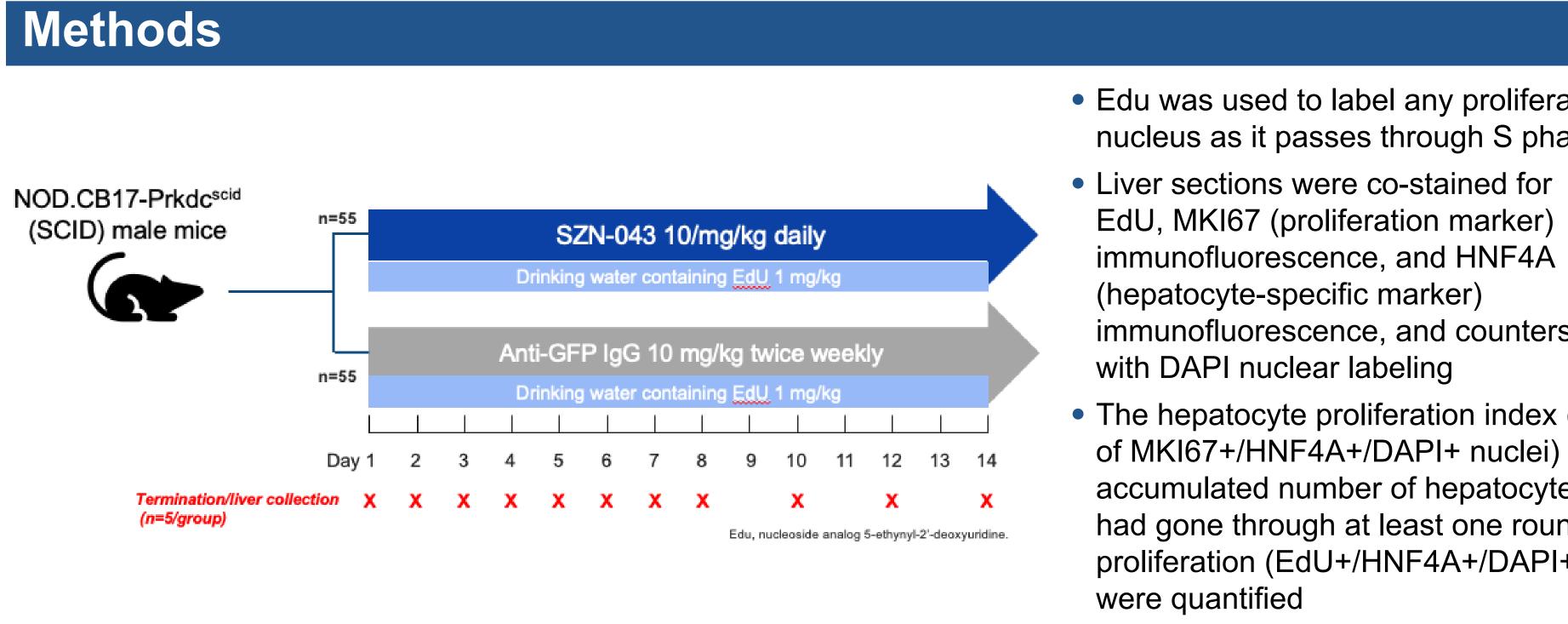
SZN-043 Induced Quick and Robust Hepatocyte Proliferation in a 14-Day Daily Dosing Edu-labeling Study in SCID Mice

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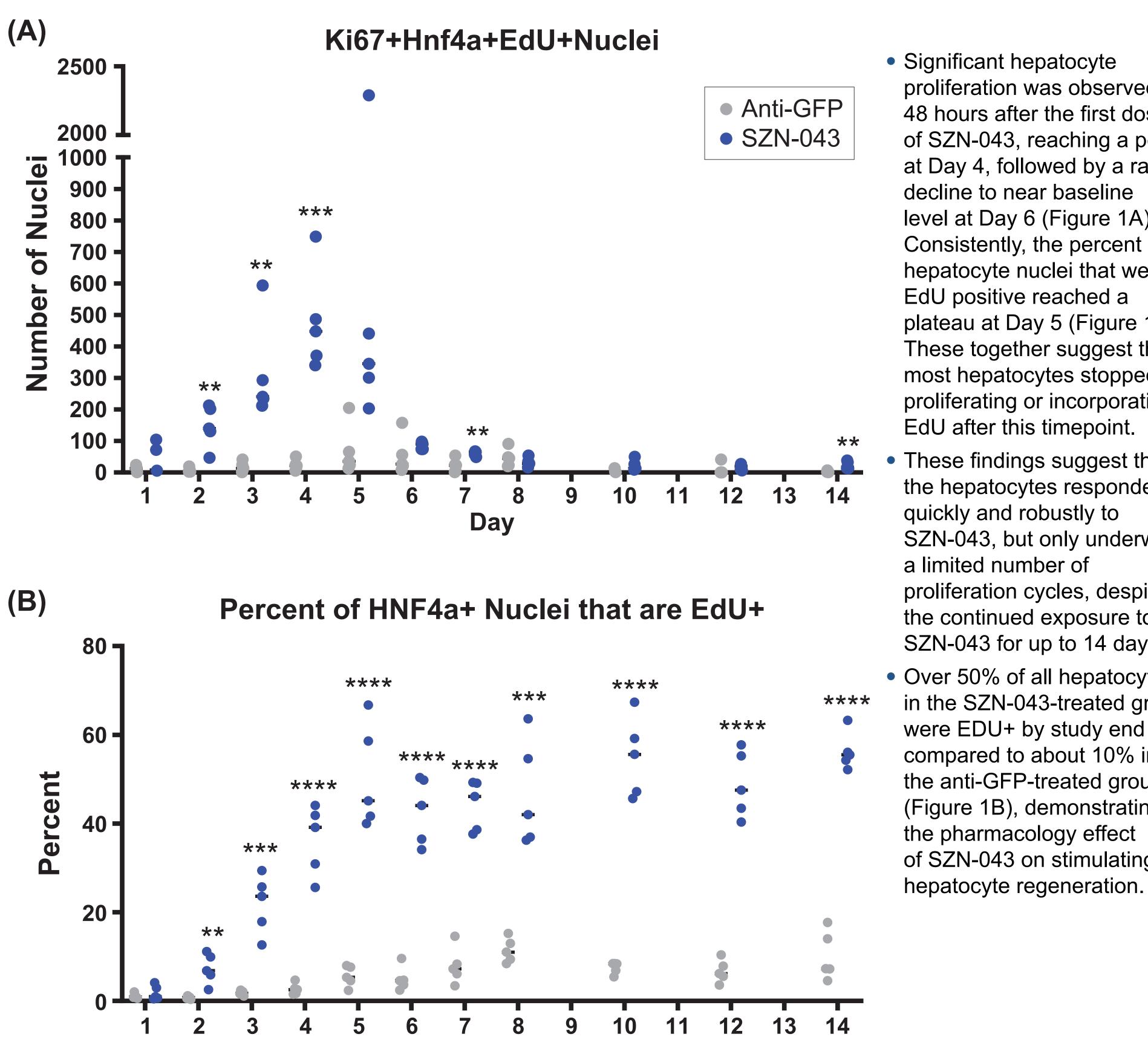
Background

- Wnt/β-catenin signaling plays a key role in hepatocyte regeneration in homeostasis and after liver injury
- R-spondins (RSPO) amplify the signal of Wnt ligands by stabilizing and increasing the amount of Wnt receptors on the cell surface
- We previously showed that our hepatocyte-targeted RSPO mimetic, SZN-043, specifically induced Wnt target gene expression in the liver and promoted hepatocyte proliferation in mice
- This study aimed to evaluate the kinetics and the overall effects of SZN-043-induced hepatocyte proliferation in a 2-week daily SZN-043 dosing schedule



Results

Figure 1. Significant Proliferation of Hepatocytes in Response to SZN-043 Was Quick and Transient



Day

Figure 2. Liver mRNA Expression of Axin2 was Significantly Increased with SZN-043

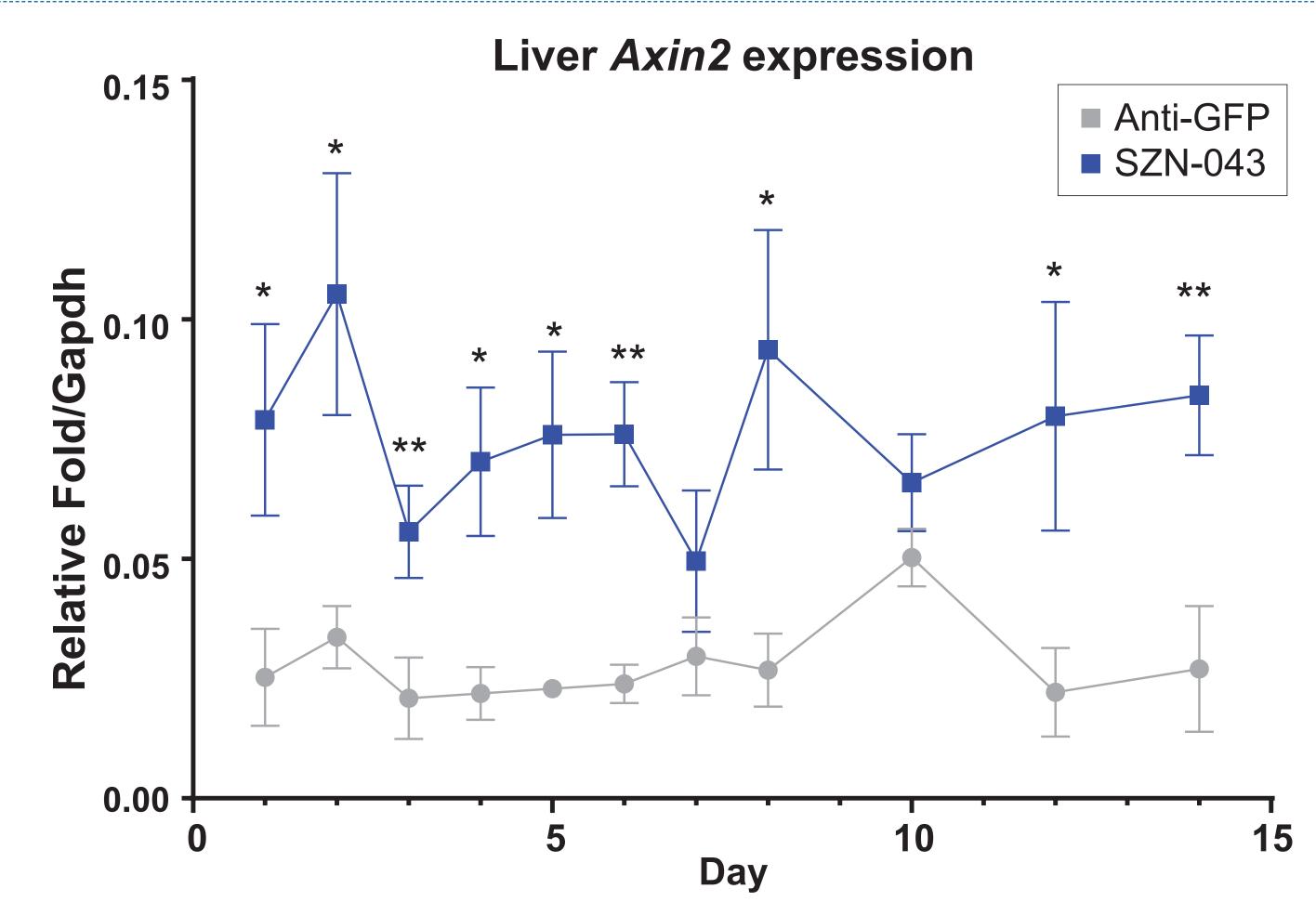
 Edu was used to label any proliferating nucleus as it passes through S phase

- Liver sections were co-stained for
- immunofluorescence, and HNF4A
- immunofluorescence, and counterstained with DAPI nuclear labeling

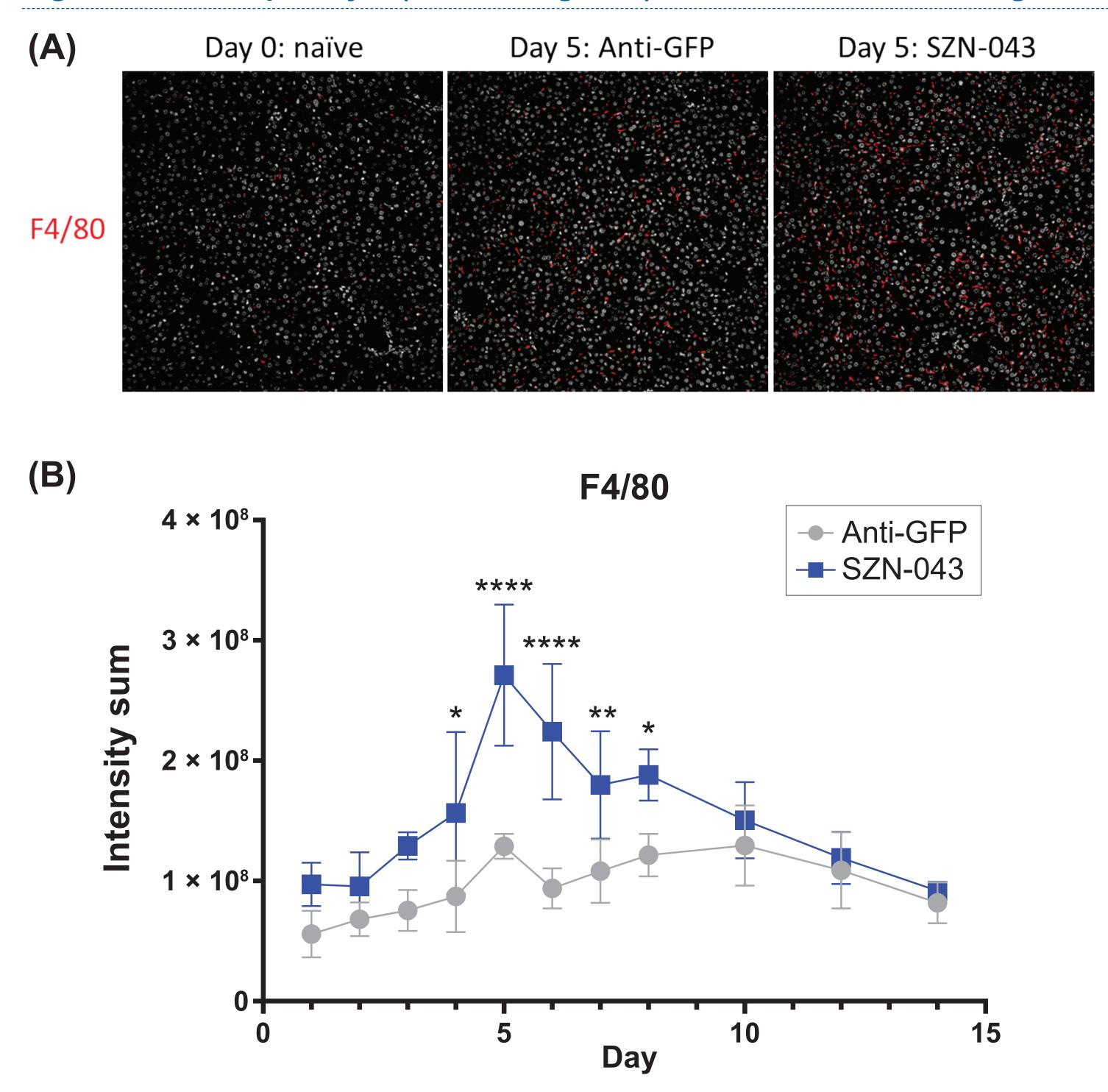
 The hepatocyte proliferation index (number of MKI67+/HNF4A+/DAPI+ nuclei) and the accumulated number of hepatocytes that had gone through at least one round of proliferation (EdU+/HNF4A+/DAPI+ nuclei)

 Significant hepatocyte proliferation was observed 48 hours after the first dose of SZN-043, reaching a peak at Day 4, followed by a rapid decline to near baseline level at Day 6 (Figure 1A). Consistently, the percent of hepatocyte nuclei that were EdU positive reached a plateau at Day 5 (Figure 1B). These together suggest that most hepatocytes stopped proliferating or incorporating EdU after this timepoint.

These findings suggest that the hepatocytes responded quickly and robustly to SZN-043, but only underwent a limited number of proliferation cycles, despite the continued exposure to SZN-043 for up to 14 days. Over 50% of all hepatocytes in the SZN-043-treated group were EDU+ by study end compared to about 10% in the anti-GFP-treated group (Figure 1B), demonstrating the pharmacology effect of SZN-043 on stimulating



Mean with SD. Statistical Analyses: Two-way ANOVA, with Holm-Šídák correction. All comparisons made with the anti GFP group. *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001.



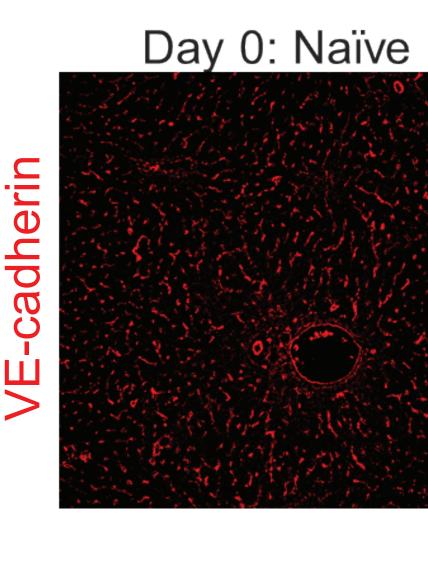
Mean with SD. Statistical Analyses: One-way ANOVA, Holm-Šídák test. All comparisons made with the anti GFP group. *p < 0.05, **p < 0.01, ***p < 0.001, **** p < 0.0001.

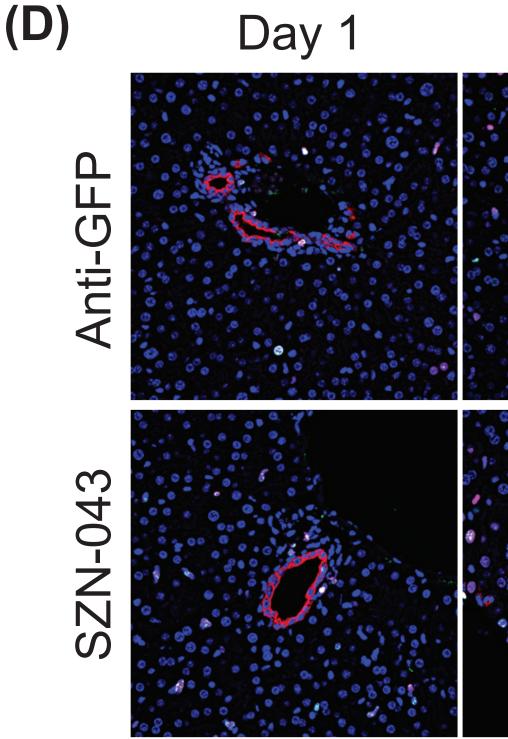
- Axin2 is a Wnt/β-catenin target gene and an indicator of SZN-043 activity in the liver.
- Liver mRNA expression of Axin2 was significantly increased with SZN-043 treatment compared to anti-GFP throughout the study, indicating that continuous dosing of SZN-043 maintained β-catenin signaling induction despite the low level of hepatocyte proliferation after day 5.
- This result together with Figures 1A and 1B suggested hepatocytes only go through a limited number of cell cycles when continuously exposed to SZN-043 in the 14-day study.

Figure 3. Non-Hepatocyte (HNF4A-negative) Cell Proliferation was Significant with SZN-043

- Significant non-hepatocyte (HNF4A-negative) cell proliferation was also observed with SZN-043
- Proliferation of nonhepatocyte appeared to have peaked after hepatocyte proliferation
- Mostly of Kupffer cells/ macrophages
- F4/80 (macrophage and monocyte marker; red) expression peaked significantly at Day 5 compared to anti-GFP (Figure A and B). (liver sections counted stained with DAPI; white)
- After Day 8, F4/80 expression was comparable between SZN-043 and anti-GFP, with no visible differences observed.
- Endothelial cell marker staining did not reveal differences between SZN-043 and anti-GFP groups (Figure 3C).





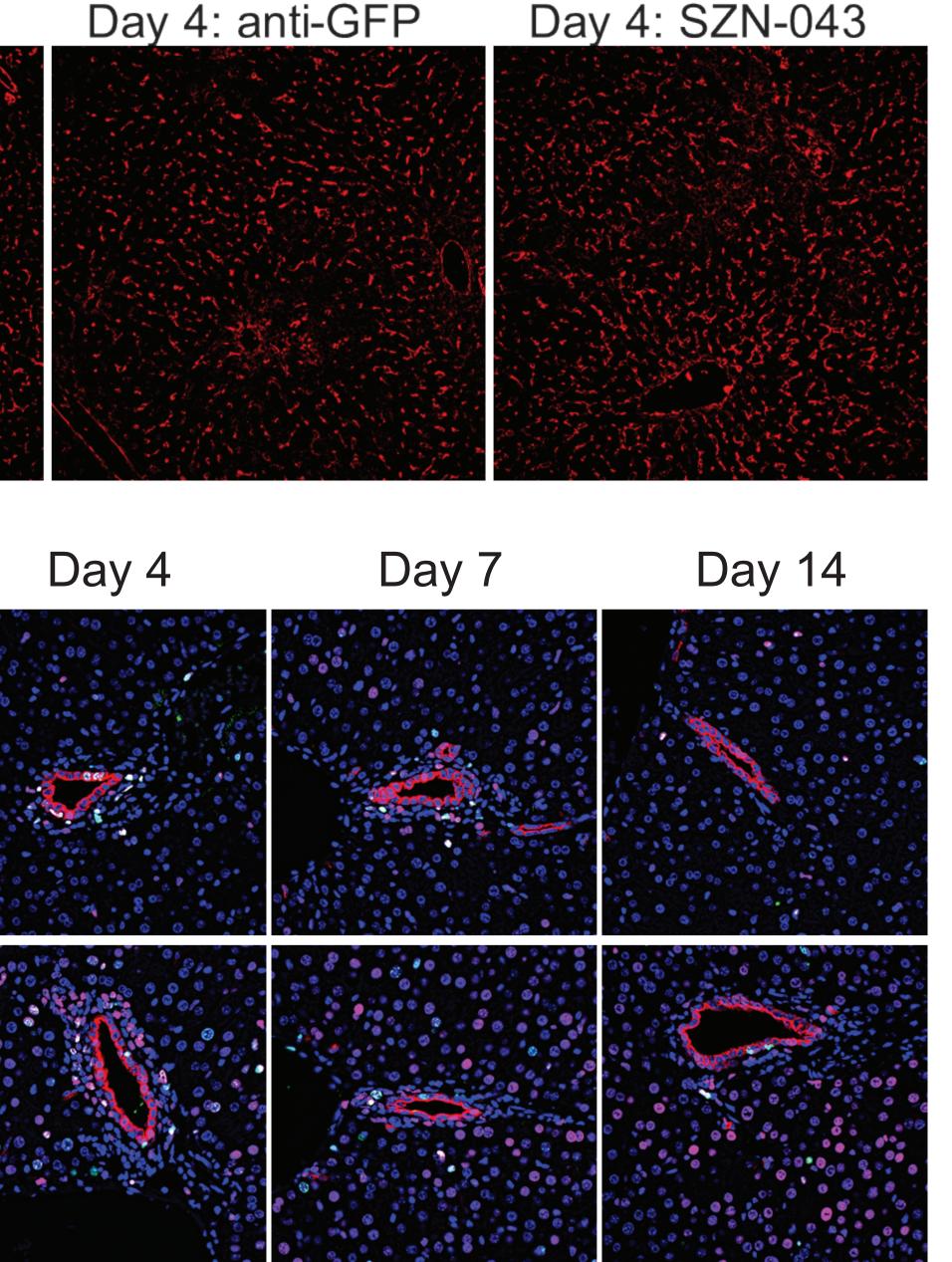


Conclusions

- for up to 14 days.
- hepatocytes.

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- There was negligible proliferation of cholangiocytes (epithelial cells of the bile duct; labeled with CK19) with SZN-043 (Figure 3D).
- As previous studies with SZN-043 have shown no direct impact on cells other than hepatocytes (data on file), the proliferation observed in the HNF4Anegative cells in this study was likely a secondary effect in response to and in support of the increasing number of hepatocytes.

• SZN-043 induced a rapid transient increase in hepatocyte proliferation, followed by rapid a decline to baseline despite continued exposure to SZN-043 and β-catenin signaling induction

 Hepatocyte proliferation induced by SZN-043 was self-limited and likely controlled by other mechanisms controlling cell proliferation (Sarkar A. et al., 2021).

 Proliferation observed in the HNF4A-negative cells was likely due to the tissue responding to the large number of proliferating