Suppression of OATP1B1, OATP1B3, and OATP2B1 Transporters in Primary Cryopreserved Human Hepatocytes Following Lipid Delivery of Stealth siRNA™

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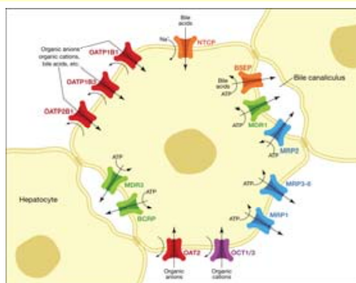
Introduction

The Organic Anion Transporting Polypeptide (OATP) transporters play an important role in the hepatic uptake of a wide range of drugs and endogenous compounds. Primary hepatocytes (freshly isolated or cryopreserved) cultured appropriately retain the major hepatic membrane transporters and drug metabolizing enzymes (Figure 1) (Sahi, 2005). The absence of transporter-specific substrates and inhibitors makes it difficult to characterize the contribution of specific transport proteins. Cell lines exist that have been stably transfected with individual transporters; however, they require maintenance and lack the normal metabolic capabilities and gene programming of that of primary hepatocytes. RNAi is an effective tool for studying the effects of gene repression (Tuschl, 2001). An RNAi model that specifically targeted particular OATP transporters in primary human hepatocytes would serve as an extremely valuable research tool for investigating a drug's hepatic disposition. Cryopreserved hepatocytes would be advantageous from the standpoint of convenience, reproducibility and pre-characterization (Li *et al*, 1999). We have developed a model using cryopreserved human hepatocytes and Stealth Select siRNA™ to specifically knock down OATP1B1, OATP2B1 and OATP1B3 mRNA expression, without affecting the other two transporters. Stealth Select siRNA™ was chosen due to minimal non-specific and off-target effects, such as interferon response and toxicity. Lipofectamine RNAiMax™ was chosen as the lipid delivery reagent because of its proven performance in primary cells, as well as its reduced toxicity and off-target effects. Optimizations presented in this poster include 1) determining the appropriate ratios of Lipofectamine RNAiMax™ to Stealth Select siRNA™ for maximum OATP suppression 2) determining whether knockdown of one OATP transporter could cause up-regulation of the other similar OATP transporters, 3) determining the appropriate time to assess mRNA knockdown, 4) determining which of the Stealth Select siRNA™ oligos worked best for each of the three OATP transporters, and 5) investigating a combined Stealth Select siRNA™ approach to knock down all three OAPT transporters simultaneously.

Figure 1 - Human hepatic transporters



Methods

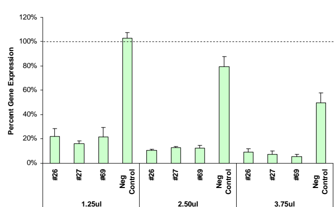
Hepatocyte culture and siRNA transfection. Cryopreserved human hepatocytes that were isolated using a two-step collagenase/perfusion method previously described by LeCluyse *et al* (2005), were obtained from CellzDirect/Invitrogen. Lot #Hu8063 was thawed according to CellzDirect's standard thawing and plating protocol, using CellzDirect's complete (antibiotic-containing) thawing media, plating media, and maintenance media. Cells were plated at a concentration of 600,000 cell/mL in 24 well plates (0.5mL total, resulting in approximately 70% confluency), as previous studies have shown sub-confluent monolayers result in the highest transfection efficiency. Cells were allowed to attach for 5 hours prior to transfection.

Stealth Select siRNA™ RNAi transfection. Stealth Select 3 RNAi™ sets were obtained from Invitrogen for OATP1B1 (**SLCO1B1:** SLCO1B1HSS116326 **(#26),** SLCO1B1HSS116327 **(#27)**, SLCO1B1HSS173769 **(#69)**; OATP2B1 (**SLCO2B1:** SLCO2B1HSS117559 **(#59)**, SLCO2B1HSS174240 **(#40)**, SLCO2B1HSS174241 **(#41)**; and OATP1B3 (**SLCO1B3:** SLCO1B3HSS120703 **(#03)**, SLCO1B3HSS120704 **(#04)**, SLCO1B3HSS120705 **(#05)**. Lipofectamine RNAiMax™ (Invitrogen) was complexed with Stealth Select siRNA™ in OptiMem™ media (GIBCO) for 30 minutes prior to addition to cell media. Cells were exposed to the siRNA for approximately 15 hr (overnight), before replacing media to Invitrogen's standard hepatocyte maintenance media. Media was then replaced every 24 hr. Final cellular monolayer confluencies were determined at 48 hr (just prior to real-time PCR) by light microscopy.

mRNA isolation and real-time PCR. mRNA isolation was performed using mRNA Catcher™ PLUS Kit (Invitrogen). cDNA synthesis and real-time PCR were performed using EXPRESS One-Step Superscript® qRT-PCR Universal (Invitrogen). TaqMan® assays (SLCO1B1 TaqMan® Assay # Hs00272374_m1; SLCO2B1 TaqMan® Assay #Hs00299670; and SLCO1B3 TaqMan® Assay # Hs00251986_m1) (Applied Biosystems) were used for mRNA quantification on an ABI 7900 instrument.

RNAiMax™ optimization. Initial experiments were performed to determine the optimal ratios of RNAiMAX™ and Stealth siRNA. Stealth Select siRNA™ #26, 27, and 69 (OATP1B1) (100nM) was combined with different amounts of RNAiMAX™ (1.25, 2.50, and 3.75µL) in 24 well plates. Optimal doses were determined based on knockdown efficiency of the OATP1B1 and minimal disruption of normal cellular expression when treated with a negative control siRNA.

Figure 3 - Optimization of RNAiMax™ ratios with Stealth Select siRNA™



Methods (cont.)

Oligo optimization and time course determination of mRNA knockdown. Optimal dose combinations of RNAiMax™ and siRNA were then used to determine whether knockdown of OAPT1B1 (Stealth Select siRNA™ #26, 27, and 69), could cause up-regulation of the other two similar OATP transporters OATP2B1 and OATP1B3. Additional experiments were done to determine whether 24 hr or 48 hours demonstrated better knockdown at the mRNA level. Optimal doses of Stealth Select siRNA™ (100nM) and RNAiMax™ (1.25µL) were used to evaluate effects of oligos #26, 27, 69 (OATP1B1), #40, 41, 59, (OATP2B1), and #03, 04, 05 (OATP1B3) on knockdown for each specific transporter.

Combined approach to OATP family knockdown. Final studies investigated an approach to suppress all three OAPT transporters simultaneously using a combination of the best oligos for each transporter (33nM individual or 33nM each of 1B1#26, 2B2 #59, 1B3 #03).

Figure 4 – Potential up-regulation of related OATP transporters

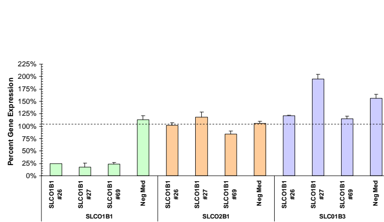


Figure 5 – Time dependent assessment of OATP mRNA suppression

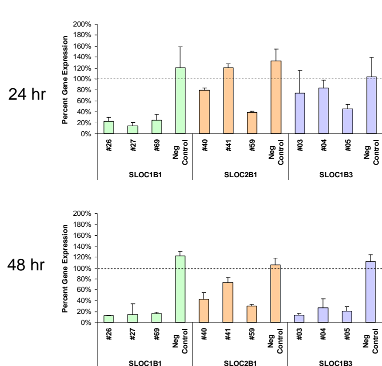


Figure 6 – Combination approach to OATP suppression

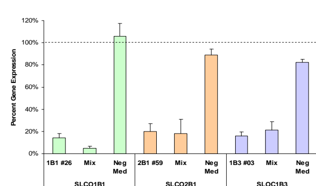


Figure 7 - Effects of RNAi on hepatocyte health

Effects on siRNA transfection on primary hepatocyte cultures. Treatments include A) untreated cells, B) 48 hr post-transfection with 33 nM OATP1B1 Stealth Select RNAi™ siRNA, and C) 48 hr post-transfection with 100 nM OATP1B1/2B1/1B3 Stealth Select RNAi™ siRNA. No toxicity was detected from any of the siRNA transfection conditions tested.

Results

Current studies focused on optimizing the appropriate combination of RNAiMax™ and Stealth Select siRNA™ in cryopreserved plateable human hepatocytes. For knockdown of the OATP transporters tested, the best ratio was 1.25ug RNAiMax™ and 100nM Stealth Select siRNA™ (Figure 3).

OATP1B1 Stealth Select siRNA™ oligo #27 caused the greatest knockdown of OATP1B1, however it also up-regulated the other transporters (almost 2-fold for OATP1B3) (Figure 4). Therefore, oligo #26 (which caused 78% knockdown of OATP1B1, without significantly effecting OATP2B1 and OATP1B3 mRNA levels) was chosen for further optimization of OATP1B1 knockdown.

Greater knockdown of OATP mRNA levels occurred at 48 hr post-siRNA transfection: OATP1B1 (87.7% knockdown with 100nM oligo #26), OATP2B1 (70.2% with 100nM oligo #59), and OAPT1B3 (87.0% with 100nM oligo #03) (Figure 5), compared to that of 24 hr post-transfection.

Significant siRNA knockdown of each of the three OATP transporters was also achieved with a combination of all three oligos used simultaneously: 33nM each of oligo #26 (OATP1B1), #50 (OATP2B1) and #03 (OATP1B3) (Figure 6).

These optimized conditions did not effect overall cell health, as determined by morphology assessment, (Figure 7), and negative control effects on the different transporter expression (Figures 5 and 6).

Conclusions

siRNA knockdown of specific OATP transporters serves as an excellent *in vitro* tool in studying transporter substrate activity of a drug in primary human hepatocytes.

In combination with RNAiMax™, Stealth Select siRNA™ can be used in primary hepatocytes to efficiently suppreress a single OATP uptake transporter and/or the entire family of OATP transporters. However, while choosing the best oligo for knocking down a specific transporter, it is important to also assess the possibility of up-regulating the other OATP transporters.

Cryopreserved plateable hepatocytes serve as an ideal model for siRNA delivery, in regards to convenience, reproducibility, as well as pre-characterization. Invitrogen has already optimized Cryopreserved Hepatocyte Recovery Media (CHRM™), Plating Media, and Maintenance Media for culturing primary cryopreserved hepatocytes. This poster demonstrates how these established research tools can also be used for efficient knock down of the human OATP uptake transporters.

The confluency in which the cells are plated has an important effect on transfection efficiency. The optimal seeding density for siRNA knockdown experiments was previously found to be sub-confluent (approximately 70% in a 24 well plate). This is contrary to standard hepatocyte studies in ADME research, in which 100% confluency is ideal. Primary hepatocytes do not divide in culture, but they are social epithelial cells that will spread out like fibroblasts to reach other cells. It is speculated that the lesser confluency allows for greater surface area exposure to the siRNA, and therefore, increases the transfection efficiency.

The best time to assess knockdown at the RNA level was determined to be 48 hr following transfection. This is believed to be related to the half-life of OAPT transporters, which is approximately 24 hr. Based on these results, and the time it takes mRNA to typically convert to protein, it is recommended that OATP functional assays be performed at 72 hr.

References

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