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**Discovery of Osmo-sensitive Transcriptional Regulation of Human
Cytochrome P450 3As (CYP3As) by the Tonicity-Responsive Enhancer
Binding Protein (TonEBP/ NFAT5)**

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Manuscript # MOL 34504

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d) Abbreviations

CAR, constitutive androstane receptor; ChIP, chromatin immunoprecipitation; CYP, cytochrome P450; EMSA, electrophoretic mobility shift assay; ER6, everted repeat 6; FBS, Fetal bovine serum; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; HNF4 α , hepatocyte nuclear factor 4 alpha; HNF3 γ , hepatic nuclear factor-3-gamma; Luc, firefly luciferase; NFAT5, the nuclear factor of activated T-cells 5; OREBP, osmotic response element-binding protein; PCR, polymerase chain reaction; PXR, pregnane X receptor; siRNA, small interfering RNA; SMIT, sodium/myoinositol cotransporter; SV, simian virus; TonE, tonicity-responsive enhancer; TonEBP, TonE binding protein; VDR, vitamin D receptor; XREM, xenobiotic-responsive enhancer module

Manuscript # MOL 34504

ABSTRACT

We report the discovery of an osmo-sensitive transcriptional control of human CYP3A4, CYP3A7, and CYP3A5. Ambient hypertonicity (350 – 450 mOsm/kg) increased mRNA expressions of the CYP3As by ~10-20-fold in human-intestinal C₂bbe1 cells, followed by an increase of CYP3A protein. Hypotonicity, on the other hand, suppressed CYP3A mRNA levels, indicating that physiological isotonic conditions may regulate the basal expression of CYP3A. Similar responses to ambient tonicity were observed in other human-derived cell lines (intestinal LS180, and hepatic HepG2) and human primary colonic cells. The 11-bp tonicity-responsive enhancer (TonE) is an osmo-sensitive regulator that is activated by the transcription factor, the nuclear factor of activated T-cells 5 (NFAT5). Luciferase-based reporter assays of 13 consensus TonE motifs within ±10kb from the transcription start sites of *CYP3As* showed that only the *CYP3A7* intron 2 region (~5kb downstream from the transcription start site) which contains two TonE motifs (+5076/+5086 and +5417/+5427) was responsive to hypertonicity stimuli. This observation was confirmed upon cotransfection with an NFAT5 expression vector, siRNA or dominant-negative NFAT5. Deletion and mutation analyses suggested that the TonE (+5417/+5427) is indispensable for the enhancer activity. NFAT5 binding to the *CYP3A7* intron 2 TonE motif was demonstrated with electrophoretic mobility shift assay and in a native cell context by chromatin immunoprecipitation. We conclude that transcription of the human *CYP3As* is influenced by ambient tonicity. The physiological significance of the tonic regulation of CYP3A enzymes remains to be determined.

Manuscript # MOL 34504

The human cytochrome P450 3A (CYP3A) subfamily represents the most abundant CYP drug-metabolizing enzymes in the liver and intestine. Together with membrane-bound transporters, they constitute a crucial component for drug elimination and excretion. Of the 3 major isoforms (CYP3A4, CYP3A5, and CYP3A7), CYP3A4 is the most abundant adult form, while CYP3A7 is the main fetal form. These isozymes collectively metabolize nearly half of all currently used medications.

The human *CYP3A* genes reside in a cluster on chromosome 7 (Nelson *et al.*, 2004), and their expression is characterized by wide inter-individual variations. Significant co-expression of the major CYP3A isoforms suggests the presence of common regulatory pathways. CYP3A induction by drugs and chemicals is known to be mediated by pregnane X receptor (PXR) (Bertilsson *et al.*, 1998; Blumberg *et al.*, 1998; Kliewer *et al.*, 1998), constitutive androstane receptor (CAR) (Xiew *et al.*, 2000), the vitamin D receptor (VDR) (Schmiedlin-Ren *et al.*, 1997; Thummel *et al.*, 2001), and hepatocyte nuclear factor-4-alpha (HNF4 α ; Tirona *et al.*, 2003). In contrast, the regulation of CYP3A basal expression in the absence of an inducing agent is poorly defined, although these nuclear factors and other regulatory proteins, such as C/EBP and hepatic nuclear factor-3-gamma (HNF3 γ ; Rodriguez-Anton *et al.*, 2003), may also play a role. While examining the dietary effects on human CYP3A expression, we made an unexpected observation that osmotic environments appear to influence the expression of these CYP3As.

In mammalian cells, ambient tonicity affects the function of a transcription factor called tonicity-responsive enhancer binding protein (TonEBP), which is also known as the nuclear factor of activated T-cells 5 (NFAT5) or the osmotic response element-binding protein (OREBP). TonEBP/NFAT5/OREBP (NFAT5 hereafter in this article) is a newly-discovered fifth member of the NFAT family of transcription factors (Miyakawa *et al.*, 1999; Lopez-Rodriguez *et al.*, 1999; Stroud *et al.*, 2002; Ko *et al.*, 2000), which forms an obligatory homodimer and trans-activates its target genes via the tonicity-responsive enhancer (TonE). NFAT5 is the only known mammalian

Manuscript # MOL 34504

transcription factor that responds to changes in osmolality, where an increase in ambient tonicity provokes NFAT5 translocation from the cytoplasm to the nuclear compartment (Woo *et al.*, 2000). NFAT5 controls the expression of osmotic stress response genes such as the sodium/myo-inositol cotransporter (SMIT: SLC5A3) (Yamauchi *et al.*, 1993) and aldose reductase (Ko *et al.*, 1997). These proteins synthesize organic osmolytes or transport them into the cell, thereby counterbalancing the ambient hypertonic stimuli (Ho SN, 2006). Constitutive nuclear localization of NFAT5 has been shown, suggesting a regulatory role under isotonic conditions (Miyakawa *et al.*, 1999; Woo *et al.*, 2000).

NFAT5-mediated gene regulation has been examined extensively in kidney which faces intense osmotic stresses. Its role in other tissues, except for immune cells, is virtually unknown. Gastrointestinal epithelia are exposed to elevated postprandial osmolality (Ladas *et al.*, 1983; Houpt, 1991; Kalantzi *et al.*, 2006). Moreover, the osmotic microenvironment in the liver is also not static, but rather active and dynamic (Go *et al.*, 2004). Thus the NFAT5 regulation may be present in these tissues. In this regard, it is intriguing that exposures to salt-rich diet in human subjects for several days have been associated with increased pre-systemic elimination of CYP3A substrates, such as quinidine (Darbar *et al.*, 1997) and verapamil (Darbar *et al.*, 1998).

In this study, we have characterized the tonicity-dependent expression of human CYP3A4, CYP3A7 and CYP3A5. In addition, a series of promoter-reporter gene assays were conducted to identify any active tonicity-responsive enhancer (TonE) in the *CYP3A* gene cluster. Our findings show that expressions of human CYP3As are highly dependent on ambient tonicity in various cell lines and primary cells. Furthermore, we found that, of the multiple consensus TonE motifs within the \pm 10kb from transcription start sites of each of the major *CYP3As*, only the *CYP3A7* intron 2 region harbours an active osmo-sensitive TonE element which is responsive to NFAT5.

Manuscript # MOL 34504

Materials and Methods

Cell culture. Cell lines were purchased from ATCC (American Type Culture Collection: VA, USA), and the human primary colonic cells were purchased from CELPROGEN (CA, USA). Human C₂bbe1 and mouse CMT93 were grown in Dulbecco's minimal essential medium containing 1.5 g/L sodium bicarbonate, 10 mg/L human holo-transferrin and 10% fetal bovine serum (FBS). Human LS180 and HepG2 cells, and mouse Hepa-1c1c7 were grown in alpha MEM containing 10% fetal bovine serum. Human normal colonic epithelia obtained from 4 Caucasian male subjects aged from 35 y.o. to 55 y.o. (CELPROGEN, CA, USA: Catalogue number: 36037-08; Lot #: 60749-05; 6050-05; 6049-05; and 60750-05) were processed and propagated in the Human Colon Complete growth medium with 10% FBS (CELPROGEN). The primary cultured cells were 95% positive for the epithelial marker, cytokeratin 19, and used for experiments at approximately 70% confluency.

Ambient osmolality modification. Ambient tonicity was increased to 350-450 mOsm/kg by adding either NaCl or sucrose to the regular culture media, and cells were incubated for 24 h unless otherwise stated. For example, the addition of 50mM NaCl to the regular media (300mOsm/kg) increases the osmolality by 100mOsm/kg to 400mOsm/kg. To decrease ambient osmolality, cells were cultured in water-diluted hypotonic media (200mOsm/Kg) (1/2: v/v:water to media). To account for the differences caused by nutrient dilution in the water-diluted hypotonic condition, PBS was used as a diluent (1/2: v/v PBS to media) as a second isotonic reference condition in some experiments. Glycerol was used as a tonicity-neutral control, since it readily crosses the plasma membranes without eliciting osmotic pressure.

Real time PCR. Total RNA was extracted using RNeasy Kit (Qiagen). cDNA was generated using random hexamers and M-MLV (Invitrogen). The ABI Prism 7700 Sequence Detection System was used for PCR employing TaqMan Universal PCR Master Mix and Assays-on-Demand Gene Expression probes (Applied Biosystems). The delta-delta Ct method (Livak and Schmittgen, 2001) was used to calculate the amplification difference, with Ct value of target

Manuscript # MOL 34504

genes being normalized to respective GAPDH value. Measurements were done in triplicate and repeated at least three times. Pre-designed primers and probe sets are as follows: Human GAPDH (Hs99999905_m1), CYP3A7 (Hs00426361_m1), CYP3A4 (Hs00430021_m1), CYP3A5 (Hs00241417_m1), SMIT (Hs00272857_s), NFAT5 (Hs00232437_m1), PXR (Hs00243666_m1), CAR (Hs00231959_m1), VDR(Hs00172113_m1); and mouse Nfat5 (Mm00467257_m1), Cyp3a11 (Mm00731567_m1), Cyp3a13 (Mm00484110_m1), Cyp3a16 (Mm00655824_m1), Gapdh (Mm99999915_g1). Primers used in SYBR Green real-time PCR for drug transporter genes are as follows: MDR1 (Forward 5'-cag agg gga tgg tca gtg tt; Reverse 5'-cct gac tca cca cac caa tg); BCRP (Forward 5'-ccc gtt ctg agc ttt ttc ag; Reverse 5'-caa ggg taa ccg cag tca tt); MRP1 (Forward 5'-agg tgg acc tgt ttc gtg ac; Reverse 5'-tcc acc aga agg tga tcc tc); MRP2 (Forward 5'-tga aag gct aca agc gtc ct; Reverse 5'-tcc acc aga agg tga tcc tc); MRP3 (Forward 5'-aca tgc tgc ccc agt taa tc; Reverse 5'-cac act ctg ggg gtc aag tt); MRP4 (Forward 5'-tgt ttg atg cac acc agg at; Reverse 5'-gac aaa cat ggc aca gat gg); OCTN1 (Forward 5'-gac cga gtg gaa tct ggt gt; Reverse 5'-tct tcc tgc caa acc tgt ct); OCTN2 (Forward 5'-ctg gtg gtt cat ccc tga gt; Reverse 5'-agt gga agg cac aac aat cc); OCT1 (Forward 5'-cct gcc tgc tca tga ttt tt; Reverse 5'-acg aat gtg ggg tac agc tc); and GAPDH (Forward 5'-caa tga ccc ctt cat tga cc; Reverse 5'-gac aag ctt ccc gtt ctg ag).

Immunoblotting/immunohistochemistry. C₂bbe1 cells were lysed in RIPA buffer with protease inhibitor cocktail and centrifuged at 10000 r.p.m. at 4°C. The supernatants containing 50 µg protein were used for blotting. Nuclear proteins were extracted with Nuclear Extraction Kit (Panomics, CA, USA). Immunoblotting was performed as described previously (Muntane-Relat *et al.*, 1995). The NFAT5 antibody (Affinity BioReagents) was used at a 1000-fold dilution. The CYP3A4 antibody (Research Diagnostics), which also recognizes CYP3A5 and CYP3A7, was used at a 500-fold dilution. β-actin (1:2000 dilution; Santa Cruz Technology) was used to ensure equal loading. For immunohistochemical analysis, after 4 h exposure to hypertonicity (NaCl-

Manuscript # MOL 34504

induced 400 mOsm/kg), C₂bbe1 cells on glass slip were fixed in 4 % paraformaldehyde with 0.2 % TritonX-100 in PBS, and incubated with the above NFAT5 antibody at a 500-fold dilution in 5 % donkey serum - PBS for 1 h at room temperature. Secondary antibody, Cy3 conjugated anti-rabbit IgG, was then used for 1 h at room temperature for visualization with fluorescent imaging microscopy.

Expression plasmids, siRNA and reporter constructs. NFAT5 expression plasmid was made from KIAA0827 clone (a gift from Dr. Nagase, Kazusa Institute, Japan) by digestion with *NotI* and *XhoI*, and ligated into pTARGET (Invitrogen). Human PXR expression plasmid (pEF-hPXR: Tirona *et al.*, 2003) was kindly provided by Dr. Kim (University of Western Ontario). Dominant negative NFAT5 $_{\Delta 1-156}$, which lacks the first 156 amino acids, was derived by in-frame insertion of KIAA0827 cDNA corresponding to amino acid residues 157-581 into *NotI* and *BamHI* restriction sites of pFLAG-CMV-2 mammalian expression vector (Sigma, St. Louis, MO) as reported (Tong *et al.*, 2006). This region of NFAT5 was shown to function in a dominant negative manner when expressed in transgenic mice (Lam *et al.*, 2004; Wang *et al.*, 2005). Small interfering RNA (siRNA) against NFAT5 was prepared as described by Na *et al.* 2003; we synthesized siRNA_{569R} (Na *et al.*, 2003) targeting exon 5 of NFAT5, which was reported to show specific silencing of NFAT5 mRNA and protein. The negative control siRNA was an inverted sequence of 569R (inv569R). In separate experiments, we used a mixture of 4 siRNAs against NFAT5 (Dharmacon SMARTpool: cat #M-009618-01; Thermo Fisher Scientific, Lafayette, CO, USA) or control non-targeting mismatch siRNA (cat #D-001206-13).

*CYP3A7*_[-9302/+53] and *CYP3A4*_[-10466/+53] plasmids (Bertilsson *et al.*, 2001) were kindly provided by Dr. Blomquist (Karolinska Institute). The *CYP3A7* promoter fragment encompassing -370/+55 of the transcription start site was cloned by PCR from the *CYP3A7*_[-9302/+53] plasmid using cloning primers (forward 5'-tcc gct agc gca cac tcc agg cat agg taa-3'; reverse 5'-cat gga tcc tgc tgc tgt ttg ctg ggc tgt-3'). Similarly, the 478 bp *CYP3A4* promoter plasmid from -424 to +54

Manuscript # MOL 34504

of the transcription start site was generated from the *CYP3A4*_[-10466/+53] plasmid (forward 5'-aca gct agc ctg ggt ttg gaa gga tgt gt-3'; reverse 5'-cat gga tcc tgt tgc tct ttg ctg ggc tat gt-3'). These promoter fragments introduce a *NheI* and a *BamHI* restriction site at the 5'- and 3'-end, respectively, and were inserted into the *NheI* and *BglIII* sites of the pGL3-Basic luciferase reporter gene vector, thereby destroying the 3'-restriction site (Goodwin *et al.*, 1999) for subsequent reporter constructs. The 737 bp *CYP3A5* promoter fragment (-688/+49 from the transcription start site) (Burk *et al.*, 2004) was generated from C₂bbe1 genomic DNA using cloning primers (forward 5'-aca gct agc aga tct atc acc aca gag tca gag ggg atg-3'; reverse 5'-cat gga tcc gct gtt tgc tgg gct gtt tgc ctg g-3'), introducing a *NheI* - *BglIII* tandem restriction site at the 5'-end, and *BamHI* site at the 3'-end. This was similarly inserted into the *NheI* and *BglIII* sites of pGL3-Basic vector. The *BglIII* digestion sequence in the tandem restriction site was used for the *CYP3A5* promoter-driven constructs.

The fragments of *CYP3A7* intron 2 for the deletion/mutation assays were made by PCR from genomic DNA of C₂bbe1 cells using following primers: Backbone *CYP3A7*_[+4910/+5590] (Forward 5'-tcg gta cca ggc aga atc aca tgc aaa a-3'; Reverse 5'-gaa gat ctt gag caa tct tac gac att cca-3'); *CYP3A7*_[+4910/+5204] (Forward 5'-tcg gta cca ggc aga atc aca tgc aaa a-3'; Reverse 5'-gaa gat ctc aac aaa gcc ctc act tag ga-3'); *CYP3A7*_[+4910/+5453] (Forward 5'-tcg gta cca ggc aga atc aca tgc aaa a-3'; Reverse 5'-gaa gat ctc tga caa tgg ata acc acc tta act-3'); *CYP3A7*_{[+4910/+5453]mutant} (Forward 5'-tcg gta cca ggc aga atc aca tgc aaa a-3'; Reverse 5'-gaa gat ctc tga caa tgg ata acc acc ttt aac tTt Tac ttt cca-3'), where "T" indicates mutations; and the backbone reverse *CYP3A7*_{[+4910/+5590]reverse} (5'-gaa gat cta ggc aga atc aca tgc aaa a-3' / 5'-tcg gta cct gag caa tct tac gac att cca-3'); and *CYP3A7*_[+5088/+5590]: Forward 5'-tcg gta cca gct tat ttc cac agg gcc a -3'; Reverse 5'-gaa gat ctt gag caa tct tac gac att cca -3'). These fragments were inserted into *KpnI* and *BglIII* sites of the *CYP3A* promoter-driven reporter plasmids (see above). The fragment of

Manuscript # MOL 34504

*CYP3A7*_[+4910/+5590]3' position was made using primers (Forward: 5'-ttc gga tcc agg cag aat cac atg caa aa-3'; reverse: 5'-ctc gtc gac tga gca atc tta cga cat tcc a-3'). They were inserted into the *Bam*HI and *Sal*I sites of the reporter. Other reporter constructs were similarly made, and inserted into the appropriate restriction sites of luciferase reporters containing either the respective *CYP3A* or SV40 minimal promoter. All constructs were confirmed by sequencing (TCAG DNA sequencing facility, Hospital for Sick Children).

Transient transfection and luciferase-based reporter assay. C₂bbe1 cells were used unless otherwise stated. Cells were seeded onto 6-well plates at 0.5 x 10⁶ cells/well. After 48 h, cells were transfected with 0.3-0.5 µg of the firefly luciferase reporter plasmids and 0.08-0.2 µg pRL-TK plasmids (Promega, Madison, WI, U.S.A.) containing a *Renilla* luciferase gene by Lipofectamin 2000 (Invitrogen) in Opti-MEM (Gibco). In some experiments, cells were cotransfected similarly with NFAT5 expression vector, hPXR expression vector (pEF-hPXR: Tirona *et al.*, 2003), siRNA against NFAT5 (569R and inv569R: Na *et al.*, 2003), a combination of 4 gene-specific siRNAs (SMARTpool NFAT5: Dharmacon), the dominant negative NFAT5, or empty expression plasmids. At 24 h - 48 h post-transfection, cells were incubated in various experimental conditions for another 16-24 h unless otherwise stated. SMARTpool NFAT5 siRNA experiments were conducted as follows. Overnight-seeded HepG2 cells at 50% confluence were transfected for 48 h with 32.5 nM siRNA against NFAT5 (siNFAT5) or equal molar mismatched siRNA controls. These siRNAs were earlier suspended in liposome carrier Dharmafect 1,2,3,4 transfection reagent (Dharmacon) at 0.1 µL/nM siRNA concentration in serum-free Opti-MEM (Invitrogen). Cells were then treated with different tonicity for 16 h.

Luciferase activities of the cell extracts were determined using the Dual-Luciferase Reporter Assay System (Promega). Relative luciferase activity was calculated as observed relative light units from firefly luciferase normalized to *Renilla* luciferase values, and expressed as ratios to its minimal promoter construct under isotonic conditions, unless otherwise stated. In some

Manuscript # MOL 34504

experiments, the ratios were further normalized to those of the respective reporter in a control isotonic condition. All experiments were done in triplicate and repeated at least three times.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from confluent C₂bbe1 cells treated for 4h at NaCl-induced hyperosmolality (400 mOsm/kg) using Nuclear Extraction Kit (Panomics) according to the manufacture's instruction. EMSA was performed using the LightShift Chemiluminescent EMSA kit (Pierce) with modifications. 0.5 pmol of the 27bp 5'biotinylated probe (+5409/+5435 from CYP3A7 transcriptional start site) was used to detect protein/DNA interaction with 100x, 200x or 400x fold increase of competitor probes (unbiotinylated) or mutant (tAAaGagA-aG, where capitalized letters represent base changes and dash represents a 1 bp deletion from the original "tggaaagtac"). NFAT5 antibody used for supershift was from Affinity Bioreagents at a concentration of 2.5µL/reaction. The binding reaction consists of 10µg of nuclear extract, 1x binding buffer, 2µg of poly dI-dC, 3µg of random primers (invitrogen), 5mM MgCl₂, 0.05% NP40 and 1pmol of biotinylated probe, with or without the stated amount of competitor or mutant probes, or NFAT5 antibody, at a final volume of 10µL. Incubation was carried out for 40 min at room temperature for all reactions. The 6% PAGE was allowed to run for 2 hours before transfer to the nylon membrane followed by UV crosslinking (Ultraviolet Crosslinker, UVP). Detection by chemiluminescence was carried out according to manufacturer's instructions.

Chromatin immunoprecipitation (ChIP). ChIP assay was done using the ChIP kit (Upstate-cell signaling solutions: NY, USA). Briefly, C₂bbe1 cells were incubated under NaCl-induced hypertonic condition (400mOsm/kg) for 16 hours and proteins are crosslinked to DNA by 1% formaldehyde for 10 minutes at 37°C. The cells were then lysed in SDS and sonicated using a probe sonicator to obtain sheared DNA fragments ranging from 200 to 1000 bps. A 200µL aliquot was taken for subsequent reverse-linking with 8µL 5M NaCl, and the DNA phenol/chloroform extracted and ethanol precipitated. 1% of fraction was used as the input control. Another aliquot of 200µL was then diluted with the ChIP dilution buffer and incubated

Manuscript # MOL 34504

with salmon sperm DNA/protein A agarose beads for 1 hour at 4°C to remove non-specific DNA that initially binds to the beads. The supernatant was incubated with 2µL (1:500) rabbit polyclonal IgG NFAT5 antibody (SC-13035X: SantaCruz, CA, USA), CYP1A1 antibody (SC-20772: SantaCruz, CA, USA) as irrelevant target antibody or no antibody at 4°C overnight, and then fresh beads (60µL) were added with agitation at 4°C for 1 hour. The beads were washed twice according to the buffer systems supplied in the kit, and the protein/DNA complex is eluted with 250µL elution buffer (1% SDS, 0.1M NaHCO₃) and only 200µL supernatant is collected after 30 min shaking in room temperature. This step was repeated twice to obtain 400µL eluted samples. The samples were reverse-crosslinked with 20µL of 5M NaCl for >4 h at 65°C and treated with proteinase K. DNA of the samples was then phenol/chloroform extracted and ethanol precipitated using standard techniques. DNA was resuspended in 100 µL DEPC water for subsequent PCR reaction. All PCR reactions were done in a 50 µL reaction mix using Mastercycler (Eppendorf) with 1% template (1µL). All PCR conditions were as follows (95°C, 2 min); (95°C; 45 sec, 60°C; 1 min, 72°C; 30 sec) x 40 cycles; (72°C; 2 min) except for NFAT5 coding region (annealing temperature at 55°C; 1 min). The *CYP3A7* intron 2 TonE regions were assessed as fragment A and fragment B. Fragment A contains an antisense TonE motif +4688/+4698; and Fragment B with a sense TonE +5417/+5427. The primers for Fragment A (288 bp) are as follows: forward, 5'– gtc att tgc acc tgc ttg aa; and reverse, 5'– tgc atg tga ttc tgc ctt tg. Those for Fragment B (271 bp) were as follows: forward, 5'– aac agg ctt tgt gtg agc aa; and reverse, 5'– atg act tgt tcc tgc cct gt. For positive control, we detected a 194 bp PCR product of the SMIT promoter which contains an active TonE sequence at -21622/-21611 from the start site. This site was originally characterized as TonEp (Rim *et al.*,1998). The primers used for SMIT-TonEp site are: forward 5' - cgc gaa ggt ccc tag ctc; reverse 5' - gac cct gcc tgc ccc tac. NFAT5 coding region (exon 14: the third terminal exon lacking a TonE motif) was used as the negative PCR control (Lopez-Rodriguez *et al.*, 2001) with the following primers: forward, 5' gtt gcc atg cag agt aac tct and reverse 5' cat tgg att ttg att ggg ttg aat atc ctg for an 180 bp product.

Manuscript # MOL 34504

Statistical analysis. Results of mRNA levels are expressed as fold induction compared to isotonic conditions. Luciferase-based reporter activity is shown as ratios to appropriate controls as described above. Data are shown as mean \pm s.e.m., unless otherwise stated. When appropriate, data were analyzed by the Student's t-test or one-way ANOVA followed by Dunnett's multiple comparison test.

Manuscript # MOL 34504

Results

Increased ambient hypertonicity caused a substantial increase of CYP3A expression in the human intestinal C2bbe1 cells. In C₂bbe1 cells, a subclone of the Caco-2 human colon carcinoma cell line with relatively homogeneous brush-border epithelial characteristics, ambient hypertonicity (400 mOsm/kg) increased CYP3A mRNA expression by >10-fold in 12 h (Fig. 1a) followed by an increase in protein levels (Fig. 1b). (Note that C2bbe1 cells have wild-type CYP3A7*1A/*1A and CYP3A5*3/*3 genotypes: personal communication from Dr. C. Vyhldal). Relative baseline mRNA levels of each CYP3A in C2bbe1 cells were roughly 1 : 3 : 0.5 (CYP3A4 : CYP3A5 : CYP3A7). The dose response of tonicity-induced mRNA levels was also evident in hypertonic conditions created by adding NaCl or sucrose (Fig 1c). In contrast, the tonicity-neutral membrane-permeable compound, glycerol, did not have such an effect, which was also reflected in protein expression (Fig. 1d). NFAT5 is accumulated in the nuclear compartment in 4 h post hypertonic treatment with NaCl or sucrose, showing clear demarcation of the nuclei, whereas cytoplasmic NFAT5 remains to be seen in cells with isotonic or glycerol treatment, obscuring cytoplasm-nuclear boundaries (Fig. 1e). In addition, hypertonicity did not significantly prolong CYP3A mRNA decay in the presence of actinomycin D (data not shown), suggesting that hypertonicity-triggered *CYP3A* gene expression cannot be explained by increased mRNA stability. Together with CYP3A, intestinal xenobiotics transporters such as P-glycoprotein (MDR1) and breast cancer resistance protein (BCRP: ABCG2) constitute a functional unit of drug and toxin absorption barrier. However, in contrast to CYP3A and an osmo-sensitive gene, SMIT, hypertonicity caused only marginal changes, if any, in expression of these transporters (Fig 1f).

Osmotic stress (i.e., hypertonicity) provokes the activation of NFAT5, which binds to its cognate response DNA element, called tonicity-responsive enhancer (TonE), thereby facilitating transcription of target genes such as SMIT. To investigate NFAT5-dependency of tonicity-induced CYP3A expression, we examined tonicity responses of C₂bbe1 cells transfected with

Manuscript # MOL 34504

NFAT5 expression plasmids (Fig. 1g). CYP3A and SMIT (positive control) mRNA expressions were increased in parallel with increased NFAT5 levels at isotonic condition (CYP3A4: 2.54 ± 0.47 , $p=0.046$; CYP3A7: 2.32 ± 0.17 , $p=0.03$; CYP3A5: 2.08 ± 0.16 , $p=0.01$; SMIT: 1.68 ± 0.07 , $P<0.01$; fold-increase compared to empty vector transfection, $M \pm SEM$, $n=4$). This trend was also observed in hypertonic conditions, but statistical significance was not reached due to large variations among cell preparations. On the other hand, hypertonicity/NFAT5-responsiveness in mRNA expression of the known CYP3A regulators, PXR, CAR and VDR, was unremarkable (Fig 1g).

To further characterize involvement of NFAT5 in CYP3A expression, loss-of-function assays were conducted using 2 different siRNA approaches against NFAT5: a combination of 4 different siRNA against NFAT5 (siNFAT5: see Methods), or siRNA_{569R} (Na *et al.*, 2003). Because conditions could not be sufficiently optimized for C2bbe1 cells in these experiments, we used HepG2 cells. As shown in Fig 1h, siRNA-mediated knockdown of NFAT5 with siNFAT5 caused significant reduction in CYP3A mRNA levels in both isotonic and NaCl-induced hypertonicity. NFAT5 knockdown using 569R NFAT5 siRNA (Na *et al.* 2003) showed similar tendency under hypertonicity conditions (Fig 1h: inset), although the magnitude of reduction was milder than siNFAT5. In these experiments, siRNA_{569R} caused approximately 50% reduction of NFAT5 mRNA, and siNFAT5 caused ~80% reduction (not shown). Taken altogether, these findings indicate that human CYP3A expression is influenced by a tonicity-driven mechanism, possibly involving NFAT5.

CYP3A expression parallels ambient tonicity in human-derived cells. NFAT5-mediated gene control is bidirectional (Woo *et al.*, 2000). In other words, levels of target genes increase as ambient tonicity increases, while they decrease as ambient tonicity decreases. Changes of CYP3A expression in C2bbe1 cells followed this pattern (Fig. 2: left column), paralleling ambient tonicity changes from hypo- (200 mOsm/kg) to hyper-tonicity (450 mOsm/kg). So were other human cells

Manuscript # MOL 34504

(primary human colon epithelia, a colon carcinoma-derived cell line [LS180], and a hepatoma-derived cell line [HepG2]) (Fig. 2), suggesting that this is likely a universal phenomenon among human cells. Unlike human CYP3A, however, mouse *Cyp3a13* mRNA levels changed inversely to ambient tonicity, whereas *Smit*, an established NFAT5 target gene, showed similar responses in human (Fig. 2) and mouse cells (intestinal CMT93 and hepatic Hepa1c1c7 cells: not shown). It is unclear if the tonicity effect on mouse *Cyp3a13* represents a system-wide phenomenon in mice, or a response specific to *Cyp3a13*, as other mouse *Cyp3a* mRNAs were undetectable or not examined in these cell lines.

Tonicity-responsive enhancer (TonE) is located in the *CYP3A7* intron 2 region. We sought the *CYP3A* gene locus for existence of any tonicity-responsive enhancer (TonE), which is characterized by an 11 bp consensus sequence (tggaanNNYNY [N: any nucleotide; and Y: any pyrimidine]). Analyses of the genome database revealed that there are 85 consensus TonE motifs in the *CYP3A* gene cluster. In our experiments (Fig. 1 and 2), tonicity dependency was observed in all 3 major CYP3As, and therefore, we postulated that a functional TonE sequence is located in a relative vicinity of the transcription start site of each *CYP3A* gene cassette. In order to identify a responsible TonE(s) in the 230 kb-wide *CYP3A* gene locus, our first approach was to screen ± 10 kb of the transcription start site of the 3 major *CYP3As*. As shown in Fig 3a, 11 sense and 2 anti-sense TonE consensus sequences were located in these regions (*CYP3A4*: 3 sense and 1 anti-sense; *CYP3A5*: 4 sense; and *CYP3A7*: 4 sense and 1 anti-sense). Of those, 6 putative TonE consensus motifs were localized in the -10kb of the 5'-flanking region of each gene; one motif for *CYP3A4* (-7913/-7903 from the *CYP3A4* transcription start site); two for *CYP3A7* (-7900/-7890, and -551/-541); and three for *CYP3A5* (-6341/-6331, -3051/-3041, and -1924/-1914). Some of their localizations were close to XREM and ER6, which contain known enhancers *trans*-activated by PXR and CAR. However, luciferase reporter assays in C2bbe1 cells showed that constructs containing these motifs (Fig. 3a: *CYP3A4* [-7979/-7140], [-7979/-7831], [-7850/-7140], [-638/+53]; *CYP3A7* [-7994/-7155], [-7994/-7831], [-630/+55], [-7856/-7155]; and *CYP3A5* [-

Manuscript # MOL 34504

6543/-5985], [-3245/-2940], [-2235/-1862]), driven by SV40 promoter or each *CYP3A* promoter, were unresponsive to hypertonicity (data not shown). In order to further explore these negative findings, we examined tonicity responsiveness of the 2 reporter constructs, *CYP3A4*_[-10466/+53] and *CYP3A7*_[-9302/+53] (Bertilsson *et al.*, 2001), which span approximately 9 kb of the 5'-flanking regions of *CYP3A4* or *CYP3A7*, respectively. As shown in Fig. 3b, these reporters were unresponsive to hypertonic stimuli in hepatic HepG2 cells. In contrast, rifampicin-induced PXR-dependent responses were clearly observed as expected, indicating that the PXR-mediated pathway is intact in these constructs. These reporters transfected in C2bbe1 cells were not responsive to either rifampicin ± PXR or hypertonicity treatment (not shown). Taken together, these findings suggest that a tonicity responsive element does not exist at least in the -10 kb 5'-flanking regions of the *CYP3A* genes.

We then examined the remaining 7 TonE consensus motifs within 10kb downstream from the transcription start site of each of those *CYP3A* genes (Fig. 3a): two sense TonE sequences in the *CYP3A4* exon 3/intron 3 (+6144/+6154, and +6169/+6179 from the *CYP3A4* transcription start site), 1 antisense sequence in the *CYP3A4* intron 2 (+5636/+5646), 2 sense sequences in the *CYP3A7* intron 2 (+5076/+5086 and +5417/+5427 from the *CYP3A7* transcription start site), 1 antisense sequence in the *CYP3A7* intron 2 (+4688/+4788); and one sense sequence in *CYP3A5* exon 3 (+5437/+5447 from the *CYP3A5* transcription start site). Screening with SV40 promoter-driven luciferase reporters in C2bbe1 cells (Fig. 3a: *CYP3A4*_[+5971/+6352], *CYP3A7*_[+4910/+5590], and *CYP3A5*_[+5318/+5669]) showed that only *CYP3A7*_[+4910/+5590] with the *CYP3A7* intron 2 sense TonE sequences resulted in robust responses under isotonic conditions, and more so after hypertonicity exposures (not shown). We then further examined comparable regions of *CYP3A4* and *CYP3A7* around 5-6 kb downstream of the transcription start sites (Fig. 3a). The *CYP3A4*_[+5971/+6352] reporter driven by the *CYP3A4* promoter, which contains 2 TonEs at +6144

Manuscript # MOL 34504

and +6169, was not responsive (Fig. 3c). Moreover, the *CYP3A4*_[+5403/+6429] construct containing the 3 TonEs (+5636, +6144, and +6169) did not show activity (Fig. 3c: +*CYP3A4*_[+5403/+6429]). In contrast, the *CYP3A7*_[+4910/+5910] with the 2 sense intronic TonEs (*CYP3A7* +5076 and +5417: see Fig. 3a), driven by the *CYP3A7* promoter, was active under isotonic condition, and responded to hypertonicity. However, inclusion of the antisense TonE (*CYP3A7* +4688) did not modify tonicity responsiveness (Fig. 3c: +*CYP3A7*_[+4658/+5587]), and the reporter containing the antisense TonE (*CYP3A7* +4688) alone was inactive (Fig. 3c: +*CYP3A7*_[+4658/+4909]). These findings indicate that the *CYP3A7* intron 2 region containing the 2 sense TonE motifs (+5076/+5086 and +5417/+5427) has a tonicity-responsive enhancer activity.

Transactivation of *CYP3A7* intron 2 TonE is dependent on NFAT5. The *CYP3A7* intron 2 region (+4910/+5590) was placed in each of the *CYP3A* promoters in luciferase constructs and tested for its transactivation activity with and without NFAT5 expression vector cotransfection (Figure 3d). Results showed that this region is capable of activating all *CYP3A* promoter constructs in response to hypertonicity or NFAT5 overexpression. Loss-of-function assays were also conducted using the *CYP3A7*_[+4910/5590] luciferase reporter cotransfected with dominant-negative NFAT5 (dnNFAT5_{Δ1-156}: Tong *et al.*, 2006) or empty pFLAG-CMV-2 expression vector (pFLAG). As shown in Fig. 3e, dnNFAT5_{Δ1-156} decreased reporter activity in both isotonic and hypertonic conditions (p<0.01; n=4). Similarly, reporter activity was examined with siRNA_{569R} against NFAT5 (Na *et al.*, 2003). siRNA_{569R} significantly reduced reporter activity (p<0.05; n=4: Fig. 3e). In these experiments, siRNA_{569R} caused approximately 50% reduction of NFAT5 mRNA (not shown). Together, these findings suggest that *CYP3A7* intron 2 region may work in conjunction with other *CYP3A* promoters and that NFAT5 is required for enhancer activation.

The TonE motif at +5417 in the *CYP3A7* intron 2 region is required for transactivation.

Serial deletions of the *CYP3A7* intron 2 fragment were conducted to determine the minimal

Manuscript # MOL 34504

sequence responsible for enhancer activity (Fig 4a). Compared to the *CYP3A7*_[+4910/+5590] reporter plasmid with two TonE sense motifs, serial upstream deletions showed a gradual reduction in reporter signals. Constructs without the upstream TonE motif (constructs +5088/+5590, and +5361/+5590) still retained the reporter activity, suggesting that the upstream TonE motif and neighbouring regions are dispensable, but required for a full response. We found that the reporter +5361/+5590 was the shortest construct that responds to hypertonicity or NFAT5. Further deletion (construct +5428/+5590), which is devoid of both TonE motifs, abolished the response. This observation supports that the downstream motif (+5417/+5427) is required in the NFAT5-mediated transactivation. This notion is further supported by the +4910/+5204 construct, where the upstream motif without the downstream segment was unresponsive. This indicates that the upstream TonE motif and immediate neighbouring regions are insufficient for enhancer activity. Furthermore, mutations in the +5417/+5427 TonE motif (tggaagtAA; the 2 “A”s in the mutant represent adenine replacing thymine and cytosine, respectively) drastically reduced tonicity responsiveness (Fig. 4b), reaffirming requirement of the downstream motif for robust enhancer activity. General enhancers have the ability to transactivate promoter constructs independent of their location. To test if the *CYP3A7* intron 2 region has such an enhancer characteristics, it was placed downstream of the reporter gene (+4910/+5590 3' position). Note that the *CYP3A7*_[+4910/+5490] 3' position reporter construct may mimic actual *CYP3A7* promoter – intron 2 DNA configuration to some extent. Results show that this reporter plasmid still retained tonicity/NFAT5 responsiveness, although the magnitude of the response was smaller (Fig 4b).

NFAT5 specifically binds to the *CYP3A7* intron 2 TonE at +5417. EMSAs showed in Fig. 5 that competition by unlabeled TonE DNA sequence (lanes 2 and 3), lack of effects of mutant TonE (lanes 4 and 5) and shift of NFAT5-TonE complex by NFAT5 antibody (lane 6), indicating specific binding of NFAT5 to the DNA sequence containing the +5417 TonE motif. The mutant

Manuscript # MOL 34504

TonE sequence shown to have low but detectable activity in the earlier reporter assay (Fig. 4b) was weakly competitive in inhibiting NFAT5-TonE binding in EMSA (not shown). To further explore NFAT5 binding to the *CYP3A7* intron 2 TonE site at +5417 in a native chromatin context, ChIP assay was performed (Fig. 6). As shown in Fig. 6a, Fragment B represents a DNA region surrounding the active TonE (+5417), while Fragment A contains a region with an antisense TonE further upstream (+4688) which is inactive in luciferase reporters (Figure 3C; *CYP3A7* [_{+4658/+4909}]). In our ChIP assay, Fragment B containing the TonE at +5417 is clearly detectable, and so is the TonE motif located upstream of the *SMIT* promoter (Rim *et al.*, 1998), one of the NFAT5 target genes (Figure 6b, lane 1). Neither the TonE-absent exon 14 region of *NFAT5* gene (Fig. 6b, lane 4) nor antibody against CYP1A1 (Fig. 6b, lane 2 and 5) produced an amplicon, validating specificity of NFAT5-TonE binding in our assay. Moreover, Fragment A containing the neighbouring inactive TonE motif is undetectable (Figure 6c). Taken together, this is consistent with the notion that NFAT5 binds to the *CYP3A7* intron 2 region at +5417 in a native DNA setting.

Manuscript # MOL 34504

Discussion

We have discovered that the expressions of human CYP3A4, CYP3A5 and CYP3A7 are under the influence of ambient tonicity. Moreover, the phenomenon is not restricted to immortalized cell lines, as it is also seen in primary colonic cells (Fig. 2), suggesting that this unexpected link between ambient osmotic environment and human CYP3A expression may represent a process of physiological significance. Although highly speculative, CYP3As may mediate osmolyte production, or catabolism of osmolytes to counter-balance increased intracellular concentrations of organic osmolytes upon ambient hypertonicity challenges. Further studies are required to elucidate biological and *in vivo* significance of this phenomenon. This is important particularly because basal CYP3A expressions in these cells *in vitro*, including primary cells, are lower than those *in vivo*.

Our data suggest that the increased CYP3A expression is the result of transcriptional activation mediated by NFAT5. Several transcriptional factors, such as PXR and CAR, have been well established in the *CYP3A* regulation network. It remains to be defined if the tonicity-mediated CYP3A expression is modified by other transcription factors. Specifically, it awaits further studies to elucidate a hierarchy of these factors in regulation of CYP3As, which determines system characteristics such as additivity, synergism, or antagonism among them. As shown in Fig 1e, ambient hypertonicity may cause mild alterations in mRNA expression for some of these transcription factors, which may contribute to tonicity-triggered CYP3A induction. However, relatively rapid induction of CYP3A mRNA within 4 hours by ambient hypertonicity (Fig 1a) suggests a direct tonicity-mediated transcriptional induction rather through a secondary mechanism, if any.

Compared to human CYP3As, responses of mouse *Cyp3a13* were exactly opposite in the two cell lines tested (CMT93 rectal cells and hepe1c1c7 liver cells), although mouse *Smit*, a known NFAT5 target gene, responded in the same manner as does human SMIT. Presently, tonicity responses of other mouse *Cyp3a* remain elusive, because only *Cyp3a13* was detectable in this

Manuscript # MOL 34504

study. No orthologous *CYP3A* pair exists between mouse and human, suggesting a species-specific independent expansion of the ancestral *CYP3A* gene cassette over the last 75 million years (Nelson *et al.*, 2004). Rodent and human *CYP3A* share many regulatory factors such as the PXR and CAR, but distinct species differences still exist. Whether mouse *Cyp3a* in vivo similarly responds to tonicity remains to be examined.

Mammalian cellular responses to osmotic stresses are mediated by the osmoregulatory transcription factor called tonicity enhancer binding protein (NFAT5) (reviewed by Ho SN, 2006). Upon activation by increased osmotic pressure, NFAT5 is translocated to the nucleus and binds to the tonicity responsive-enhancer (TonE) of target genes. Our NFAT5 siRNA experiments (Fig. 1h) suggest that NFAT5 plays a central role in the tonicity-CYP3A pathway. There are 13 consensus TonE motifs within the ± 10 kb from the transcription start sites of the *CYP3As* (Fig. 3a). The present study shows that among these DNA elements, only the 0.7 kb region within *CYP3A7* intron 2 has robust enhancer activity, responsive to hypertonicity and NFAT5 overexpression or knockdown (Fig. 3b-e). Deletion and mutation analyses showed that the *CYP3A7* intron 2 TonE at +5417 is indispensable for the minimal enhancer activity, although a full response requires the neighbouring region (Fig. 4a, b). Using EMSA and ChIP assays, we further showed NFAT5-binding to this TonE motif (Fig. 5 and 6). Altogether, our findings indicate specific binding of NFAT5 to the region surrounding *CYP3A7* intron 2 TonE at +5417 in a native cell context.

The *CYP3A7* intron 2 region containing the responsive TonE site (+5417) placed immediate 3'-side of the luciferase reporter gene (*CYP3A7*_[+4910/+5590] 3'position) in order to mimic the natural genomic configuration is responsive to hypertonicity and NFAT5 over-expression (Fig. 4b). An enhancer located in the intron 3 of the human TNF- α gene, which interacts with the TNF- α promoter, has been characterized in a similar experimental approach (Barthel and Goldfeld, 2003). The magnitude of response of the *CYP3A7*_[+4910/+5590] 3'position, however, was lower than

Manuscript # MOL 34504

when placed at 5' of the *CYP3A7* promoter (Fig. 4b). It is presently unknown whether this quantitative difference is of any biological significance in native DNA context, or simply an experimental limitation in an artificial environment of gene reporter assays.

Our studies show that expressions of *CYP3A4*, *CYP3A5* and *CYP3A7* are all dependent on ambient tonicity, and that the *CYP3A7* intron 2 region houses the only TonE consensus motif within the \pm 10kb from the transcription start sites of the *CYP3As*. Based on these findings, we speculate that the *CYP3A7* intronic TonE segment serves as an enhancer for *CYP3A7*, and possibly as a long-range enhancer for *CYP3A4* and *CYP3A5* as seen in some genes including *β -globin*. Alternatively, there may be unidentified TonEs, or similar enhancers, separately for each *CYP3A* gene. Involvement of other transcriptional factors in this phenomenon is largely speculative at this point. We think that at least PXR is not involved because overexpression of PXR does not increase tonicity-responses of *CYP3A* mRNA (not shown), and because XREM-containing 5'-flanking regions of *CYP3A4* and *CYP3A7* do not respond to tonicity (Fig 3b). If PXR, CAR or VDR is involved, lack of tonicity responses in genes such as *MDR1* is also difficult to explain. Further studies are required to address these questions.

The implications of our findings are several-fold. First, because NFAT5 shows basal activity under physiological osmolality (Miyakawa *et al.*, 1999; Woo *et al.*, 2000), normal osmotic environment may be one of the baseline stimuli for *CYP3A* expression. This implies that the tightly-regulated constitutive osmotic environment provides consistency and stability to the basal transcriptional drive, thereby sustaining *CYP3A* basal expression. Second, intestinal *CYP3A* expression may be affected through this pathway by osmolality/tonicity changes within the intestinal lumen due to food or fluid intake. Intestinal lumen faces periodic surges of osmolality related to food intake (Haupt, 1991; Kalantzi *et al.*, 2006; Ladas *et al.*, 1983). Therefore, *CYP3A* mRNA expression in the intestine may increase after food intake, which then elevates *CYP3A* protein levels. This may explain clinical observations where human subjects receiving high-salt diets for 7 to 10 days showed significant increase in presystemic elimination and metabolite

Manuscript # MOL 34504

formation of orally-administered CYP3A substrates: quinidine, and verapamil (Darbar *et al.*, 1997 and 1998). Third, the mechanism of urine volume control by NFAT5, which is exemplified in dehydration (Lam *et al.*, 2004), may be supported by an increased CYP3A5 level because of its role in renal Na⁺ and water retention (Givens *et al.*, 2003; Kuehl *et al.*, 2001; Thompson *et al.*, 2004). Given the potential role of kidney-predominant CYP3A5 in salt retention and hypertension by converting corticoids to 6 β -hydroxysteroids with mineralocorticoid action, this pathway is likely to be part of a regulation loop for salt homeostasis. The renal NFAT5-CYP3A5 pathway may be crucial to the hypertension pathogenesis theory associated with genetic variation in renal CYP3A5 expression (Kuehl *et al.*, 2001).

In summary, we discovered the ambient tonicity-driven, NFAT5-mediated expression of human CYP3A4, CYP3A5 and CYP3A7. Whether the CYP3A7 intron 2 TonEs are responsible for propagation of the tonicity-NFAT5 signal toward all 3 isoforms of CYP3A await further studies.

Manuscript # MOL 34504

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Manuscript # MOL 34504

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Manuscript # MOL 34504

FOOTNOTES

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Manuscript # MOL 34504

FIGURE LEGEND

Figure 1. Ambient hypertonicity induces CYP3A expression in C₂bbe1 human intestinal cells. **a**, Time course of gene expression induced by ambient hypertonicity. C₂bbe1 cells were cultured under hypertonic conditions (400 mOsm/kg), and mRNA levels of CYP3A4 (closed circle), CYP3A7 (open circle), CYP3A5 (open square), SMIT (closed square) and NFAT5 (open triangle) were measured with real-time PCR. Results are normalized to respective GAPDH levels, and expressed as ratios to the value at time 0 of each gene (mean \pm s.e.m.: n=3). **b**, Time-dependent expression of CYP3A protein in hypertonic (400 mOsm/kg) or isotonic (300 mOsm/kg) conditions. Cell lysates were obtained from C₂bbe1 cells incubated for the indicated periods, and immunoblotting was performed. **c**, Dose-response of mRNA levels to increasing ambient osmolality (left: isotonic 300 mOsm/kg; middle left: 350 mOsm/kg; middle right: 400 mOsm/kg; and right: 450 mOsm/kg). C₂bbe1 cells were treated for 24 h in media of increasing osmolality using NaCl (white bars), sucrose (black bars) or glycerol (shaded bar), and mRNA levels were measured with real-time PCR. GAPDH-standardized results are expressed as ratios to those of respective isotonic condition (mean \pm s.e.m.: n=3). **d**, Solute-dependent increase of NFAT5 and CYP3A protein expressions in hyperosmotic conditions. Western blotting was performed on cell lysates obtained from C₂bbe1 cells after 24 h incubation under abovementioned isotonic or hyperosmolality conditions (400 mOsm/kg) created with different solutes: NaCl, sucrose or glycerol. **e**, Immunohistochemical detection of NFAT5 intracellular distribution. After 4 h treatment with iso- or hyper-osmolality medium (400 mOsm/kg), C₂bbe1 cells were fixed, incubated with the NFAT5 antibody, and visualized with Cy3 conjugated anti-rabbit IgG. Arrows show NFAT5 staining, which is confined almost exclusively in the nuclear compartment under NaCl or sucrose treatment, but is scattered in cytoplasm in isotonic or glycerol treatment. **f**, mRNA expression of transcription factors and intestinal xenobiotics transporters upon hypertonicity challenges. C₂bbe1 cells were incubated in hypertonic conditions

Manuscript # MOL 34504

for 24 h, and mRNA levels were quantified with real-time PCR. GAPDH-standardized results are expressed as ratios to those of respective isotonic condition (mean \pm s.e.m.: n=3). **g**, NFAT5/tonicity-responsiveness of mRNA expressions of CYP3As, SMIT, PXR and CAR. C2bbe1 cells transfected with NFAT5-pTARGET or pTARGET empty vector were grown in regular isotonic or hypertonic (400 mOsm/kg) medium for 24 h. mRNA was extracted and measured with real-time PCR. Results are normalized to individual GAPDH, and expressed as ratios to those of isotonic conditions with pTARGET empty vector (mean \pm s.e.m.: n=4). **h**, Effects of NFAT5 knockdown with siRNA on mRNA expressions of CYP3As and SMIT in HepG2 cells. HepG2 cells were treated with siRNA NFAT5 (siNFAT5) or control (mismatch non-target siRNA) for 48 h, and incubated for another 16 h with isotonic or NaCl-induced hypertonic medium. Results are standardized to respective GAPDH levels, and expressed as ratios to the value of each gene in cells transfected with mismatch non-target siRNAs under isotonic conditions (dotted line), and shown as mean \pm s.e.m.: n=3 (*p<0.05; **p<0.01). *Inset*: Similar treatment with siRNA 569R against NFAT5 or control (inv569R), followed by hypertonic treatment for 18 h. Representative figures are shown.

Figure 2. Ambient tonicity elicits parallel changes in mRNA levels of CYP3As in human cell lines and primary human colonic cells. CYP3A mRNA levels in human-derived cell lines and primary colonic cells in response to ambient hyper- and hypo-tonicity. Cells were incubated for 24 h in media with osmolality ranging from high (400 mOsm/kg) to low (200 mOsm/kg) levels. mRNA was extracted and measured with real-time PCR. Red bar 1: +50 mM NaCl (400 mOsm/kg); red bar 2: +100 mM sucrose (400 mOsm/kg); open bar 3: medium/PBS (67/33 v/v%: isotonic 300 mOsm/kg); and blue bar 4: medium/water (67/33 v/v%: hypotonic 200 mOsm/kg). Results are standardized to respective GAPDH levels, and expressed as ratios to the value of each gene in control isotonic condition (mean \pm s.e.m.: n=3).

Manuscript # MOL 34504

Figure 3. Tonicity-responsive enhancer (TonE) exists in *CYP3A7* intron 2. **a**, A scheme of the *CYP3A* gene cluster and corresponding reporter constructs, which shows the ± 10 kb of the *CYP3A* transcriptional start sites. Eleven sense and 2 antisense consensus TonE motifs in the regions are depicted as an open column on either upper (sense) or lower (antisense) side of the genome (horizontal line) with the starting 5' (sense TonE) and 3' - base pair positions (antisense TonE) relative to the transcription initiation site of each *CYP3A* gene. The reporter constructs (thick horizontal lines with or without TonE as a white dot) are shown with the 5'-/3'- ends of the sequence. The constructs with an asterisk (*) were responsive to hypertonicity and NFAT5. **b**, Activity of the reporter constructs encompassing 9-10 kb of the 5'-flanking regions of *CYP3A4* or *CYP3A7*. Luciferase reporter assays were conducted in hepatic HepG2 cells with cotransfection of human PXR or empty expression vector after 24 h incubation in the presence of 10 μ M rifampicin (solid bar) or 50 mM NaCl (shaded bar: hypertonic 400 mOsm/kg). The normalized reporter responses are shown here as a ratio to the respective reporter activity value in control isotonic conditions (open bar). Results are expressed as mean \pm s.e.m. (* $p < 0.05$, ** $p < 0.01$; n=6-9). **c**, Reporter assays for constructs harbouring regions within the transcription units of *CYP3A4* and *CYP3A7*. Activity of the reporter constructs was measured in C2bbe1 cells after 24 h of control isotonic (open bar) or hypertonic conditions (solid bar). Results are expressed as ratios to activity of the respective minimal promoters (mean \pm s.e.m.: n=3). **d**, Activity of the reporter construct containing the *CYP3A7* intron 2 region (+4910/+5590) with different *CYP3A* promoters in hypertonic or NFAT5 overexpression conditions. C2bbe1 cells were co-transfected with respective reporter constructs and either NFAT5 expression plasmids (shaded bar) or empty vector (open and solid bars). After 24 h incubation under isotonic (open or shaded bar) or hypertonic (solid bar) condition, luciferase activity was measured. In this experiment, results are expressed as values relative to those of the promoter-less construct with empty expression plasmid under isotonic condition (mean \pm s.e.m.: n=3). **e**, Loss-of-function assays on the

Manuscript # MOL 34504

*CYP3A7*_[+4910/+5590] reporter using dominant negative (dnNFAT5: upper) or siRNA (lower) against NFAT5. C2bbe1 cells were cotransfected with the reporter plasmid and either dnNFAT5_{Δ1-156} (open bar) or pFLAG empty vector (solid bar). On another experiments, siRNA_{569R} (open bar) or an inverted 569R sequence (siRNA_{inv569R}: solid bar) was used. Cells were treated with isotonic or hypertonic medium for 24 h. Results are normalized against the values of the *CYP3A7* minimal promoter reporter under isotonic conditions (means ±SEM, *p<0.05; **p<0.01, compared to respective controls: n=4).

Figure 4. The *CYP3A7* intron 2 TonE (+5417) is indispensable for the enhancer activity. a, Deletion analyses of the *CYP3A7* intron 2 region, using the reporter *CYP3A7*_[+4910/+5910], which harbours an upstream (+5076, open triangle) and downstream (+5417, black triangle) TonE site. C2bbe1 cells co-transfected with the reporter constructs and either NFAT5 expression vector or empty plasmid were treated with isotonic or hypertonic conditions for 24 h. The 5' -/3'-ends of the construct sequences are shown on the left side as base positions from the *CYP3A7* transcription start site. Luciferase activities are expressed as ratios to those of the minimal promoter in isotonic conditions (means ± s.e.m.: n=3). **b,** Mutation and positioning analyses of the *CYP3A7* intron 2 region. The mutant downstream TonE (shaded triangle labelled “*mut*”), the reversed configuration and the 3'-positioned constructs were similarly tested in C2bbe1 cells. Results were normalized to that of *CYP3A7* minimal promoter under isotonic conditions (means ± s.e.m.: n=3).

Figure 5. NFAT5 binds to the *CYP3A7* intron 2 TonE motif (+5417/+5427) in *in vitro* binding assay. Nuclear extracts from C₂bbe1 cells treated with hypertonic medium for 4 h were incubated with biotinylated DNA probes derived from the *CYP3A7* intron 2 TonE motif (+5417) containing the surrounding region (+5409/+5435). EMSA was conducted with increasing concentrations of unlabeled competitor DNA or mutant DNA probes (see Methods). TonE probe

Manuscript # MOL 34504

signals in the absence of competing or mutant DNA sequences (lane 1), and those in the presence of increasing concentrations of the competing unlabelled TonE region (lane 2 and 3) or mutant DNA sequence (lane 4 and 5) are shown (open triangle). A super-shift band driven by NFAT5 antibody is demonstrated (solid triangle: lane 6). A representative figure is shown.

Figure 6. ChIP assay for NFAT5 binding to the *CYP3A7* intron 2 TonE motif (+5417) in

native cell context. a, A scheme of the *CYP3A7* intron 2 region shown with TonE motifs.

Fragment A and B were amplified in ChIP assay. Fragment A contains an inactive antisense

TonE at +4688, and Fragment B holds the functional TonE at +5417. **b,** NFAT5 binding to

Fragment B containing the TonE motif at +5417. *C₂bbe1* cells exposed to NaCl-induced

hypertonicity (400 mOsm/kg) for 16 h were subjected to ChIP analyses using NFAT5 antibody to

immunoprecipitate and amplify 271 bp Fragment B (see Methods). Output lanes: 1 and 4, NFAT5

antibody; 2 and 5, CYP1A1 antibody; and 3 and 6, no antibody. A 180-bp region within exon 14

of NFAT5 was used as a PCR negative control. **c,** NFAT5 binding to a TonE site of SMIT, one of

the NFAT5 target genes. *C₂bbe1* cells were similarly treated and a 194 bp sequence was

amplified after immunoprecipitation using NFAT5 antibody, CYP1A1 antibody or no antibody.

Lane designations are the same as above. **d,** Binding of NFAT5 is specific to TonE motif at

+5417 within Fragment B. *C₂bbe1* cells were treated similarly, and NFAT5-DNA complex was

immunoprecipitated using NFAT5 antibody, negative control antibody (CYP1A1 ab) or no

antibody. Fragment A with inactive TonE and Fragment B with functional TonE were subjected

to PCR amplification. **a-c,** Representative results of 2-4 experiments are shown.

Figure 1a-e

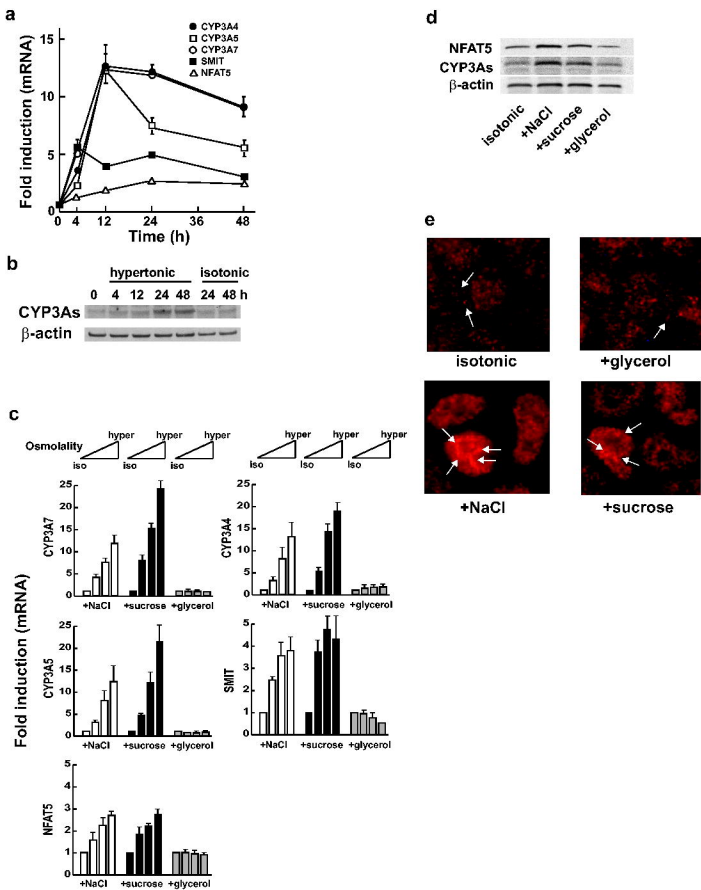
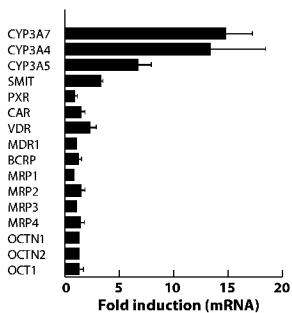
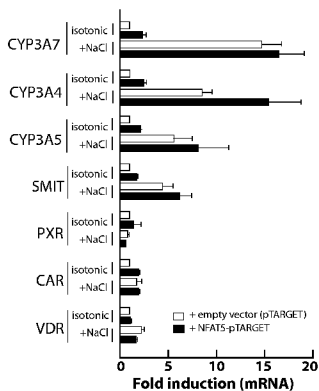


Figure 1f, 1g and 1h

f



g



h

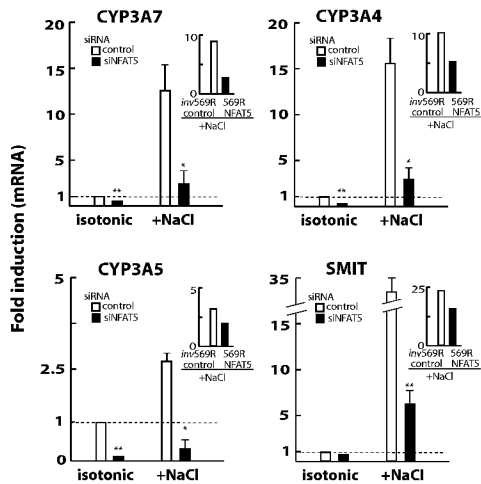


Figure 2

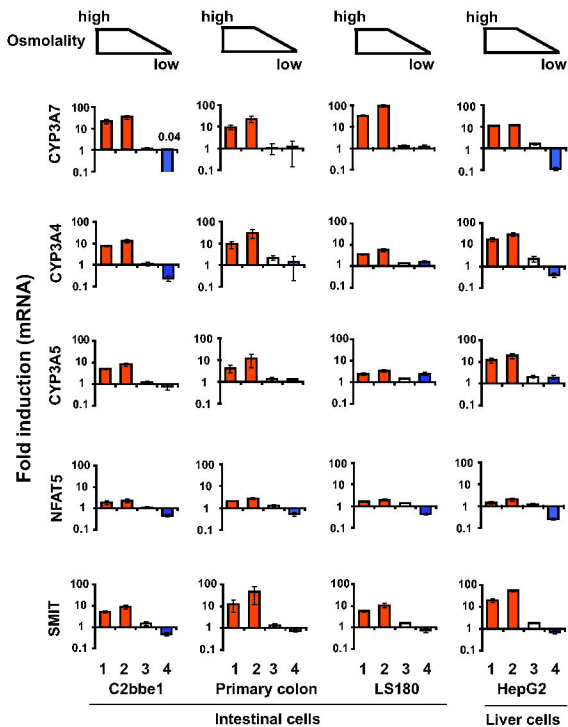
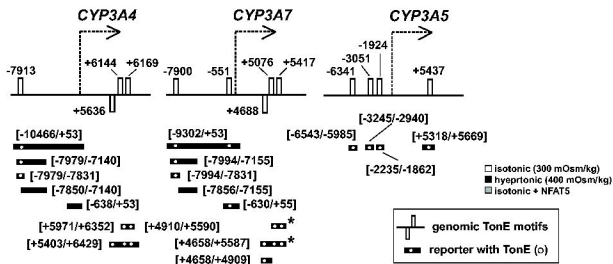
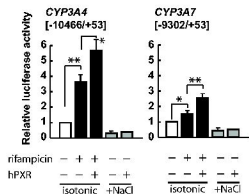


Figure 3

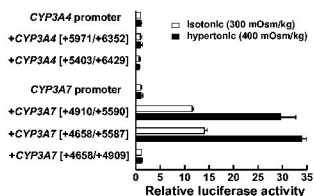
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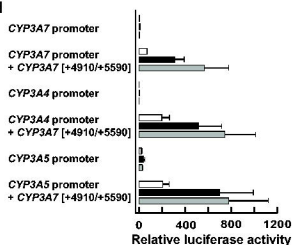
b



c



d



e

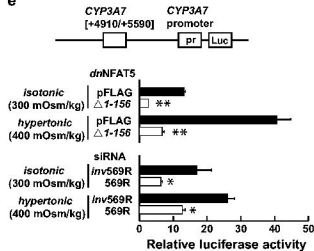
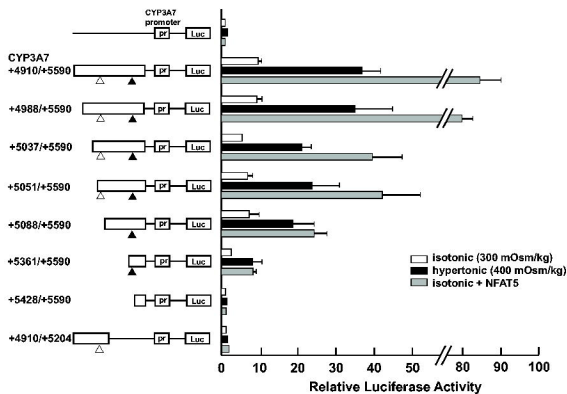


Figure 4

a



b

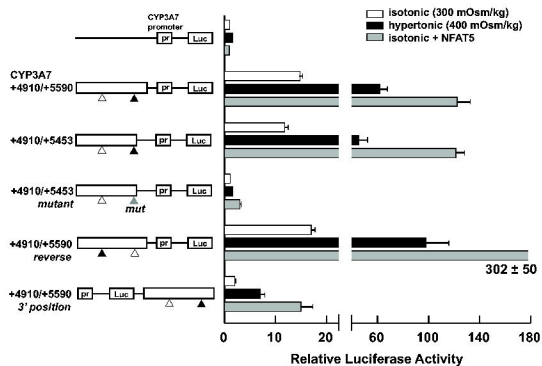


Figure 5

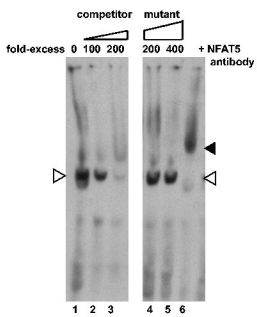


Figure 6

