Targeting polyamine biosynthesis to stimulate beta cell regeneration in zebrafish

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ABSTRACT
Type 1 diabetes (T1D) is a disease characterized by destruction of the insulin-producing beta cells. Currently, there remains a critical gap in our understanding of how to reverse or prevent beta cell loss in individuals with T1D. Previous studies in mice discovered that pharmacologically inhibiting polyamine biosynthesis using difluoromethylornithine (DFMO) resulted in preserved beta cell function and mass. Similarly, treatment of non-obese diabetic mice with the tyrosine kinase inhibitor Imatinib mesylate reversed diabetes. The promising findings from these animal studies resulted in the initiation of two separate clinical trials that would repurpose either DFMO (NCT02384889) or Imatinib (NCT01781975) and determine effects on diabetes outcomes; however, whether these drugs directly stimulated beta cell growth remained unknown. To address this, we used the zebrafish model system to determine pharmacological impact on beta cell regeneration. After induction of beta cell death, zebrafish embryos were treated with either DFMO or Imatinib. Neither drug altered whole-body growth or exocrine pancreas length. Embryos treated with Imatinib showed no effect on beta cell regeneration; however, excitingly, DFMO enhanced beta cell regeneration. These data suggest that pharmacological inhibition of polyamine biosynthesis may be a promising therapeutic option to stimulate beta cell regeneration in the setting of diabetes.

Introduction
Type 1 diabetes (T1D) is a disease characterized by destruction of the insulin-producing beta cells in the pancreas. To date, virtually all therapies designed to improve beta cell function have targeted autoimmunity. While several of these therapies were reported to initially stabilize beta cell function, there was an eventual decline that proceeded at a rate comparable to untreated controls. These results highlight a critical gap in our understanding of how to reverse or prevent the beta cell damage and loss present in individuals with T1D.

Previous studies revealed that pharmacologically inhibiting polyamine biosynthesis in non-obese diabetic (NOD) mice, using the drug difluoromethylornithine (DFMO) resulted in preserved beta cell function and enhanced beta cell mass. DFMO targets the polyamine biosynthesis pathway by directly inhibiting ornithine decarboxylase (ODC), the rate-limiting enzyme in the production of polyamines (putrescine, spermidine, and spermine). Polyamines are polycationic, low molecular weight aliphatic amines that are required for cell proliferation and have been reported to have a crucial role in the proliferation of multiple cancers. Given that high concentrations of polyamines are found in endocrine and exocrine cells in the pancreas, our previous work investigated the role of polyamine biosynthesis in the developing pancreas. This work identified a direct connection to pancreatic cellular differentiation; however, the requirement for polyamine biosynthesis in cellular regeneration, and specifically beta cell regeneration, remains unclear.

Similar to the study performed with DFMO, treatment of NOD mice, either prediabetic or new onset, with the first-in-class tyrosine kinase inhibitor Imatinib mesylate prevented and reversed
diabetes. Whereas the most common application for Imatinib is its use as a therapeutic to inhibit the activity of the novel BCR-ABL tyrosine kinase constitutively active in chronic myeloid leukemia (CML), the findings in the study by Louvet and colleagues pose an interesting alternative application for the drug. That said, the impact of Imatinib on beta cell regeneration remains uncharacterized.

Interestingly, the treatment of NOD mice with either DFMO or Imatinib reported reductions in inflammation; however, the positive effects on diabetes outcomes in both studies were attributed to endocrine changes. Resultantly, these preliminary studies in rodents increased evidence for the initiation of clinical trials to investigate the effects of these drugs on human disease. In particular, a trial in new-onset T1D using DFMO (Eflornithine) was approved to determine the effect of polyamine biosynthesis inhibition on the progression of disease (NCT02384889). Similarly, a clinical trial in recent-onset T1D was approved to investigate the effects of Imatinib (Gleevec) on long-term remission of T1D (NCT01781975). Despite the initiation of these clinical trials, the biological effect of DFMO or Imatinib on beta cell growth remains unclear.

Given that the aforementioned clinical trials are investigating DFMO and Imatinib treatment for improved diabetes outcomes, the goal of this study is to determine the effect of these drugs specifically on beta cell growth. Therefore, we utilized a zebrafish regeneration model system to assess the effect of DFMO or Imatinib treatment on beta cell regeneration. The identification of an FDA-approved therapeutic that regenerates functional beta cells in the setting of diabetes would represent a landmark advance in the treatment of T1D.

Results

DFMO treatment enhances beta cell regeneration

The zebrafish pancreas develops from two distinct (dorsal and ventral) buds, with the principal islet developing from dorsal pancreatic progenitor cells and the exocrine developing from ventral pancreatic progenitors. Insulin-expressing cells are first detected in the dorsal pancreatic bud at the 12–14 somite stage (approximately 14 hours post fertilization; hpf), with the remainder of the endocrine cell types appearing by 24 hpf. By 3 days post fertilization (dpf), the principal islet has coalesced and exocrine cells have differentiated, expanded, and express characteristic markers including the transcription factor Pancreatic transcription factor 1a (Ptf1a) and the digestive enzymes CarboxypeptidaseA and Trypsin.

To test the hypothesis that DFMO promotes beta cell regeneration, we utilized double-transgenic zebrafish Tg(insa:flag-NTR;cryaa:mCherry);Tg(ptf1a-gfp), which fluorescently mark the exocrine cells with green fluorescent protein (GFP) and express the nitroreductase (NTR) gene in the insulin-expressing beta cells. In this system, the expression of NTR is induced in all cells that express insulin and subsequent treatment of the zebrafish with Nifurpinol (NFP) induces cellular ablation. Whereas this model lacks the immune cell infiltration reported in T1D, the targeted destruction of the beta cells can be used as a model to examine growth and regeneration following extreme beta cell loss. As such, previous studies have used this model to better understand the mechanism by which beta cells naturally regenerate in the zebrafish, including neogenesis and transdifferentiation.

At 3 dpf, zebrafish embryos were treated with NFP for 24 hours to induce beta cell ablation; this experimental timeline allowed for sufficient islet and exocrine differentiation to occur before beta cell death was induced. To determine the effect of DFMO on beta cell regeneration, beta cell-ablated embryos at 4 dpf were then treated with varying doses of DFMO (0.5%, 1%, 1.5% wt/vol) and cellular effects were evaluated at 7 dpf. DFMO doses of 0.5% and 1% wt/vol were well tolerated; however, the 1.5% wt/vol dose showed toxicity and death at 7 dpf (Figure 1A). All subsequent experiments analyzed only zebrafish treated with 0.5% and 1% wt/vol DFMO. GFP expression in the pancreas was used to measure exocrine pancreas length (Figure 1B); both whole-body and pancreas length were unchanged in response to DFMO treatment (Figure 1C).

Morphometric analysis of endocrine cell populations was performed on DFMO-treated zebrafish compared with no treatment and NFP-ablated controls. Immunofluorescence was used to visualize cells in the principal islet in each embryo; NFP mediated a significant reduction in insulin-expressing beta cell...
number at 4 dpf (Figure 2A). Following DFMO treatment for 3 d, no significant changes in the number of glucagon-expressing cells (Figure 2B) or insulin/glucagon co-expressing cells (Figure 2C) were observed. However, the number of insulin-expressing cells was significantly increased in both the 0.5% and 1% wt/vol DFMO-treated zebrafish compared with NFP-ablated controls at 7 dpf (Figure 2D). Excitingly, the beta cell number in the 0.5% and 1% wt/vol DFMO-treated zebrafish was not significantly different compared with untreated control embryos at 7 dpf (Figure 2D), indicating that DFMO not only stimulates beta cell regeneration but does so at an increased rate resulting in a complement of beta cells equivalent to normal development at 7 dpf.

**Imatinib treatment does not enhance beta cell regeneration**

Similar to the analysis of DFMO-treated zebrafish, we investigated the effect of Imatinib on beta cell regeneration. We tested a serial dilution (500 to 0.005 µM) of Imatinib and observed minimal to no death in all doses except 500 µM (Figure 3A).
Using GFP expression, exocrine pancreas length was measured at 7 dpf in all treatment groups and controls; no changes were observed in either the exocrine pancreas or whole-body development (Figure 3B). Whereas NFP mediated a significant reduction in insulin-expressing beta cell number at 4 dpf, no significant change in the number of insulin-expressing cells, glucagon-expressing cells, or insulin/glucagon co-expressing cells was observed at 7 dpf with Imatinib treatment compared with NFP-ablated controls (Figure 4AE). In short, there was no evidence that Imatinib treatment stimulated beta cell regeneration above that of the baseline level of regeneration observed in this zebrafish diabetes model.

Discussion

Zebrafish are a versatile model organism for answering questions related to pharmacological impact on cell growth. Using this system, we have identified that treatment with DFMO, and thus inhibition of polyamine biosynthesis, in the setting of diabetes in zebrafish results in enhanced beta cell regeneration. Moreover, our data show no effect on regeneration with Imatinib treatment. Whereas other studies have utilized zebrafish to screen molecules to identify targets that impact beta cell growth or development, our study has exploited this system to understand the impact on beta cell growth of drugs that have been shown to potentially alter diabetes onset and progression in rodents. In particular, our data suggest that the preserved beta cell function and enhanced beta cell mass reported with DFMO treatment of NOD mice may be as a result of polyamine biosynthesis inhibition stimulating beta cell regeneration.

In addition to linking polyamine biosynthesis with beta cell regeneration, our results have clarified a time window for the effects of DFMO treatment on the pancreas. Previous studies determined that pharmacological inhibition of polyamine biosynthesis during early embryonic organogenesis negatively impacted pancreas development, suggesting that inhibition of polyamine biosynthesis during the...
developmental stages could have a marked impact on the whole organism. However, once development is complete, and in a normal setting, our data show that DFMO treatment does not alter pancreas development or whole-body growth. Rather, it is only under conditions of depleted beta cell mass that the effects of DFMO are observed, namely induction of beta cell regeneration.

In stark contrast to that observed with DFMO treatment, treatment with Imatinib following beta cell ablation resulted in no increased beta cell regeneration. This lack of effect on beta cell growth was somewhat unexpected given the reported reversal of diabetes observed in NOD mice treated with Imatinib.14 That said, a potential caveat to this conclusion is the need to directly examine the inhibition of tyrosine kinase activity in zebrafish following treatment with Imatinib. Therefore, further analyses are required to fully understand if there is an effect of Imatinib treatment on the preservation or regeneration of beta cells in the setting of diabetes.

Questions also remain with respect to how DFMO stimulates beta cell growth. We speculate that inhibition of polyamine biosynthesis in the setting of diabetes may induce a mechanism that stimulates beta cell neogenesis or endocrine cell trans-differentiation, which were previously identified mechanisms that regenerate beta cells in mouse and zebrafish;27,32 however, further study is required to resolve this unknown. Moreover, results from the DFMO clinical trial (NCT02384889) will also clarify if the positive effects observed in our zebrafish model of beta cell loss translate to significant outcomes for individuals with new-onset T1D.

**Materials and methods**

**Zebrafish maintenance and strains**

Zebrafish were raised in standard laboratory conditions at 28.5°C and all procedures conducted in accordance with OLAW guidelines and approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee. The transgenic lines Tg(insa:flag-NTR;cryaa:mCherry)26 and Tg(ptf1a-gfp)24 were intercrossed to produce experimental embryos that were then cultured in standard conditions at 28.5°C in egg water, which was supplemented with 4 mM 1-phenyl 2-thiourea (PTU)(Acros) to inhibit pigment formation. Only Tg(insa:flag-NTR;cryaa:mCherry);Tg(ptf1a-gfp) embryos were used in experiments; these double transgenic embryos were sorted at 3 dpf by identification of GFP-expressing cells and mCherry-expressing eye lenses using a Leica M205 fluorescent dissecting microscope.

**Beta cell ablation and pharmaceutical treatment of zebrafish**

Beta cell ablation was achieved in Tg(insa:flag-NTR;cryaa:mCherry);Tg(ptf1a-gfp) embryos by treatment with 2.5 µM Nifurpirinol25 (Sigma) dissolved in DMSO (Fisher) supplemented with PTU (as
above) from 3–4 dpf. Embryos were then treated for 3 d (4–7 dpf) with either difluoromethylornithine (DFMO) (0.5%, 1%, or 1.5%wt/vol; generous gift from Dr. P. Woster) or Imatinib (500 µM, 50 µM, 5 µM, 0.05 µM, 0.01 µM, or 0.005 µM; LC Laboratories), dissolved in egg water, as both drugs have been reported to be soluble in water,\(^{33,34}\) thus removing the requirement for the use of an additional chemical vehicle. Drug treatments were refreshed each day for 3 d (4–7 dpf). Embryo health and survival were assessed daily. At 7 dpf, embryos were removed from drug treatments, washed with
egg water, and preserved overnight at 4°C in 2.5% formaldehyde (Fisher) in buffer (50 mM PIPES, 2 mM EGTA, 1 mM MgSO₄ (Fisher)).

**Immunofluorescence analysis of wholemount zebrafish embryos**

Immunofluorescent staining was performed as previously published³⁵ with modifications to account for age of the embryos analyzed. Briefly, embryos were blocked in 4% Bovine Serum Albumin (Gemini Bio Products) for 90 minutes at room temperature followed by incubation with primary antibodies for 48 hours at 4°C. The following primary antibodies were used: guinea pig anti-insulin (Agilent; 1:200), mouse anti-glucagon (Cell Signaling Technology; 1:100), and chicken anti-GFP (Aves Labs; 1:500). For visualization, embryos were incubated overnight at 4°C with secondary antibodies (Alexa-488, Cy3, or Alexa-647 (Jackson Immunoresearch)); DAPI (Sigma; 1:1000) was used to visualize nuclei. Images were acquired with a Zeiss 710 confocal microscope. For each embryo, images were captured every 1 µm across the principal islet. Individual endocrine cell types were quantified using ImageJ. Statistical significance was determined by one-way ANOVA with multiple comparisons using Tukey’s post hoc test (Prism7, GraphPad).

**Pancreas length measurements**

Fluorescent wholemount images were captured (Leica) such that the whole embryo and GFP-expressing pancreas could be visualized. Length was measured using ImageJ. Statistical significance was determined by one-way ANOVA with multiple comparisons using Tukey’s post hoc test (Prism7, GraphPad).

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**Keypoints**

- This study evaluates two drugs in clinical trial for new/recent-onset type 1 diabetes, DFMO, and Imatinib, to determine their effect on beta cell growth;
- In the diabetes setting in zebrafish, DFMO not only stimulates beta cell regeneration but does so at an increased rate restoring a complement of beta cells equivalent to normal development;
- Imatinib treatment did not stimulate beta cell regeneration in this zebrafish diabetes model;
- This study identifies an FDA-approved therapeutic, DFMO, with the capacity to regenerate beta cells in the setting of diabetes in zebrafish.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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