Online Appendix A

1. Differential therapeutic window of rapamycin analogs

Human aortic endothelial cells (EC, Promocell, Heidelberg, Germany) and human aortic smooth muscle cells (SMC, ATCC, Manassas, VA) were cultured in EGM-2 medium (Lonza) and in low glucose DMEM (Life Technologies, Carlsbad, CA), respectively. Media was supplemented with serum, growth factors and antibiotics as recommended by the cell manufacturers, and replaced every 48h during culture. Cells were incubated at 37ºC and 5% CO_2 in a humidified incubator and used for experiments while in passages 4-6.

The antiproliferative activity of Ridaforolimus (Ariad Pharmaceuticals, MA), Sirolimus, Everolimus and Zotarolimus (MedChem Express, NY) in SMC and EC were examined. Cells were seeded at $5*10^3$cell/well in 96 well plates to perform MTS assay, and at $2*10^4$ cell/well in 12 well plates to determine cell number with an automatic Coulter cell counter. Prior to the incubation with drug cells were allowed to adhere to the cell culture plates for 24 hours. Then, to synchronize cells by growth arrest smooth muscle cells were cultured in basal DMEM with 0.2% bovine serum albumin for 48 hours, and endothelial cells were cultured in basal EBM with 0.1% FBS for 24 hours. Thereafter, cultures were treated for 4 days with each of the individual drug under study in supplemented media. In one of the experiments PDGF (10 ng/ml) was given as well to stimulate cell growth and to contrast cell responsiveness with and without stimulus. Cell viability and density were assessed to ensure that cells had not detached or become superconfluent and consequently unresponsive. The experiments were performed three separate times and each condition within each experiment was performed in triplicate.

The four drugs tested exhibited differential suppression of endothelial cells and smooth muscle cells, with superior performance of Ridaforolimus and Zotarolimus over Everolimus and Sirolimus. There was no difference as to whether cells were stimulated with serum alone or serum and PDGF simultaneously – the differential effects remained (data not shown). There was a reproducible

effect and modest dose response on smooth muscle cell growth control over concentrations 10^{-16} to 10^{-4} M (Figure 1 Suppl.).

The effects of Ridaforolimus were preferential for smooth muscle cells by four log orders, equivalent to Zotarolimus, and superior to Everolimus and Sirolimus. While the IC50 for Ridaforolimus and Zotarolimus on smooth muscle cells was approximately 10^{-8} M Everolimus and Sirolimus achieved the same effects only at 1000 to 10,000 times higher doses (10^{-5} and 10^{-4} M). Moreover, Ridaforolimus and Zotarolimus exhibited a differential effect on endothelial cell proliferation – reducing cell number by 50% only at the highest dose tested (10^{-4} M). This endothelial cell IC50 of 10^{-4} M, was similarly observed for Sirolimus and Everolimus (Table 1 Suppl.) leaving no dose differential between the two cell types for these latter two drugs. Higher drug concentrations were not assayed due to the fact that all drugs become insoluble in cell culture media at higher concentrations.

Similar effects were noted with PDGF stimulation. Ridaforolimus inhibited human aortic smooth muscle cell proliferation exposed to fetal bovine serum alone and serum with 10 ng/ml of PDGF equivalent to literature values and here to Zotarolimus, and significantly more efficiently than Sirolimus and Everolimus. At the same time Ridaforolimus had far less of an effect on endothelial cells with measurable toxicity noted only at 10-4 M and minimal dose response until that point. This was true for fetal bovine serum alone or replete with PDGF. Only Zotarolimus behaved similarly, Sirolimus and Everolimus had no effective discriminatory effects with identical IC50 for endothelial cells as for smooth muscle cells. These latter two drugs could only inhibit vascular smooth muscle cells at doses that are also inhibitory of endothelial cells leaving no therapeutic window for the two cell types.

These data confirm the antiproliferative effects of Ridaforolimus on vascular smooth muscle cells and a significant therapeutic window with far greater preservation of vascular endothelial cells than native Sirolimus or Everolimus.

Supplemental Table 1 (cumulative results of experiments)

IC_{50} [M]	SMC	EC
SIROLIMUS	10^{-4}	10^{-4}
EVEROLIMUS	10^{-5}	10^{-5}
ZOTAROLIMUS	10^{-8}	10^{-4}
RIDAFOROLIMUS	10^{-8}	10^{-4}

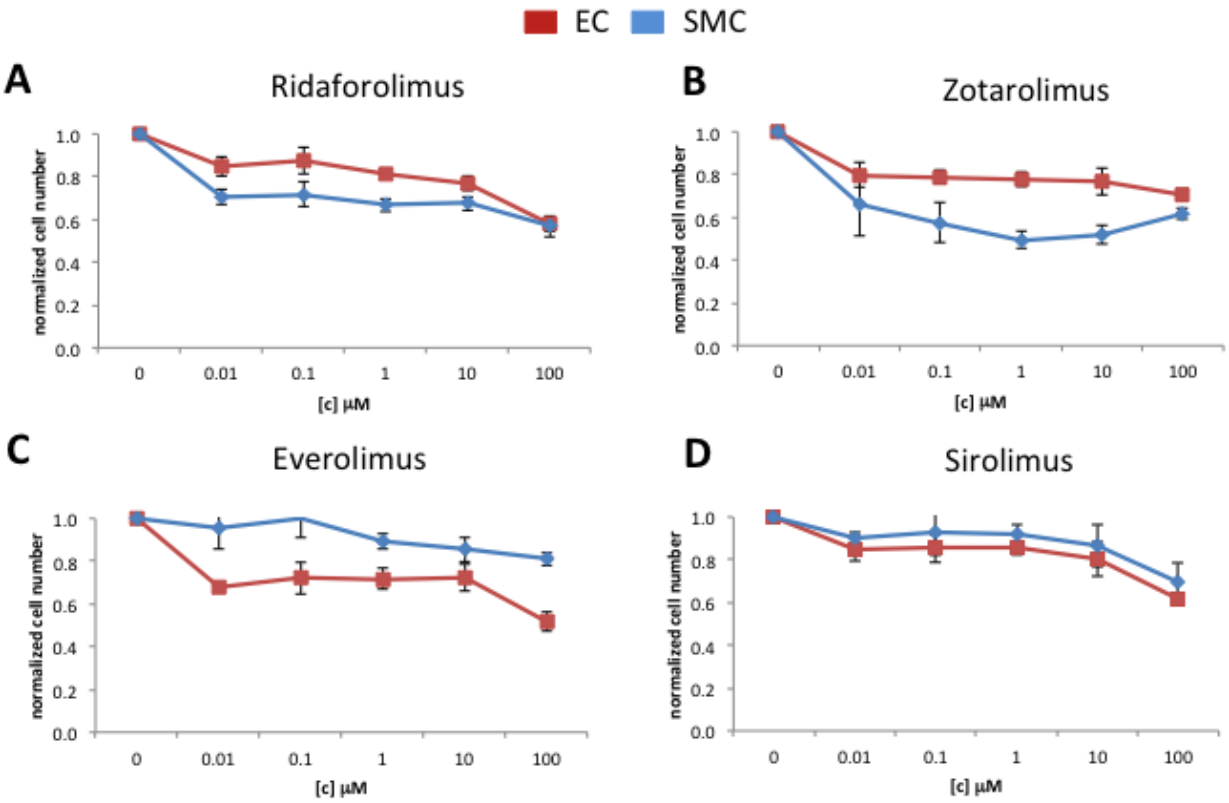

Figure 1 supplement – Effect of various rapamycin analogs on cell survival.

Figure 1 supplement legend: Smooth muscle cells (SMC) and Endothelial cells (EC) were exposed to escalating doses of rapamycin analogs. Cell survival (normalized to baseline) is shown as a function of drug dose. Ridaforolimus (A) and Zotarolimus (B) exhibited a greater inhibitory effect on SMC than EC at equivalent doses, whereas for Everolimus (C) and Sirolimius (D) the situation is reversed with greater inhibition of EC.

2. In-Vivo Pharmacokinetics of the BioNIR Stent

Stent elution and tissue pharmacokinetics of the Ridaforolimus Eluting Stent were tested in a porcine coronary artery model. This study enrolled 27 Yucatan swine, 3 animals per time point, for determination of Ridaforolimus concentration in stented arteries and tissue on Days 1, 3, 7, 14, 30, 60, 90, 180, and 456.

All animals underwent a single interventional procedure on Day 0 in which stents were implanted in up to 3 coronary arteries. Angiography was performed and recorded on Day 0 before stent placement, during stent deployment (balloon), and after stent deployment. Seventy-three Ridaforolimus eluting stents were implanted in the coronary arteries of these 27 animals with no procedural events or complications associated with the implantation and no stent or procedural-related significant clinical abnormalities. Activated clotting times (ACT) were monitored during the interventional procedures. The ACTs ranged from 267 seconds to 411 seconds at the time of stent implantation. Average balloon to artery ratios ranged from 1.12 to 1.19.

Animals were euthanized on Days 1 (24 hours), 3, 7, 14, 30, 60, 90, 180, and 456. The hearts and representative samples of the kidneys, liver, and lungs were collected. Following necropsy, stents were manually separated from the arterial tissue to yield proximal, distal, and stented arterial segments for analysis. Blood samples, stents, arterial tissue, and subjacent and downstream myocardial tissue samples were analyzed by liquid chromatography-mass spectrometry/mass spectometry to determine Ridaforolimus concentration. Kidney, liver, and lung samples were analyzed at the Day 30 and Day 456 time points.

Serial blood samples were collected at 0, 15, 30 minutes, 1, 2, 4, 6 hours and 1, 2, 3, 4, 6, 8, 14, 30 days for determination of systemic exposure to Ridaforolimus. Mean Ridaforolimus levels in

whole blood levels peaked at 2 hours post-implant (2.855 ±0.528 ng/mL), and then declined steadily. At 144 hours (and thereafter), the systemic Ridaforolimus concentration was below the quantitation limit ([BQL] <0.500 ng/mL) in all samples.

Stent release of Ridaforolimus occurred in a bi-exponential fashion with more than half of the Ridaforolimus released from the stents by 14 days post-implant (56.48 ±3.8%), and nearly 90 percent (87.97 ±6.4%) released by Day 90. At 180 days a mean of 98.07 ±2.6% of the drug was released with mean detectable levels remaining on the stents of 2.1 ±2.9 µg (Figure 2 Suppl.).

Ridaforolimus concentrations in arterial tissue within the stented segment reached a mean maximum of 6.523 ±7.635 ng/mg at 7 days, and then declined approximately 2-fold to 3.138 ±2.578 ng/mg by Day 30 with concentrations of 1.410 ±0.807 ng/mg on Day 90 and 0.874 ±0.579 ng/mg on Day 180. Proximal and distal unstented segments showed maximal concentrations of 0.052 ±0.073 ng/mg and 0.600 ±1.317 ng/mg, respectively. Ridaforolimus concentrations in myocardium subjacent to stented arteries reached a maximum of 0.151 ±0.082 ng/mg at Day 1 and then rapidly declined through Day 14 to near or BQL (<0.0120) and were mostly BQL thereafter (Figure 3 Suppl.).

In summary, these data show bi-exponential elution of Ridaforolimus from the Medinol stent with approximately 98.07% of the loading dose eluted by Day 180. Resultant mean Ridaforolimus concentrations in arterial tissue reached a maximum at 7 days of 6.523 ±7.635 ng/mg and remained within a mean range of ≥0.874 ±0.579 ng/mg and ≤3.138 ±2.578 ng/mg for up to 180 days post-implant. Ridaforolimus was not detected in any stent, artery, myocardial, or tissue sample on Day 456.

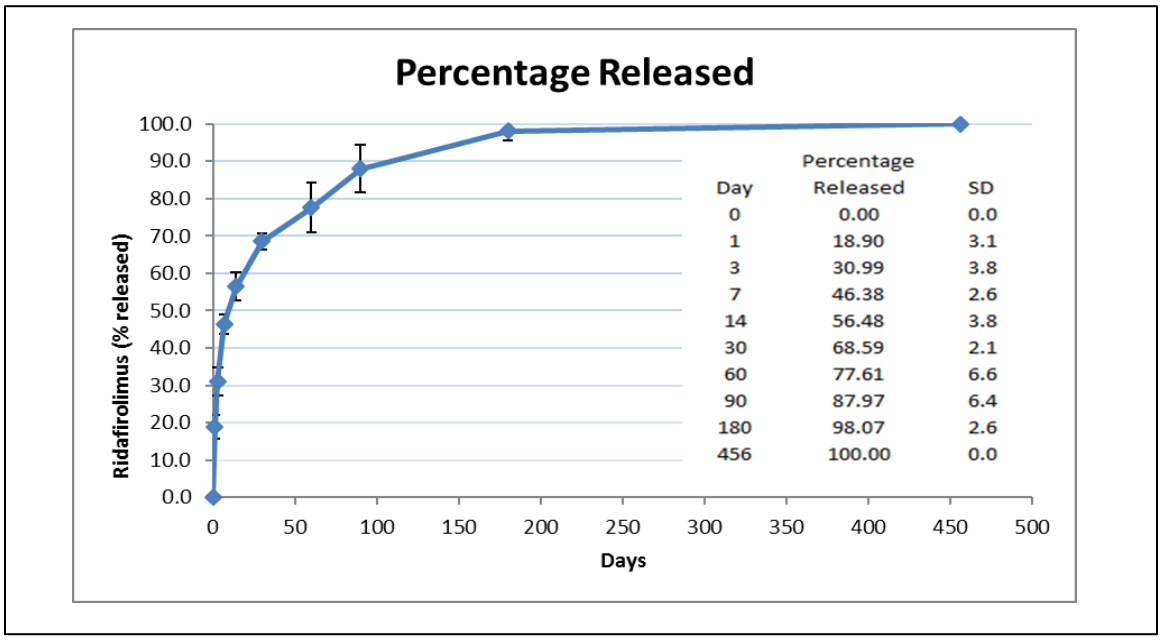

Figure 2 supplement – In-vivo drug release in a porcine coronary model

Figure 2 supplement legend: More than half of the Ridaforolimus content was released from the stents by 14 days post-implant (56.48%), by 90 days nearly 90 percent was released (87.97%), and by 180 days nearly all Ridaforolimus was released (98.07%). By Day 456, all stent samples were below the quantitation limit.

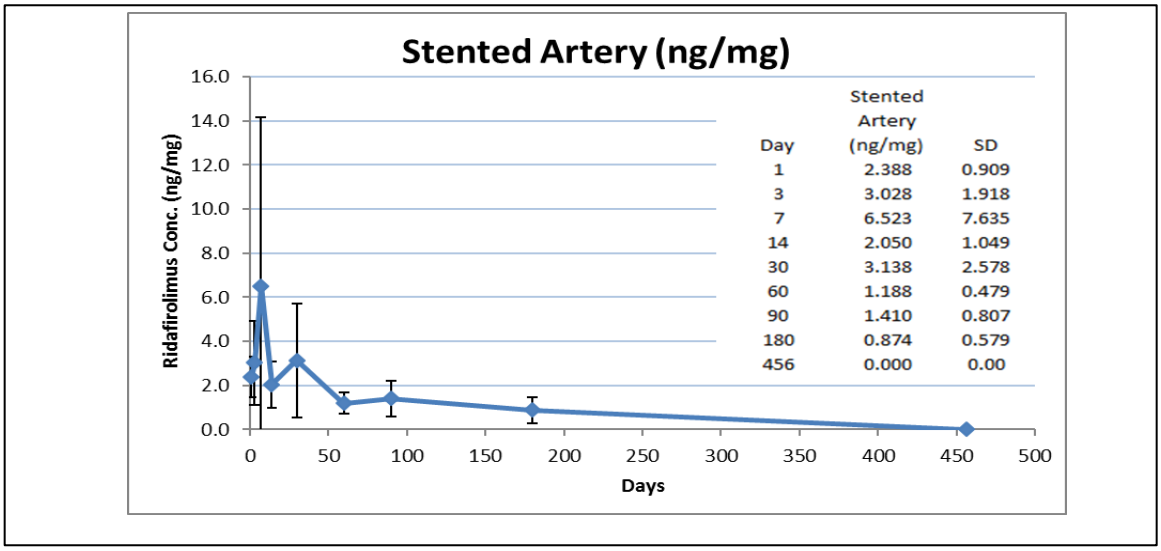

Figure 3 supplement – Drug deposition in arterial tissue in a porcine coronary model

Figure 3 supplement legend: Ridaforolimus concentrations in arterial tissue surrounding the stent are between 1-3 ng/mg for the first 90 days (with the exception of one outlying point at 7 days) and then gradually decline, with levels reaching zero between 180 and 456 days