ALDH2 mediates 5-nitrofuran activity in multiple species

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Running title: ALDH2 mediates 5-nitrofuran activity

SUMMARY

Understanding how drugs work *in vivo* is critical for drug design and for maximizing the potential of currently available drugs. 5-nitrofurans are a class of pro-drugs widely used to treat bacterial and trypanosome infections, but despite relative specificity 5-nitrofurans often cause serious toxic side-effects in people. Here, we use yeast and zebrafish, and human *in vitro* systems to assess the biological activity of 5-nitrofurans, and identify a conserved interaction between aldehyde dehydrogenase (ALDH) 2 and 5-nitrofurans across these species. In addition, we show that the activity of nifurtimox, a 5-nitrofuran anti-trypanosome pro-drug, is dependent on zebrafish Aldh2 and is a substrate for human ALDH2. This study reveals a conserved and biologically relevant ALDH2–5-nitrofuran interaction that may have important implications for managing the toxicity of 5-nitrofuran treatment.

HIGHLIGHTS

- Zebrafish provide a viable assay for the biological toxicity of 5-nitrofurans
- ALDH2 inhibitors prevent 5-nitrofuran toxicity in zebrafish and yeast
- Genetic dependence on ALDH2 for 5-nitrofuran toxicity in zebrafish and yeast systems
- 5-Nitrofurans bind to and are substrates of human ALDH2

INTRODUCTION

Drugs often have multiple targets *in vivo* that can lead to unintended side-effects. Identifying unintended drug targets and their *in vivo* relevance is a fundamental challenge in chemical biology. 5-Nitrofurans are a class of drugs that save thousands of lives as front line treatments for parasitic trypanosome infections in Latin America and Africa, and are also effective antibiotics in human and veterinary medicine (Castro, et al., 2006; Coura, 2010; Nussbaum, et al., 2010; Priotto, et al., 2009). 5-Nitrofurans are of such importance to human health that the World Heath Organization deems the 5-nitrofuran, nifurtimox, an essential medicine and Bayer HealthCare provides nifurtimox free of charge for trypanosome infections. 5-Nitrofurans are pro-drugs, and their relative specificity comes from parasitic and bacterial specific nitroreductases (NTRs) that reduce the 5-NO_2 functional group to a toxic anion radical thereby generating reactive oxygen species and inducing cell death. Despite their wide-spread use 5-nitrofurans have serious toxic side-effects (Castro, et al., 2006). For nifurtimox, toxic side-effects lead to treatment cessation in over 30% of patients with Chagas disease, caused by *Trypanosoma cruzi* infection (Castro, et al., 2006). Clinical side-effects are complex and can vary between populations but include polyneuropathy, depression, forgetfulness, alcohol intolerance and headaches, as well as gastrointestinal complications. There is currently no treatment strategy available to reduce the off-target toxic side-effects of 5-nitrofurans.

Decades of research have identified multiple human enzymes capable of 5-nitrofuran reduction *in vitro*, in cells or tissues (Dubuisson, et al., 2001; Rao, et al., 1987; Rao and Mason, 1987). However, whether these enzymes are relevant to 5-nitrofuran side-effect activity and the potential for therapeutic intervention to inhibit their activity *in vivo* is undetermined. Drug mechanism of action is readily examined in the zebrafish model system, in which clinically active compounds can be directly assayed in the transparent embryo (Zon and Peterson, 2005). Within two to five days of zebrafish development most tissues and organs have formed thereby enabling the identification of tissue-specific drug activities and/or bioactivation. These features allow facile phenotypic chemical screens within the whole animal. Phenotypic small molecule screens in zebrafish have enabled the identification of new biological pathways, novel bioactive chemicals, and unexpected potential for known drugs (Taylor, et al., 2010). Drugs often have multiple targets *in vivo* and examining the effects of small molecules on the developing zebrafish can also identify unintended drug targets (Ishizaki, et al., 2010; Ito, et al., 2010; Laggner, et al., 2012; Rihel, et al., 2010).

Here, we use a multi-species approach to identify ALDH2 as a mediator of 5-nitrofuran toxicity in yeast and zebrafish, and show that 5-nitrofurans are substrates for human ALDH2 *in vitro*. In a zebrafish phenotypic screen, we found that 5-nitrofurans are melanocytotoxic. We exploited this highly visible *in vivo* activity to generate a 5-nitrofuran probe, identify ALDH2 as a

5-nitrofuran target, and validate the interaction *in vivo*. This interaction is conserved from yeast to human, and is also relevant for the clinically active 5-nitrofuran, nifurtimox. We propose that this new interaction may be relevant to some of the 5-nitrofuran toxicity observed in the clinic.

RESULTS

5-Nitrofurans are active in zebrafish

Melanocytes are pigment producing cells that generate black melanin, and pigmented melanocytes are clearly visible in the developing zebrafish beginning at 28 hours post fertilization (hpf; Fig. 1A). We identified four 5-nitrofuran compounds (NFN1 [Maybridge BTB05727], NFN2 [SEW00138], NFN3 [BTB13657], NFN4 [BR00087]) in a chemical screen for modulators of melanocyte development in zebrafish embryos (Fig. 1A, C; see Methods). We also found zebrafish were sensitive to the clinically active 5-nitrofuran nifurtimox (Fig. 1B, C). 5-Nitrofuran treatment directly affected the melanocyte and melanocyte progenitor viability in a dose-dependent manner, and was independent of tyrosinase activity (Fig. S1, Movie S1). Thus, 5-nitrofurans are melanocytotoxic in zebrafish, and unlike prodrugs that are bioactivated by pigmentation enzymes (Jawaid, et al., 2009; Yang and Johnson, 2006) their activity is independent of tyrosinase. Altered pigmentation is not a feature of 5-nitrofuran toxicity in humans, however, melanocyte specificity in zebrafish provided a rapid, convenient and highly visible assay to study 5-nitrofuran activity in an animal model, independent of trypanosome infection.

5-Nitrofuran activity requires the 5-NO_2 moiety

5-Nitrofurans are pro-drugs, and the 5-NO_2 moiety is essential for bioactivation in parasites and bacteria (Maya, et al., 2007). We modified NFN1 by replacing the NO_2 moiety with a hydrogen atom (Fig. 1C; NFN1.1; Table 1; Supplemental Information). In contrast to NFN1, treatment with NFN1.1 had no effect on zebrafish melanocytes, and the melanocyte remained pigmented and intact (Fig. 1A; Table 1). Nitrofuran activity in melanocytes is therefore dependent upon the 5-NO_2 functional group. As in humans, zebrafish do not have NTRs (which are present in trypanosomes) to process the 5-NO_2 functional group, and thus the effects of NFN1 on zebrafish melanocytes may provide information about alternative methods of 5-nitrofuran processing.

Nitrofurans bind ALDH2 in zebrafish

To identify the possible targets of the 5-nitrofurans, we performed affinity purification to capture 5-nitrofuran interacting proteins in zebrafish extracts. First, we generated a series of 5-nitrofuran derivatives and tested their activity on zebrafish (Table 1; Supplemental Information). Importantly,

5-nitrofuran derivatives containing a phenyl ring (NFN5, 5.1, 5.2) effectively targeted zebrafish melanocytes (Table 1). As substitution at the *para*-position of the phenyl ring in NFN5.1 and NFN5.2 was tolerated, a 5-nitrofuran probe was generated by linking to biotin through the *para*-position of the phenyl ring (Pr-NFN; Fig. 2A). Next, the 5-nitrofuran probe was bound to streptavidin beads and protein complexes captured from zebrafish extract derived from 3-day embryos were subjected to tandem mass spectrometry. A 57-kD binding protein was identified as aldehyde dehydrogenase (Aldh) 2b (Fig. 2B; Table S1). Zebrafish have two *aldh2* (Lassen, et al., 2005; Song, et al., 2006) genes (*a* and *b*) that are orthologs of human ALDH2 (Fig. S2); *aldh2b* is expressed in neural crest derived cells, including presumptive melanocytes (Thisse, 2001). To confirm the identity of the 57-kD protein, we repeated our affinity purification protocol, and performed western blotting with anti-Aldh2 zebrafish antibodies raised against both Aldh2 a and b forms (Lassen, et al., 2005) (Fig. 2C). As a control, we generated a furan probe that was identical to the nitrofuran probe except that it lacked the 5-NO_2 functional group (Pr-FN; Fig. 2A). Aldh2 (either 2a or 2b) bound more strongly to the 5-nitrofuran probe than to the control probe, and not to streptavidin beads alone (Fig. 2C). These experiments validate Aldh2 as a 5-nitrofuran binding protein.

Aldh2 is required for 5-nitrofuran activity in zebrafish

Aldh2 catabolizes toxic aldehydes in the liver after alcohol consumption (Druesne-Pecollo, et al., 2009), in the heart after ischemia (Chen, et al., 2008), and in dopamine metabolism (Yao, et al., 2010). We asked if the 5-nitrofuran toxicity was dependent on Aldh2 in zebrafish. The natural product daidzin, found in the Kudzu vine (*Pueraria lobata*), is a potent and specific inhibitor of human ALDH2 and has long been used in traditional medicines as an antidipsotropic (Keung and Vallee, 1993; Keung and Vallee, 1993; Lowe, et al., 2008). More recently, ALDH2 inhibitors have been shown to reduce anxiety associated with treatment of cocaine and alcohol addiction (Arolfo, et al., 2009; Yao, et al., 2010). We reasoned that ALDH2 inhibitors were likely to prevent the toxicity of 5-nitrofurans in zebrafish because i) human ALDH2 is closely related to zebrafish Aldh2 (a and b) (Fig. S2), and ii) computational modeling of zebrafish Aldh2b bound to daidzin suggested that critical drug-protein interactions are conserved between species (Fig. 3A). Treatment of zebrafish embryos with daidzin protected melanocytes from the cytotoxicity of the co-administered 5-nitrofuran NFN1 (Fig. 3B), as well as the clinically active 5-nitrofuran, nifurtimox (Fig. 3C). Thus, co-administration of the Aldh2 inhibitor daidzin abrogates the activity of NFN1 and nifurtimox in zebrafish.

To provide additional evidence that the action of daidzin was by inhibition of Aldh2 and not an additional unintended target, zebrafish embryos were co-treated with NFN1 and a second

ALDH1/2 inhibitor, disulfiram (DSF). DSF, also called Antabuse and Antabus, is used to treat chronic alcoholism by preventing the ALDH2 dependent metabolism of alcohol and producing enhanced sensitivity to alcohol. DSF also chelates copper, and we and others have found that DSF prevents pigmentation of zebrafish melanocytes prior to melaninization most likely due to inhibition of copper dependent pigmentation enzymes (Fig. S3; O'Reilly-Pol and Johnson, 2008). Treatment of three dpf embryos with fully pigmented melanocytes with DSF had no effect on melanocyte integrity, while DSF prevented melanocyte toxicity upon co-treatment with NFN1 (Fig. 3B). Taken together, these experiments with two chemically independent ALDH2 inhibitors support a biological role for Aldh2 in the bioactivation of 5-nitrofuran melanocytotoxicity in zebrafish.

ALDH2 is regulated in a tissue specific manner, and in particular εPKC can directly modulate ALDH2 during ischemic preconditioning in the heart (Chen, et al., 2008; Chen, et al., 2010). We identified the PKC inhibitors PKC412 and Ro318220 as chemical suppressors of 5-nitrofuran activity in zebrafish by screening a library of 80 known kinase inhibitors. Treatment of 3 dpf zebrafish embryos with PKC412 or Ro318220 had no effect on melanocyte viability (Fig. 3B). However, treatment with PKC412 or Ro318220 prevented NFN1 activity in melanocytes (Fig. 3B). We tested a third PKC inhibitor GF109203X, that can inhibit ethanol or dopamine D2 receptor agonist NPA induced intracellular translocation of εPKC (Yao, et al., 2008). GFX109203X had no effect on melanocytes alone, but we found that it could also suppress NFN1 melanocytotoxicity (Fig. S3). GFX109203X was also effective at preventing the activity of nifurtimox in zebrafish melanocytes (Fig. 3C). Although we do not know if PKC directly enhances Aldh2b activity or expression in zebrafish, these results suggest that PKC activity is important for 5-nitrofuran cytotoxicity within the melanocyte.

ALDH2 contributes to background adaptation in zebrafish melanocytes

We wanted to understand why zebrafish melanocytes were sensitive to 5-nitrofuran treatment, when this is not a feature of 5-nitrofuran toxicity in patients. Unlike human melanocytes, zebrafish melanocytes respond to environmental conditions by concentrating or dispersing their melanosomes in light or dark conditions respectively (Logan, et al., 2006). This effect is termed background adaptation, and is a dopaminergic response (Logan, et al., 2006). A role of Aldh2 in zebrafish background adaptation has not been previously identified, however, *aldh2b* is specifically expressed in developing pigment cells (Thisse, 2001), and ALDH2 is required for dopamine metabolism in mammals (Chen, et al., 2010). We tested the effects of ALDH2 inhibition on background adapation in zebrafish and found that daidzin treatment blocked dispersal of melanin in zebrafish melanocytes in the dark (Fig. 3D). These observations suggest Aldh2 activity is required for regulation of zebrafish background adaptation and explain the melanocyte sensitivity to 5-nitrofurans.

Multi-species conservation of the 5-Nitrofuran-ALDH interaction

Chemical-genetic and chemical-chemical interactions identified in yeast are often conserved in multi-cellular species including zebrafish and mammals (Ishizaki, et al., 2010). Budding yeast have five aldehyde dehydrogenase genes (*ALD2-6*) that all share 42-48% similarity with human *ALDH 1/2* (Fig. S2). Yeast also have two fungal-specific nitroreductase-like proteins, but these share little similarity with the nitroreductases that are known to reduce nitrofurans (de Oliveira, et al., 2007). To establish that 5-nitrofurans also showed activity in yeast, liquid cultures were treated with increasing concentrations of NFN1 (Fig. 4A). Yeast were highly sensitive to NFN1, which inhibited growth even at a sub-micromolar concentrations. In contrast, treatment with the control furan compound, NFN1.1, had no effect on yeast growth, even at 100 μM. These data indicate that the toxicity of 5-nitrofurans in yeast is dependent on the 5-NO_2 moiety. To test if NFN1 toxicity was dependent on ALDH activity, we tested drug combinations in yeast cultures. Increasing concentrations of daidzin rescued the effects of 50 μM NFN1 on yeast growth rate in a dose-dependent fashion, whereas daidzin alone had no effect on growth (Fig. 4B).

Mutations that render yeast resistant to a specific compound can provide direct links to the target pathway (Ishizaki, et al., 2010). We determined if yeast strains bearing deletions in each of the *ALD* genes (orthologs of human and zebrafish *ALDH1/2*) were resistant to 5-nitrofuran treatment. The *ald2Δ, ald3Δ, ald4Δ* and *ald5Δ* deletion strains each exhibited the same sensitivity to NFN1 as wild type (*data not shown*). In contrast, an *ald6Δ* strain was significantly less sensitive to NFN1 treatment, as was an *ald2Δald3Δ* double deletion strain (Fig. 4C, D). These effects of different *ald* mutations appeared to be additive as a triple *ald2Δ ald3Δ ald6Δ* deletion strain was almost completely resistant to 50 μM NFN1 treatment (Fig. 4D). Once activated 5-nitrofurans cause DNA damage, and consistent with this we find chemical-genetic profiles in yeast indicate that disruption of DNA damage repair pathways causes hypersensitivity to 5-nitrofurans (Fig. S4).

To further validate the genetic dependence of 5-nitrofuran bioactivity on Aldh2, we used morpholino oligonucleotides (MOs) to knockdown *aldh2b* in zebrafish. Single-cell embryos were injected with a splice-site blocking *aldh2b* MO, and at 2 dpf were treated with NFN1. PCR analysis of the splice-site MO indicated that *aldh2b* morphants had reduced levels of correctly spliced *aldh2b* transcript in addition to a mis-spliced transcript indicating that the *aldh2b* morphants are hypomorphic for *aldh2b* (Fig. S4). We consistently found that the splice-site blocking *aldh2b* MO conferred partial resistance to a low treatment dose (0.8μM) of NFN1 melanocytotoxicity (Fig. 4E). An *aldh2b* translation block MO also conferred partial resistance to a short NFN1 treatment (Fig. S4). We conclude that there is a genetic dependence on Aldh2b for 5-nitrofuran activation in zebrafish, in line with genetic studies in yeast.

5-Nitrofurans are substrates for human ALDH2

There are 19 ALDH enzymes in humans, each with specific targets and additional activities (Marchitti, et al., 2008). To determine if the 5-nitrofuran-ALDH2 interaction is conserved in humans we asked if human ALDH2 could bind 5-nitrofurans directly. Purified human ALDH2 was added to the 5-nitrofuran probe (Pr-NFN), a furan control probe (Pr-FN), or streptavidin beads alone. In an analogous manner to the experiments using zebrafish extracts, human ALDH2 binding was strongly enriched in association with the 5-nitrofuran, while the control furan and the streptavidin beads alone did not bind ALDH2 (Fig. 5A).

Given our results with diadzin in yeast and zebrafish, we proposed that NFN1 was probably a substrate of ALDH enzymes. ALDH2 enzymes have reducing potential as well as dehydrogenase activity (Chen, et al., 2002; Marchitti, et al., 2008), and ALDH2 has been shown, in the absence of a reducing agent, to inactivate itself during the bioactivation of substrates such as nitroglycerine (GTN) (Chen, et al., 2010; Wenzel, et al., 2007). Consistent with this, we found that in the absence of a reducing agent, NFN1, but not the no-nitro NFN1.1, inactivated recombinant human ALDH2 *in vitro* (Fig. 5B-D). Likewise, we found that following 10 minutes incubation with nifurtimox, ALDH2 activity was reduced by 39.6% (at 5μM nifurtimox), 77.6% (at 16.7μM nifurtimox), and by 96.5% (at 50μM nifurtimox) (Fig. 5C). Importantly, like the zebrafish studies, these experiments were performed with nifurtimox at concentrations that are within the range of those recorded in the serum of nifurtimox-treated patients (Paulos, et al., 1989; Saulnier Sholler, et al., 2011). For both NFN1- and nifurtimox-inactivated ALDH2, the subsequent addition of a reducing agent (TCEP) led to partial reactivation of the enzyme in line with literature studies using the accepted substrate GTN (Fig. 5D). We observe that the NFN1-ALDH2 interaction is stronger than the nifurtimox-ALDH2 in zebrafish and in our biochemical assay. This raises the possibility that the mechanism of action of nifurtimox is more complex than NFN1, or that NFN1 may in fact be a more effective 5-nitrofuran compound than nifurtimox.

Daidzin does not affect nifurtimox trypanocidal activity

In an attempt to develop a clinically testable hypothesis, we examined the genome sequence of the trypanosomatids to identify possible ALDH enzymes in *T. brucei*, *T. cruzi* and *Leishmania* (Fig. S2) (Aslett, et al., 2010; Cross, 2005; Lowe, et al., 2008; Marchitti, et al., 2008; Sobreira, et al., 2011). Given the absence of an obvious ALDH2 in *Trypanosoma* we hypothesized that while Aldh2 inhibition would protect the zebrafish melanocytes and yeast cells from 5-nitrofuran activity, ALDH2 inhibitors might not protect trypanosomes from 5-nitrofuran sensitivity (Fig. 6A). We grew the bloodstream-form *T. brucei* (strain 427) in HMI9 media, and determined the trypanocidal

activity of nifurtimox in the absence and presence of daidzin. Trypanosomes were stained with an Alamar Blue vital dye as an indicator of *Trypanosoma* survival. We found nifurtimox was equally effective in the absence (ED_{50} = 2.12 ± 0.17 μM; slope 1.00) and presence (ED_{50} = 2.18 ± 0.10 μM; slope 0.98) of daidzin (Fig. 6B). The trypanocidal effect of nifurtimox against bloodstream *T. brucei* obtained in these assays was comparable to previously observed effects (Priotto, et al., 2009; Sokolova, et al., 2010). Daidzin treatment alone showed no trypanocidal affect up to 100μM (data not shown). We conclude that daidzin does not interfere with 5-nitrofuran trypanocidal activity, consistent with a lack of an *ALDH2* in trypanosomes.

DISCUSSION

We have used a multi-species, chemical-biology approach to identify 5-nitrofurans as new substrates for ALDH2. We have identified a series of 5-nitrofuran compounds by phenotypic screening in zebrafish, and shown that 5-nitrofuran specific melanocytotoxicity *in vivo* is mediated at least in part by Aldh2 (Fig. 1, 3). Zebrafish gene products are usually conserved in humans and are often sensitive to clinically active drugs at physiological concentrations (Zon and Peterson, 2005). As shown here, phenotypic chemical screens in zebrafish are effective because i) the rapid and cell type specific toxicity of 5-nitrofurans can be visualized in real time (Movie S1), ii) the whole animal is amenable to pharmacological studies (Fig. 1A, B), and iii) initial structure activity relationships can be determined to enable the design of biologically relevant probes for affinity purification (Fig. 2; Table 1).

Despite the benefits of phenotypic screens in zebrafish, target identification remains a challenge in chemical biology (Laggner, et al., 2012; Taylor, et al., 2010; Zon and Peterson, 2005). Here, we use parallel approaches to enable identification of an important new target of 5-nitrofurans. First, we used affinity chromatography to identify Aldh2 as a 5-nitrofuran binding partner, and confirmed the dependence on the 5-NO_2 functional group using an inactive furan probe (Fig. 2). Second, we used computational modelling to predict that the ALDH2 inhibitor daidzin would be active in zebrafish (Fig. 3A), and used two chemically distinct ALDH2 inhibitors (daidzin and DSF) to confirm the biological relevance of the 5-nitrofuran-Aldh2 interaction *in vivo* (Fig. 3B,C). Third, we showed cross-species conservation of the drug-drug interactions in the evolutionarily distant budding yeast system (Fig. 4A, B). Fourth, we used genetic mutants in yeast and gene knockdowns in zebrafish to validate a genetic dependence on ALDH activity for 5-nitrofuran activity *in vivo* (Fig. 4C-E). Fifth, we show that the 5-nitrofuran-ALDH2 interaction is maintained with human ALDH2 (Fig. 5A). Finally, using a literature-precedent method, we show that 5-nitrofurans are direct substrates of human ALDH2 (Fig. 5B-D).

We find that zebrafish melanocytes are sensitive to the 5-nitrofurans because unlike human melanocytes, zebrafish melanocytes use ALDH2 to elicit a melanocyte background adaptation response (camouflage; Fig. 3D). While additional host enzymes, including the possibility of other ALDHs, may bioactivate 5-nitrofurans in patients, we speculate that, in line with our studies in zebrafish and yeast, daidzin may protect cells that specifically express ALDH2, such as the liver and dopaminergic neuronal cells (Fig. 6A). Although 500 million individuals worldwide have an ALDH2 inactive variant (Druesne-Pecollo, et al., 2009), it is unknown if these genetic variants contribute to the variability of 5-nitrofuran associated side effects; our chemical-genetic data in yeast and zebrafish (Fig. 4) suggest that this hypothesis could be examined in the clinic. 5-Nitrofurans have also recently become anti-cancer agents, and nifurtimox is currently in clinical trials for relapsed/refractory pediatric neuroblastoma and medulloblastoma (Saulnier Sholler, et al., 2011). It is possible that 5-nitrofuran bioactivation by ALDH2 explains the sensitivity of these dopaminergic cancers to nifurtimox. We find that human melanoma cells are also sensitive to nitrofurans, that DNA damage occurs, and that this activity is dependent on the NO_2 functional group present in NFN1 (Fig. S4). Taken together with the hypersensitivity of yeast DNA damage mutants to NFN1, these results suggest that once activated, the cytotoxic effects of 5-nitrofurans arise through a similar DNA damage-dependent mechanism across species. Although, it is unclear at this time whether NTR- and ALDH2-mediated activation of 5-nitrofurans leads to exactly the same toxic intermediates.

We argue that NFN1, but not the no-nitro NFN1.1, is a substrate for recombinant human ALDH2 *in vitro* (Fig. 5). Analogous observations have been made in ALDH2 bioactivation of nitroglycerin (Chen, et al., 2010; Wenzel, et al., 2007), thereby raising the interesting question of how 5-nitrofurans are bioactivated by ALDH2. ALDH2 enzymes have reducing potential as well as dehydrogenase activity (Chen, et al., 2002; Marchitti, et al., 2008), and we envision that ALDH2 may reduce the nitro group of 5-nitrofurans potentially generating nitroso-, hydroxylamine and/or amine intermediates with concomitant oxidation of the enzyme. Interestingly, DTT can react with 5-nitrofurans leading to oxidation of DTT to the corresponding disulfide (L.Z., N.W. unpublished data). As DTT contains two thiols in close proximity, in an analogous manner to the active site of ALDH2, we suggest that the reaction of 5-nitrofurans with ALDH2 and DTT may be linked by a common mechanism.

5-Nitrofurans are important therapeutic agents, yet many patients suffer from unacceptable drug induced toxic side effects. One approach to solving this problem is to identify new anti-trypanosome drug targets, such as the recently identified *N*-myristoyltransferase inhibitors (Frearson, et al., 2010) that have been validated in mouse trypanosomiasis models. Based on our studies in model systems and *in vitro*, we propose a complementary approach that involves

targeting and minimizing the toxic side effects of current therapies, thereby allowing more patients to benefit from approved treatment regimes that are already available (Fig. 6A). If the 5-nitrofuran-ALDH2 interaction is conserved in patients, then combination therapy to treat 5-nitrofuran toxic side-effects may be testable because i) ALDH2 is a targetable enzyme, ii) the ALDH2 inhibitors daidzin and DSF are both currently available at low cost and show activity in humans with limited toxicity, and iii) our analysis indicates that *T. brucei* and *T. cruzi* have neither a close ALDH2 homolog (Fig. S2) nor is *T. brucei* protected from nifurtimox by daidzin (Fig. 6B). Our findings provide impetus for addressing the role of ALDH2 in 5-nitrofuran activation in the pre-clinical and clinical setting.

SIGNIFICANCE

How drugs work *in vivo* and identifying unintended drug targets is a fundamental challenge in chemical biology. Nifurtimox is one of only two drugs used to treat Chagas disease caused by *Trypanosoma cruzi* infection, which is estimated to affect over 10 million people per year and to kill between 15,000 and 50,000 annually. Like other 5-nitrofurans, nifurtimox is a pro-drug that is activated by parasite specific nitroreducatases to a toxic form. Despite the absence of nitroreductases in humans, 5-nitrofurans cause significant clinical off-target toxic side-effects in patients that interfere with the ability to complete the treatment course. There has been no significant improvement in trypanosome disease treatment for 40 years, and there is currently no treatment strategy to reduce the burden of these toxic side-effects of existing drugs in patients.

Here, we use model organism chemical-genetics to explore the basis for this toxicity. We use the zebrafish model system to 1) identify toxic effects of 5-nitrofuran compounds, 2) as a platform for structure-activity relationships and target identification, and 3) to show that the toxicity of 5-nitrofurans in zebrafish can be prevented with the co-treatment of ALDH2 inhibitors. We then show that the ALDH2-5-nitrofuran interaction is conserved in yeast and with human ALDH2, and argue that 5-nitrofurans are a direct substrate of human ALDH2. We extend these finding to show that the 5-nitrofuran, nifurtimox, also has Aldh2 dependent activity in zebrafish, and that it is a direct substrate of human ALDH2. Thus, we show in model systems that combination drug treatments of ALDH2 inhibitors with 5-nitrofurans block the 5-nitrofuran unintended biological activity, and propose that similar treatments based on a readily available combination of inexpensive approved drugs may prevent some of the clinical side-effects caused by 5-nitrofurans.

ACKNOWLEDGEMENTS

We are grateful to I. Jackson, M. Frame, K. Ball, D. Harrison and N. Hastie for discussions and reading of the manuscript, V. Vasiliou for the zebrafish Aldh2 antibody, G. Sholler and S.

Wilkinson for nifurtimox reagents. This work was funded by the NIH (SLJ), the Wellcome Trust project (TKS), a Royal Society (MT, NJW), a Scottish Universities Life Sciences Alliance Research Chair (MT), the European Research Council (MT), Cancer Research UK (LZ, NJW), the European Commision FP-7 ZF-CANCER project (EEP), Medical Research Scotland (EEP, HI), and the Medical Research Council (EEP, KT, ZZ, PG).

FIGURE LEGENDS

Figure 1. 5-Nitrofurans promote melanocytotoxicity in zebrafish. (**A**) Examples of two day old zebrafish embryos treated for 48 hours with DMSO as a control, 5 μM NFN1, 5 μM NFN1.1, or (**B**) 50 μM nifurtimox. Black melanocytes (red arrows) and melanocytes detritus are indicated (blue arrows). (**C**) Chemical structures of the four 5-nitrofurans (NFN1-4; Maybridge compounds BTB05727, SEW00138, BTB13657, BR00087) identified in a chemical screen for modulators of melanocyte development. The 5-NO_2-furan functional group shared between the 5-nitrofurans, including nifurtimox, is indicated (red). Chemical structure of NFN1.1. is identical to NFN1 but lacks the 5-NO_2 functional group required for activity (blue). See also Figure S1, Movie S1.

Figure 2. 5-Nitrofurans bind Aldh2 in zebrafish. (**A**) Biotinylated probes linked to a 5-nitrofuran (Pr-NFN) and a control furan (Pr-FN). Biotin is labelled in blue, and the 5-nitro or modification moeity in red. (**B**) Silver stain of protein bands identified using Pr-NFN probe, or streptavidin beads alone as a control (No Probe). The red box indicates the region of the gel that was isolated for mass spectrometry analysis (arrow) at 57 kD. **(C)** Western blot of zebrafish protein bound to the no probe control, the furan (Pr-FN) control or the 5-nitrofuran probe (Pr-NFN), and probed with zebrafish anti-Aldh2 antibodies. A band corresponding to 57 kDa is indicated (arrow). MW: molecular weight. See also Fig. S2, Table S1.

Figure 3. Aldh2 is responsible for 5-nitrofuran activity in zebrafish. (**A**) A predicted binding model of daidzin to zebrafish ALDH2b, based on key residues involved in the human ALDH2-daidzin (PDB 2vle) protein-ligand interaction (Lowe, et al., 2008). The equivalent residues in zebrafish Aldh2b are shown. Human ALDH2→ Zebrafish Aldh2 2b (Asp457→ Asn473; Phe170→ Phe186; Trp177→ Trp193; Val120→ Val 136; Phe296→ Phe312; Phe292→ Ile308; Asp457→ Asn473; Cys303→ Cys319). (**B**) Aldh2 and PKC inhibitors prevent 5-nitrofuran activity in zebrafish. Examples of 2 d pf zebrafish embryos treated with 20 μM of the ALDH inhibitors daidzin or DSF for one hour, or with 20 μM of the PKC inhibitors PKC412 or Ro318220, and then treated with 5 μM NFN1 or 0.1% DMSO alone for two days. Experiments were repeated at least 3 times, with n > 10 embryos per condition. (**C**) Examples of 2 dpf zebrafish embryos pretreated with

DMSO, 30 μM of daidzin or the PKC inhibitor GFX 109203X for one hour, and then treated with 50 μM nifurtimox for 7 hours. Punctate melanocytes are indicated. Experiments were repeated at least 3 times (n = 5-10 embryos per condition) and treatment condition cohorts blind scored. (**D**) Daidzin alters background adaptation in zebrafish embryos. (Left) Images of fixed zebrafish embryos (5 dpf) treated with 0.1% DMSO or 10 μM daidzin, and shifted from a dark environment to a light environment (light), or *vice versa* (dark). The average percentage of melanin coverage (within the area indicated by red dotted outline) for each treatment condition ± standard deviation is indicated. (Right) Box plot of melanin coverage (y-axis) for each embryo in different treatment conditions (x-axis). Individual values taken from one of three experiments are shown as red circles. The box depicts the lower quartile and the upper quartile, with the median depicted by the intersecting line. Whiskers extend between the minimum and maximum of all the data. In DMSO treated embryos, melanocytes are significantly contracted in the light and expanded in the dark ($P<0.001$, n=20 for each condition); ANOVA, [95% CI 11.081(5.966,16.195)]. Zebrafish treated with daidzin contract their melanin in response to light environment, but do not significantly expand their melanin in response to dark environments [95% CI 0.563(-4.552, 5.677)]. The experiment was repeated three separate times with embryos at 5 dpf (n=5-20 embryos per condition) and once at 4 dpf (n= 10 embryos per condition). See also Fig. S3.

Figure 4. Cross-species conservation of 5-nitrofuran-ALDH2 interaction in yeast. (**A**) Yeast cultures were treated with NFN1 (red) or NFN1.1 (blue). OD values were normalized against DMSO treated controls. The mean of two experiments with three replicates is shown; error bars represent standard error. (**B**) Daidzin-NFN1 drug interaction was assessed by combination matrix assays in 96 well plates. Cultures were treated with NFN1 (red) or with daidzin in the absence (blue) or presence of 50 μM NFN1 (black). The average normalized growth of three experiments is shown; error bars represent standard error. (**C**) Normalized growth in the presence of NFN1 was determined for wild type (blue) and the *Δald6* strain (red). Data points are the mean of four replicates; error bars represent standard error. (**D**) NFN1 dose response curves for *Δald2 Δald3* (red) and the *Δald2 Δald3 Δald6* (black) strains, as well as wild type control (blue) were generated and normalized against DMSO-treated controls. The average of three replicates is shown; error bars represent standard error. (**E**) Three dpf control (n=24) or *aldh2b* splice-site morphants (n=62) without NFN1 treatment (left panels) or with 0.8μM NFN1 treatment (right). Embryos were scored as class I (strong) or class II (mild) sensitivity to NFN1 (bar graph). *aldh2b* morphant embryos were less sensitive to NFN1 treatment compared to control morphants [P=0.007; 95% CI (0.139, 0.528); Fisher's exact test]. See also Fig. S4.

Figure 5. 5-Nitrofurans bind and are substrates for human ALDH2 *in vitro*. (**A**) Binding of purified human ALDH2 by 5-nitrofuran probe (Pr-NFN), a furan control probe (Pr-FN) or streptavidin beads alone (No Probe). Arrow indicates ALDH2 protein, ALDH2 input lane (0.5μg). (**B**) Schematic overview of chemical reaction used to monitor recombinant human ALDH2 activity and experimental design. In experiment **C** (red arrow) ALDH2 was incubated with 1% DMSO, NFN1, NFN1.1 or Nifurtimox for 10 mins., and then ALDH2 activity was assessed. In experiment **D** (red + blue arrows), ALDH2 was incubated with 1% DMSO, NFN1, or Nifurtimox for 10 mins., incubated with 0.5mM TCEP or buffer alone for a further 15 mins., and then ALDH2 activity was assessed. (**C**) Bar graph of spectrophotometric analysis of the rate of production of NADH (monitored at 341nm) by ALDH2 (expressed as a percentage of DMSO control treatment) with DMSO, NFN1, NFN1.1 and Nifurtimox. (**D**) Bar graph of spectrophotometric analysis of the rate of production of NADH by ALDH2 after combined treatment of DMSO, NFN1, and Nifurtimox with TCEP or buffer. Enzyme buffer = 50 mM sodium phosphate, pH 7.4. Error bars are S.D.; experiments repeated in triplicate.

Figure 6. ALDH2 in trypanosomes. (**A**) Schematic of a 5-nitrofuran-daidzin combination treatment strategy. ALDH2 can cause 5-nitrofuran bioactivtion in ALDH2 expressing cells (*e.g.* zebrafish melanocytes), but not in trypanosomes because they lack ALDH2 (see also Fig. S2). We propose that co-treatment with an ALDH2 inhibitor such as daidzin could limit 5-nitrofuran toxicity without interfering with anti-trypanosome activity. (**B**) Viability of *Trypanosoma brucei* (bloodstream-form) at 37°C after 72 hour treatment with increasing concentrations of nifurtimox in the absence or presence of daidzin (100μM). Experiments were conducted twice in replicates of four; a representative set of data from one experiment containing four replicates is shown. ED: effective dose.

Methods

Zebrafish small molecule screens and treatments. The chemical library was a collection of 1576 Maybridge compounds (Ishizaki, et al., 2010). Two 4-hpf embryos were arrayed in 96-well plates containing 10 μM of compound in 1% DMSO in 300 μl of E3 embryo medium. Embryos were assessed and imaged for phenotypic changes at 28, 36, 48 and 56 hpf. For the screening of The Screen-Well™ Kinase Inhibitor Library, (Enzo Life Sciences), 5 embryos (24 hpf) were placed into each well of a 24-well plate (Corning Incorporated) containing 20μM NFN1 (BTB05727, Maybridge Screening compounds) and 5, 10 or 20 μM of a corresponding compound (total volume 1 ml per well). For co-treatments experiments, five 36-48 hpf embryos were arrayed in 24-well

plates in 600ul – 1ml of E3 embryo medium and pretreated with ALDH or PKC inhibitors (1 - 7 hours), and then treated with 0.5 - 5 μM NFN1 or 50 μM nifurtimox.

Affinity purification and co-immunoprecipitation with 5-nitrofuran beads. Lysate was generated from approximately 900 3 dpf zebrafish in 300 μL of RIPA buffer [2 M Tris pH 7.5, 5 M NaCl, 1% NP40, Na-deoxycholate, 10% SDS, 0.5 M NaF, 1 M β-glycosyl phosphate and protease-inhibitor cocktail tablet (Roche)], centrifuged at 4ºC (25mins), transferred to a new tube and kept on ice. Protein capture was performed using a pull-down biotinylated protein: protein interaction kit (Pierce) using the biotinylated chemical probe (5uL 10mg/mL DMSO solution), and bead complexes were washed with 0.1 M NaCl TBS buffer 4X to reduce non-specific binding. Beads were boiled in 3x Laemmli buffer with DTT for 5 minutes and run on 10% SDS-PAGE gel for electrophoresis. Captured proteins were visualized with Silverquest silver staining kit and/or Colloidal blue staining kit (Invitrogen). The mass spectroscopy was analysed in University of Dundee 'FingerPrints' Proteomics Facility. For western blotting, protein was detected using rabbit anti-zebrafish Aldh2 (1:1000) and goat anti-rabbit antibody (1.5:5000; Calbiochem).

In vitro binding assay. ALDH2 Human Recombinant protein (ProSpec) was added to 4 μL 10 mg/mL of chemical probe with 100 μL TBS buffer, and incubated at room temperature for 1 hour. 50 μL streptavidin beads suspension was added to the mixture (room temperature; 1h), supernatant removed and beads washed with 4 x 0.1M NaCl TBS buffer, boiled in 3x Laemmli buffer with DTT for 5 minutes and run on 10% SDS-PAGE gel for electrophoresis. The bands were detected by silver staining (Invitrogen).

Molecular modelling. Using analogous methods to those previously used (Medda, et al., 2009), the zebrafish Aldh2b homology model was generated using the Swiss model server using bovine ALDH2 (PDB code 2AG8). The daidzin structure was generated using the PRODRG server. The docking studies were performed using the program GOLD. All visualisation and analysis was performed using Pymol.

Yeast growth assays

Overnight *S. cerevisiae* BY4741 cultures in SC media were diluted (OD_{600} 0.025) and dispensed into 96-well Corning Costar assay plates. Quantitative growth curves were generated in Tecan Sunrise plate readers at 30˚C 564 rpm with automated absorbance reads every 15 minutes. Growth curve data was used to determine when control cultures reached late log phase and OD values of the entire plate at that time point were used to calculate normalized growth values. Data was analyzed with custom R scripts to generate plots. For the deletion strain growth curves, normalization was

performed against control wells for each strain.

Trypanocidal studies

The trypanocidal activity of nifurtimox in the absence and presence of daidzin (100 μM) against *Trypanosoma brucei* bloodstream-form (strain 427) were cultured at 37°C in HMI9 medium supplemented with 2.5 μg ml^{-1} G418, and viability determined using the Alamar Blue^{TM} test as described previously (Mikus and Steverding, 2000). The data was fitted using GraFit software to obtain ED50 ± standard deviations and slope factors.

Supplemental Methods

The synthesis of all the NFNs and NFN-based affinity probes is described in the Supplementary Information.

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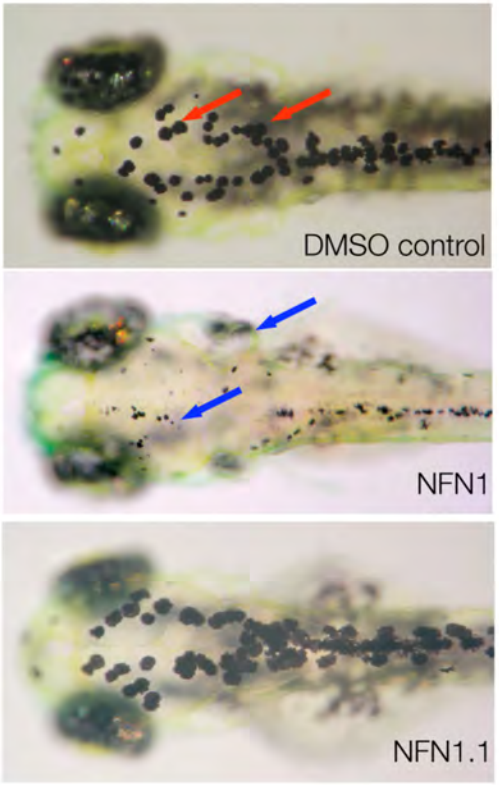

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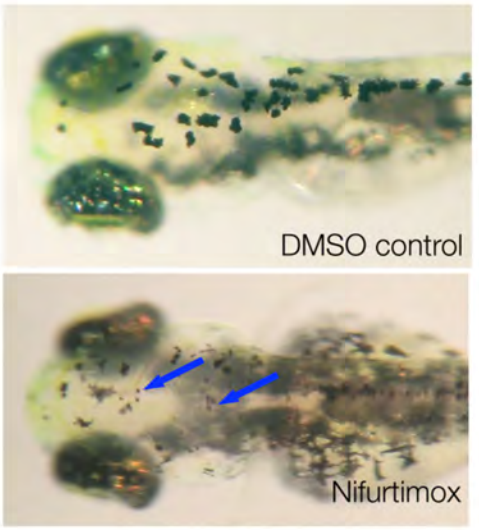

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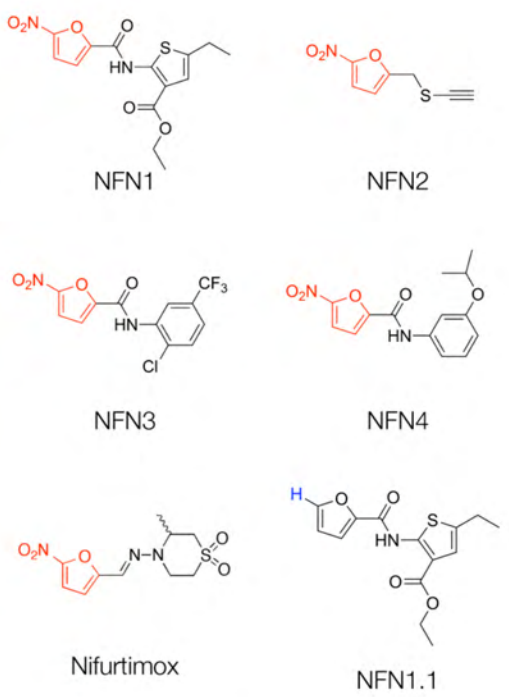

Zhou et al., Figure 1

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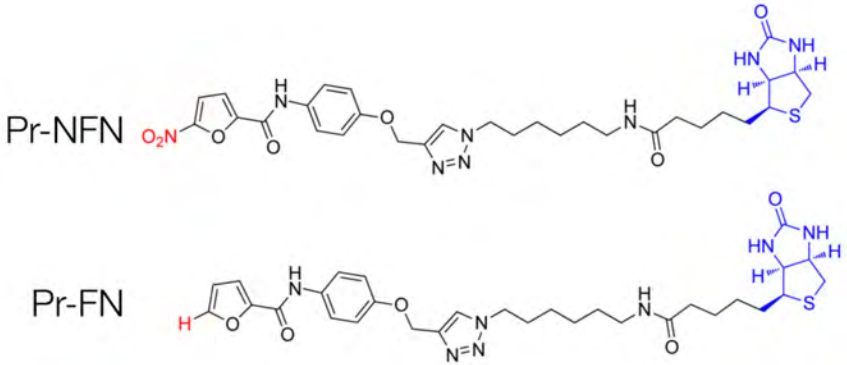

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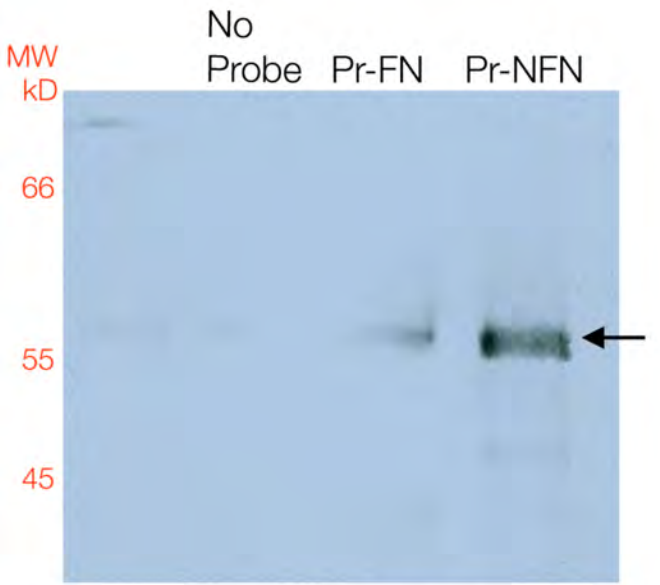
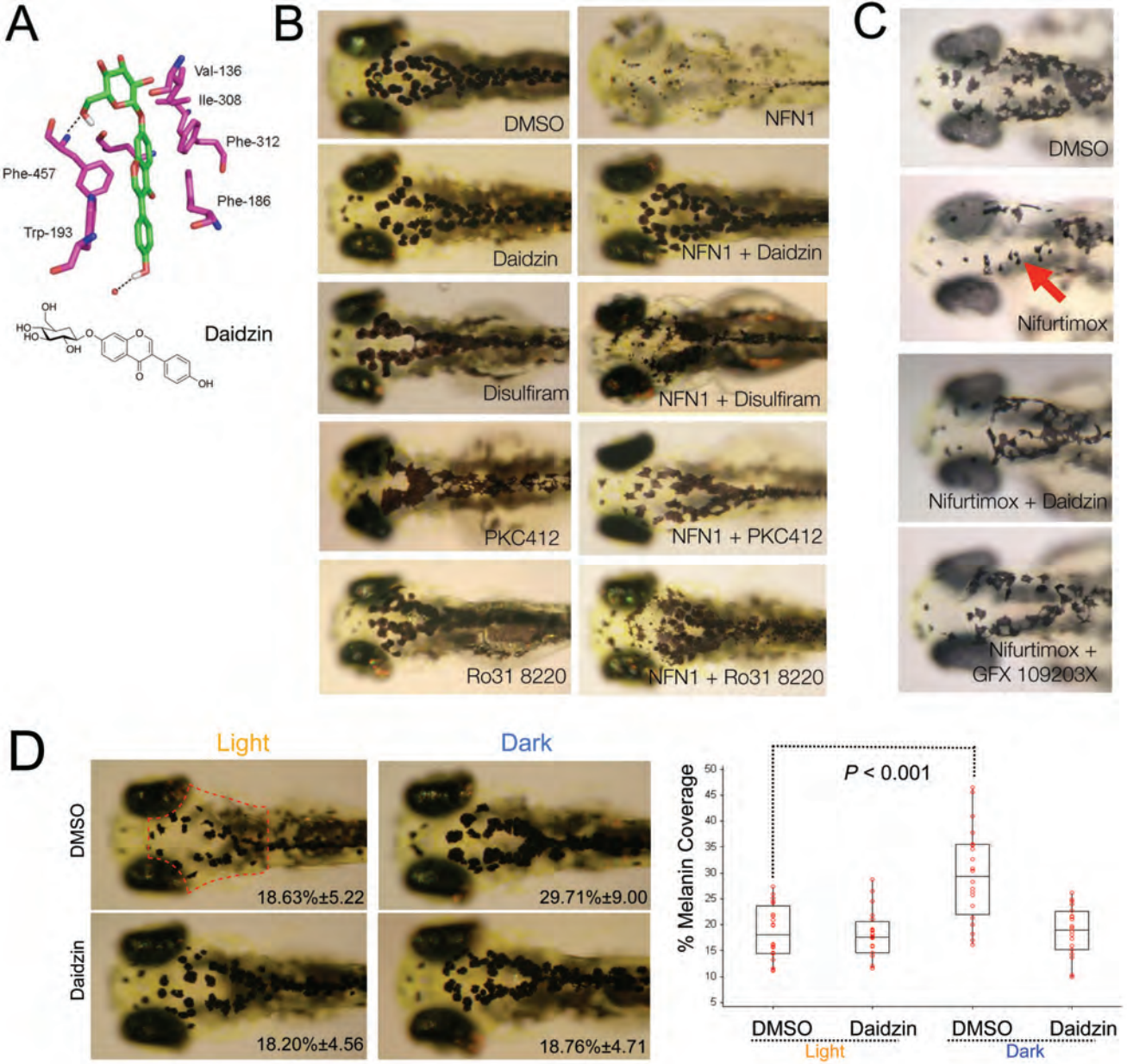


α-zebrafish Aldh2

Zhou et al., Figure 2



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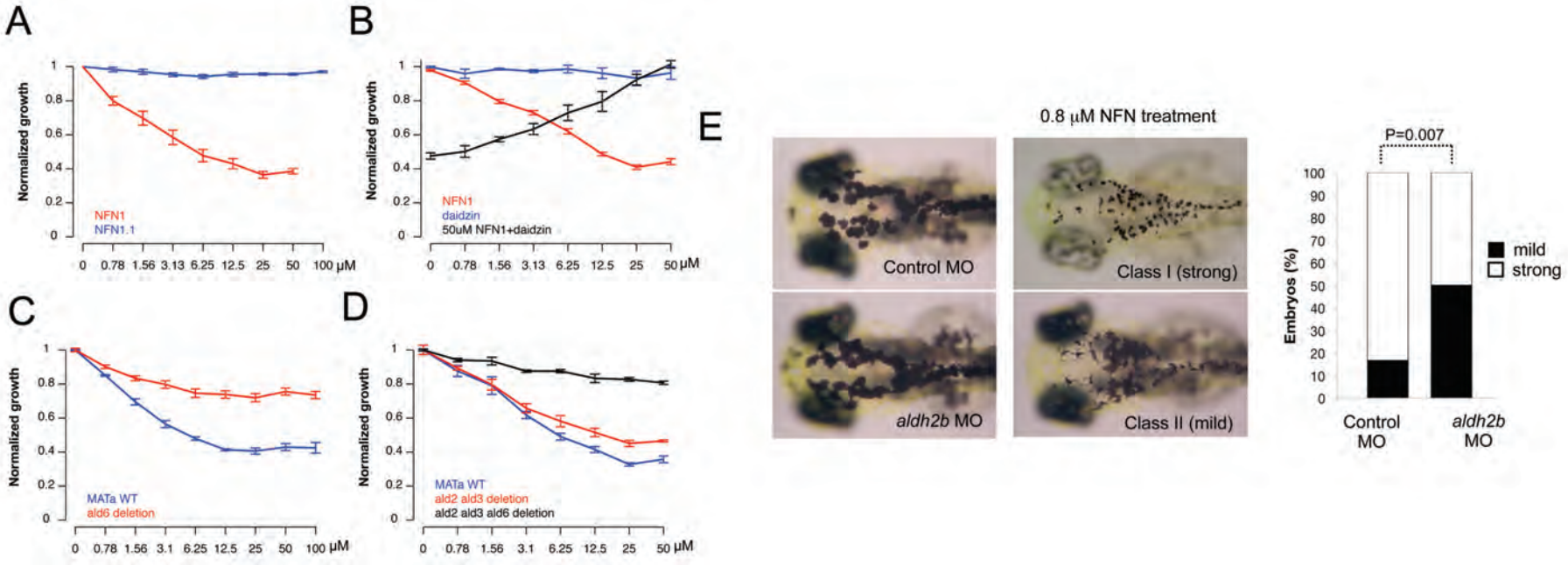

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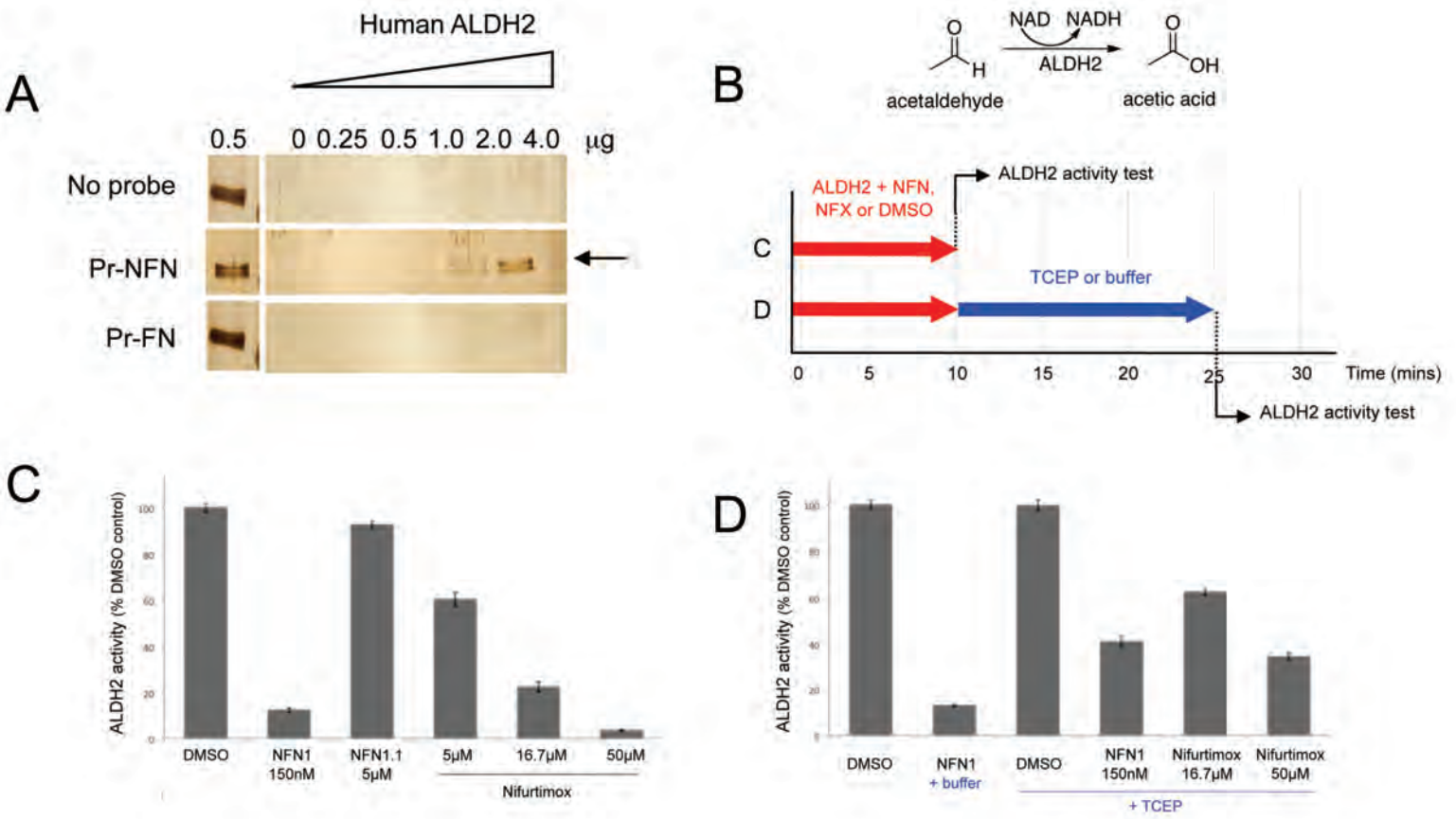

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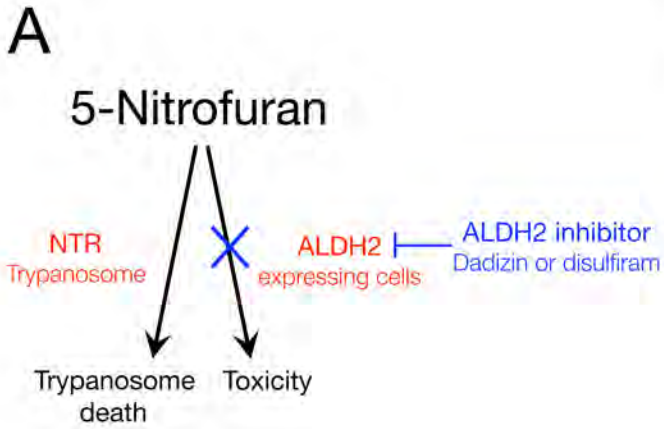

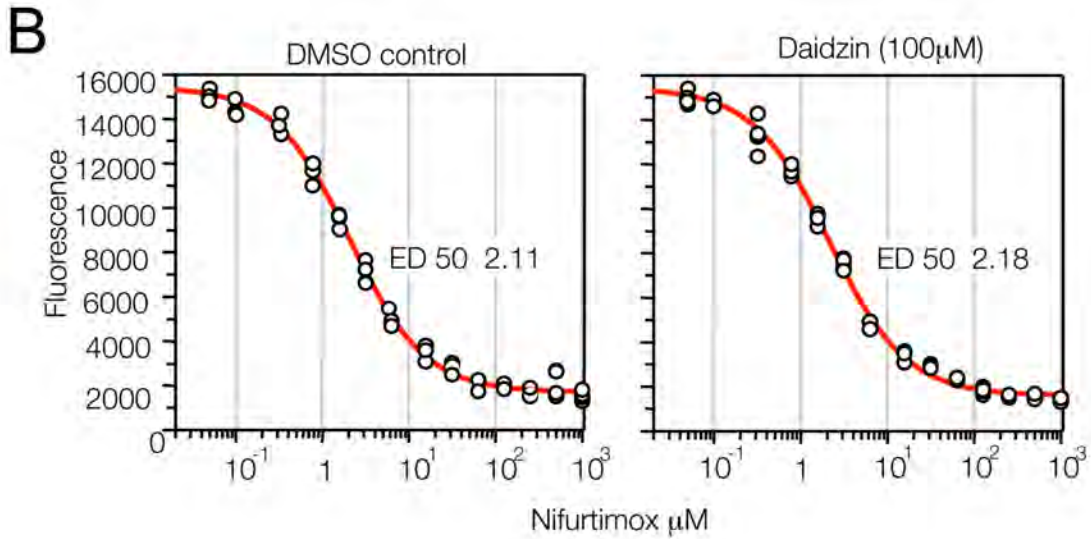

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