MODERATE HYPERBILIRUBINEMIA IMPROVES RENAL HEMODYNAMICS IN ANGIOTENSIN II DEPENDENT HYPERTENSION.

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Running Title: plasma bilirubin and renal hemodynamics

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ABSTRACT

We have previously demonstrated that moderate hyperbilirubinemia decreases blood pressure in Angiotensin (Ang) II-dependent hypertension through mechanisms that decrease oxidative stress and increase nitric oxide levels. Since decreases in renal hemodynamics play an important role in mediating the hypertensive actions of Ang II, the goal of the present study was to examine the effect of moderate hyperbilirubinemia on glomerular filtration rate (GFR) and renal blood flow (RBF) in a mouse model of Ang II hypertension. Mice were made moderately hyperbilirubinemic by two methods: Indinavir or specific morpholino antisense oligonucleotides against UGT1A1, which is the enzyme responsible for the conjugation of bilirubin in the liver. GFR and RBF were measured in mice after implantation of an osmotic minipump delivering Ang II at a rate of 1 µg/kg/min. GFR was measured by continuous infusion of I\textsuperscript{125}-iothalamate on days 5-6 of Ang II infusion in conscious mice. RBF was measured on day 7 of Ang II infusion in anesthetized mice. Blood levels of unconjugated bilirubin were significantly increased in mice treated with Indinavir or anti-UGT1A1 (P=.002). Ang II decreased GFR by 33% of control (n=9, P=.004) and this was normalized by moderate hyperbilirubinemia (n=6). Next, we examined the effect of moderate hyperbilirubinemia on RBF in Ang II infused mice. Ang II infusion significantly decreased RBF by 22% (P=.037) of control and this decrease was normalized by moderate hyperbilirubinemia (n=6). These results indicate that improvement of renal hemodynamics may be one mechanism by which moderate hyperbilirubinemia lowers blood pressure in this model.
KEY WORDS

Bilirubin, Renal Blood Flow, Glomerular Filtration Rate, Morpholino, Indinavir, UGT1A1
INTRODUCTION

Population studies have revealed that individuals with moderately elevated plasma bilirubin have reduced incidence of hypertension, renal disease and coronary artery disease (3; 4; 7; 9; 12; 15). Despite this strong correlation, the mechanism(s) that links mild increases in plasma bilirubin with the prevention of cardiovascular disease remains unknown. The Gunn rat is a model of severe hyperbilirubinemia due to lack of the hepatic enzyme responsible for the conjugation of bilirubin into the bile salts, UGT1A1. Recent studies have reported that Gunn rats are resistant to both Angiotensin (Ang) II and deoxycorticosterone (DOCA) salt induced hypertension (14; 16). One significant limitation of the Gunn rat is the extremely high plasma bilirubin levels (>10 fold increase) present in this model, which are much higher than the protective levels observed in human population studies (16). We recently reported that a two-fold increase in plasma unconjugated bilirubin levels achieved via antagonism of UGT1A1 with the drug Indinavir or through direct infusion of bilirubin attenuate Ang II dependent hypertension (31). Indinavir is a protease inhibitor that competitively inhibits UGT enzymatic activity with a K_i of 183 μM resulting in an increase in the plasma levels of unconjugated bilirubin (37).

While we have previously demonstrated the antihypertensive actions of moderate increases in plasma levels of unconjugated bilirubin, the mechanisms whereby moderate elevations in plasma bilirubin attenuate Ang II dependent hypertension remain unexplored. Studies by Coffman and colleagues have elegantly demonstrated that the chronic hypertensive actions of Ang II are mediated via its renal effects (5). In these studies, it was found that transplantation of a kidney deficient in Angiotensin type 1 (AT_1) receptors into a normal mouse completely prevented the development of chronic Ang II hypertension even though the peripheral AT_1 receptors were
intact. Likewise, transplantation of a normal mouse kidney into an AT\textsubscript{1} receptor deficient mouse restored the chronic hypertensive actions of Ang II infusion despite the mouse lacking any peripheral actions of Ang II (5). Ang II can act to increase blood pressure chronically by decreasing renal hemodynamics and increasing the tubular reabsorption of sodium (8). We recently reported that moderate hyperbilirubinemia reduces blood pressure in mice chronically infused with Ang II (31); however, it was not known what effect moderate hyperbilirubinemia has on renal hemodynamics in this model. The goal of this study was to test the hypothesis that a moderate increase in plasma bilirubin improves renal hemodynamics in Ang II dependent hypertension. This improvement in renal vascular function by moderate increases in plasma bilirubin is manifested by preservation of GFR and RBF in mice treated chronically with Ang II.

METHODS

Animals

Experiments were performed on 12- to 16-week-old male C57BL/6J mice obtained from Jackson Labs (Bar Harbor, Me). The mice were fed a standard diet containing 0.29% NaCl and were provided water ad libitum. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center and performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The mice were randomly divided into 6 treatment groups and were treated with vehicle (0.9% saline), Indinavir (500 mg/kg per day, oral gavage), UGT1A1 antisense morpholino oligonucleotide (Vivo morpholinos, AGCTCCAGCACACCACAGTCATGGT, 16 μg/kg every third day, i.v., Gene Tools, Philomath, OR), Angiotensin II (1μg/kg per minute, sc), Ang II plus Indinavir and Ang II plus UGT1A1 antisense morpholino. Mice were administered Indinavir or
UGT1A1 antisense morpholino 3 days before exposure to Ang II. Ang II was delivered via an osmotic minipump implanted subcutaneously in mice under light isoflurane anesthesia as previously reported (32).

Measurement of Plasma Bilirubin

Plasma samples were collected from mice of each experimental group at the end of the experimental protocol. Mice were euthanized by CO₂ asphyxiation and the heart immediately removed. Pooled whole blood was then collected from the chest cavity and placed in tubes containing 5 μL of an EDTA solution (0.5 M). The blood was then centrifuged at 3,000 g for 5 min, plasma collected and stored at -20°C. Total bilirubin and conjugated bilirubin concentrations were measured from 150 μL using the QuantiChrom Bilirubin Assay Kit (BioAssay Systems, Hayward, CA) according to the manufacturer instructions. The bilirubin assay was calibrated with a solution equivalent to 5 mg/dL and provided by the manufacturer. Unconjugated bilirubin was calculated as the difference between total bilirubin and conjugated bilirubin. The concentrations are expressed as mg/dL.

Glomerular Filtration Rate (GFR)

The GFR was measured by continuous infusion of ¹²⁵I iothalamate on days 5 and 6 following implantation of Ang II osmotic minipump as previously described (28). ¹²⁵I iothalamate was infused intravenously at a rate of 0.015 μCi/kg/min for 24 hours to reach steady state. Once steady state is achieved the infusion rate of ¹²⁵I iothalamate will be equal to the urinary excretion rate. An arterial plasma sample (50 μL) was collected via retro-orbital bleed in isoflurane anesthetized mice and 25 μL was measured in an Auto-Gamma counter (Cobra II, Packard
Instruments Co., Downers Gr, IL). GFR was calculated from counts obtained from both the plasma and $^{125}\text{I}$ iothalamate infusate. Two consecutive GFR measurements were averaged for each individual mouse and expressed as ml/min/g kidney weight.

**Renal Blood Flow**

Acute measurements of RBF were made in anesthetized mice (2% isoflurane) on day 7 following implantation of Ang II osmotic minipump using the 0.5PSB perivascular flow probe (Transonic Systems, Ithaca, NY) as previously described (21; 25). Mice were instrumented with carotid artery and jugular vein catheters to monitor MAP and perform infusions, respectively. To maintain constant pressure, the mice were infused with 10 $\mu$L/min of 2.38g/dL bovine serum albumin dissolved in normal saline. An incision was made on the right flank and a 0.5PSB renal flow probe positioned around the right renal artery using a micromanipulator. The signal was collected and recorded using a Transonic flowmeter (TS420) and a computerized chart recorder system (PowerLab, ADInstruments, LabChart Pro v7 software). After implantation of the catheters and flow probe the mice were allowed 30 minutes to equilibrate, after which time renal blood flow and arterial pressure measurements were made over a 20 minute period. The renal flow probe was calibrated to 0 and 1.5 ml/min at the beginning of each experiment by placing the probe in saline and recording in Labchart software with built-in calibration functions on the Transonic system TS420 blood flow meter. RBF measurements were normalized to kidney weight measured when mice were sacrificed and expressed as ml/min/g kidney weight. Renal vascular resistance (RVR) was calculated from pressure and flow data and reported as resistance units (RU= mmHg ml$^{-1}$ min$^{-1}$ g$^{-1}$).
Western Blot

Western Blots for UGT1A1 and OATP2 proteins were performed on liver lysates. 30 µg of protein were separated on 7.5% SDS-polyacrylamide gels and blotted onto nitrocellulose membrane. Membranes were blocked with Odyssey blocking buffer (LI-COR, Lincoln, Neb) for 2 hours at room temperature and were incubated with rabbit anti-UGT1A1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-OATP2 polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The membranes were then incubated with mouse anti-ß-actin antibody for 1 hour followed by both Alexa 680 donkey anti-rabbit IgG (Molecular Probes) and IRDye 800 donkey anti-mouse IgG (Rockland, Gilbertsville, Pa) for 1 hour at room temperature and visualized using an Odyssey infrared imager (Li-COR), which allows for the simultaneous detection of 2 proteins. Densitometry analysis was performed using Odyssey software (LI-COR). Levels of UGT1A1 and OATP2 proteins were expressed as the ratios of the protein to ß-actin for each sample.

Statistical Analysis

Mean values + SE are presented. Significant differences between mean values were determined using an ANOVA followed by a post hoc test (Dunnett’s). A $P<0.05$ was considered to be significant.

RESULTS

_Intra-venous treatment with a UGT1A1 antisense mopholino oligonucleotide decreases UGT1A1 protein levels in the liver._
In order to determine effects of Indinavir and the UGT1A1 antisense morpholino oligonucleotide on the levels of UGT1A1 and OATP2 protein levels in the liver, Western blots were performed on hepatic lysates in mice treated with Indinavir or antisense as outlined above. Treatment with the UGT1A1 antisense oligonucleotides resulted in a 54% decrease in liver UGT1A1 protein expression in Ang II infused mice (Figure 1A). Indinavir in combination with Ang II did not change the levels of UGT1A1 protein in the liver. Treatment with Indinavir or the antisense morpholino oligonucleotide did not result in any changes in the levels of OATP-2 protein in the liver (Figure 1B).

*Indinavir or UGT1A1 antisense morpholinos increase plasma unconjugated bilirubin levels in Ang II treated mice.*

The effects of experimental treatments on plasma bilirubin levels were determined in control mice as well as mice treated with UGT1A1 antisense morpholino, Ang II, Ang II + Indinavir, and Ang II + UGT1A1 antisense morpholino. All of the experimental treatments resulted in an increase in total bilirubin levels as compared to control (Figure 2A). UGT1A1 treatment alone or in combination with Ang II resulted in a significant (P <0.01) increase in unconjugated bilirubin levels (Figure 2B), and a significant decrease in the percent of conjugated to total bilirubin in the plasma which averaged 80 ± 3 vs. 53 ± 4 vs. 88 ± 5 vs. 75 ± 3 vs. 40 ± 6% in control, UGT1A1 antisense, Ang II, Ang II + Indinavir, and Ang II + UGT1A1 antisense, respectively. Ang II treatment resulted in an increase in total bilirubin levels in the plasma (Figure 2A) as a result of a significant increase in conjugated as opposed to unconjugated bilirubin levels (Figure 2C). Indinavir treatment of Ang II infused mice resulted in an increase in the levels of both unconjugated (Figure 2B) and conjugated bilirubin (Figure 2C). While all of the experimental treatments resulted in increased total bilirubin levels, only Indinavir and UGT1A1 antisense
treatment resulted in moderate but significant increase in unconjugated bilirubin levels in the plasma. This would support a role for increased unconjugated bilirubin playing a primary role in mediating the observed effects on GFR and RBF.

Moderate hyperbilirubinemia restores GFR in Ang II treated mice.

GFR measurements were made conscious, freely-moving mice via continuous intravenous infusion of $^{125}$I iothalamate as described above. GFR was significantly ($P = .004$) decreased in Ang II infused mice as compared to control and averaged, $1.2 \pm 0.2$ vs. $1.9 \pm 0.1$ ml/min/g kidney weight in each group, respectively (Figure 3). Moderate hyperbilirubinemia induced by either treatment with Indinavir or UGT1A1 antisense morpholino increased GFR in Ang II infused mice to levels observed in control mice with GFR averaging, $2.0 \pm 0.3$ and $1.9 \pm 0.1$ ml/min/g kidney weight in Ang II + Indinavir, and Ang II + UGT1A1 antisense morpholino, respectively (Figure 3).

Moderate hyperbilirubinemia attenuates Ang II induced decrease in RBF.

To assess the effect of moderate hyperbilirubinemia on renal blood flow in Ang II hypertensive mice, acute RBF measurements were made in anesthetized mice 7 days after implantation of Ang II containing osmotic minipumps. Ang II infusion resulted in a significant ($P = .037$) decrease in renal blood flow as compared to control with RBF averaging $3.7 \pm 0.4$ vs. $4.7 \pm 0.2$ ml/min/g kidney weight in each group, respectively (Figure 4A). Moderate hyperbilirubinemia induced by Indinavir treatment normalized RBF in Ang II infused mice to levels that were similar to those observed in control mice with RBR averaging $4.8 \pm 0.3$ ml/min/g kidney weight in Ang II +
Indinavir treated mice (Figure 4A). Indinavir treatment by itself had no significant effect on RBF as compared to control averaging $4.8 \pm 0.3$ ml/min/g kidney weight. Renal vascular resistance (RVR) calculated from RBF and blood pressure was significantly ($P=.037$) decreased in Ang II + Indinavir treated mice as compared to Ang II treated mice (Figure 4B). When normalized to kidney weight, RVR was significantly ($P=0.041$) increased in Ang II infused mice as compared to control and normalized in Ang II infused mice treated with Indinavir (Figure 4C).

We have previously demonstrated the antihypertensive actions of moderate hyperbilirubinemia in conscious mice via direct measurement of blood pressure with arterial catheters (31). In the present study, mean arterial pressure measured under isoflurane anesthesia was not different between the groups averaging $83 + 4$, vs. $83 + 3$, vs. $77 + 5$, vs. $73 + 3$ mmHg in control, Indinavir, Ang II and Ang II + Indinavir treated mice, respectively. However, we determined cardiac mass as an index of hypertension in each of the groups. The ratio of heart weight to body weight was significantly ($P=.002$) increased in Ang II infused mice and completely normalized in moderately hyperbilirubinemic mice treated with Indinavir averaging; $5.5 + 0.2$ vs. $5.2 + 0.2$ vs. $6.5 + 0.2$ vs. $5.7 + 0.1$ mg/g in control, Indinavir, Ang II, and Ang II + Indinavir treated mice, respectively.

**DISCUSSION**

Our data demonstrate that moderate hyperbilirubinemia similar to protective increases observed in large scale human population studies (4; 12) is able to restore renal hemodynamics in Ang II dependent hypertension. We have previously reported that moderate (2 fold) elevations in plasma unconjugated bilirubin reduce blood pressure in mice chronically infused with Ang II (31);
however, the effect of moderate increases in plasma unconjugated bilirubin on renal hemodynamics in Ang II infused mice was not known. Chronic Ang II infusion resulted in a significant increase in cardiac hypertrophy a marker of established hypertension, as well as decreases in both GFR and RBF which were consistent with previous reports (19; 30; 36).

However, moderate hyperbilirubinemia induced prior to Ang II infusion was able to prevent the increase in cardiac hypertrophy, maintain GFR and RBF and decrease RVR. The effect of moderate hyperbilirubinemia to preserve GFR in response to chronic Ang II infusion was similar to that observed in Gunn rats which are a model of severe hyperbilirubinemia (16). The reduction in RVR observed in the present study is supported by our previous report that moderate hyperbilirubinemia lowers blood pressure in Ang II dependent hypertension by decreasing vascular oxidative stress and increasing NO bioavailability (31). This observation is similar to studies which have demonstrated an important role for heme oxygenase derived bilirubin in the protection of endothelial and renal tubule cells from oxidative injury (17; 33). Previous studies by us and others have demonstrated that chronic infusion of Ang II results in diminished vascular sensitivity to NO which may be responsible for the decrease in GFR and RBF observed in the present study (20; 24). The increased NO bioavailability following moderate hyperbilirubinemia could result in improvement of endothelial function and preserve renal vasodilatory capacity in response to Ang II infusion. However, the precise role for increased NO bioavailability in the normalization of GFR and RBF with moderate hyperbilirubinemia during Ang II dependent hypertension will need to be addressed in future studies in which vascular NO production is chronically blocked.
In this study, we induced moderate hyperbilirubinemia in mice using two different approaches. Indinavir is a protease inhibitor developed for the treatment of human immunodeficiency virus (HIV) patients that was also found to increase the levels of unconjugated bilirubin via antagonism of hepatic UGT1A1 (6; 37). While Indinavir is well tolerated by rodents, its use in humans has been linked to the development of renal stone formation leading to renal nephropathy in some cases (2; 11; 18; 23; 29). The renal effects of chronic Indinavir treatment in humans limit its potential as an anti-hypertensive drug. Given this limitation, we explored an alternative approach to create moderate hyperbilirubinemia via targeting of hepatic UGT1A1 with morpholino antisense oligonucleotides. Morpholinos offer several advantages over traditional antisense oligos or siRNA in that they are resistant to nucleases allowing them to last longer in vivo and they exhibit less “off target” effects as compared to traditional antisense DNA molecules that contain sulfur backbones (13; 27). Morpholinos are also more specific than other steric blocking antisense because of they require greater number of contiguous bases to achieve blockade of transcription than traditional antisense DNA (27). In this study, we report that hepatic UGT1A1 can be knocked down in vivo using intravenous bolus infusions of UGT1A1 antisense morpholinos administered every 72 hours. We observed a 54% decrease in the levels of UGT1A1 protein in the liver which was associated with a three-fold increase in unconjugated plasma bilirubin levels. One limitation with the current approach is the repetitive administration of the UGT1A1 antisense morpholinos in order to achieve sufficient decreases in UGT1A1 to induce moderate increases in plasma bilirubin. Given the cost and limited ability to deliver the morpholinos in concentrated solutions, this approach may not be feasible in larger animal models or in human patients. An alternative approach could be to develop viral vectors that contain
siRNAs to heptatic UGT1A1. This approach could be highly feasible given the relative ease in delivering virus to the liver and the long lasting effects of certain viruses such as lentiviruses.

While this is the first study that has successfully attenuated Ang II dependent renal hemodynamic changes by manipulation of hepatic UGT1A1 levels, alternative targets of hepatic bilirubin metabolism may also be potential candidates to induce moderate hyperbilirubinemia chronically. UGT1A1 is the major enzyme responsible for the conjugation of bilirubin in the liver; however, this enzyme is also responsible for the glucuronidation of other drugs in the liver which aids in their metabolism and elimination from the body (35). It is possible that targeting of UGT1A1 in certain hypertensive patients taking drugs like warfarin could be problematic due to alterations in metabolism (10). The effect that decreases in UGT1A1 observed with antisense morpholinos in the current study would have on metabolism of other xenobiotic drugs is not known and merits further investigation.

An alternative approach to increase the levels of unconjugated bilirubin in the plasma is to combine UGT1A1 inhibition with induction of heme oxygenase-1 (HO-1) activity. Several studies have documented the ability of systemic induction of HO-1 either chemically or genetically to decrease blood pressure in animal models of hypertension (1; 22; 34). The effect of inhibition of UGT1A1 with Indinavir or antisense morpholinos on HO-1 expression and activity is not known. However, one must be careful to titrate the level of HO-1 induction and the degree of UGT1A1 inhibition to ensure moderate (~2 fold) increases in unconjugated bilirubin levels in order to achieve optimal therapeutic effects of moderate hyperbilirubinemia.
We observed increases in total serum bilirubin levels in mice treated with Indinavir, UGT1A1 antisense morpholino oligonucleotide and Ang II. Interestingly, the increase in total serum bilirubin observed in the mice treated with Ang II was due to the increase in conjugated bilirubin levels as opposed to increases in unconjugated bilirubin levels observed in Indinavir and UGT1A1 antisense morpholino treated mice. The effect of Ang II to increase conjugated bilirubin levels was not affected by Indinavir treatment but was not observed in mice treated with UGT1A1 antisense morpholino. This finding along with our observation of a trend towards increased hepatic UGT1A1 protein levels would suggest a potential role for UGT1A1 in the increase in plasma conjugated bilirubin levels observed in Ang II treated mice. Our results would suggest that increases in unconjugated bilirubin mediate the beneficial actions of moderate hyperbilirubinemia induced by Indinavir and UGT1A1 antisense morpholino. This is in line with experimental evidence indicating that conjugated bilirubin as opposed to unconjugated bilirubin is unable to inhibit the NADPH oxidase-dependent production of superoxide anion (26).

**Perspectives**

Our results demonstrate that increases in total plasma bilirubin due to moderate elevations in unconjugated bilirubin are able to preserve renal hemodynamics in Ang II dependent hypertension. These results also reveal that moderate hyperbilirubinemia lowers renal vascular resistance in Ang II hypertension and this may play an important role in the resetting of pressure-natriuresis and lowering of blood pressure in this model. However, other non-renal mechanisms
of moderate hyperbilirubinemia cannot be ruled out at this time. We have also demonstrated for the first time that selective targeting of hepatic UGT1A1 using antisense morpholino oligonucleotides increases plasma unconjugated bilirubin levels in mice. Targeting hepatic UGT1A1 may be a novel therapeutic approach for improving renal hemodynamics and lowering blood pressure in hypertensive patients.
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FIGURE LEGENDS

**Figure 1**- Representative Western blots of A) hepatic UGT1A1 and B) hepatic OATP2 protein levels in control, Ang II, Ang II + Indinavir, and Ang II + UGT1A1 antisense morpholino treated mice. Mice were treated as described in the *Methods* and livers collected at the end of the experimental protocol. Treatment with the UGT1A1 antisense morpholino resulted in a significant decrease in hepatic UGT1A1 protein expression (*P=0.002 vs. control, n=6). None of the treatments had any effect on hepatic OATP2 protein levels (n=6).

**Figure 2**- Plasma bilirubin levels in control (n=10), UGT1A1 antisense morpholino (n=4), Ang II (n=7), Ang II + Indinavir (n=6) and Ang II + UGT1A1 antisense treated (n=6) mice. Plasma samples were collected at the end of the experimental protocol as described in the *Methods*. Plasma samples of mice treated with UGT1A1 antisense were collected on day after two i.v. injections of the UGT1A1 antisense morpholino given 72 hours apart. A) Total plasma bilirubin levels were significantly increased in all groups. B) plasma unconjugated bilirubin levels were significantly increased in UGT1A1 and Indinavir treated mice. C) Plasma conjugated bilirubin levels were significantly increased in Ang II and Ang II + Indinavir treated mice. *=p<0.005 as compared to control.

**Figure 3**- Effect of moderate hyperbilirubinemia induced by either Indinavir or UGT1A1 antisense morpholino treatment on glomerular filtration rate (GFR) in mice. Mice were treated and GFR measured as described in the *Methods*. Ang II treatment resulted in a significant
decrease in GFR and this was completely normalized by moderate hyperbilirubinemia. *=p<0.005 as compared to control.

Figure 4- A) Effect of moderate hyperbilirubinemia induced by Indinavir on renal blood flow (RBF) in mice. Mice were treated RBF measured as described in the Methods. Ang II treatment resulted in a significant decrease in RBF and this was completely normalized by moderate hyperbilirubinemia. B) Effect of moderate hyperbilirubinemia induced by Indinavir on renal vascular resistance (RVR) in mice. Moderate hyperbilirubemia resulted in a decrease in RVR in Ang II treated mice. † =p<0.05 as compared to Ang II treated. C) Effect of moderate hyperbilirubinemia induced by Indinavir on RVR in mice normalized to kidney weight. Ang II treatment resulted in a significant increase in RVR which was normalized by moderate hyperbilirubinemia. *=p<0.05 as compared to control.
Figure 1

A

UGT1A1

β-Actin

UGT1A1/β-actin (A.U.)

Control Ang II Ang II + Indinavir Ang II + Morpholino

B

OATP2

β-Actin

OATP2/β-actin (A.U.)

Control Ang II Ang II + Indinavir Ang II + Morpholino

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Figure 2
Figure 3
Figure 4