2015). Further, ALDH1 inhibitors, although able to reduce enzyme activity, are not designed to eradicate ALDH
\textsuperscript{H} subpopulations (Koppaka et al., 2012; Tomita et al., 2016).

We hypothesized that ALDH1 might bio-activate certain 5-nitrofuran derivatives and thereby eradicate ALDH
\textsuperscript{H} melanoma-initiating subpopulations in tumors. Here, we report that 5-nitrofurans are bio-activated by ALDH1 and show that nifuroxazide, a clinically approved 5-nitrofuran pro-drug, is selectively bio-activated by ALDH1 enzymes over ALDH2 enzymes. This interaction is critical to mediate nifuroxazide anti-tumor activity. Nifuroxazide-induced loss of ALDH
\textsuperscript{H} subpopulations has a direct impact on the melanoma-initiating (cancer stem) cell potential in tumors. This is of clinical importance because we show that BRAF inhibitors lead to an increase in ALDH
\textsuperscript{H} melanoma cells in matched patient biopsies in recurrent metastatic melanoma, and that BRAF inhibitors plus MEK inhibitors lead to an increase in ALDH1A1 mRNA in matched patient biopsies while on treatment.

Our work argues that an important mechanism of action for nifuroxazide in cancer is through the eradication of ALDH
\textsuperscript{H} melanoma-initiating subpopulations in tumors, rather than inhibit ALDH1 enzymatic activity, and that the tumor-initiating and stem cell properties of cancer subpopulations can be drug targets, rather than targeting the molecular activity of cancer mutations.

RESULTS

ALDH1 is a Selective Target for Nifuroxazide

We have previously demonstrated that 5-nitrofuran pro-drugs can be substrates for, and bio-activated by, ALDH2 enzymes, leading to DNA damage and reactive oxygen species to kill cancer cells (Zhou et al., 2012). This prompted us to test if 5-nitrofurans might be bio-activated by ALDH1, yielding an opportunity to specifically target ALDH
\textsuperscript{H} melanoma subpopulations.

To establish the range of concentration of drug activity in cells, we first tested four clinical 5-nitrofurans (nifuroxazide, nitrofurantoin, furazolidone, and nifurtimox), our 5-nitrofuran tool compound NFN1, and the inactive no-nitro control compound NFN1.1 (in which a hydrogen atom replaces the nitro moiety) (Figures 1 A, S1 A, and S1 B). Among the clinical compounds, we found nifuroxazide to have the lowest half maximal effective concentration (EC
\textsubscript{50}) value in A375 melanoma cell lines and used nifuroxazide as a clinical 5-nitrofuran in our subsequent studies.

Next, we tested the potential for nifuroxazide and NFN1 to be substrates for ALDH1 and ALDH2 enzymes in vitro. If 5-nitrofurans are bio-activated by ALDH1, they will compete for ALDH activity toward aldehyde substrates. In this assay, ALDH enzymes metabolize aldehydes and convert NAD+ to NADH. Test substrates that compete for ALDH activity toward aldehydes lead to a reduction in NADH production. As positive controls, we used the ALDH1/2 inhibitor disulfiram, or the potent ALDH2 inhibitor daidzin (Koppaka et al., 2012). We found that both nifuroxazide and the NFN1 tool compound were effective substrates for the ALDH1 isoform ALDH1A1 (Figure 1 B), which is highly expressed in melanoma-initiating cell lines.

Surprisingly, while the NFN1 tool compound was a substrate for both ALDH1 and ALDH2 enzymes, nifuroxazide was not an effective substrate for ALDH2 (Figure 1 B). These results were validated in a zebrafish ALDH2 activity assay (Figure S1 C) (Zhou et al., 2012). Zebrafish melanocytes express ALDH2, and treatment with nifuroxazide (up to 30 \(\mu\)M) did not show any toxicity toward zebrafish melanocytes, whereas treatment with NFN1 (10 \(\mu\)M) significantly reduced the number of melanocytes. To further explore the different selectivity for ALDH2 at equivolent concentrations, we purified ALDH2 and tested its activity with increasing concentrations of NFN1 and nifuroxazide. Dose-curve analysis revealed NFN1 is a highly selective for ALDH2 (NFN1 IC
\textsubscript{50} = 63.9 nM), while nifuroxazide had no activity toward ALDH2 up to 10 \(\mu\)M (Figure S1 D). Thus, the antibiotic nifuroxazide is a selective substrate for ALDH1.

Next, we used the Aldefluor assay to determine if nifuroxazide might be a substrate of, and modulate, ALDH1 activity in melanoma cells. The Aldefluor assay is based on ALDH enzyme activity to convert the freely diffusible fluorescent BODIPY-aminocetaldehyde to the negatively charged BODIPY-aminoacetaldehyde that is retained and accumulates in the cell. A375 melanoma cell lines are heterogeneous for ALDH activity, with cells presenting low, intermediate, and high fluorescence for ALDH activity (Figure 1 C) and used to isolate ALDH
\textsuperscript{H} and ALDH
\textsuperscript{L} subpopulations by fluorescence-activated cell sorting (FACS) (Figure 1 D). ALDH activity heterogeneity is due, at least in part, to increased mRNA expression of ALDH1A3 in the ALDH
\textsuperscript{H} subpopulation compared with the ALDH
\textsuperscript{L} subpopulation (Figure 1 E). Nifuroxazide and NFN1 effectively reduced Aldefluor activity (Figure 1 F), indicating that nifuroxazide inhibits ALDH enzyme activity in cells. Inhibition of Aldefluor activity was dependent on the 5-nitro moiety because our no-nitro control compound (NFN1.1) had no effect on ALDH activity in cells (Figure S1 E).

To test if 5-nitrofuran activity toward ALDH was linked to the mechanism of melanoma cell death, we tested if N,N-diethylami-no-nobenzaldehyde (DEAB) could prevent 5-nitrofuran cytotoxic activity in cells. DEAB is a potent inhibitor of ALDH1, but also has broad inhibitor activity toward other ALDH enzymes (Koppaka et al., 2012; Luo et al., 2012; Moreb et al., 2013). We found that DEAB pre-treatment protected the cells from the cytotoxicity of NFN1 (Figure 1 G). These data indicate that 5-nitrofuran pro-drug cytotoxicity is dependent on ALDH activity.
Nifuroxazide Bio-activation Leads to Oxidation and Inhibition of ALDH1 Enzymes

ALDH1A1 and ALDH1A3 are closely related enzymes and are the predominant ALDH1 isoforms in primary melanoma and melanoma cell lines (Luo et al., 2012). To address the molecular mechanism of how nifuroxazide interacts with ALDH1 enzymes, we first used molecular modeling. The ALDH1A3 structure has recently been solved (Moretti et al., 2016) and our analysis
revealed that nifuroxazide effectively fits into the substrate pocket of ALDH1A3, predicting direct interactions with cysteines 313 and 314 in the active site (Figures 2A and 2B).

To address the potential of 5-nitrofurans to interact with ALDH1A1, we incubated ALDH1A1 with the 5-nitrofurans and performed mass spectrometry of the intact protein (Figure 2C). The results showed ALDH1A1 mass increasing by +35 atomic mass units (amu), consistent with a di-oxidation with nifuroxazide plus NAD+ (42% oxidation of total protein) or NFN1 plus NAD+ (80% oxidation of total protein). In comparison, incubation
Figure 3. ALDH1A3 Mediates Nifuroxazide Activity

(A) Western blot of ALDH1A3 in ALDH1A3 siRNA transfected A375 (si1A3) cells, siRNA control (siCon), and wild-type (WT) control cells. GAPDH, loading control.

(B) Aldefluor activity in si1A3 cells or siCon cells. DEAB was used as a negative control (n > 3).

(C) Sensitivity of si1A3 and siCon cells to NFN1 measured by levels of Draq7 expression using IncuCyte Zoom. Values are means ± SEM (n = 3, ****p < 0.0001, ANOVA with Tukey’s test).

(D) Western blot of ALDH1A3 protein in ALDH1A3 knockout single-cell clones. GAPDH, loading control (n > 3).

(E) Aldefluor activity in ALDH1A3 knockout clones and A375-Cas9 WT cells. DEAB is used as negative control.

(F) Sensitivity of ALDH1A3Cpr3 and A375-Cas9 cells to 10 μM nifuroxazide measured by levels of Draq7 expression using IncuCyte Zoom. Values are means ± SEM (n = 4, ***p < 0.001, ANOVA).

(G) Representative images of WT and ALDH1A3Cpr3 cells treated with nifuroxazide or DMSO, imaged with IncuCyte Zoom. Note cell death in upper panel.
of ALDH2 with nifuroxazide plus NAD+ did not generate the observed mass shift, while NFN1 plus NAD+ generated both a 31-amu shift and a possible ALDH2-NFN1 adduct (+338 amu) (Figure 2D). The nitro moiety is essential for this process because incubation of NFN1.1 did not generate any additional species with either enzyme (Figures 2C and 2D). Likewise, oxidation of ALDH enzymes by 5-nitrofurans was dependent on NAD+ (Figure S2).

We then used liquid chromatography-tandem mass spectrometry to determine the site of nifuroxazide-catalyzed modification. Quantitative analysis of the modification revealed a significant increase in the oxidation of the catalytic cysteine in the active center of ALDH1A1 upon the addition of nifuroxazide (Figure 2E).

Together, these results demonstrate that nifuroxazide directly and specifically interacts with the ALDH1A1 and ALDH1A3 isoforms leading to oxidation and inactivation of ALDH1 isozymes at the catalytic core. Thus, unlike ALDH1 enzyme inhibitors, nifuroxazide has a unique two-hit mechanism whereby bio-activation of nifuroxazide by ALDH1 enzymes leads to cytotoxicity concomitant with ALDH1 inactivation (Figure 2F). Nifuroxazide pro-drugs provide an opportunity to move beyond inhibiting ALDH1 enzymes in cancer cells, to selectively eradicating ALDH1-expressing cells in melanoma.

**Nifuroxazide Bio-activation Is Mediated by ALDH1A3**

Primary melanoma samples express high levels of ALDH1A1 and ALDH1A3, but ALDH1A3 is the predominant form in A375 melanoma cell lines (Luo et al., 2012). If the 5-nitrofurano-ALDH1 interaction is relevant *in vivo*, we would expect that loss of ALDH1 would confer resistance to 5-nitrofuran treatment. Small interfering RNA (siRNA) knockdown of ALDH1A3 as the dominant ALDH enzyme responsible for Aldefluor activity, and validates ALDH1A3 as the ALDH enzyme inactive in xenografts (Figure S3D), consistent with the concept that ALDHHigh subpopulations have functional tumor-initiating characteristics in melanoma cells. Importantly, we found that ALDH1A3Cpr3 and ALDH1A3Cpr21 cell lines were resistant to nifuroxazide and NFN1 in cell growth (2D culture) and colony cultures (3D culture) (Figures 3F–3H and S3E–S3H).

Given that loss of ALDH1A3 made cells resistant to nifuroxazide, we hypothesized that overexpression of ALDH1 should increase cell sensitivity to nifuroxazide. We overexpressed ALDH1A3 in A375 and C089 melanoma cells by transient transfection, and found that the cells were more sensitive to nifuroxazide than control cells (Figures 3I–3L).

Thus, our genetic analysis confirms that the cytotoxic activity of nifuroxazide in these melanoma cells is mediated by ALDH1, and not an otherwise unknown target enriched in the ALDHHigh subpopulation. By extension, and in combination with our *in vitro* assays in Figures 1 and 2, it is likely that nifuroxazide is bio-activated in cancers by other ALDH1 isoforms including ALDH1A1.

**Nifuroxazide Selectively Kills ALDHHigh Subpopulations**

Having established that ALDH1 enzymes are targets for nifuroxazide in cells, we wanted to test if nifuroxazide is selectively cytotoxic for ALDHHigh tumor-initiating (stem cell-like) subpopulations. First, we FACS sorted the top 5% of cells highly expressing ALDH enzyme activity (ALDHHigh cells) and the bottom 5% of cells with the lowest ALDH enzyme activity (ALDHLow cells) (see Figure 1D). The ALDHHigh cell population had increased sphere-forming potential, which was further increased following serial passage (Figure S4A) and were more efficient at forming colonies in soft agar (Figure 4A). ALDHHigh cells also had the potential to differentiate into ALDHIntermediate and ALDHLow cells over time while maintaining an ALDHHigh population, whereas ALDHLow cells were unable to form ALDHHigh populations (Figure S4B). In addition, *in vivo*, ALDHHigh cells were more tumorigenic than the ALDHLow cells (Figure S4C). Together, these data validate that the top 5% of ALDHHigh cell populations are enriched for phenotypic properties associated with stemness and melanoma tumor-initiating potential.

Having separated the cells into ALDHLow and ALDHHigh populations, and validated their cellular phenotypes, we then tested soft agar colonies derived from each population to increasing concentrations of nifuroxazide and NFN1 (Figures 4B and S4D). We used lower concentrations of drug treatments for the colony assay because the single cells are grown in anchorage-independent conditions and are treated over 21 days to develop into colonies. ALDHHigh-derived colonies were highly sensitive to nifuroxazide and NFN1. In contrast, colonies generated from ALDHLow cells, while producing fewer colonies overall, were resistant to NFN1 and nifuroxazide treatment.

(H) Clonogenic potential of ALDH1A3Cpr3, ALDH1A3Cpr21, and control A375-Cas9 cells in soft agar. Values are means ± SEM (n = 3, ***p < 0.001, ****p < 0.0001, ANOVA with Dunnett’s test).

(I) Western blot image of ALDH1A3 protein levels in A375 cells overexpressing ALDH1A3 (transient transfection ALDH1A3 [tALDH1A3]) and WT control cells. GAPDH, loading control.

(J) Aldefluor activity in tALDH1A3 and WT control. DEAB was used as negative control.

(K and L) Sensitivity of A375 (K) and C089 (L) cells, control and overexpressing ALDH1A3 to nifuroxazide. Viability was measured using SRB assay. Values are means ± SEM (n = 3). See also Figure S3.
We tested if the paradigm of ALDH<sup>High</sup> activity and nifuroxazide sensitivity can be extended to melanoma cell lines representative of different melanoma genetic subtypes. We tested Aldefluor activity and nifuroxazide sensitivity in four additional melanoma cell lines: C089 (BRAF<sup>V600E</sup>), C077 (NF1<sup>mut</sup>), MEWO (NF1<sup>mut</sup>), and C092 (triple wild-type) (Ranzani et al., 2015). Aldefluor activity varied widely among cell lines, independently of genetic subtype (Figures 4C and S4E). We found that cell lines with relatively high median levels of ALDH activity formed colonies and were highly sensitive to nifuroxazide (Figures 4D and S4F). In contrast, cells with relatively low median levels of ALDH activity had very low colony formation potential and were resistant to nifuroxazide (Figures 4D and S4F). Thus, nifuroxazide has selective activity toward multiple cell lines with overall relatively high ALDH activity, and this is independent of melanoma genetic subtype.

**Nifuroxazide Targets ALDH<sup>1High</sup> Subpopulations in Tumors**

Ultimately, we wanted to establish if ALDH<sup>High</sup> subpopulations are selective targets for nifuroxazide in vivo and impact tumor growth. We performed a melanoma xenograft assay with human melanoma cells that express a tdTomato transgene (A375-L2T cells) enabling us to isolate melanoma cells following treatment. Once the tumors were established, mice were treated with nifuroxazide or with oil vehicle. Nifuroxazide significantly attenuated tumor growth (>10 tumors) (Figures 5A and 5B). We also tested if nifuroxazide could be effective at lower treatment concentrations, and treated A375 melanoma xenografts with 50 or 150 mg/kg for 13 days. Both drug treatments attenuated tumor growth, and in some mice led to partial melanoma regression suggesting that ALDH<sup>High</sup> subpopulations may be required to support continued tumor growth (Figure S5A). These data suggest that near-maximal drug absorption or efficacy may already be achieved at 50 mg/kg.

Following treatment, we isolated the tumors and analyzed Aldefluor activity in A375-L2T melanoma cells. Tumors treated with nifuroxazide had reduced numbers of ALDH<sup>High</sup> cells evaluated by Aldefluor assay, indicating that nifuroxazide can target ALDH<sup>High</sup> subpopulations directly in the tumor (Figure 5C). Strikingly, immunohistochemistry revealed that ALDH<sup>High</sup> cells were absent in tumors treated with nifuroxazide (Figures 5D and 5E), accompanied by increased levels of cleaved caspase-3, an apoptosis marker (Figures 5F and 5G). Therefore, nifuroxazide does not just inhibit ALDH enzyme activity in cells, but specifically eradicates ALDH<sup>1High</sup> tumor subpopulations.

To assess the phenotypic outcome of targeting ALDH<sup>1High</sup> tumor-initiating subpopulations, we tested if melanoma cells treated with nifuroxazide could initiate new tumors in serial transplantation (Figure 5H). We removed tumors from mice that had been treated with nifuroxazide or oil vehicle, FACS sorted 10,000 melanoma cells per tumor, and injected these into recipient mice without further treatment. Melanomas from mice that had been treated with the oil vehicle control rapidly grew tumors while, in contrast, melanomas from nifuroxazide-treated tumors were significantly inhibited in forming new tumors (Figure 5I). These results were validated in tumors generated from A375 cells without the tdTomato marker, whereby, due to a lack of fluorescence, melanoma cells were selected by excluding...
Figure 5. Nifuroxazide Targets ALDH1A3^High^ Subpopulations in Melanoma Tumors

(A) Sensitivity of A375-L2T tumors to nifuroxazide in vivo. Mice were treated with 150 mg/kg nifuroxazide or vehicle for 9 continuous days. Values are means ± SEM (n = 3 mice/condition; experimental replicates >3, *p < 0.05, two-way ANOVA with Sidak’s test).

(B) Tumor weights of control or nifuroxazide-treated mice at the end of drug treatment. Values are means ± SEM (n = 3 mice/condition; experimental replicates >3, **p < 0.01; Student’s t test). Image of tumors from control or nifuroxazide-treated mice at the end of drug treatment duration.

(C) Aldefluor activity in A375-L2T cells in tumors treated with nifuroxazide or vehicle for 9 days. Note the significant shift to the left in the tumors from nifuroxazide-treated mice indicating reduced Aldefluor activity (geometric mean of Aldefluor activity; n = 3 p < 0.05; Student’s t test).

(legend continued on next page)
endothelial and immune cells (Figure S5B). Therefore, nifuroxazide effectively inhibits tumor growth in vivo by selectively targeting ALDH1High subpopulations directly in the tumor, while surviving ALDH1Low cells lack melanoma initiation potential, and are unable to form new tumors.

**Targeted Therapy Increases ALDHHigh Cells and Sensitivity to Nifuroxazide**

Little is known about the clinical importance of ALDH1High subpopulations in human clinical samples that have recurrent following BRAF inhibitor (BRAFi) and/or MEK inhibitor (MEKi) treatment. First, we performed immunohistochemistry for ALDH1 isoforms on seven patient melanoma samples before BRAFi treatment and in recurrent melanoma from the same patient. The ALDH1 antibody detects both ALDH1A1 and ALDH1A3 isoforms, and thus detects ALDH1 proteins that mark ALDH1High subpopulations in melanoma (Luo et al., 2012). As shown in Figures 6A–6D, four of the melanomas that relapsed after treatment showed a dramatic increase in the number of ALDH1-positive cells and an increase in ALDH1 expression compared with their matched pre-treatment samples. These observations indicate that ALDH1High subpopulations may play an important role during melanoma relapse for some patients.

Next, we tested the potential for nifuroxazide to synergize with BRAFi inhibitors. In cell culture, BRAFi lead to an adaptive increase in the number of cells expressing stemness markers, such as ALDH1High 24 hr post-treatment (Figure 6E) (Ravindran Menon et al., 2015). Building on these results, we found that treatment with BRAFi dramatically increased the sensitivity of A375 melanoma colonies to nifuroxazide (Figure 6F).

BRAF signaling depends on MEK activation, and the combination of BRAFi plus MEKi has improved clinical benefit for patients with BRAF mutant melanoma (Luke et al., 2017). To establish if BRAFi plus MEKi increases ALDH1 levels in melanoma, we analyzed RNA sequencing (RNA-seq) data of a melanoma patient cohort before treatment and 12 ± 5 days into BRAFi plus MEKi combined treatment (European Genome-phenome Archive: S00001000992) (Kwong et al., 2015). Of nine patients, six showed an increase in ALDH1A1 expression while on BRAFi plus MEKi treatment (Figure 6G). These data indicate that for some patients, BRAFi plus MEKi treatment is associated with an increase in ALDH1A1 expression.

Next, we sought to address if vemurafenib (BRAFi) plus trametinib (MEKi) treatment could lead to an increase in ALDH activity in cell line models and provide a window of opportunity to synergize with nifuroxazide. We found cell lines varied in response and sensitivity to the combined treatment. A375 cells were highly sensitive to MEKi, and the addition of MEKi (2 nM) inhibited the increase in ALDH activity promoted by the BRAFi. In contrast, low concentrations of BRAFi (50 nM) plus MEKi (0.2 nM) lead to an increase in Aldefluor activity in A375 cells (Figure 6H). In C089 cells, BRAFi (1 μM) alone did not significantly increase ALDH1 mRNA (Figure S6A), while the addition of MEKi alone, or BRAFi plus MEKi, increased ALDH activity at all MEKi concentrations (0.04–2 nM) and was associated with an increase in mRNA expression of ALDH1A1 (Figures 6H and S6A). Inhibition of the MAPK pathway with MEKi did not increase ALDHHigh in D38 NRASQ61K mutant cells at any concentration (0.04–30 nM) (Figure 6H). These data indicate that BRAFi plus MEKi combined therapy can lead to an adaptive increase in ALDHHigh subpopulations in a subset of melanoma cell lines, although the underlying drug sensitivities may vary. Ultimately, we tested if the therapy-induced ALDH activity increase primed the cells for sensitivity to nifuroxazide. We found that both A375 and C089 cell lines had increased sensitivity to nifuroxazide treatment when combined with the low doses of BRAFi plus MEKi that increase ALDH activity (Figure 6I). These results strengthen the concept that targeted therapy leads to an adaptive increase in ALDH1 in melanoma and provides an opportunity to treat with nifuroxazide.

**DISCUSSION**

We discover that ALDH1 is a target for the widely used antibiotic nifuroxazide, and that this mechanism selectively targets ALDH1High cells to inhibit tumor growth and initiation potential.

Our results indicate that higher levels of ALDH1 activity in melanoma are associated with increased sensitivity to nifuroxazide, independent of melanoma genotype. This may permit cancer patient stratification for nifuroxazide responsiveness based on ALDH1 activity. Analysis of TCGA RNA expression across tumors reveals melanoma to be among the cancers with the highest ALDH1A3 overall expression (Figure S6B) (Cancer Genome Atlas Network, 2015; Cerami et al., 2012; Gao et al., 2013b). Targeting ALDH1High subpopulations may be especially important for the 50% of melanoma patients that do not have BRAF mutations and so far have limited options for targeted therapy (Luke et al., 2017).

The clinical evidence we present here indicates that ALDH1High subpopulations increase from primary tumor to BRAFi-resistant metastatic melanomas in some patients, suggesting that melanoma cells may change their cellular state in response to drug treatments. This is supported by analysis of the RNA-seq dataset from patients that reveals some patient melanomas
Figure 6. ALDH\textsuperscript{High} Cells Are Enriched in Melanoma Patient Samples Following Targeted Therapy

(A–D) Immunohistochemistry of ALDH1 expression in melanoma patient samples. Matched patient samples were taken before and after vemurafenib treatment. Images are shown at 2.5× and 40× magnification. Boxes indicate the region in which the image has been captured with a 40× magnification. Scale bars, 1 mm (2.5×) and 50 µm (40×). The four of seven matched patient samples that showed an increase in ALDH1 post-treatment are presented.
increase ALDH1A1 expression while on BRAFi plus MEKi. Further analysis of a published RNA microarray dataset (GEO: GSE35230) (Greger et al., 2012) of A375 clone 18R6-4 revealed that, despite being resistant to BRAFi plus MEKi, combined treatment led to an increase in ALDH1A3 mRNA expression, suggesting that resistant cells are still sensitized to therapy, and that ALDH1 expression may contribute to resistance mechanisms (Figure S6C). This drug-induced increase in ALDH1 protein and mRNA underscores the importance in targeting ALDH1High cells, and suggests that an important therapeutic window may be during or following targeted therapy.

Because ALDH1High subpopulations are present in many cancer types, major efforts are underway to generate ALDH1 isoform-specific inhibitors. Given that 19 ALDH enzymes are present in the human genome (including at least 6 within the ALDH1 family) (Koppaka et al., 2012), it is not clear at this stage if specific ALDH1 isoform inhibitors will be sufficient to effectively target ALDH1High subpopulations in human cancers, or if other ALDH isoforms might be able to compensate and lead to drug resistance. By contrast, we show that nifuroxazide has a two-hit mechanism, whereby it first inactivates ALDH1 through its bio-activation, and, secondly, the bio-activated species generates cytotoxicity to kill the cell. Therefore, rather than transiently inhibiting ALDH1High activity, nifuroxazide selectively kills ALDH1High subpopulations.

Nifuroxazide has been commonly used to treat intestinal infections such as colitis and diarrhea. Bio-activation of 5-nitrofurans in bacteria and trypanosomes generates reactive nitro metabolites and causes DNA damage. Repurposing nifuroxazide as an anti-cancer drug will require the identification of a therapeutic window that selectively targets ALDH1High cells in cancer without eradicating ALDH1High stem cell populations in somatic tissues. Evidence from clinical use of nifuroxazide indicates that nifuroxazide is generally well tolerated (Begovic et al., 2016; Bourree et al., 1989). Nifuroxazide is administered at 800 mg/day in human patients when used as an antibiotic, while in veterinary medicine, animals are often treated at 50 mg/kg, and higher doses (>200 mg/kg) have been described in animals without side effects (Cagliero, 1976; Gao et al., 2013a). We find nifuroxazide is well tolerated in mice at 50 and 150 mg/kg, with no signs of side effects or weight loss. All drug administrations in our in vivo studies are oral, as administered for patients when used as an antibiotic. This is a similar dose range used for nifurtimox, which is used at 800 mg/day for trypanosome infection (albeit for 30–90 days), 150 mg/kg/day for pre-clinical neuroblastoma xenograft studies for 28 days (Saulnier Sholler et al., 2009), and at 30 mg/kg/day for cancer treatment (NCT00601003) (Saulnier Sholler et al., 2011). While we cannot directly compare the pharmacokinetics of nifurtimox with nifuroxazide, our studies suggest that nifuroxazide may be effective at targeting ALDH1High cells in a clinically relevant dose range. Importantly, if the combined BRAFi (plus MEKi) and nifuroxazide drug synergy we observe in cell culture holds true in the clinical setting, this may reveal BRAFi (plus MEKi) and nifuroxazide to be a highly beneficial drug combination and permit lower drug doses with reduced overall drug toxicity.

Despite the evidence for melanoma subpopulations in cancer progression and drug resistance, there is an absence of drugs that specifically target phenotypic tumor-initiating cell populations available in the clinic. Indeed, despite the use of nifurtimox in patients with neuroblastoma and medulloblastoma, the mechanism for the anti-cancer activity is unknown. Given that ALDH1High activity marks functional stem cell properties that specifically target phenotypic tumor-initiating cell populations available in the clinic, indeed, despite the use of nifurtimox in patients with neuroblastoma and medulloblastoma, the mechanism for the anti-cancer activity is unknown. Given that ALDH1High activity marks functional stem cell properties, the mechanism for the anti-cancer activity is unknown. Given that ALDH1High activity marks functional stem cell properties, the mechanism for the anti-cancer activity is unknown.

In summary, we have provided substantial mechanistic insights into how a specific 5-nitrofuran used as an antibiotic can act through ALDH1 to reduce melanoma initiation and progression. We believe that such insights are critical to expand the pipeline of cancer drug discovery as it allows us to rationally reposition existing drugs for therapy. The discovery that ALDH1 is a target for nifuroxazide, and that this enables the specific killing of ALDH1High melanoma cells, goes beyond approaches of reducing the tumorigenic potential of such cells by interfering with their ALDH1 activity, which may be limited and transient. This is an important alternative therapeutic strategy to target phenotypically distinct tumor-initiating populations that contribute to melanoma growth and are marked by ALDH1High activity.

SIGNIFICANCE

One of the major challenges for cancer treatment and therapeutic design is intratumor heterogeneity, shaped in part...
by phenotypically distinct tumor cell subpopulations. In melanoma, and in many other cancers, high levels of ALDH activity (ALDH1A3High) mark a subpopulation of cells with increased tumor-initiation potential and cancer stem cell properties. Here, we show that ALDH1 is a target for 5-nitrofuran pro-drugs that are widely used to treat bacteria infections, and that have recently been shown to have anti-cancer potential.

We have previously shown that 5-nitrofurans are bio-activated by ALDH2, the enzyme responsible for alcohol metabolism. We now show that the 5-nitrofuran, nifuroxazide, is selective for ALDH1, and that the cytotoxic mechanism of action in melanoma cells is mediated by eradicating ALDH1A3High subpopulations. This is significant because no clinically available drugs have so far emerged to specifically eradicate ALDH1A3High subpopulations. We show that ALDH1High subpopulations are enriched in patients with recurrent melanoma following BRAF inhibitor treatment and that ALDH1A1 mRNA is increased in patient melanomas during BRAF plus MEK inhibitor treatment. We demonstrate that nifuroxazide activity in melanoma cells is via a two-hit mechanism: bio-activation by ALDH1 leads to toxicity in cells, coupled with oxidation and inhibition of ALDH1. Thus, unlike ALDH inhibitors, we discover that bio-activation of 5-nitrofuran pro-drugs can selectively kill ALDH1A3High cell populations. Targeting subpopulations in melanoma based on the distinctive phenotypic properties of tumor-initiating (stem) cells, rather than targeting the molecular activity of cancer mutations, is an orthogonal therapeutic approach to current targeted and immune therapies.

STAR* METHODS

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**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and can be found with this article online at https://doi.org/10.1016/j.chembiol.2018.09.005.

**ACKNOWLEDGMENTS**

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**AUTHOR CONTRIBUTIONS**

S.S., R.C., and Y.L. designed and performed biological experiments. D.R.H. performed in silico modeling. T.D.H., L.Z., and A.V.K. performed mass spectrometry. C.-H.C., D.M.-R., M.R., D.J.A., A.U.-B., N.O.C., V.G.B., and M.F. provided reagents and cell lines, and contributed to experimental direction and design. L.S., W.X., and X.X. collected clinical samples and performed immunohistochemistry. M.E.M. is a clinical NIH pathologist and performed the pathology analysis for Figure 6. E.E.P. conceived, designed, and directed the experiments. E.E.P. wrote the manuscript with assistance from S.S., and with input from all authors.

**DECLARATION OF INTERESTS**

D.J.A. and M.R. have filed a patent application no. 1704267.2. However, the content of the patent is not related to the current manuscript. M.R. currently works at Artios Pharma and own shares options of the company. However, none of the work described in this study is related to, based on or supported by the company. T.D.H. holds significant financial equity in SAJE Pharma. However, none of the work described in this study is related to, based on or supported by the company. D.M.-R. and C.-H.C. are advisor to Foresee Pharmaceuticals, who hold their patents related to ALDH2 activators. However, none of the work described in this study is related to, based on or supported by the company. All other authors declare no competing interests.

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by nifuroxazide improves antitumor immunity and impairs colorectal carcinoma metastasis. Cell Death Dis. 8, e2534.
## STAR METHODS

### KEY RESOURCES TABLE

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### Experimental Models: Organisms/Strains

| Mouse: NOD SCID | Charles River | RRID:IMSR_ARC:NODSCID |

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents should be directed to the corresponding author E. Elizabeth Patton (e.patton@igmm.ed.ac.uk).

EXPERIMENTAL MODEL DETAILS

**Chemicals**

Vemurafenib (LKT labs), Trametinib (Selleckchem), Nifuroxazide (EMD Millipore), NFN1 (Maybridge), and clinical 5-nitrofurans were prepared as 100mM solutions in 100% dimethyl sulfoxide (DMSO). NFN1.1 was generated in house following Zhou et al., 2012. Working solutions were freshly prepared before addition to the cell media.

**Cell Lines**

A375 cells were obtained from America Type Culture Collection (ATCC). To generate A375-L2T cells, A375 cells were transfected with ubiquitin promoter-driven L2T (pFU-L2T) and sorted by flow cytometry every week for 3 weeks. L2T lentivirus vector was kindly given by Dr. Sanjiv S. Gambhir (Stanford University) to M.F. through an MTA. DMEM medium, l-glutamine, penicillin/streptomycin and fetal bovine serum (FBS) were purchased from Life Technologies. Cells were maintained in DMEM medium supplemented with 5μg/mL l-glutamine, 1% penicillin/streptomycin and 10% FBS and cultured in a humidified 5% CO₂ incubator at 37°C. C077, C092, C089, and MEWO cells were maintained in RPMI-1640 medium (Life Technologies, Thermo Fisher Scientific) supplemented with 1% penicillin/streptomycin and 10% FBS and cultured in a humidified 5% CO₂ incubator at 37°C. All cell lines are routinely tested for mycoplasma by PCR every three months (date of last test June 20, 2018).

**In Vivo Animal Studies**

Female 6–8 week-old NOD/SCID mice were purchased from Charles River Laboratories. Mice were used for tumor xenograft and drug treatment studies. Mice were housed in individually ventilated cages (IVC) in groups of 5-6 mice per cage. All animal experiments were approved by the University of Edinburgh Animal Welfare and Ethical Review Body (approval PL01-16) and the UK Home Office (PPL 70/8897).

METHOD DETAILS

**Generation of ALDH1A3 Mutant Lines Using CRISPR/Cas9**

We used two vector system in which the LentiCas9-Blasta vector (Addgene; 52962) contained the human codon-optimized Streptococcus pyogenes Cas9-expression cassette and the LentiGuide-Puro (Addgene; 100000049) contained the gRNA expression cassette. The lentiviral vector was produced by transfection of 293T (ATCC) cells with the lentiviral vector plasmid and packaging plasmids pCMVPax2 (Addgene; 36052) and VSV-G (Addgene; 14888) with polyethylenimine transfection reagent following manufacturer’s instructions. 24 hours post transfection, the medium was replaced with fresh media and the cells were cultured for 48 hours. The lentiviral vector containing supernatant was filtered (0.45μm filter) and A375 cells (1×10⁴ cells) were transduced with 2ml of LentiCas9-Blast virus particles in 5ml culture media and 5μg polybrene. Transduced cells were selected with 1ng/ml blasticidin to obtain stable cell line expressing Cas9 (A375-Cas9).

SgRNAs sequence was cloned in to LentiGuide-Puro in 5ml culture media and 5μg polybrene. Transduced cells were selected with 1ng/ml blasticidin to obtain stable cell line expressing Cas9 (A375-Cas9). To this, oligos (100μM) were annealed and phosphorylated in 1× T4 ligation buffer with 1× T4 PNK (New England Biolabs) and were ligated into the BsmB1 site of the LentiGuide-Puro vector. One Shot™ Stbl3™ chemically competent E. coli were transformed by adding 100ng of DNA and heat-shocked for 45 seconds at 42°C. Sample was spread on agar with puromycin plates and incubated for 24 hours and single colonies were selected.

SgRNAs were expressed by a transient transfection of the A375-Cas9 cells with LentiGuide-Puro vector described above. Briefly, 5 × 10⁴ A375-Cas9 cells were plated in a 6 well/plate. After 24 hours A375-Cas9 cells were transduced with ALDH1A3-LentiGuide-Puro using Lipofectamine 3000 (0.3μl/well) (Invitrogen) and P3000™ reagent (0.2μl/well). Medium was changed after 24 h. 48 h later, 0.8 μg/ml puromycin (Sigma) was added into transected cells. Single-cell clones were isolated for sequencing and positive clones (ALDH1A3Cpr3, and ALDH1A3Cpr21) were expanded in culture.
ALDH1A3 Overexpression
A375 cells were seeded in 96 well plate at 2.5 × 10^3 cells/well and incubated for 24 hours. Cells were treated 0.1μg ALDH1A3 over-expressing vector (ALDH1A3/pCMV6-XL4) (Origene) or pCMV6-XL4 empty vector (control) in Opti-MEM™ media with Lipofectamine 3000 (0.3μl/well (Invitrogen) and P3000™ reagent (0.2μl/well). Medium was changed after 72 hours.

siRNA Transfection
A375 cells were seeded in 96-well plate at a density of 2.5 × 10^3 cells/well or in 6-well plate at a density of 5 × 10^4 cells/well and incubated for 24 hours. Cells were then treated with 5pmol ALDH1A3 siRNA or scrambled control in Opti-MEM™ media with Lipofectamine RNAiMAX (1.5μl/well)(Invitrogen). Medium was changed after 96 hours.

Flow Cytometry and Aldefluor Assay
The aldehyde dehydrogenase (ALDH) enzyme activity was measured using the ALDEFLUOR assay kit (STEMCell Technology) according to the manufacturer's protocol. In brief, cells were incubated with the fluorescent bodipy-aminoacetaldehyde (BAAA) reagent for 30 minutes at 37°C. As a negative control, ALDH enzyme activity was blocked using diemethylamino-benzaldehyde (DEAB). Aldefluor activity was measured using FACS Fortessa (BD Biosciences). For cell sorting, the Aldefluor stained cells were analyzed using the FACS Aria II (BD Biosciences) and the top 5% of cell population with highest ALDH activity (ALDH High cells), detected on the green fluorescence channel (515–545 nm), and the bottom 5% of cells with lowest ALDH activity (ALDHLow cells) were cell sorted. Data were analyzed using FlowJo software program. Dead cells were excluded using Propidium iodide (PI, 13μM, Roche) or 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI, 3μM, Sigma-Aldrich).

Cell Viability Assay
Cytotoxicity of the 5-nitrofurans on melanoma cell lines was determined using the sulforhodamine-B (SRB) cytotoxicity assay (Sigma-Aldrich). 1000 cells per well were plated in 96-well plates overnight. Cells were then treated either with a vehicle control (DMSO) or increasing concentrations of compounds (1nm - 100μM) for 72 hours. SRB assay was carried out as described previously (Skehan et al., 1990). In brief, cells were fixed with Trichloroacetic acid for 1 hour at room temperature, followed by 0.4% SRB for 30 minutes at room temperature. Plates were then washed and the SRB was dissolved by 10mM Tris buffer and read at 570nm on a microplate reader. Experiments were performed in three independent series and the mean half maximal effective concentration (EC50) was used to compare cytotoxicity.

Cytotoxicity and Apoptosis Assays Using IncuCyte ZOOM Live-Cell Imaging System
IncuCyte ZOOM Live-Cell Imaging system (Essen Bioscience) was used for kinetic monitoring of cytotoxicity and apoptotic activity of Nifuroxazide or NFN1 on melanoma cells. 1000 cells/well were seeded in 96 well plate and allowed to attach overnight. Cells were treated with increasing concentrations (1nm - 100μM) of compounds in the presence of Draq7™ (3μM, Abcam) and IncuCyte® NucLight BacMam 3.0 (2% v/v) (Essen Bioscience). Draq7™ stains the nuclei in dead and permeabilized cells and IncuCyte® NucLight BacMam 3.0 enables nuclear labelling of living cells. Plates were scanned and fluorescent and phase-contrast images were acquired in real time every 3 hours from 0 to 72 hours post treatment. Normalized green and red object count per well was quantified at each time point and time-lapse curves were generated by IncuCyte ZOOM software. Ratios of Draq7™ level in treated cells compared to vehicle were plotted in Microsoft Excel.

Apoptosis Detection Using Annexin V/PI
10^5 cells were seeded in 6-well plates overnight. Cells were then treated with compounds or vehicle for 48 hours. For detection of apoptotic cells, cells were dissociated with cell dissociation buffer and 500μl of binding buffer was added to each sample followed by 5μg/ml of Annexin V–FITC and 5μg/ml of PI. Samples were gently vortexed and incubated for 15 minutes at room temperature in the dark. Samples were analysed using FACS Fortessa (BD Biosciences).

Colony Formation Assay in Soft Agar
5000 cells were suspended in serum free DMEM media supplemented with 10ng/ml EGF, 10ng/ml FGF, B27 supplement (1x, Thermo Fisher Scientific), 5μg/ml L-glutamine, and 0.3% low melting agarose (Sigma-Aldrich) in the presence or absence of compounds. Cells were then layered over a solid base of 0.5% low melting agarose in 35mm plastic dishes. The cultures were incubated at 37°C for 12 days. Colonies (>6 cells) from 10 separate fields were counted using a microscope with a 4X objective.

Sphere Formation Assay and Differentiation Assay
1000 sorted ALDH<sup>High</sup> and ALDH<sup>Low</sup> cells were cultured in serum free DMEM media supplemented with 10ng/ml EGF, 10ng/ml FGF, B27 supplement (1x), 5μg/ml L-glutamine and then plated in low adhesion round bottom plates (Corning®). Cell proliferation was measured by counting disaggregated single cells using a haemocytometer. Dead cells were excluded by using 0.4% Trypan blue solution (Sigma–Aldrich).

For differentiation assay, cell sorted ALDH<sup>High</sup> and ALDH<sup>Low</sup> cells were cultured in serum containing (10% FBS) DMEM media supplemented with 5μg/ml L-glutamine, 1% penicillin/streptomycin and cultured in tissue culture plates for 5 days. Differentiation of cells evaluated by Aldefluor assay.
Western Blot Analysis
Cells were plated at 100,000 cells/well in 6-well plates and allowed to adhere overnight. Cells were lysed with RIPA lysis buffer (Sigma-Aldrich), supplemented with a complete protease inhibitor (Thermo Fisher Scientific Inc.). Protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories) and 25μg protein per lane was electrophoresed on a 12% SDS-polyacrylamide gel in running buffer (Bio-Rad). Gels were transferred onto nitrocellulose membranes using the semi-dry Turbo Transfer system with Turbo Transfer buffer (Bio-Rad). Membranes were probed using rabbit polyclonal ALDH1A3 (1:1000, Invitrogen), and GAPDH (1:5000, Abcam) followed by goat anti-rabbit IRDye 680- or 800- labelled secondary antibodies (LI-COR Biosciences). Membranes were imaged using an Odyssey infrared scanner (LI-COR Biosciences).

In Vivo Studies
The tumorigenicity of ALDH1A3high vs ALDH1A3low cells was compared by injecting 50 A375 sorted cells suspended in 100μl of HBSS containing 30% matrigel (Corning). Cells were introduced via subcutaneous injection to the right flank of mice. Tumor size was measured twice a week with a calliper.

To evaluate the cytotoxicity of nifuroxazide in vivo, NOD/SCID mice were injected with 10,000 A375-L2T cells in 30% matrigel via subcutaneous injections. Once tumors were established, animals were allocated to experimental group randomly so that the average tumor size within the experimental group would be the same. Mice were treated with vehicle or 150mg/kg or 50mg/kg nifuroxazide suspended in sunflower oil via oral gavage each day. For serial transplantation studies, once drug treatment was completed, tumors were removed and digested into single cells. For tumor digestion, tumors were minced with a surgical blade and single cell suspensions were generated by enzymatic digestion with 2mg/ml Collagenase D (Roche) for 1 hour at 37°C with intermittent vortexing, followed by passage through 40μm filters (Fisher Scientific). Red blood cells were lysed using Ammonium chloride solution (STEMCELL technology). Cells were washed twice and A375-L2T cells were cell sorted using FACS Aria. 10,000 sorted A375-L2T cells excised from vehicle treated (DMSO) tumor or nifuroxazide treated tumor was suspended in HBSS containing 30% standard matrigel and injected subcutaneously into 6-8 weeks old NOD/SCID mice. Tumor growth was measured twice a week using calliper.

Immunohistochemistry
Paraffin-embedded tissue samples were first de-paraffinized in xylene and re-hydrated in graded washes of ethanol and water. Samples were boiled in pre-warmed 10mM sodium citrate (pH 6.0) for 10 minutes, followed by incubation in 3% H2O2 for 20 minutes and ples were boiled in pre-warmed 10mM sodium citrate (pH 6.0) for 10 minutes, followed by incubation in 3% H2O2 for 20 minutes and blocked in DAKO® Protein Block Serum-Free (DAKO Agilent pathology solutions) for 1 hour at room temperature. Samples were incubated with ALDH1A3 (1:10000, invitrogen) overnight at 4°C, followed by HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Slides were counterstained with Mayer’s hematoxylin for 5 minutes.

Patient samples were strained using Leia Bond-III instrument using the IHC J protocol with Bond Polymer Refine Red Detection System (Leica Microsystems). Antigen retrieval was performed using ER1 solution (Leica Microsystems) for 20 minutes. Sections were then incubated with ALDH1A3 at 1:300 (BD Biosciences) for 15 minutes, followed by 20 minutes post-primary AP and 30 minutes incubation with polymer AP. Sections were then incubated with Bond Polymer Refine Red Detection System for 15 minutes and counterstained with hematoxylin for 5 min. Slides were washed three times between each step with either bond wash buffer or water.

ALDH1A3 and ALDH2 In Vitro Activity Assay
The ALDH1A3 in vitro assay was conducted using commercially bought ALDH1A3 (Life Technologies), and purified ALDH2-His was synthesized in-house and used for ALDH2 (see below). ALDH (5ug) was pre-incubated at 25°C in 50mM sodium phosphate buffer (pH7.4), 0.4mM NAD+ and drug (1μM NFN1, NFN1.1; 10μM nifuroxazide) or vehicle (1% DMSO) for 10 minutes. Disulfiram or Daidzin (10μM; ALDH1A3 & ALDH2 respectively) was used as a negative control. The assay was initiated by the addition of 0.4mM acetaldehyde. NADH turnover was measured using NanoDrop™ 2000 UV-Vis spectrophotometer at 340nm (ε = 6.22mM/cm) after 10mins to determine ALDH activity against DMSO. Normalized ANOVA were used for statistics.

ALDH2 Protein Purification and Activity
Expression from pTrcHis-TOPO® His-tagged human ALDH2 plasmid was achieved through transformation of the plasmid into BL21 E. coli and transduction with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). Proteins were extracted through Constant ONESHOT Cell Disruption lysis and lysates, then centrifuged. ALDH2-His was purified on ÄKTApurifier™ UPC 100, first through the HiTrap IMAC FF 1mL column and then through size exclusion using HiLoad™ 16/600 Superdex™ 200 pg size exclusion. ALDH expression was confirmed by gel chromatography and purified ALDH fractions pooled together.

Purified His-tagged ALDH2 (5ug) was pre-incubated at 25°C in 50mM sodium phosphate buffer (pH7.4), 2.5mM NAD+ plus logarithmic dilution of 5-nitrofuran (NFN1 or NAZ) or 1% DMSO for 10mins. The assay was initiated by the addition of 2.5mM acetaldehyde. NADH turnover was measured using Spectramax M5 plate reader at 340nm (ε = 6.22mM·cm⁻¹) for a total of 30mins to determine ALDH activity. The enzymatic rate (V) and IC50 values determined were determined using the initial linear change of absorbance between 60 – 300secs. Daidzin (10μM) was used as a positive control and NFN1.1 (10μM) as a negative control.

In Silico Modeling ALDH1A3 with Nifuroxazide
Water molecules and other hetero atoms were removed from the structure of ALDH1A3 (PDB 5FHZ) and the program PDB2PQR 2.1.1 used to assigned position-optimised hydrogen atoms, utilising the additional PropKa algorithm with a pH of 7.4 to predict protonation
The MGLTools 1.5.6 utility prepare_receptor4.py was used to assign Gasteiger charges to atoms. Hydrogen atoms were assigned to compound structures using OpenBabel 2.4.1, utilising the -p option to predict the protonation states of functional groups at pH 7.4. The MGLTools utility prepare_ligand4.py was used to assign Gasteiger charges and rotatable bonds. Autodock 4.2.6 was used to automatically dock the compounds into the retinoic acid binding pocket of the crystal structure. A grid box that encompassed the maximum dimensions of the cognate ligand plus 12 Å in each direction was used. The starting translation and orientation of the ligand and the torsion angles of all rotatable bonds were set to random. The Autogrid grid point spacing was set at 0.2 Å. The Autodock parameter file specified 50 Lamarckian genetic algorithm runs, 7,625,700 energy evaluations and a population size of 300.

Mass Spectrometry Analysis
To analyze ALDH-5-nitrofuran interactions by mass spectrometry, complexes for analysis were formed from 10.0 μM of the ALDH isoenzyme with 100 μM compound in 2% (v/v) DMSO (final) with or without 500 μM NAD+ and incubated for 1 hour at room temperature in 10mM HEPES, pH 7.5. Samples (2 μL) were injected using an Agilent 1200SL HPLC with a low rate of 0.3 mL/min consisting of 70% H2O and 30% acetonitrile with 0.1% formic acid into an Agilent 6520 quadrupole-time of flight (Q-TOF) mass spectrometer operating in TOF mode. The spectra were extracted and deconvoluted using the software packages MassHunter (B.08.00) and Bioconfirm (B.08.00). The abundance levels of the peaks were calculated on the individual deconvoluted spectra using the Bioconfirm (B.08.00) software package.

To analyze the oxidation of ALDH cysteines by 5-nitrofuran, first all unmodified cysteines were alkylated to prevent oxidation during the processing: cysteines already oxidized would not be alkylated, and instead would cascade towards the triply oxidized cysteine sulfonic acid. Next, ALDH1A1 was digested into peptides, and the modified and unmodified peptides were identified on an Agilent Model 6520 QTOF. Proteins from the in vitro reaction were suspended in a digest buffer with a final concentration of 0.8 M Urea, 10mM dithiotreitol and 100mM Tris-HCl pH 7.5. Protein solutions were then incubated for 30 minutes at 50°C. Reduced cysteine residues were alkylated by adding iodoacetamide solution to a final concentration of 50mM and incubated 30 minutes at room temperature, in the dark. Proteins were digested with by adding 0.1 μg Trypsin and GluC 0.1 μg (Promega) per sample for 16 hours at 37°C. Trypsin activity was inhibited by acidification of samples to a concentration of 1% TFA. Digests were desalted on C18 Stage tips and eluates were analysed by HPLC coupled to a Q-Exactive mass spectrometer as described previously (Turriziani et al., 2014). Peptides and proteins were identified and quantified with the MaxQuant software package (1.5.7.4), and label-free quantification was performed by MaxLFQ (Cox et al., 2014). The search included variable modifications for oxidation (M, C, Y, W), dioxidation of (M,C,W), trioxidation (C), carbamidomethylation (C) and carbamidomethylation+oxidation (C) as variable modifications. The false discovery rate, determined by searching a reverse database, was set at 0.01 for both peptides and proteins. Peptide oxidations were relatively quantified by [PeptideOX]/[Peptides].

QUANTIFICATION AND STATISTICAL ANALYSIS

Graphpad Prism software was used for statistical analyses. Statistical and quantification details of experiments can be found in the figure legends. Data are presented as ±SEM of at least 3 independent experiments. Significance was defined a p < 0.05 between comparison groups.