Report on Pathology

Study: The effect of Compound X on pancreatic islets in rhesus macaques

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January 1, 2013

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REPORT ON PATHOLOGY

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Study Contact Person:

Study Pathologist:

Conclusions:

Under the conditions of the study, Compound X when administered orally at 150 mg/kg into non-diabetic rhesus macaques significantly increased islet size and density in normal rhesus macaques compared to placebo-treated controls. In addition this treatment at the 150 mg/kg dose also resulted in a significantly higher relative islet mass and islet cell proliferation compared to placebo-treated controls.

These effects were not seen when Compound X was used at a lower dose suggesting that this dose did not reach therapeutic levels.

These findings suggest that Compound X would potentially be beneficial in upregulating insulin production in some diabetic patients thus improving the clinical outcome. However, this study was performed in normal rhesus macaques without clinical or pathologic evidence of diabetes. Repeating this experiment in animals identified as diabetic or prediabetic would potentially be worthwhile to determine if Compound X effectively upregulates insulin production in diabetic animals.

Study Design: 18 rhesus macaques (9 males and 9 females) aged 3-4 years old were enrolled in this study and randomly assigned to one of four study groups representing placebo, low-dose, and high-dose of compound X according to the table below. Animals were dosed orally twice daily approximately 8 hours apart for 120 days. On day 120, all animals were euthanized and the pancreas was harvested and fixed in formalin. Formalin-fixed pancreases were shipped for processing.

Group #	Group Name	# Females	# Males	Daily Dose (mg/kg/day)	Dose Level (mg/kg/dose)
1	Placebo	3	3	0	0
2	Low-Dose	3	3	500	50
4	High Dose	3	3	1500	150

Tissues examined: Each pancreas was embedded in paraffin and stained with hematoxylin and eosin (HE). In addition, Ki67 and insulin immunohistochemistry were performed on all pancreata for all animals. All slides were evaluated for the following criteria by a pathologist at Charter Preclinical Services.

1) **Cell Proliferation:** A cell proliferation index was calculated for each slide. Ten random islets were assessed at 200x magnification for the presence of Ki67+ cells. The number of Ki67+ cells was reported for each field. These data were then used to generate a mean for each slide.

2) **Relative Acinar/Islet Cell Mass**: The relative proportion of acinar cells, and islets was estimated by the pathologist. This was generally accomplished with the HE stained slides. For each cell type, the percentage of the overall pancreatic mass was estimated to the nearest 5-10%. Blood vessels, fat, and other supportive structures were not included.

3) **Islet Density**: The number of islets in each of five 100x (10x objective) fields was counted and reported. This was used to generate the mean number of islets/100x field for each slide. Individually stained beta cells were not counted, only areas which had multiple insulin+ cells together forming an islet.

4) **Beta Cell Area/Representative 20x Field**: For each slide, a photograph of a single 20x field (2x objective) was obtained with a Nikon DS-Ri1 camera attached to a Nikon Eclipse-Ci microscope. Images were analyzed with Nikon Elements-D image analysis software. Images were thresholded using hue, saturation, and intensity to highlight immunostained beta cells in pancreatic islets. In general, the same basic parameters were used for most slides, but it was sometimes necessary to adjust the threshold parameters between slides due to changes in the staining profile of the slides. Background staining was minimal and excluded as necessary. The thresholded area (positively stained beta cells) was calculated by the software and reported as μm^2 for each slide.

Graphs and statistical analysis were generated using GraphPad Prism software and statistical significance is noted in the chart, figure legend, and/or tables when appropriate. A one-way ANOVA was used to compare groups.

Results: The graphs below depict the data generated for the study. In all graphs bars represent group means with standard errors shown.

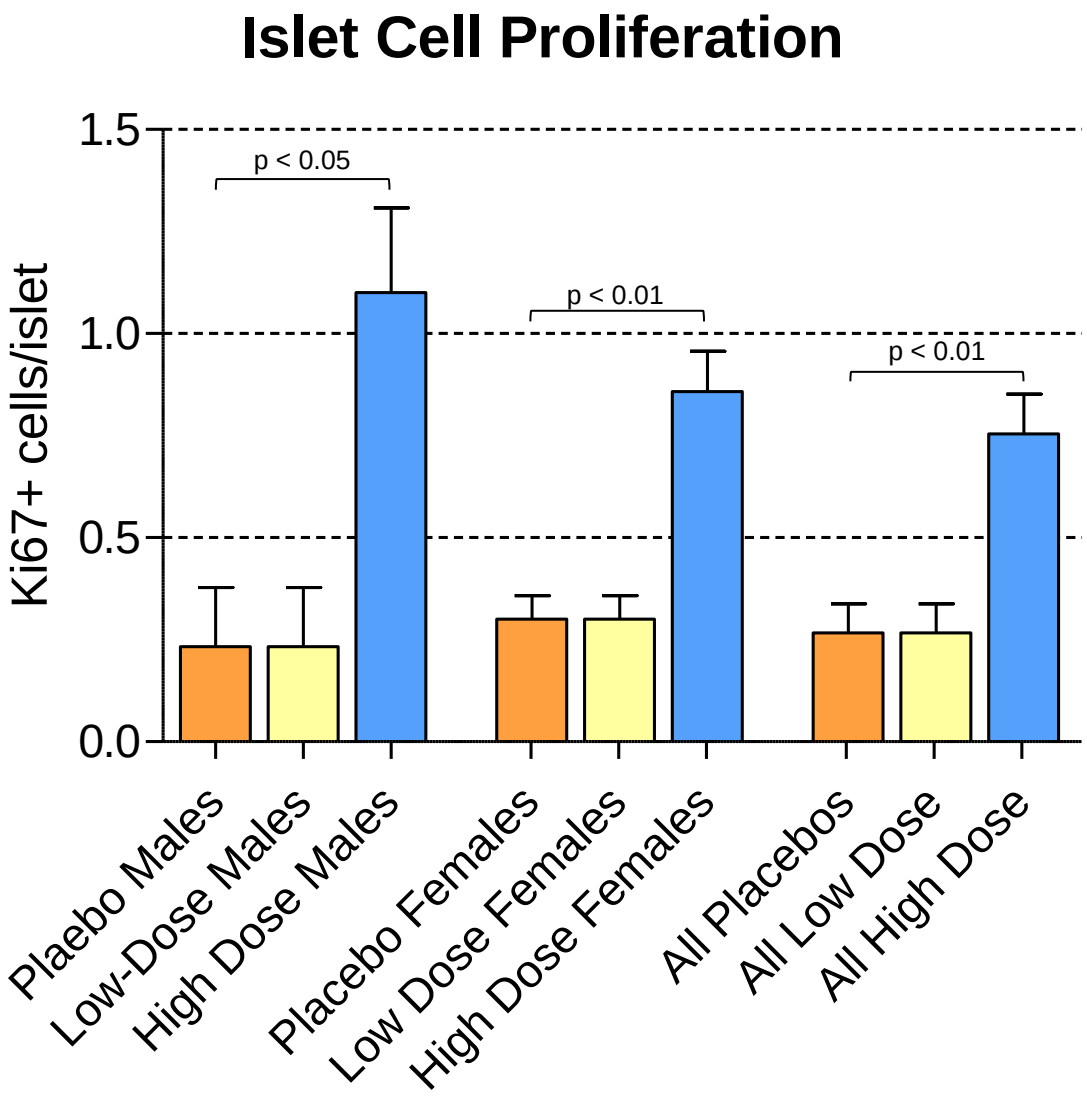

Figure 1: *Islet cell proliferation index for sample study*

Islet cell proliferation was calculated as the number of Ki67+ cells/islet. Treatment significantly effected islet density for males (one-way ANOVA, p = 0.0165), females (one-way ANOVA, p = 0.0025), and when all animals were considered together (one-way ANOVA, p = 0.0008).

Compound X at a low dose did not have any effect on the islet proliferation index. However, at a high dose, Compound X significantly increased cellular proliferation compared to both placebo and low dose treatment (Bonferroni Multiple Comparison Test).

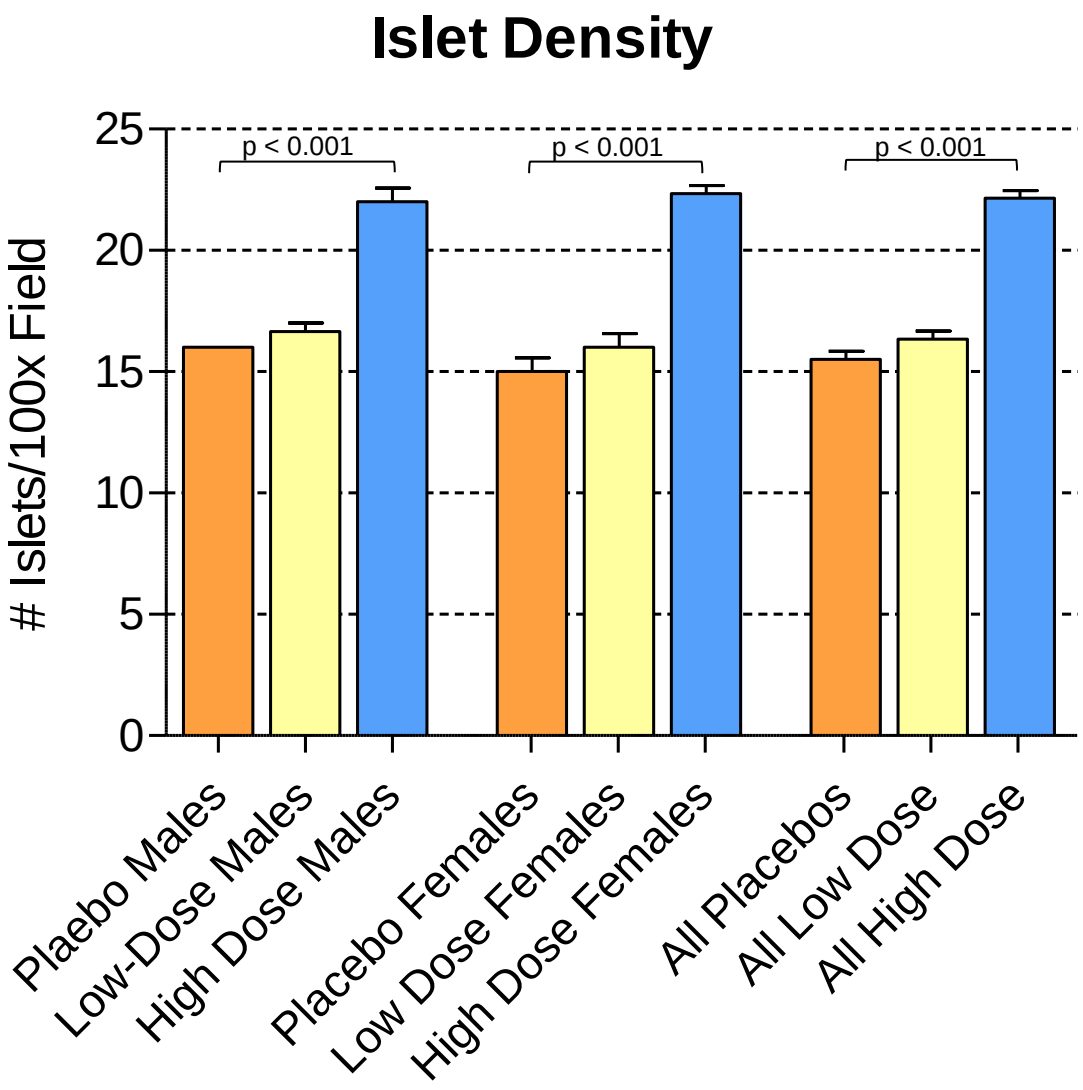

Figure 2: *Relative islet density in pancreases for Sample Study*

Islet density was calculated as the number of islets/representative 100x (10x objective) field). Treatment significantly effected islet density for males (one-way ANOVA, $p < 0.0001$), females (one-way ANOVA, $p = 0.0001$), and when all animals were considered together (one-way ANOVA, $p < 0.0001$).

Compound X at a low dose did not have any effect on the islet density. However, at a high dose, Compound X significantly increased the number of islets in each field compared to both placebo and low dose treatment (Bonferroni Multiple Comparison Test).

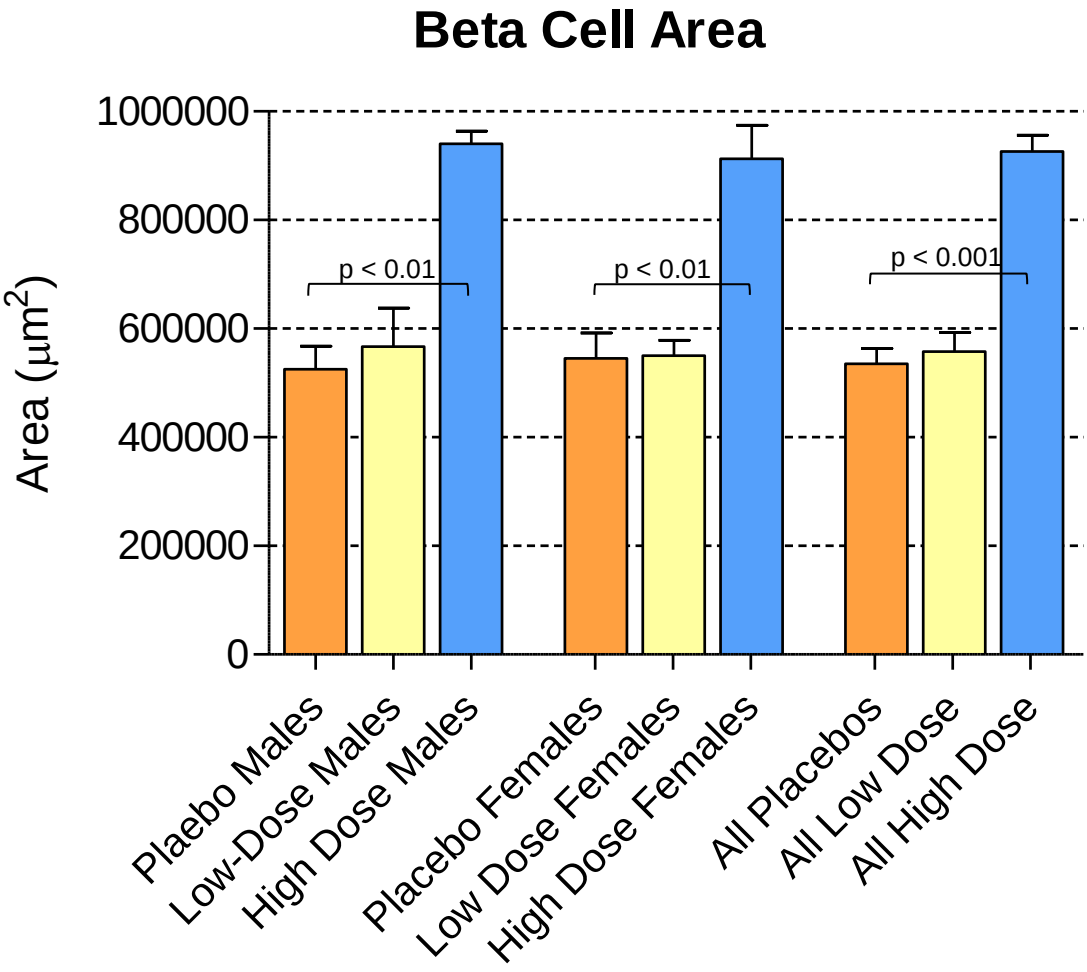

Figure 3: *Beta cell area in representative 20x fields from pancreases for sample study*

Beta cell area was calculated using quantitative image analysis. Treatment significantly effected overall beta cell area for males (one-way ANOVA, $p = 0.0020$), females (one-way ANOVA, $p = 0.0024$), and when all animals were considered together (one-way ANOVA, $p < 0.0001$).

Compound X at a low dose did not have a significant effect on the beta cell area compared to placebo. However, at a high dose, Compound X significantly increased the beta cell area in each field compared to both placebo and low dose treatment (Bonferroni Multiple Comparison Test).

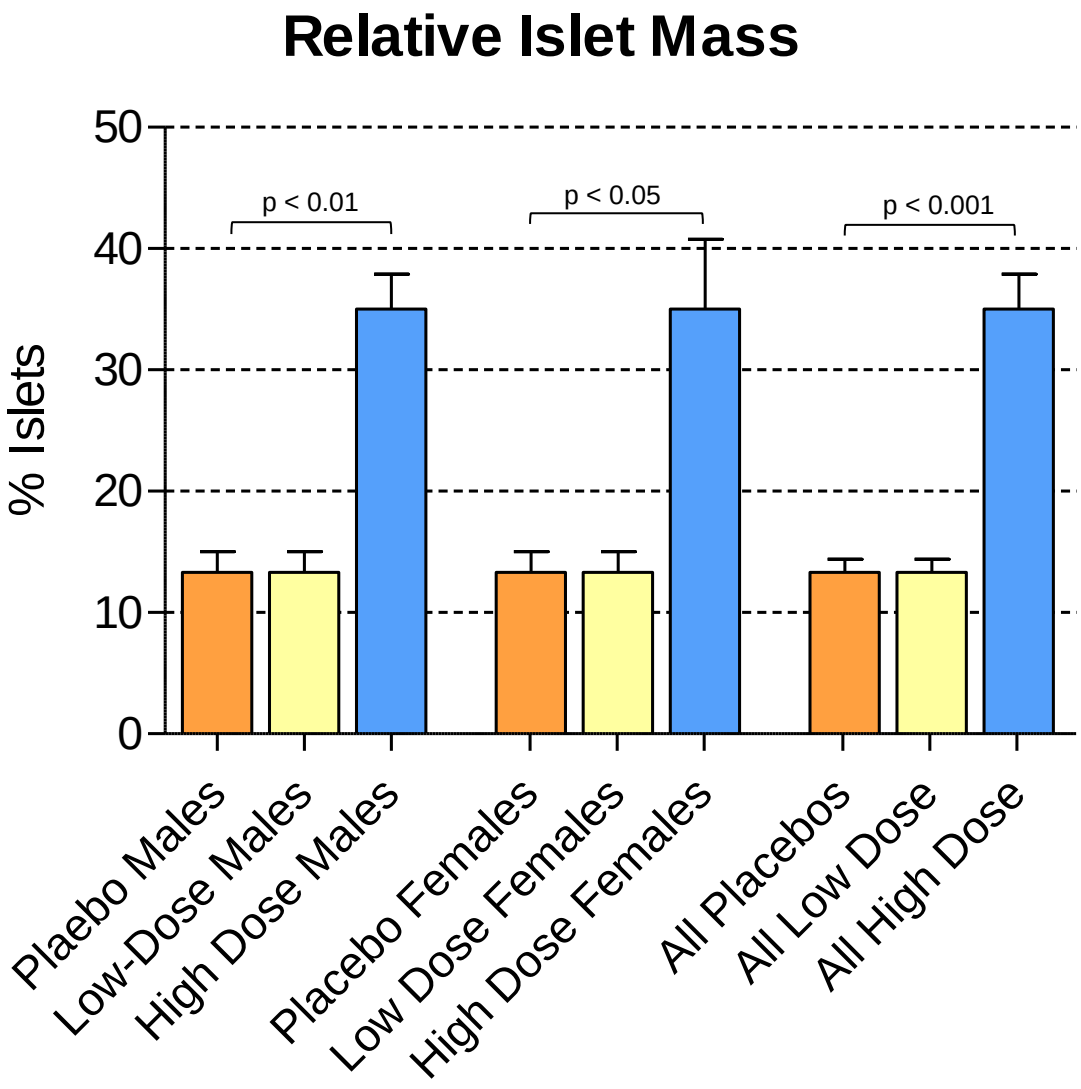

Figure 4: *Islet area from pancreases for sample study*

The relative percentage of the overall pancreatic mass occupied by islets was estimated to the nearest 5%. Treatment significantly effected overall islet mass for males (one-way ANOVA, $p = 0.0005$), females (one-way ANOVA, $p = 0.0079$), and when all animals were considered together (one-way ANOVA, $p < 0.0001$).

Compound X at a low dose did not have a significant effect on the relative islet mass compared to placebo. However, at a high dose, Compound X significantly increased the area occupied by islets compared to both placebo and low dose treatment (Bonferroni Multiple Comparison Test).

Description and Representative Photomicrographs:

Placebo: Animals treated with placebo had essentially normal pancreata. There were a normal number of islets which were generally a normal size. Islets were scattered throughout the pancreas randomly. There was no significant inflammation or pancreatic necrosis and cell proliferation within islets as measured by Ki67 immunoreactivity was rare. Insulin immunostaining was moderate to strong in most islets. In most animals, immunostaining was seen diffusely through the islets indicating an even distribution of beta cells within the islet. This is considered the normal beta cell distribution in nonhuman primates.

Low-Dose: There were no notable differences between animals treated with a low-dose of Compound X and placebo.

High-dose: Animals treated a high dose of Compound X had, compared to placebo-treated animals, larger pancreata and more cellular proliferation. Compound X appeared to increase both the number (islet density) and size (beta cell area) of islets compared to placebo. Additionally, the relative percentage of islet area was increased compared to placebo and low-dose treatment. Pancreata were still morphologically normal and insulin immunostaining was moderate to strong in most islets.

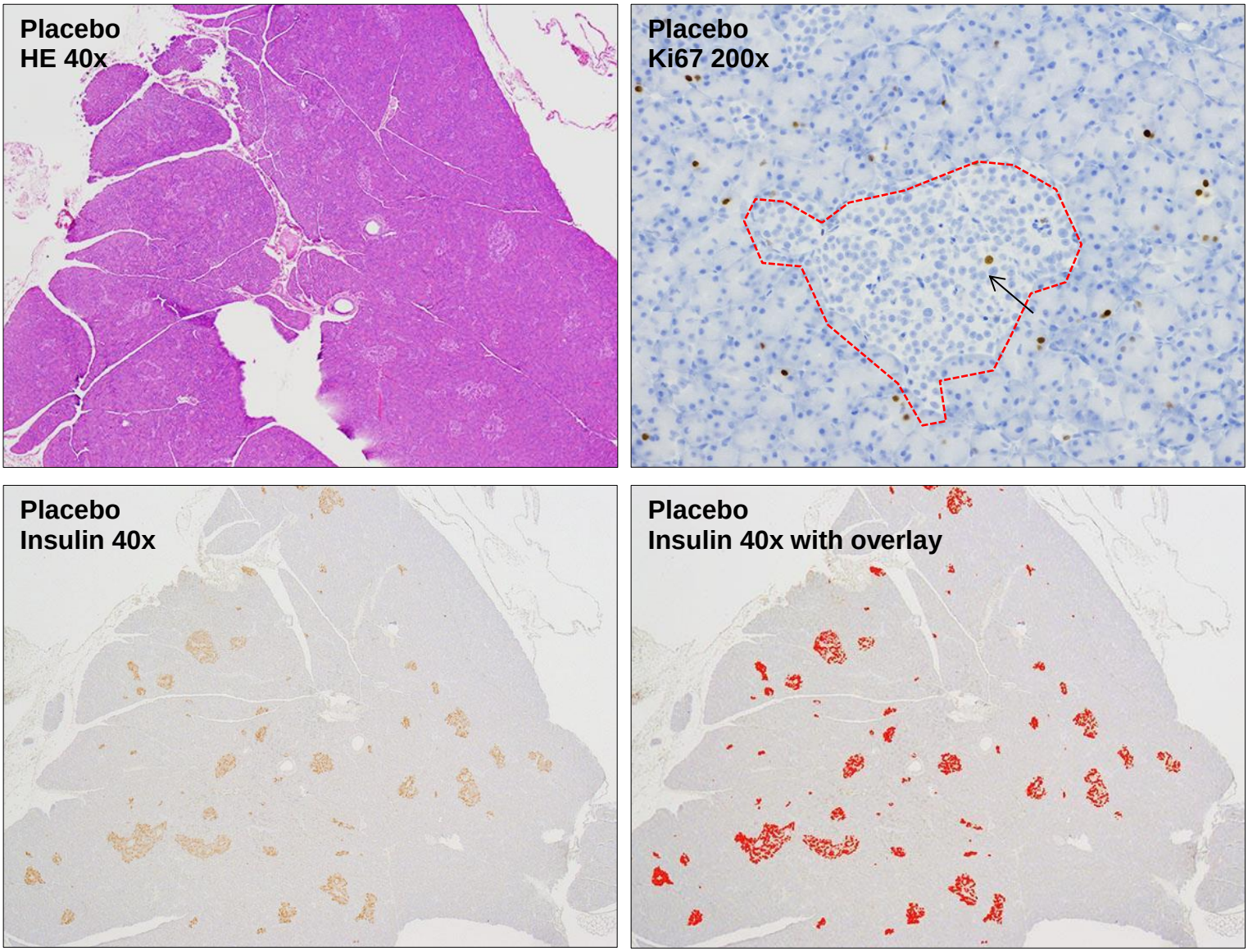

Figure 5: *Representative photomicrographs of placebo and low-dose animals for sample study*

Placebo-treated animals had a normal number of islets of normal size. Islets were evenly scattered within the pancreas and were generally small in size. There were few Ki67+ cells limited to scattered cells in some islets. The islet is outlined in a dashed red line and the arrow points to a Ki67+ cell. Insulin immunostaining was present in all islets. Most animals had normal diffuse immunostaining, which has been reported to be normal in cynomolgus macaques[1] and marmosets.[2] Areas in red on the overlay image represent areas that were included in the quantification of beta cell area.

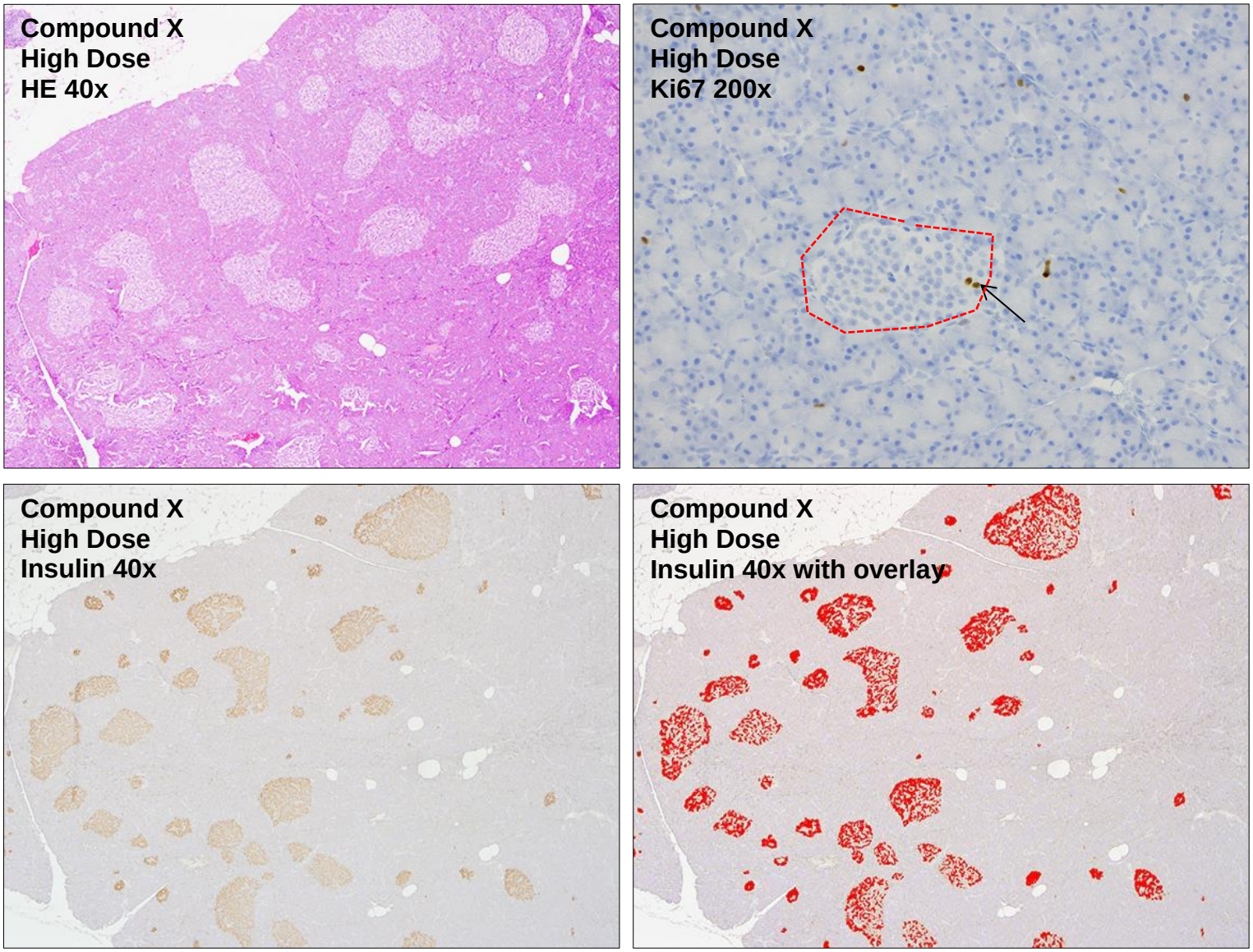

Figure 6: *Representative photomicrographs of animals treated with high dose Compound X*

Compound X administered at a high dose resulted in a significant increase in islet area and density. Islets were more numerous and larger and occupied a larger percentage of the total area of the pancreas. Ki67 immunoreactivity (islet proliferation) was also increased compared to placebo.

Conclusions:

Under the conditions of the study, Compound X when administered orally at 150 mg/kg into non-diabetic rhesus macaques significantly increased islet size and density in normal rhesus macaques compared to placebo-treated controls. In addition this treatment at the 150 mg/kg dose also resulted in a significantly higher relative islet mass and islet cell proliferation compared to placebo-treated controls.

These effects were not seen when Compound X was used at a lower dose suggesting that this dose did not reach therapeutic levels.

These findings suggest that Compound X would potentially be beneficial in upregulating insulin production in some diabetic patients thus improving the clinical outcome. However, this study was performed in normal rhesus macaques without clinical or pathologic evidence of diabetes. Repeating this experiment in animals identified as diabetic or prediabetic would potentially be worthwhile to determine if Compound X effectively upregulates insulin production in diabetic animals.

References:

1. Cabrera et al. The unique cyto-architecture of human pancreatic islets has implications for islet cell function. *Proceedings of the National Academy of Sciences of the United States of America.* 2006. **103** (7): 2334–9.
2. Wachtman et al. Differential Contribution of Dietary Fat and Monosaccharide to Metabolic Syndrome in the Common Marmoset (*Callithrix jacchus*). Obesity. 2011.

Raw Data Follows on Next Page:

Group 1 - Placebo																				
Animal ID	**Cell proliferation (#Ki67+ cells/islet)**											**Acinar Cell Mass (%)**	**Beta Cell Mass (%)**	**Islet Density (# islets/10x field)**						**Islet Area / Rep. 2x field (µm)**	**Comments**
	Islet 1	Islet 2	Islet 3	Islet 4	Islet 5	Islet 6	Islet 7	Islet 8	Islet 9	Islet 10	Mean			Field 1	Field 2	Field 3	Field 4	Field 5	Mean		
Male 1	0	0	0	0	0	0	0	0	0	0	0	85	15	16	16	16	16	16	16	600000	
Male 2	1	0	1	0	1	0	1	0	1	0	0.5	85	15	16	16	16	16	16	16	450000	
Male 3	1	0	0	1	0	0	0	0	0	0	0.2	85	10	16	16	16	16	16	16	525000	
Mean											0.23	85.00	13.33						16.00	525000.00	NA
Standard Deviation											0.25	0.00	2.89						0.00	75000.00	NA
Female 1	1	0	0	1	0	0	0	0	0	0	0.2	85	15	15	17	14	15	14	15	475000	
Female 2	1	0	0	1	1	0	0	0	0	0	0.3	90	10	14	17	15	16	18	16	525000	
Female 3	1	1	1	1	0	0	0	0	0	0	0.4	80	15	14	14	14	14	14	14	635000	
Mean											0.30	85.00	13.33						15.00	545000.00	NA
Standard Deviation											0.10	5.00	2.89						1.00	81853.53	NA
Total Group Mean											0.27	85.00	13.33						11.00	408836.22	NA
Total Group SD											0.18	3.16	2.58						7.21	230730.95	NA

Group 2 - Low Dose Compound X																				
Animal ID	**Cell proliferation (#Ki67+ cells/islet)**											**Acinar Cell Mass (%)**	**Beta Cell Mass (%)**	**Islet Density (# islets/10x field)**						**Islet Area / Rep. 2x field (µm)**	**Comments**
	Islet 1	Islet 2	Islet 3	Islet 4	Islet 5	Islet 6	Islet 7	Islet 8	Islet 9	Islet 10	Mean			Field 1	Field 2	Field 3	Field 4	Field 5	Mean		
Male 1	0	0	0	0	0	0	0	0	0	0	0	85	15	17	17	17	17	17	17	625000	
Male 2	1	0	1	0	1	0	1	0	1	0	0.5	85	15	17	17	17	17	17	17	425000	
Male 3	1	0	0	1	0	0	0	0	0	0	0.2	85	10	16	16	16	16	16	16	650000	
Mean											0.23	85.00	13.33						16.67	566666.67	NA
Standard Deviation											0.25	0.00	2.89						0.58	123322.07	NA
Female 1	1	0	0	1	0	0	0	0	0	0	0.2	85	15	16	16	16	16	16	16	500000	
Female 2	1	0	0	1	1	0	0	0	0	0	0.3	90	10	17	17	17	17	17	17	550000	
Female 3	1	1	1	1	0	0	0	0	0	0	0.4	80	15	15	15	15	15	15	15	600000	
Mean											0.30	85.00	13.33						16.00	550000.00	NA
Standard Deviation											0.10	5.00	2.89						1.00	50000.00	NA
Total Group Mean											0.27	85.00	13.33						11.75	419998.39	NA
Total Group SD											0.18	3.16	2.58						7.51	230589.43	NA

Group 3 - High Dose Compound X																				
Animal ID	**Cell proliferation (#Ki67+ cells/islet)**											**Acinar Cell Mass (%)**	**Beta Cell Mass (%)**	**Islet Density (# islets/10x field)**						**Islet Area / Rep. 2x field (µm)**	**Comments**
	Islet 1	Islet 2	Islet 3	Islet 4	Islet 5	Islet 6	Islet 7	Islet 8	Islet 9	Islet 10	Mean			Field 1	Field 2	Field 3	Field 4	Field 5	Mean		
Male 1	1	1	1	1	1	1	1	1	1	1	1	70	30	22	22	22	22	22	22	895000	
Male 2	1	2	1	2	1	2	1	2	1	2	1.5	60	40	23	23	23	23	32	24.8	950000	
Male 3	1	1	1	1	1	1	1	1	0	0	0.8	65	35	21	21	21	21	21	21	975000	
Mean											1.10	65.00	35.00						22.60	940000.00	NA
Standard Deviation											0.36	5.00	5.00						1.97	40926.76	NA
Female 1	1	1	1	1	1	1	1	1	0	0	0.8	65	35	23	23	23	23	23	23	789000	
Female 2	1	1	1	1	1	1	1	1	1	1	1	75	25	22	22	22	22	22	22	984000	
Female 3	1	1	1	1	1	1	1	0	0	0	0.7	55	45	22	22	22	22	22	22	965000	
Mean											0.83	65.00	35.00						22.33	912666.67	NA
Standard Deviation											0.15	10.00	10.00						0.58	107518.99	NA
Total Group Mean											0.97	65.00	35.00						16.35	677016.06	NA
Total Group SD											0.29	7.07	7.07						10.32	417001.71	NA