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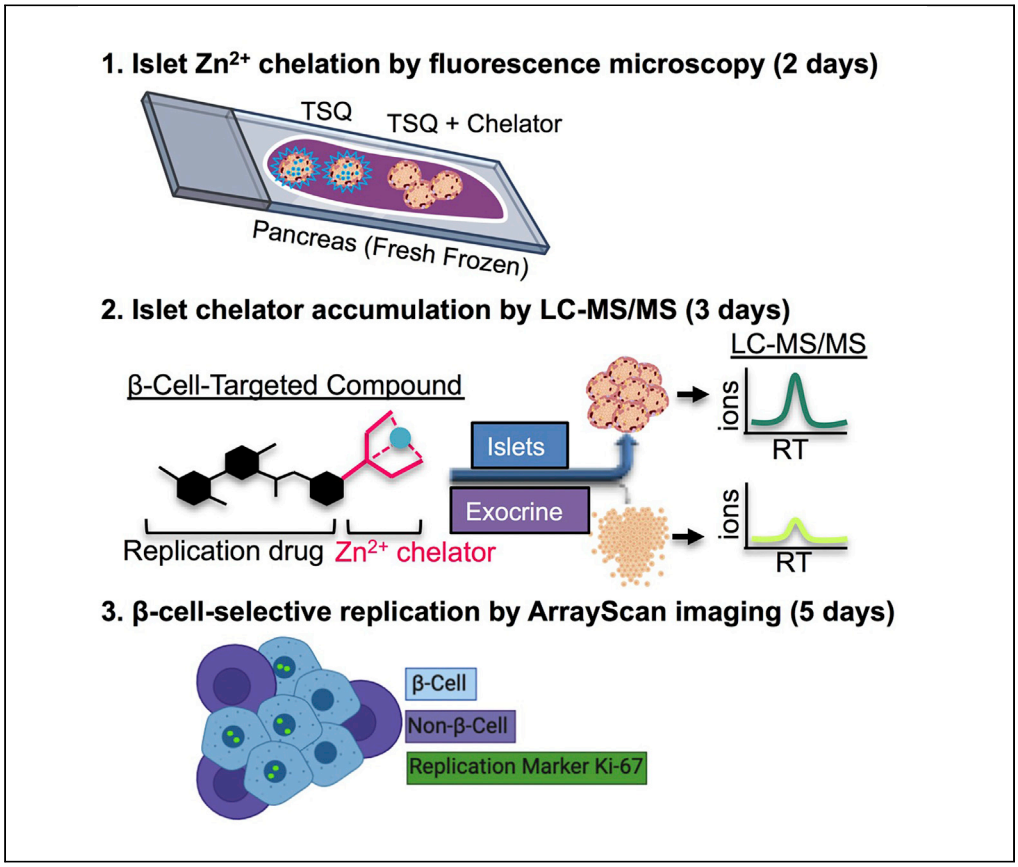
Protocol for determining zinc-dependent β cell-selective small-molecule delivery in mouse pancreas



Targeted drug delivery to pancreatic islet β cells is an unmet clinical need. β cells possess a uniquely high Zn^{2+} concentration, and integrating Zn^{2+}-binding activity into a small molecule can bias drug accumulation and activity toward β cells. This protocol can be used to evaluate a molecule's capacity to chelate islet Zn^{2+}, accumulate in islets, and stimulate β cell-selective replication in mouse pancreas. One obstacle is establishing an LC-MS/MS-based method for compound measurement. Limitations include target compound ionizability and the time-sensitive nature of some experimental assay steps.

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HIGHLIGHTS

Protocol to measure zinc-dependent β cell-selective small-molecule delivery

Islet Zn^{2+} chelation assessed by fluorescence microscopy via TSQ competition

Islet chelator accumulation can be assessed by LC-MS/MS

β cell-selective replication can be assessed by high-content cell imaging

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Protocol for determining zinc-dependent β cell-selective small-molecule delivery in mouse pancreas

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SUMMARY

Targeted drug delivery to pancreatic islet β cells is an unmet clinical need. β cells possess a uniquely high Zn^{2+} concentration, and integrating Zn^{2+}-binding activity into a small molecule can bias drug accumulation and activity toward β cells. This protocol can be used to evaluate a molecule's capacity to chelate islet Zn^{2+}, accumulate in islets, and stimulate β cell-selective replication in mouse pancreas. One obstacle is establishing an LC-MS/MS-based method for compound measurement. Limitations include target compound ionizability and the time-sensitive nature of some experimental assay steps.

For complete details on the use and execution of this protocol, please refer to Horton et al. (2019).

BEFORE YOU BEGIN

Compounds

Timing: variable

1. Obtain or synthesize compounds containing Zn^{2+}-binding and β cell replication moieties as well as non-Zn^{2+}-binding control compounds.
   a. The Zn^{2+}-binding/chelator group dipicolylamine has been exemplified, though other moieties may be effective.
   b. Replication compounds include CC-401 (Abdolazimi et al., 2018), 5-IT (Annes et al., 2012; Dirice et al., 2016), OTS167 derivatives (Allegretti et al., 2020), GNF-4877 (Shen et al., 2015), and harmine derivatives (Kumar et al., 2020; Wang et al., 2015).
2. If desired, assess DYRK1A inhibition by these compounds by either biochemical means (K_I or IC_{50}) or by a cell-based assay (DYRK-NFAT luciferase; Abdolazimi et al., 2018).

Primary tissue acquisition and cell culture

Timing: variable

3. Perform islet isolation from mice or rats (Zhao et al., 2016).
4. Prepare 804-G cell conditioned media.

a. Culture 804-G in RPMI medium with 10% FBS and 1× penicillin/streptomycin to confluence.
b. Collect conditioned media (48 h), spin down and filter, then freeze in 10 mL aliquots and store at −20°C (up to 1 year).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Guinea pig insulin 1° antibody	DAKO	#A0564
Rabbit insulin (C27C9) 1° antibody	Cell Signaling	#3014S
Goat PDX-1 1° antibody	R&D Systems	#AF2419
Mouse Ki-67 1° antibody	BD Pharmingen	#550609
Anti-mouse 2° antibody	Jackson Immunoresearch	715-545-150
Anti-goat 2° antibody	Jackson Immunoresearch	705-175-147
Anti-guinea pig 2° antibody	Jackson Immunoresearch	706-295-148
Anti-rabbit 2° antibody	Jackson Immunoresearch	711-295-152
Chemicals, peptides, and recombinant proteins		
GNF4877	xcessbio	M60294-1s
OTS167	Cayman Chemical	16873
TSQ	Santa Cruz Biotechnology	Sc-471927
Dithizone	Santa Cruz Biotechnology	Sc-206031A
TPEN	Santa Cruz Biotechnology	Sc-200131
Hoechst 33342 viability stain	Thermo	#62249
Nuclear Green (DCS-1)	abcam	ab138905
Isoflurane (liquid)	Henry Schein	1182097
Paraformaldehyde (32%)	Electron Microscopy Services	15714-S
Deposited data		
Raw and analyzed data	N/A	Horton et al., 2019; Mendeley Data
Experimental models: cell lines		
804-G bladder carcinoma cells	N/A	RRID: CVCL_J122
Mouse (C57BL/6J) islet cells	N/A	Laboratory of Justin Annes
Mouse (C57BL/6J) exocrine pancreas	N/A	Laboratory of Justin Annes
Experimental models: organisms/strains		
C57BL/6J male mice (8–12 weeks)	The Jackson Laboratory	RRID:CVCL_AW02
Sprague-Dawley male rats (250–300 g)	Charles River	RRID:CVCL_0352
Other		
DC Protein Assay Kit I	Bio-Rad	5000111
OCT compound	Fisher Scientific	23-730-571
ImmEdge hydrophobic barrier PAP pen	Vector Laboratories	H-4000
Histopaque-1119	Sigma-Aldrich	11191
Collagenase A (CIzyme)	VitaCyte	005-1030
DMEM low glucose	GE Healthcare	SH30021.02
Penicillin-streptomycin (100×)	Corning	30-002-CI
Fetal bovine serum	Corning	35010CV
Falcon Black 384-well plates	Corning	353962
Cryostat blades	Thermo Fisher	DT315M50
Microscope slides	Fisher	22-230-892
ArrayScan VTI HCS reader	Thermo Fisher	N01002INF
Photoshop	Adobe	N/A
Prism 7.0 or 8.0	GraphPad	N/A

MATERIALS AND EQUIPMENT

Phosphate-buffered saline (PBS)		
Reagent	Final concentration	Add to 50 mL
NaCl	37 mM	108.114 g
KCl	2.7 mM	10.1 g
Na_2HPO_4	10 mM	59.99 g
KH_2PO_4	1.8 mM	12.24 g
ddH_2O	n/a	Up to 50 mL

Note: Can be stored at 20°C–24°C indefinitely.

DCS-1 working solution		
Reagent	Final concentration	Amount
DCS-1 (5 mM)	1 μM	1 μL
Phosphate-buffered saline	n/a	5 mL

Note: Can be stored at 4°C for at least 1 month

TSQ staining solution		
Reagent	Final concentration	Amount
TSQ (10 mM)	10 μM	0.5 μL
DCS-1 working solution	n/a	500 μL

Note: Must be prepared freshly before each use

8% PFA		
Reagent	Final concentration	Amount
PFA (32%)	8%	1 mL
Phosphate-buffered saline	n/a	3 mL

Note: Must be prepared freshly before each use

Sodium citrate buffer		
Reagent	Final concentration	Amount
Sodium citrate trihydrate	0.5 M	7.8 g
HCl (11.6 M)	n/a	To pH 6.0
ddH_2O	n/a	Up to 50 mL

Note: Can be stored at 20°C–24°C for up to 1 month

Antigen retrieval solution		
Reagent	Final concentration	Amount
Sodium citrate buffer	5%	1 mL
Formamide	95%	19 mL

Note: Must be prepared freshly before each use

Blocking solution		
Reagent	Final concentration	Amount
Tx-100	0.3%	150 µL
Donkey serum	6.25%	3.12 mL
Phosphate-buffered saline	n/a	47 mL

Note: Can be stored at 4°C for at least 1 month

Antibody dilution solution		
Reagent	Final concentration	Amount
Blocking solution	20%	1 mL
Phosphate-buffered saline	80%	4 mL

Note: Can be stored at 4°C for at least 1 month

Hoechst 33342 staining solution		
Reagent	Final concentration	Amount
Hoechst 33342	4 µM	1 µL
Phosphate-buffered saline	n/a	5 mL

Note: Can be stored at 4°C in aluminum foil for at least 6 months

STEP-BY-STEP METHOD DETAILS

Assess islet Zn^{2+} chelation by microscopy

Timing: 1–2 days per compound

This competition assay with TSQ is a readily accessible experimental system to establish the capacity of small molecules to chelate Zn^{2+} in islets. TPEN, an exceptionally strong Zn^{2+} chelator, serves as a positive control for Zn^{2+} competition.

1. Prepare frozen mouse pancreas sections.
   a. Label plastic blocks and fill with OCT at 20°C–24°C.

   Note: Avoid introducing air bubbles.
      i. Have dry ice ready in an insulated container.
   b. Dissect out mouse pancreas.
      i. Perform euthanasia by anesthetizing a mouse with isoflurane overdose, then cervical dislocation.

   Note: Humanely sacrifice mice under appropriate conditions to minimize pain and distress.
      ii. Open the body cavity and cut the pancreas away from the spleen and duodenum.

   ⚠ CRITICAL: Take special care to include the islet rich pancreatic tail in the dissection.
   c. Place pancreas in OCT.
      i. Use forceps to shape the pancreas into a dense ball.

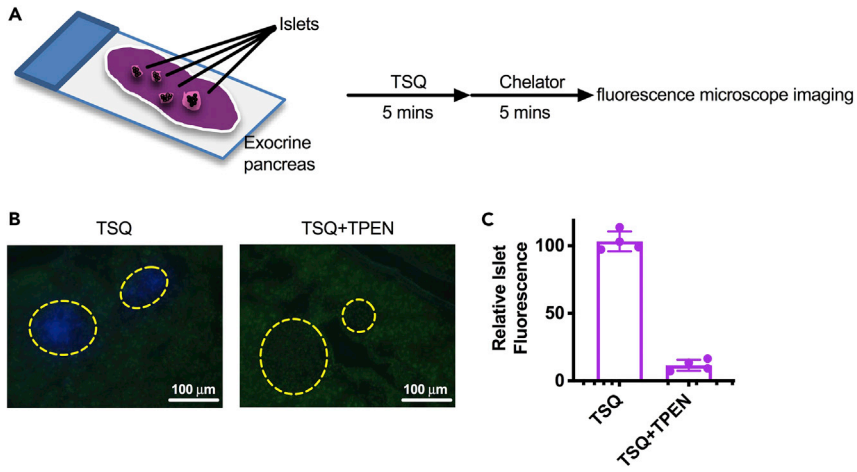

Figure 1. Assessment of islet Zn^{2+} chelation by fluorescence microscopy

(A) Experimental workflow consists of treatment of pancreatic frozen sections with TSQ, followed by treatment with a competing chelator, and fluorescent imaging.

(B) Representative imaging results of TSQ-treated sections, without and with TPEN as a competing chelator. Islets are highlighted (yellow).

(C) Representative result of islet section treated without and with TPEN, with constant TSQ (10 µM). Relative fluorescence is defined as the normalized ratio of islet TSQ fluorescence to exocrine TSQ fluorescence, where sections treated with TSQ alone are 100% and sections treated with TSQ and TPEN are 0%. Data are represented as means ± SD.

ii. Place pancreas in middle of OCT and press the pancreas toward the center of the OCT so it is fully submerged.

iii. Place mold on dry ice.

⚠ CRITICAL: Do not fix the tissue at any stage, as TSQ/Zn^{2+} staining will be removed.

Pause point: Transfer to −80°C for storage or continue to tissue sectioning.

Note: Sections can be stored at −80°C for at least 12 months without loss in quality.

2. Cut frozen sections.
   a. Have clear slides at 20°C–24°C next to a cryostat for section mounting and dry ice available for storing completed slides.

   Note: Use a fresh blade for the best results.
   b. Set cryostat blade to cut 10 µm sections, mount frozen block in cryostat, and cut into the block until the pancreas is included on sections.
   c. Place intact pancreatic sections on (+)-charged slides. Place as many as two sections/slide.
   d. Store sections on dry ice while cutting, or place directly at −80°C.
   e. Prepare at least 8 sections for testing individual compounds, and at least 2 sections for control treatments.

Pause point: Either transfer to −80°C for storage or continue to staining.

3. Conduct competition assays with experimental compounds and TSQ (Figure 1A).
   a. Prepare staining solutions.
      i. Prepare phosphate-buffered saline (PBS) (37 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4).

ii. Prepare DCS-1 solution by diluting Nuclear Green (DCS-1) in PBS at 1 μM final concentration.
iii. Prepare TSQ staining solution by diluting TSQ to 10 μM in DCS-1 staining solution. Make at least 500 μL per compound to be tested.
iv. Use DCS-1 solution to make compound dilutions for experimental testing. For compounds without known chelating activity, start by testing a single concentration (500 μM or 1 mM). Test experimental compounds at a range of concentrations from 500 μM to 1 μM.

Note: Approximately 50 μL of diluted compound is needed per experimental concentration.

v. As a positive control, dilute TPEN to 500 μM in DCS-1 solution. In parallel, make a vehicle control by making a 5% solution of DMSO in DSC-1 solution.

b. Stain tissue sections.
i. Bring section to 20°C–24°C, wash by dipping the section in PBS, and carefully dry off extra liquid using a lint-free wipe.
ii. Demarcate pancreas using a hydrophobic marker in a small circle.
iii. Add the TSQ-containing staining solution (50 μL) to the section and incubate 5 min, covered with aluminum foil.
iv. Remove solution with a pipette without disturbing tissue.
v. Pipette the experimental or control compound solution (50 μL) onto the TSQ-stained section and incubate 5 min, covered with aluminum foil.
vi. Remove solution and replace with 100 μL PBS per tissue sample.

c. Determine islet TSQ fluorescence (Figure 1B).
i. Place slides on a fluorescent microscope at 10–20× magnification.
ii. Find islets by locating areas with sparse nuclei as seen in the green channel (see Figure 1B).

Note: Green fluorescence displays DCS-1 staining of nuclei and allows islets to be located, whereas blue fluorescence reflects the presence of Zn^{2+}-TSQ complex.

iii. Obtain microscope images to capture green (Excitation 503 nm, Emission 526 nm) and blue fluorescence (Excitation 370 nm, Emission 495 nm).

Note: TPEN-treated sections should have similar TSQ fluorescence in islet and non-islet tissues; alternatively, DMSO-treated sections should exhibit robust islet TSQ fluorescence relative to non-islet pancreatic tissue.

iv. Capture at least 3 images at 20× magnification of different islets on every section.
v. Repeat the above staining protocol for every experimental compound concentration.

⚠ CRITICAL: The time duration from initiating section TSQ staining until image capture should not exceed 10 min.

Pause point: Additional compound dilutions may be evaluated on freshly thawed sections as convenient.

d. Quantify and plot islet fluorescence (Figure 1C).
i. Use Photoshop (Adobe) or equivalent software to analyze blue/green fluorescent images.
ii. Identify islets in the green channel and demarcate the areas of interest using the elliptical marquee tool.
iii. Record the median fluorescent intensity of the identified region(s) in the blue channel. This value reflects islet fluorescence.
iv. Move the marquee tool, without changing its size or shape, to a non-islet area and record blue median fluorescence.
v. Repeat for all islets in all images.
vi. Calculate the ratio of islet to exocrine median fluorescence in the blue (TSQ) channel for all islets imaged.

vii. Normalize islet/exocrine fluorescence ratio to 0% (TPEN-treated) and 100% (DMSO-treated) following the below formula:

$$Normalized\ fluorescence = \frac{\frac{Raw\ islet\ median\ fluorescence}{Raw\ exocrine\ median\ fluorescence} - \frac{TPEN-treated\ raw\ islet\ median\ fluorescence}{TPEN-treated\ raw\ exocrine\ median\ fluorescence}}{\frac{vehicle-treated\ raw\ islet\ median\ fluorescence}{vehicle-treated\ raw\ exocrine\ median\ fluorescence} - \frac{TPEN-treated\ raw\ islet\ median\ fluorescence}{TPEN-treated\ raw\ exocrine\ median\ fluorescence}} \times 100\%$$

viii. Plot the normalized fluorescence according to experimental compound treatment concentration.

ix. Using Prism (GraphPad) or equivalent software, fit the dilution data to a 4-parameter inhibition curve to obtain the 4 parameter values of Top, Bottom, IC50, and n_H.

Note: IC_{50} and n_H indicate strength of chelator's affinity for Zn^{2+} in an islet context.

Assess islet accumulation of compounds by LC-MS/MS

Timing: 3 days

This step measures zinc-dependent accumulation of Zn^{2+}-binding compounds in islets (Figure 2).

4. Obtain islets and exocrine pancreas from mice.
   a. Euthanize mice by isoflurane overdose followed by cervical dislocation.

   Note: Humanely sacrifice mice under appropriate conditions to minimize pain and distress.
   b. Isolate islets from pancreata using bile duct-based collagenase A perfusion followed by tissue extraction and histopaque centrifugation (Gotoh et al., 1985; Zhao et al., 2016).
   c. Collect and suspend isolated islets in low glucose DMEM with 1× penicillin-streptomycin and 10% fetal bovine serum.
   d. Collect and suspend pelleted exocrine tissue in low glucose DMEM with 1× penicillin-streptomycin and 10% fetal bovine serum.

   Note: Exocrine tissue must be used within 3 h of isolation. Islets can be used immediately or rested 16 h in medium at 37°C in a tissue culture incubator.

   Note: Keep pelleted exocrine tissue following histopaque centrifugation cold on ice (0°C) during the islet extraction process.

5. Quantify protein in islets and exocrine tissue.
   a. Take an aliquot of handpicked islets ($\geq$ 40 islets) and of exocrine tissue, spin down and resuspend in PBS, and lyse by at least 3 freeze/thaw cycles.
   b. Use a fraction of the collected tissues to quantify the total islet and exocrine protein using the DC Protein Assay (Bio-Rad) or an equivalent assay.
   c. Use at least 1 μg of islet or exocrine protein per experimental condition.

   Note: Islet and exocrine tissues should be kept intact for the drug accumulation assay, i.e., discrete aliquots are used for protein concentration determination.

6. Perform accumulation assay (Figure 2A).
   a. Split islet and exocrine tissue into PCR tubes at equivalent protein concentrations, at 0.1–0.5 μg protein/μL. Dilute to 10 μL (i.e., 1–5 μg) per condition.
   b. Add TPEN (1 mM final concentration) or vehicle (DMSO) to conditions.
   c. Incubate 5 min in thermal cycler at 37°C.
   d. Add test compounds in media (recommended concentration of 10 μM).

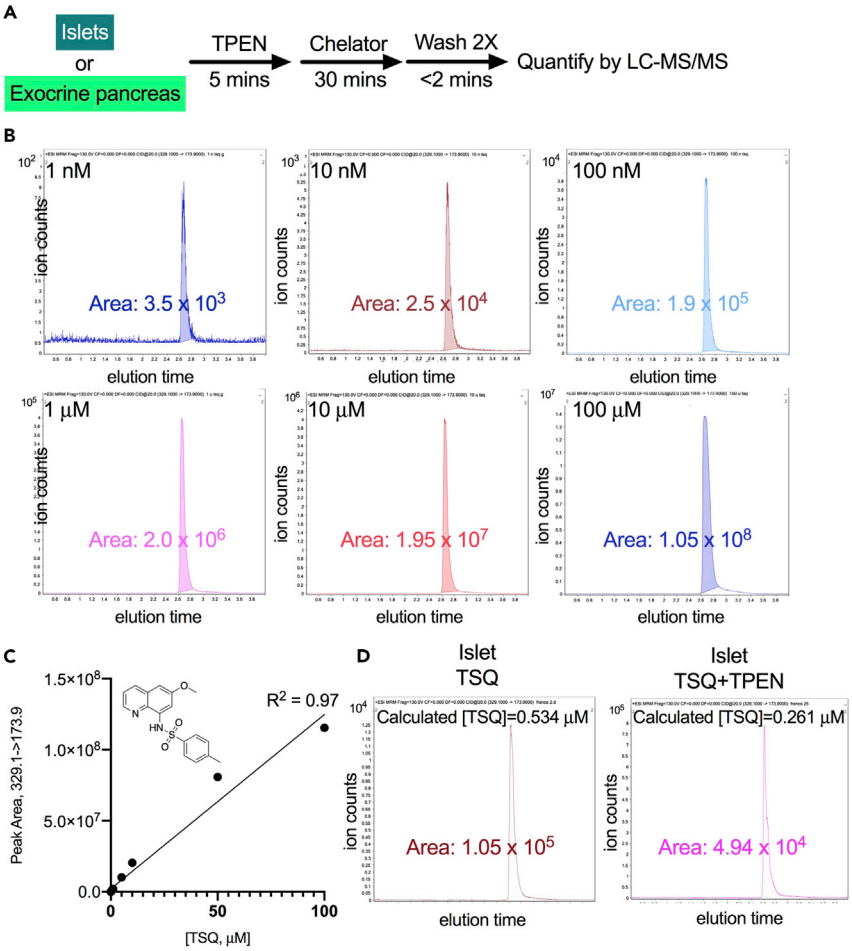

Figure 2. Assessment of islet accumulation of compounds by LC-MS/MS

(A) Experimental workflow includes incubation of isolated islets or exocrine pancreas with chelator, followed by quantification by LC-MS/MS.

(B) Representative raw data for standard concentrations of TSQ.

(C) Representative calibration curve for TSQ standards shown in (B).

(D) Representative peaks and quantification for islets treated with TSQ, without and with TPEN.

i. Perform experiment in at least quadruplicate, preferably n=6–12.
ii. Include the addition of a non-chelating compound [10 µM] in every condition (e.g., the scaffold on which the bi-functional compound is based) for normalizing zinc-dependent drug accumulation (see below, step 7h).
iii. Include TSQ (10 µM)-treated islets and exocrine tissue as a positive control.

e. Incubate islets and exocrine tissue in a thermal cycler for 30 min at 37°C.
f. Place samples on ice (0°C).
g. Rapidly (within 20 s) add 50 µL PBS, spin down (<20 s, ≤2,000 × *g*), take off PBS, and repeat.
h. After second wash, suspend tissues in 20 µL PBS total and freeze at −20°C.

Pause point: Either continue onto quantification or place in freezer. Samples may be stored for at least 4 weeks without compromising quality.

7. Quantification by LC-MS/MS (Troubleshooting) (Figures 2B and 2C)

Note: LC-MS/MS conditions using different mobile phases, extraction procedures, or liquid chromatography gradients may be used, as long as the compound(s) of interest efficiently elute from the column and display linearity of peak area *vs.* concentration.

a. Prepare a calibration curve with compounds of interest from 50 μM down to 1 nM in MeOH, water, MeCN, or other LC-MS/MS compatible solvent.

Note: Do not use DMSO as solvent.

   i. Include at least two compound concentration points per order of magnitude.

b. Prepare standards (50–100 μM) of each individual analyte for method development.

c. Establish a multi-reaction monitoring method for each compound.

d. Dilute islet and exocrine pancreas assay samples, as well as all standards in calibration curve, 1:2 in 50% MeCN/50% water with 0.1% formic acid.

e. Centrifuge all samples and place supernatant free of particulate into LC-MS/MS plates or vials.

   i. If particulate remains, adjust MeCN/water mix and/or dilute further.

f. Run standard curves and samples over LC-MS/MS using a gradient of MeCN and water.

   i. Start with a gradient of 95% water → 40% water → 50% water → 95% water over 4–6 min.

   ii. If analyte peaks appear at the beginning (initial 30 s) or end (final 30 s) of the elution, adjust gradient or change solvents.

g. Quantify each analyte peak according to its peak area in the standard curve (Figure 2D).

h. Plot compound accumulation (nmol/g protein) and ratio to non-chelator in sample for islets and exocrine tissue. Use the formulas

$$Compound\ accumulation\left(nmol\ compound/_{g\ protein}\right) = \frac{Concentration\ of\ compound\left(\frac{nmol}{L}\right)}{concentration\ of\ protein\left(\frac{g}{L}\right)}.$$

$$Accumulation\ ratio = \frac{Concentration\ of\ chelating\ compound}{Concentration\ of\ non-chelating\ compound}$$

Note: Zn^{2+}-dependent accumulation is evidenced by islet-selective, TPEN-sensitive compound accumulation. TSQ should exhibit a 2–4-fold excess accumulation in islets versus exocrine tissue that is ablated by TPEN.

Assess β cell-selective replication

Timing: 5 days

This step describes a method for assessing *in vitro* compound-stimulated β cell and non-β cell replication induction, providing a compound-specific β cell selectivity index (Figure 3A).

Note: Alternative methods for assessing β cell replication exist and include, but are not limited to, immunofluorescent imaging of intact islets (Kong et al., 2018) or FACS-based replication analysis (Carballar et al., 2017). To assess cellular replication, alternative markers to Ki-67 such as BrDU (Lindberg et al., 2005) or EDU (Salic and Mitchison, 2008) may be used.

8. Obtain islets from rats.

   a. Obtain Sprague-Dawley (250 g) rats.

Note: Other rat strains are likely acceptable. Rat islets have higher basal and compound-induced replication than mouse or human islets, facilitating determination of compound efficacy.

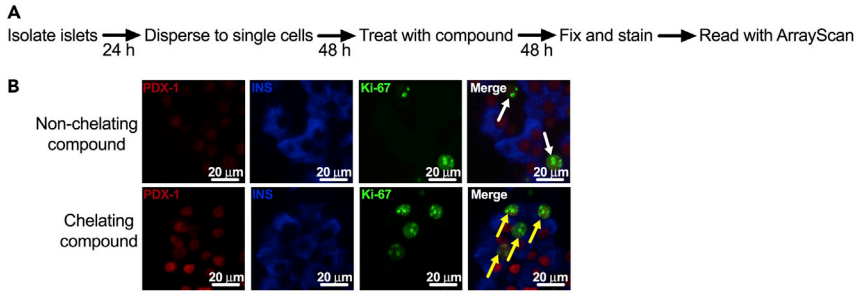

Figure 3. Assessment of β cell-selective replication

(A) Experimental workflow involves islet isolation, dispersal to single cells by trypsinization, compound treatment and incubation, fixing and antibody staining, and quantification of replication by ArrayScan methodology.

(B) Representative results for a control non-chelating and chelating replication compound, with β cell replication signified by yellow arrows, and non-β cell replication signified by white arrows.

b. Euthanize rats by isoflurane overdose and cervical dislocation.

Note: Humanely sacrifice rats under appropriate conditions to minimize pain and distress.

c. Isolate islets from pancreata using bile duct-based collagenase A perfusion followed by tissue extraction and histopaque centrifugation as described in detail (Gotoh et al., 1985; Zhao et al., 2016).

d. Collect and suspend isolated islets in low glucose DMEM with 1× penicillin-streptomycin and 10% fetal bovine serum and culture in a tissue culture incubator (12–16 h).

e. On the day of islet isolation, treat 384-well black fluorescence cell culture plates with 804-G cell conditioned medium (Preparing conditioned media) with 50 μL per well. Place plates in a cell culture incubator (37°C) 12–16 h.

9. Disperse islets into single cells.
   a. Collect islets in a 50 mL conical tube and centrifuge at 197 × *g* for 5 min.
   b. Warm Trypsin-EDTA and low glucose DMEM with 10% FBS and 1× penicillin-streptomycin to 37°C.
   c. Carefully remove supernatant using a 1 mL aspirating pipet with a 200 μL pipet tip on the end.
   d. Wash cells with PBS (30 mL), centrifuge at 197 × *g* for 5 min.
   e. Again, carefully remove supernatant as in step 9c. Suspend the islet pellet in pre-warmed Trypsin-EDTA (250 μL per rat) and place at 37°C for 5 min.
   f. Pipet up and down with a 1,000 μL pipet 25 times, then place at 37°C for another 5 min.
   g. Pipet up and down with a 200 μL pipet 30 times and place at 37°C for an additional 5 min.
   h. Pipet up and down with a 200 μL pipet 30 times.
      i. If visible chunks of islets remain, add more trypsin and repeat steps 9e–9h.
   i. Place digested islets on ice and count a 1:10 dilution (e.g., 1 μL of islets in 9 μL media) using a hemocytometer.
      i. Repeat count for precision using a fresh 1:10 dilution of digested islets.
      ii. The presence of aggregated cells indicates insufficient trypsin digestion; repeat (9h) as necessary.
   j. Calculate the number of wells that can be plated. The density must be 25,000–28,000 cells/well for optimal results. For example, if 1,000,000 cells are counted, a maximum of 40 wells can be used.
   k. Dilute the trypsinized cells in low glucose DMEM with 10% FBS and 1× penicillin-streptomycin for a final volume of 60 μL/well.
   l. Plate the cells in the coated 384-well cell culture plates from step 8e.

Note: Avoid the four wells at each edge of the plate to avoid poorly understood "edge-effects" on assay reproducibility.

m. Culture cells for 48 h at 37°C to allow attachment.

Note: At this point cells should be flattened and in small cell clusters throughout the well, as assessed under a microscope. This process has been visualized in a previously published step-by-step protocol (Zhao et al., 2016).

10. Treat cells
    a. Prepare concentrated stock solutions ([10 mM] in DMSO) of experimental compounds. Include a positive control replication-inducing compound such as GNF-4877 (>150 nM) or OTS-167 (2–20 nM).
        i. Prepare treatment solutions by diluting compounds in media to 3× the desired final concentration.
        ii. Include at least 6 DMSO-treated wells to establish a baseline replication rate.
    b. Prior to drug treatment, perform a partial media exchange by adding 50 μL per well and then removing 70 μL (40 μL final volume).
    c. Treat cells with 20 μL of the prepared β cell replication-stimulating compound or DMSO solutions (final volume 60 μL), and incubate at 37°C for 48–72 h.

11. Fix and stain cells
    a. After incubation, remove culture media by inverting the tissue culture plate and forcefully decanting.
    b. Fix cells using 8% PFA (60 μL/well) for 20 min.
    c. Replace PFA with PBS (100 μL/well).

Pause point: Plates may be kept at 4°C for up to 2 weeks without substantial loss in staining quality.

    d. Freshly prepare antigen retrieval solution by diluting 0.15 M sodium citrate pH 6.0 at a 1:20 dilution in formamide.
    e. Decant PBS solution.
    f. Immediately add antigen retrieval solution (70 μL/well) and heat at 70°C for 45 min.
        i. Use a water bath with just enough water to cover skirt of plate without submerging it.
        ii. Place a heavy object, such as a full bottle, atop the plate to prevent bowing/deformation of the tissue culture plate during heating.
    g. Remove from water bath and allow the plate to cool (15 min at 20°C–24°C).
    h. Wash wells with PBS twice (100 μL/well).
    i. Apply blocking solution (6.25% donkey serum, 0.3% Tx-100 in PBS; 50 μL/well) for 30 min at 20°C–24°C.
    j. Prepare antibody dilution solution by diluting blocking solution 1:5 in PBS.
    k. Decant blocking solution and add primary antibodies (1:100 anti-PDX-1 (goat, R&D systems), 1:200 anti-Ki-67 (mouse, BD), 1:300 anti-insulin (rabbit, Cell Signaling)) in antibody dilution solution (40–50 μL/well).
    l. Incubate (12–16 h) at 4°C.
    m. Decant primary antibody solution, wash twice with PBS (100 μL/well).
    n. Add secondary antibodies (Cy5, goat; Rhodamine Red, rabbit; AF488, mouse; Jackson ImmunoResearch, all 1:200) in antibody dilution solution (40–50 μL/well).

Note: Use of TRITC-conjugated antibodies is not recommended because they may exhibit green channel fluorescence.

    o. Cover the plate with aluminum foil and incubate 1 h at 20°C–24°C.
    p. Wash twice with PBS (100 μL/well) and treat with Hoechst 33342 [4 μM] staining solution (50 μL/well) for 20 min.

q. Remove Hoechst staining solution and replace with 100 μL PBS.

Pause point: Aluminum foil covered plates may be stored at 4°C for up to 4 weeks without substantially reducing image quality.

12. Image using ArrayScan (Thermo Fisher) (Annes et al., 2012; Zhao et al., 2016) or other high-content cell-scanning microscope (Troubleshooting).
    a. Define a four-color protocol to image Hoechst, Ki-67, PDX-1, and insulin staining.
        i. Using a test well (positive control) determine optimal focus and exposure times for the plate.

Note: If only three colors are available, use either PDX-1 or insulin instead of both.

    b. Define parameters to exclude fibroblasts.
        i. Select cells of interest by applying a nuclear (Hoechst) staining intensity threshold.
        ii. Apply a maximum value for nuclear size since islet endocrine cells have smaller nuclei than fibroblasts.
    c. Define replication events by the presence of Ki-67-positive staining in β cells or non-β cells.
        i. Define replication events based on Ki-67 staining intensity.
        ii. Define β cell events based on insulin and/or PDX-1 co-staining.

13. Acquire images (70 fields/well) and quantify the frequency of replication events (Figure 3B).
    a. Determine the average basal (DMSO-treated) β cell and non-β cell replication rates with the following formulas

$$replication\ \ rate_{\beta-cell} = \frac{\#\ \ ki-67^{+}\ \ and\ \ insulin^{+}\ cells}{\#\ \ insulin^{+}\ cells} \times 100\%$$

$$replication\ \ rate_{non-\beta-cell} = \frac{\#\ \ ki-67^{+}\ \ and\ \ insulin^{-}\ cells}{\#\ \ insulin^{-}\ cells} \times 100\%.$$

Note: Islets from Sprague-Dawley rats of this size typically exhibit a basal β cell replication rate of 0.5%–2.0%.

    b. Determine the compound-induced replication rates using the same calculations for both β cell and non-β cell populations as described in 13a.

Note: The minimal effective concentration stimulated by a small molecule can be obtained by comparing individual compound-treated replication rates to the basal replication rate using the pairwise Student's t test.

    c. Determine the fold replication induced by each treatment by dividing the induced replication rate by the average basal replication rate

$$Fold\ \ replication_{\beta-cells} = \frac{compound-treated\ \ replication\ \ rate_{\beta-cell}}{DMSO-treated\ \ replication\ \ rate_{\beta-cell}}$$

$$Fold\ \ replication_{non-\beta-cells} = \frac{compound-treated\ \ replication\ \ rate_{non-\beta-cells}}{DMSO-treated\ \ replication\ \ rate_{non-\beta-cell}}.$$

    d. Determine selectivity ratio by dividing the fold replication induced in β cells by the fold replication induced in non β cells using the formula

$$Selectivity\ \ ratio = \frac{Fold\ \ replication_{\beta-cells}}{Fold\ \ replication_{non-\beta-cells}}.$$

EXPECTED OUTCOMES

For the islet Zn^{2+} chelation assay, molecules with the ability to bind, or chelate, Zn^{2+} in islet sections will compete with TSQ fluorescence. Results of this assay allow one to determine whether a small molecule, either novel or established, can chelate Zn^{2+} in the context of pancreatic islets. When potential chelators are titrated, inhibition of Zn^{2+}-TSQ fluorescence can be used to calculate the IC_{50} and Hill slope n_H. Especially high-affinity chelators such as TPEN and DPA derivatives display a Hill slope >1. Non-chelating (or weakly chelating) molecules will not compete with TSQ for islet Zn^{2+} fluorescence. This assay enables identification of chelation motifs with the potential to promote β cell selective accumulation before investing in more resource-intensive assays.

Based on experience, molecules that robustly chelate Zn^{2+} in pancreatic islets accumulate in β cells as assessed by LC-MS/MS. This accumulation can be measured by comparing compound levels in islet and exocrine tissues. Additionally, the Zn^{2+}-dependence of accumulation may be determined by comparing compound levels in the absence and presence of the strong Zn^{2+} chelator TPEN. Thus far, only compounds that display a Hill slope >1 in the islet Zn^{2+} chelation assay exhibit TPEN-sensitive islet accumulation. Compounds that do not chelate islet Zn^{2+}, or do so weakly, are not anticipated to accumulate in islets in a TPEN-dependent or tissue-specific manner.

Extending the utility of these chelation-focused assays, the β cell replication selectivity assay allows one to determine whether a molecule displays a proclivity toward β cell replication induction relative to zinc-deficient non-β cells. For example, the molecule 4877-EXT-DPA induces 8-fold higher β cell replication compared to non-β cells. This effect is not preserved when chelation is attenuated. Thus, this assay may establish that a molecule exerts β cell selective replication-promoting activity.

Finally, these three experiments integrate well into a combined approach to identify and characterize Zn^{2+} binding-dependent β cell targeting molecules. While the first two assays are explicitly focused on characterizing the Zn^{2+} phenomena of binding/chelation and accumulation in β cells, the third assay has no explicit Zn^{2+}-dependence. Thus, the protocol for assessing β cell selective replication is broadly applicable to assessing the selectivity of β cell replication-stimulating molecules. In this protocol, β cell selective replication is the demonstrated readout for the β cell selectivity established in small molecules by Zn^{2+}-dependent binding and accumulation.

QUANTIFICATION AND STATISTICAL ANALYSIS

TSQ-Zn^{2+} fluorescence in islets after treatment with potential chelators was determined. Initially, these data were normalized to TPEN-treatment (0% fluorescence) and vehicle-treatment (100% fluorescence). Fluorescence after treatment with chelators was compared to vehicle-treated islet fluorescence with a pairwise Student's t test. For chelator accumulation studies by LC-MS/MS, chelator and non-chelator concentrations were quantified using a standard curve generated from the linear peak area portion of the curve. Concentrations (nM) were normalized to total protein (g/L) as measured by the DC Protein assay. Comparisons were made with a pairwise Student's t test. β cell replication was calculated by dividing the number of cells positive for both insulin and Ki-67 by the number of cells positive for insulin. The minimal effective (Ki-67 induction) concentration (EC_{min}) was calculated by a pairwise Student's t test relative to DMSO-treated wells. All statistical analyses were performed using GraphPad (Prism).

LIMITATIONS

If the aqueous solubility of a compound is low, assessment of islet Zn^{2+} chelation may require adjusting the initial screening concentration to below 500 μM. Compounds that autofluoresce in the 350–400 nm range are incompatible with this assay. Notably, this pancreatic section-based assay is time consuming, since it requires serial staining of sections for each test compound concentration. Additionally, given the use of unfixed frozen sections, this assay is time-sensitive and must be performed rapidly (<10 min). In contrast to DPA-based motifs, some chelators are non-Zn^{2+}-selective, which

could lead to non-β cell-selective accumulation. Thus, it is important to select molecules known to preferentially bind Zn^{2+} or establish alternative assays to assess chelation of other metals.

For LC-MS/MS-based accumulation, compounds must be ionizable by MS/MS and exhibit an acceptable LC elution pattern. Additionally, wash steps must be performed rapidly to avoid sample loss/dilution.

For determining a β cell-selective replication response, the investigator must have expertise in the islet isolation technique. Because this replication assay is relatively lengthy from start to finish, low-islet purity will favor fibroblast contamination, which impairs β cell survival and replication.

TROUBLESHOOTING

Problem 1

Autofluorescent compounds interfere with TSQ fluorescence (step 3).

Potential solution

In lieu of TSQ, the absorbent visible dye dithizone can be used, though its signal-to-noise is less optimal than TSQ.

Problem 2

Analysis with LC-MS/MS in positive mode with suggested gradient and mobile phases fails to reveal an analyte peak (step 7).

Potential solution

Switch the ionization modality from positive to negative mode. Increase analyte concentration during method development. Spectral separation can be improved by altering the column used for liquid chromatography.

Problem 3

ArrayScan automated analysis technology is unavailable (step 12).

Potential solution

Alternative quantification of fluorescently labeled islet cells with programs such as Volocity (PerkinElmer) or through manual counting of stained cells.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Justin Annes (jannes@stanford.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Data generated from this protocol and referred to in Horton et al. (2019) are posted to Mendeley Data (https://data.mendeley.com/datasets/3ycr7rs56f/1).

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AUTHOR CONTRIBUTIONS

Conceptualization, T.M.H. and J.P.A.; methodology, T.M.H. and J.P.A.; investigation, T.M.H.; writing – original draft, T.M.H. and J.P.A.; writing – review & editing, T.M.H., B.R.K., and J.P.A.; supervision, J.P.A.; funding acquisition, T.M.H. and J.P.A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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