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Pharmacological premature termination codon readthrough of *ABCB11* in bile salt export pump deficiency: an in vitro study

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List of Abbreviations: 4-PB, 4-phenylbutyrate; BA, bile acids; BSEP/Bsep, bile salt export pump; GFP, green fluorescent protein; TC, taurocholate; HEK293, Human embryonic kidney 293; LT, liver transplantation; MDCK, Madin-Darby canine kidney; MRP2, multidrug resistance protein 2; NMD, nonsense mediated mRNA decay; Ntcp, Na^+-taurocholate co-transporting polypeptide; PFIC2, progressive familial intrahepatic cholestasis type 2; PTC, premature termination codon; RT, readthrough; UDCA, ursodeoxycholic acid; wt, wild-type; ZO-1, zonula occludens 1.

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Abstract

Background & Aims**:* Progressive familial intrahepatic cholestasis type 2 (PFIC2) is a severe hepatocellular cholestasis due to biallelic mutations in *ABCB11* encoding the canalicular bile salt export pump (BSEP). Nonsense mutations are responsible for the most severe phenotypes. The aim was to assess the ability of drugs to induce readthrough of six nonsense mutations (p.Y354X, p.R415X, p.R470X, p.R1057X, p.R1090X and p.E1302X) identified in PFIC2 patients. ***Approach & Results: The ability of G418, gentamicin and PTC124 to induce readthrough was studied using a dual gene reporter system in NIH3T3 cells. The ability of gentamicin to induce readthrough and to lead to the expression of a full-length protein was studied in HEK293, HepG2 and Can 10 cells, using immunodetection assays. The function of the gentamicin-induced full-length protein was studied by measuring the [^{3}H]-taurocholate transcellular transport in stable MDCK clones co-expressing Ntcp. Combinations of gentamicin and chaperone drugs (UDCA, 4-PB) were investigated. In NIH3T3, aminoglycosides significantly increased readthrough level of all mutations studied, while PTC124 only slightly increased readthrough of p.E1302X. Gentamicin, induced a readthrough of p.R415X, p.R470X, p.R1057X, p.R1090X in HEK293 cells. The resulting full-length proteins localized within the cytoplasm, except for Bsep^{R1090X} that was also detected at the plasma membrane of HEK293 and at the canalicular membrane of Can 10 and HepG2 cells. Additional treatment with 4-PB and UDCA significantly increased the canalicular proportion of full-length Bsep^{R1090X} protein in Can 10 cells. In MDCK clones, gentamicin induced a 40% increase of the Bsep^{R1090X} [^{3}H]-taurocholate transport, which was further increased with additional 4-PB treatment. ***Conclusion***: This study constitutes a proof of concept for readthrough therapy in selected PFIC2 patients with nonsense mutations.

INTRODUCTION

Progressive familial intrahepatic cholestasis type 2 (PFIC2) is an autosomal recessive disease due to mutations in *ABCB11* encoding Bile Salt Export Pump (BSEP).(1, 2) BSEP is expressed at the canalicular membrane of hepatocytes and is the major transporter responsible for biliary bile acid (BA) secretion.(3, 4) In patients with PFIC2, impaired biliary bile acid secretion leads to decreased bile flow resulting in chronic cholestasis with severe pruritus and to bile salt accumulation responsible for severe ongoing hepatocellular damages leading to fibrosis, end-stage liver disease and high risk of hepatocellular carcinoma. Medical therapy with ursodeoxycholic acid (UDCA), rifampicin, and surgical therapy such as biliary diversion may provide some symptomatic relief. Nevertheless, in the majority of cases, liver transplantation (LT) is required because of unremitting pruritus, hepatic failure or hepatocellular carcinoma.(1) More than two hundred variants of *ABCB11* have been identified in PFIC2 patients including missense, nonsense and splice mutations.(5, 6) A genotype-phenotype correlation has been confirmed in a recent study of the largest cohort of PFIC2 patients suggesting that the severity of the disease is correlated with the impairment of the expression level and of the function of the resulting BSEP mutants.(1, 6) Accordingly, severe mutations such as nonsense mutations result in severe phenotypes (i.e. reduced response to medical and non-LT surgical therapy, very high risk of hepatocellular carcinoma) leading to a survival with native liver after age 10 years estimated around 33% in PFIC2 patients carrying a severe mutation on each allele of *ABCB11*.(6) In addition, those patients have been suggested to be at the highest risk to develop anti-BSEP alloimmunization after LT, a complication of LT specific to PFIC2 patients.(7-9) Thus, there is a clear unmet therapeutic need for PFIC2 patients carrying nonsense mutations. Nonsense mutations lead to the formation of an in-frame premature termination codon (PTC) and result in the formation of a truncated protein which is not or poorly expressed - the mRNA being degraded by the nonsense mediated mRNA decay (NMD) pathway -, in the latter case not correctly localized and/or not functional.(10) Targeted pharmacotherapy is a promising personalized therapeutic approach which aims to partially correct the consequence of the mutations identified in a specific patient. It is a field of active research especially in diseases involving protein of the ATP binding cassette (ABC) family such as BSEP.(11) We and others have shown that both *in vitro* and in selected patients, some drugs namely correctors –able to correct misstrafficking and to reduce endoplasmic reticulum associated degradation-, such as 4-phenylbutyrate (4-PB), could be of benefit in BSEP deficiency,

including PFIC2 due to specific missense mutations of *ABCB11*.(4, 12-14) Concerning nonsense mutations, it has been known for decades that aminoglycosides (e.g. gentamicin, geneticin) can bind to mammalian ribosomes and affect the fidelity of the translation process and induce a translational readthrough (RT) of the PTC allowing the partial restoration of full-length protein synthesis.(15) More recently, other drugs with some ability to induce a PTC RT were identified. Among them, PTC124 (Ataluren) has been tested in various preclinical studies as well as in clinical trials, in patients with various genetic diseases such as cystic fibrosis and Duchenne muscular dystrophy.(15)(and references therein) However, the effects of RT-inducing drugs have been studied neither *in vitro* nor *in vivo* in genetic liver diseases. Herein, we studied *in vitro* the ability of such drugs to induce RT of six nonsense mutations identified in patients with PFIC2. Our data show that treatment with aminoglycosides could induce RT of one of these mutations leading to the expression of a partially functional mutant. In addition, our data suggest that combination of aminoglycosides with correctors further improve the rescue of this mutant. This work provides new proof of concept in the field of targeted pharmacotherapy of PFIC2 resulting from nonsense mutations in *ABCB11*.

MATERIALS AND METHODS

Patients

Among the cohort of PFIC2 patients referred to the French reference network for rare liver diseases dedicated to biliary atresia and genetic cholestasis, we selected six patients carrying six different *ABCB11* nonsense mutations (p.Y354X, p.R415X, p.R470X, p.R1057X, p.R1090X and p.E1302X) at least on one allele. Results of *ABCB11* gene analysis and assessment of patients phenotype were performed as previously reported (1) and are detailed in Table 1.

Readthrough quantification in NIH3T3 cells

Complementary oligonucleotides corresponding to human (and rat when relevant i.e. different from the human sequence) nonsense mutations embedded in their natural context (Table 2) were annealed and inserted in the unique Msc1 cloning site located between lacZ and LUC (luciferase) coding sequences.(16) All translating ribosomes would thus result in galactosidase synthesis, but only those that readthrough the stop codon will synthesize luciferase. This dual gene reporter system provided an internal control for normalization of individual experiments for the overall expression level of each construct to take into account vector stability, transfection efficiency, transcriptional, translational rates and mRNA stability. An in-frame construct (TQ), with a glutamine codon replacing the stop codon between the two reporter genes, was used as a control (Table 2). Readthrough efficiency was estimated by calculating the ratio of luciferase versus galactosidase activities and was expressed as a percentage of the ratio obtained with the control TQ. NIH3T3 are easy to culture and transfect cells originating from primary mouse embryonic fibroblasts. Previous studies have validated the use of the dual gene reporter system described above to study PTC RT in NIH3T3 cells.(16, 17) Twenty-four hours after seeding in 6-well plates, NIH3T3 cells were transiently transfected with corresponding plasmids using Jet Pei (Invitrogen, California, USA; DNA/transfection reagent ratio: 1 µg/2.5 µL). Eighteen hours after transfection, cells were treated for 24 hours with 200 µg/mL of G418 (geneticin, Sigma-Aldrich Chimie, Lyon, France), 800 µg/mL of gentamicin (Sigma-Aldrich), 15 µM (i.e. 4.26 µg/mL) of PTC124 (Therapeutics–Selleckchem, South Plainfield, USA) or vehicle (PBS). These doses were selected according to published data.(16-18) In some additional experiments concerning the rat p.R1090X mutant, cells were also treated with 400 and 1200 µg/mL of gentamicin. Then, cells were

harvested and lysed, and β-galactosidase and luciferase enzymatic activities were assayed as previously described.(17)

Studies of the nonsense mutants

DNA constructs and mutagenesis

Mutations p.Y354X, p.R415X, p.R470X, p.R1057X, p.R1090X and p.K1302X, were introduced in a pEGFP-N1 vector (19) encoding a rat Bsep-green fluorescent protein (GFP) fusion protein using the QuickChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies, Massy, France) as previously described.(13) Primers sequences used for mutagenesis are detailed in Supporting Table 1. Rat Bsep was used because of its high degree of homology with the human ortholog and a higher level of expression in cultured cells.(20) The whole constructs were verified by automated Sanger sequencing.

Transfection, treatments of HEK293, Can 10 and HepG2 cells. Quantitative and qualitative studies of Bsep expression

Human Embryonic Kidney (HEK) 293 cells are human epithelial cells, easy to culture and to transfect; they were used to screen for the ability of RT-inducing drugs to lead to a full-length Bsep expression. HepG2 and Can 10 are well characterized hepatocellular polarized lines of human and rat origin, respectively. They were used to study the canalicular expression of the basal and the RT-induced full-length $Bsep^{R1090X}$. HEK293, Can 10 and HepG2 cells were plated at 0.5, 0.15 and 2.6 x 10^4 cells.cm^{-2} respectively, onto glass coverslips in 6 or 12-well plates. HEK293 and HepG2 cells were transiently transfected the day after using Fugene HD Transfection reagent (Promega, Madison, Wisconsin, USA; DNA/transfection reagent ratio: 1 µg/4 µL) and Jet Prime Transfection Reagent (Ozyme, Paris, France; transfection ratio: 2 µg/4 µL), respectively and Can 10 cells on day 2 using X-tremeGENE HP DNA Transfection Reagent (Roche Diagnostics, Meylan, France; transfection ratio: 1 µg/2 µL) with the plasmids, according to the manufacturer's instructions. Six hours after transfection, HEK293 cells were treated for 24 hours at 37°C with gentamicin (400-1200 µg/mL), PTC124 (15 µM) or vehicle (PBS). Can 10 and HepG2 cells were treated with gentamicin (800 µg/mL) or vehicle (PBS) for 24 hours at 37°C (or 27°C for Can 10). In some experiments, after 18 hours of treatment with gentamicin, Can 10 cells were co-treated with 4-PB (1 mM, Biovision Clinisciences, Montrouge, France) and/or UDCA (50 µM, Sigma-

Aldrich) or corresponding vehicle (PBS and DMSO, respectively) for 24 additional hours. Then, cells were fixed and immunostained using the following primary antibodies: mouse anti-GFP (1:80, Roche Diagnostics, Meylan, France), rabbit anti-GFP (1:500, Abcam, Cambridge, UK), mouse anti-multidrug resistance protein 2 (MRP2) (1:100, Enzo Life Sciences, Villeurbanne, France), rat anti-zonula occludens (ZO-1) (13) and rabbit anti-occludin (1:500, Thermofisher, Waltham, USA). Appropriate goat anti-immunoglobulin G Alexa Fluor 488 and 594 secondary antibodies (Molecular Probes, Eugene, OR) were used at a dilution of 1:500. Cells were observed using a Zeiss Axioskop epifluorescence microscope (Carl Zeiss, Jena, Germany) and a confocal microscope (Eclipse TE-2000-Nikon-C1; Nikon, Tokyo, Japan) as previously described.(13) HEK293, Can 10 and HepG2 cells expressing Bsep mutants were quantified as follows: 10 fields by coverslip were captured randomly with the same exposure time. For each field, the number of cells with a detectable degree of GFP was expressed as a percentage of the total number of cells. Can 10 and HepG2 cells expressing GFP were further classified according to the presence (canalicular GFP+) or the absence (cytoplasmic GFP+) of a canalicular enrichment of Bsep-GFP as previously described.(13)

Generation of MDCK clones stably expressing rat Bsep and Ntcp

Madin-Darby canine kidney (MDCK) is a well-characterized polarized cell line which was used to study Ntcp- and Bsep-mediated bile acid transcellular transport as previously reported.(21) MDCK cells were first transfected with plasmids encoding $Bsep^{wt}$-GFP or $Bsep^{R1090X}$-GFP, using Fugene HD transfection reagent according to manufacturer's instructions. Clonal selection of stably transfected cells was obtained using 600 µg/mL of G418. Expression of $Bsep^{R1090X}$ was checked by Real Time-quantitative PCR as described in Supporting Information (Supporting material). To allow BA entry in MDCK cells, the rat cDNA of the Na^+-taurocholate co-transporting polypeptide (Ntcp, *Slc10A1*), a basolateral BA transporter, was cloned into the lentiviral vector pLenti-cMyc-DDK-IRES-Puro plasmid (PS100069, OriGene, Rockville, MD). $Bsep^{wt}$ and $Bsep^{R1090X}$ expressing MDCK clones were plated into 60 mm diameter Petri dishes at 1.8 x 10^5 cells. Twenty-four hours after seeding, cells were infected with lentiviral particles containing the recombined plasmid, at a multiplicity of infection of 30 and recombinants were selected with 3 µg/mL puromycin (Ozyme, Paris, France). Expressions of Bsep-GFP and Ntcp-cMyc were examined by immunolocalization using rabbit anti-GFP (1:500, Abcam, Cambridge, UK) and mouse anti-cMyc (1:250, BD Pharmigene, San Diego, CA) antibodies as described in the previous

section and by immunoblotting. Briefly, cells were lysed as previously described (22) and immunoblotting was performed with mouse anti-GFP (1:500, Roche Diagnostics), anti-β-actin (1:3000, Sigma Aldrich) and anti-cMyc (1:500, BD Pharmigene) primary antibodies. Horseradish peroxidase-linked mouse-specific secondary antibody (GE Healthcare, Little Chalfont, UK) were used to detect peroxidase activity by chemiluminescence (ECL detection kit, BioRad, Marnes-La-Coquette, France).

Transcellular taurocholate transport assay

MDCK clones stably expressing Bsep-GFP and/or Ntcp-cMyc were seeded on polyethylene terephthalate membrane inserts (pore size 3 μm; BD Falcon) in 24-well plates at a density of 10^4 cells/insert. During the five following days, the integrity of cell monolayer was assessed by measuring transepithelial electrical resistance and Lucifer yellow permeability test.(23) Cell monolayers were treated for 24 hours with gentamicin (800 μg/mL) or vehicle at 37°C. In some experiments, cells were treated for 24 additional hours with 4-PB (1 mM) or vehicle, or grown at 27°C. Thereafter, culture medium was replaced by prewarmed transport buffer (in mM: 118 NaCl, 23.8 $NaHCO_3$, 4.83 KCl, 0.96 $KH2PO_4$, 1.2 $MgSO_4$, 12.5 HEPES, 5 glucose, and 1.53 $CaCl_2$, adjusted to pH 7.4) (21) in apical and basal compartments, in the presence of [^{3}H]taurocholate ([^{3}H]TC) (0.1 μM, 1 μCi/mL, Perkin Elmer, Waltham, MA) and taurocholate (TC) (0.9 μM, Sigma-Aldrich) in the basal compartment. After 2 hours, the apical buffer and membrane inserts with cells were collected. Transcellular transport and intracellular accumulation of [^{3}H]TC were calculated from the radioactivity measured by scintillation counter (Hidex 300 SL, Hidex Turku, Finland) in the apical buffer and cell lysates, respectively. Aliquot (50 μL) of cell lysates (120 μL 1% Triton X-100) was used to determine protein concentration (DC Protein Assay Kit, BioRad, Marnes-La-Coquette, France), with bovine serum albumin as a standard. Transport data were expressed as pmol of [^{3}H]TC/mg of protein.

Statistical analysis

Data were analyzed using GraphPad Prism 6 (San Diego, CA) and are presented as mean ± SEM (standard error of the mean). Statistical analyses were performed using the Mann-Whitney test, with a p-value < 0.05 being considered statistically significant.

RESULTS

Aminoglycosides induce a readthrough of six *ABCB11* nonsense mutations in NIH3T3

For each of the six BSEP nonsense mutations (p.Y354X, p.R415X, p.R470X, p.R1057X, p.R1090X and p.E1302X) identified in the six PFIC2 patients selected (Table1), the PTC and the surrounding nucleotide context are shown in Table 2. Because the following studies were performed using the rat Bsep cDNA, nonsense mutations were also studied in the rat nucleotide context as detailed in Table 2. As expected, basal RT levels, quantified in NIH3T3, were dramatically low for all mutations. The mean basal RT levels ranged from 0.012% for the rat mutation p.Y354X to 0.322% for the human mutation p.R1057X (Table2). Basal RT levels were in the same ranges for human and rat mutations except for the mutation p.R1057X (0.047% *vs* 0.322% in the rat and human context, respectively) (Table 2). Thereafter we studied the ability of gentamicin, G418 and PTC124 to induce the RT of these PTCs. The maximal responses were obtained with G418, which increased significantly the RT of all rat (Fig. 1A) and human (Fig. 1B) PTCs. Interestingly, G418 increased the RT of the p.R1090X mutation over a level of 10%, representing a 50-fold increase over the basal rate for the human mutation. Treatment with gentamicin also increased significantly the RT of all mutations but to a lesser extent than G418. The maximum effect was also observed for the mutation p.R1090X with a gentamicin-induced RT around 3%, representing a 14-fold increase over the basal rate for the human mutation. (Fig.1A and Fig.1B). In contrast, PTC124 did not significantly increase the RT levels, except for the mutation p.K/E1302X which RT level was slightly increased from 0.128% to 0.320% (Fig. 1A and Fig. 1B). Likewise, we studied the dose-response relationship after treatment with gentamicin of p.R1090X mutation (rat sequence). We observed a dose-dependent increase of the RT levels up to 800 µg/mL, while there was no significant difference between the 800 and the 1200 µg/mL doses (Supporting Fig. S1).

Gentamicin induces a readthrough of 4 nonsense *ABCB11* mutations and leads to the production of a full-length Bsep protein in HEK293

ABCB11 nonsense mutations have been reproduced in a pEGFP-N1 plasmid expressing a rat Bsep-GFP fusion protein. Since the pEGFP-N1 plasmid contains a resistance cassette to G418, this aminoglycoside could not be used in further studies. Basal as well as gentamicin-induced RT were studied by immunostaining after transient transfection of HEK293 cells with the

corresponding plasmids. Before gentamicin therapy, we observed variable levels of expression of full-length Bsep protein (Fig. 2A), ranging from 0.12% (p.R1090X) to 10.76% (p.R470X) of HEK293 cells (Fig. 2B). Treatment with gentamicin increased significantly the level of expression of full-length Bsep protein of 4 mutations (p.R415X, p.R470X, p.R1057X and p.R1090X) among the 6 nonsense mutations studied (Fig. 2B) while virtually neither basal nor gentamicin-induced RT was detected for the p.K1302X mutation. Interestingly, in line with the results of the dual gene reporter system, the maximum effect was observed for the mutation p.R1090X, with a gentamicin-induced RT leading to a detection of a full-length Bsep protein in 35% of HEK293 cells (Fig. 2B). Moreover, no significant effect of gentamicin treatment was observed with p.Y354X and p.K1302X mutants, these two mutations being the less responsive to aminoglycosides treatment in the dual reporter system. Furthermore, PTC124 failed to induce the production of a full-length $Bsep^{K1302X}$ protein (data not shown). Because the p.R1090X mutant was the one for which we observed the most important gentamicin-induced RT in these two systems (Fig. 1, 2), we selected this mutant for further studies.

Gentamicin-induced full-length $Bsep^{R1090X}$: immunodetection studies and effect of combination therapy with UDCA and 4-PB

In HEK293 cells, treatment of the p.R1090X mutant with gentamicin led to the expression of a full-length Bsep protein detected both within the cytoplasm and at the plasma membrane level, as indicated by the colocalization of GFP with occludin (Fig. 3A). Accordingly, western blot analysis showed that gentamicin induced the production of both an immature non-glycosylated form of Bsep and a mature form of Bsep detected at 170 kDa and 190 kDa, respectively (Fig. 3B).(24) The expression of $Bsep^{R1090X}$ was also studied in Can 10 line (Fig. 4). The basal RT level was higher than the one observed in HEK293 line (Fig. 2B, 4B). Treatment with gentamicin significantly increased the expression of $Bsep^{R1090X}$ leading to the expression of a full-length protein that was detected in more than 8% of Can 10 cells (Fig. 4B). Interestingly, confocal studies showed that this full-length protein located within the cytoplasm of Can 10 cells but also at the canalicular level after gentamicin-induced RT (Fig. 4A). Subcellular localization of the p.R1090X mutant was further studied in Can 10 cells by quantification of the cytoplasmic and canalicular expression (Fig. 4C). At 37°C, the basal RT of p.R1090X led to the expression of a full-length protein that was only detected within the cytoplasm of 3.2% of Can 10 cells. After treatment with gentamicin, the expression increased to 8.4% including 0.9% of Can 10 cells in which the protein was detected

at the canalicular level. We confirmed the gentamicin-induced expression of full-length Bsep^{R1090X} in the human hepatocellular HepG2 line (Supporting Fig. S2). The basal RT level (Supporting Fig. S2B) was similar to the one observed in Can 10 line (Fig 4B). Treatment with gentamicin significantly induced the expression of a full-length protein (detected in almost 9% of HepG2 cells, Supporting Fig. S2B). Confocal studies (Supporting Fig. S2A) showed that the full-length protein located within the cytoplasm but also at the canalicular membrane as indicated by its partial co-localization with the canalicular transporter MRP2. With gentamicin treatment, this canalicular expression increased significantly to 43% of the HepG2 cells expressing full-length Bsep^{R1090X} (*vs* 11% in the control) (Supporting Fig. S2B). Growing cells at 27°C is a well-known condition to stabilize and to improve trafficking of proteins retained in the endoplasmic reticulum such as Bsep missense mutants.(13, 19, 20, 24, 25) In this condition, we observed a trend toward an increase of the basal RT of p.R1090X in Can 10 cells, including a slight canalicular localization that was not observed at 37°C (Fig. 4C). In Can 10 cells treated with gentamicin and grown at 27°C, we observed a dramatic and significant increase of the proportion of the gentamicin-induced full-length Bsep expressed at the canaliculi compared to cells grown at 37°C, from 10% to nearly 80% (Fig. 4C). Because chaperone drugs such as 4-PB and UDCA have been shown to increase the canalicular expression of several Bsep missense mutants (13, 14, 19, 25), we studied the effect of these drugs at 37°C in combination with gentamicin. Treatment with UDCA, 4-PB and UDCA + 4-PB significantly increased the canalicular expression of the gentamicin-induced full-length Bsep^{R1090X} (24 to 30% *vs* 10% of cells expressing a full-length protein, with gentamicin alone) (Fig. 4C).

Gentamicin-induced full-length Bsep^{R1090X} is partially functional and its function can be increased with 4-PB therapy

To further study the effect of gentamicin on Bsep^{R1090X} mutant, we used a functional model to study the activity of Bsep in MDCK cells. MDCK clones expressing wild type Bsep-GFP and/or Ntcp-cMyc were characterized using immunodetection techniques (Supporting Fig. S3). The expression level of Ntcp and of Bsep^{wt} was controlled using western blot as illustrated in Supporting Fig. S3A. The subcellular localization of Bsep^{wt} and Ntcp was studied using confocal microscopy. While Ntcp was located at the basolateral membrane of MDCK, Bsep^{wt} localized at the apical membrane as illustrated in Supporting Fig. S3B. Then, we studied the functionality of Bsep and Ntcp by measuring the transcellular transport and intracellular accumulation of [^{3}H]TC

across MDCK monolayers cultured in inserts. The basal to apical flux of [^{3}H]TC was virtually not detected across MDCK Bsep^{wt} cells but was detected across MDCK Ntcp cells, the later corresponding to background activity suggesting the presence of an apical non-Bsep transporter for TC, as previously reported (Supporting Fig. S3C).(21) Intracellular [^{3}H]TC concentration in MDCK clone expressing only Bsep^{wt} was minimal, whereas in MDCK-Ntcp clone, it was significantly higher (Supporting Fig. S3D). These results indicated that the expression of Ntcp in the basolateral membrane was required to detect the transcellular transport of [^{3}H]TC in the apical direction in MDCK monolayers. Furthermore, transcellular transport of [^{3}H]TC in MDCK-Bsep^{wt} Ntcp clone was 3-fold greater and intracellular [^{3}H]TC concentration was 7-fold lower compared with MDCK-Ntcp clone indicating that Bsep^{wt} efficiently transported TC across the apical membrane.

Characterization and functional study of Bsep^{R1090X}

The BA transport activity of Bsep^{R1090X} was assessed in MDCK clones expressing Bsep-GFP (wt or R1090X) and Ntcp-cMyc. Expression level of Bsep (wt or R1090X) and Ntcp was controlled by immunoblotting (Fig. 5A). As expected, Bsep-GFP was detectable only in MDCK-Bsep^{wt} Ntcp clone confirming that MDCK-Bsep^{R1090X} Ntcp clone did not express a N-Ter GFP fusion full-length protein due to PTC in position 1090. Quantification of Ntcp-cMyc electrophoretic patterns did not display statistically different expression levels of Ntcp among the two MDCK clones (data not shown). The transcellular transport of [^{3}H]TC measured in MDCK Bsep^{R1090X} Ntcp clone was significantly lower than the one measured in MDCK Bsep^{wt} Ntcp and was comparable to the one measured in MDCK Ntcp cells (Supporting Fig. S3C) suggesting that Bsep^{R1090X} was not functional (Fig. 5B).

Effect of gentamicin and/or 4-PB on Bsep^{R1090X} expression and activity

After gentamicin-induced RT, Bsep^{R1090X} localized in the cytoplasm and also at the apical membrane of MDCK cells as illustrated in Fig. 6A. Treatment with gentamicin significantly increased the transport of [^{3}H]TC across MDCK Bsep^{R1090X} Ntcp by 40% (Fig. 6B). These data suggested that a fraction of this gentamicin-induced expression of Bsep^{R1090X} expressed at the apical membrane level is functional. Additional treatment with 4-PB or by growing cells at 27°C significantly increased the transcellular transport of [^{3}H]TC in Bsep^{R1090X} Ntcp clone compared to gentamicin treatment alone (Fig. 6B).

DISCUSSION

Herein, we studied the capability of aminoglycosides and PTC124 to induce RT of six *ABCB11* nonsense mutations identified in patients with PFIC2. Aminoglycosides induced a RT of all the mutations tested in NIH3T3 cells. Among the two types of stop codon studied, we observed a higher induction of RT in sequences containing a UGA (p.R415X, p.R470X, p.R1057X, p.R1090X) than in those containing a UAA (p.Y354X, p.E/K1302X). In HEK293, gentamicin induced a RT of the four UGA PTC - leading to a significant increase of the expression of a full-length Bsep protein - but not of the two UAA PTC. These results are in line with previous studies showing the following order of gentamicin-induced PTC RT efficiency: UGA>UAG>UAA.(26, 27) In addition, we observed a higher aminoglycoside-induced RT of the human p.R1057X sequence compared with the rat p.R1057X sequence. This difference could be due to the nature of the first nucleotide after the stop codon in position n+4; it has been shown that the C nucleotide present in the human sequence is more favorable to aminoglycoside-induced PTC RT than the A present in the rat sequence.(28) This favorable UGA-C sequence was also present in Bsep^{R1090X} which was the most responsive to gentamicin therapy and was selected for further studies. In HEK293 and in the hepatocellular Can 10 and HepG2 lines, gentamicin-induced RT led to a significant threefold increase in the expression of full-length Bsep^{R1090X} protein that localized both in the cytoplasm and at the plasma membrane, suggesting the expression of both a mature and an immature form of a full-length Bsep^{R1090X} protein as observed in HEK293. Because gentamicin-induced RT of UGA PTC can result in the incorporation of 3 amino acids, namely arginine (CGA), the wild type residue of Bsep, cysteine (UGC or UGU) or tryptophan (UGG) (29-31), the gentamicin-induced full-length Bsep^{R1090X} protein could in fact be a mixture of Bsep^{wt} and of the two missense mutants Bsep^{R1090C} and Bsep^{R1090W}. The latter two could explain the cytoplasmic expression observed in HEK, HepG2 and Can10 cells, as missense mutations can result in endoplasmic reticulum-retained immature mutants. It has been shown that corrector drugs, including 4-PB and UDCA, could partially retarget such missense mutants to the canalicular membrane.(13, 19, 25) Indeed, in Can 10 cells, therapies combining gentamicin with 4-PB and UDCA increased the expression of gentamicin-induced protein, especially at the canalicular membrane. Lastly, we showed in MDCK cells that treatment with gentamicin led to a significant increase of [^{3}H]TC transport activity of the Bsep^{R1090X} mutant, this transport activity being further increased after treatment with 4-PB. It is likely that 4-PB increased the apical expression of

gentamicin-induced full-length Bsep protein in MDCK, similar to our observations in Can 10 cells; this could account for the increased [^{3}H]TC transport activity observed. It is currently impossible to predict in which proportion each amino acid will be incorporated into the peptides synthesized during RT. It is also possible that corrector drugs might increase the canalicular expression of Bsep^{wt}.(32) Therefore, we do not know the respective contribution of Bsep^{wt} and of Bsep^{R1090C} and Bsep^{R1090W} mutants in the increase of expression and transport activity of gentamicin-induced full-length Bsep protein observed with UDCA and/or 4-PB treatments. This issue remains to be further studied.

New therapeutic strategies are needed for patients carrying nonsense mutations of *ABCB11*. Targeted pharmacotherapy inducing RT of PTC could be a promising avenue. Previous studies on the BSEP^{T1210P} missense mutant suggested that a restoration above 3% of the bile acid transport function of BSEP^{wt} could lead to a significant clinical improvement.(12, 13, 33) In a study evaluating the efficiency of gene therapy in a mice model of PFIC3, a threshold of 3% of the expression of MDR3 (the canalicular ABC transporter involved in PFIC3) lead to a significant improvement of the mice phenotype.(34) In a recent study reporting the largest cohort of PFIC2 patients, nonsense mutations were identified in nearly 20% of the patients (56 out of 264).(6) Among these 56 patients, 32 harbored one of the six mutations studied herein, including 7 patients carrying the p.R1090X mutation.(6) The potential use of a drug-stimulated translational RT of PTC has been investigated in various preclinical models of many genetic diseases showing promising results.(15) Clinical trials evaluating gentamicin as a PTC-RT inducing drug have been conducted in patients with cystic fibrosis (CF), Duchenne muscular dystrophy (DMD) and more recently dystrophic epidermolysis bullosa.(15)(and references therein) Despite encouraging results the need for repetitive, prolonged and theoretically life-long intravenous administration, together with well-known ototoxicity and nephrotoxicity, have hampered the wide application of gentamicin to induce PTC-RT in patients. To overcome the toxicity of aminoglycosides, research has been conducted to identify compounds that could mitigate the toxic effects of gentamicin (e.g. daptomycin, poly-L-aspartic acid, Melatonin) or potentiate the PTC-RT stimulating efficacy of gentamicin (e.g. CDX5-1). Alternatively, modifications of the chemical structure of aminoglycosides able to stimulate PTC-RT have led to the identification of aminoglycoside derivatives such as NB124 (or ELX-02) that are less toxic and more efficient.(15, 35) In addition, many studies have searched for RT inducing compounds that are not chemically related to aminoglycosides. PTC124 (Ataluren) was one of the first compounds identified in this category

and has been extensively studied both in preclinical models and in clinical trials including CF and DMD patients.(15) Despite many positive results in various preclinical models the effects of PTC124 in patients remain inconclusive. In our study PTC124 only slightly increased the RT levels of one out of the 10 sequences studied and it failed to induce the production of a full-length Bsep protein. In addition to PTC124, many other compounds have been identified that are not chemically related to aminoglycosides and that present PTC-RT inducing properties. Among them, RTC204, RTC219, BZ6, BZ16, Escin, and recently 2,6-diaminopurine, may constitute promising drugs.(15, 36) Of note, Escin and ELX-02 have been shown to be safe in clinical trials.(15, 35) Although it is still unknown how these drugs promote PTC readthrough, an important possibility is that these compounds act through a different mechanism that enables PTC readthrough of sequences that are currently resistant to aminoglycosides induction. Finally, the combination of compounds that block the NMD could increase the level of mutated transcript expression and enhance the effect of PTC-RT-inducing drugs. Such NMD inhibitors include wortmannin, caffeine, NMDI-1, VG1 and Amlexanox.(15) Unfortunately, the experimental models used in this study overcame the NMD phenomenon, which prevented us from studying the effect of such NMD inhibitors on the expression of the gentamicin-induced full-length Bsep. Evaluation of drugs from these different categories in monotherapy or in combination constitutes a very active field of research and opens new perspectives for the treatment of genetic diseases due to nonsense mutations. However, more work is needed to identify nontoxic, orally bioavailable, efficacious readthrough agents with a good diffusion in the liver that could be evaluated in children suffering from severe genetic liver diseases such as PFIC2.(15) When such drugs become available for clinical trials, international registries of PFIC2 patients will help identify the appropriate patients to include.(6) Would these drugs improve the outcome of such patients presenting with the most severe phenotype of PFIC2 - increased risk of hepatocellular carcinoma, liver failure, severe refractory cholestasis, requiring early LT (6) – remains a challenging question. Toxicity issues, especially liver toxicity, would also require specific attention owing the severity of the liver disease of these patients. Lastly, one can speculate that such therapies, even if they are used as a short-term course, might reduce the risk of post-LT anti-BSEP alloimmunization as the expression of a full-length BSEP protein before LT might induce BSEP tolerance.

Together, our data provide an *in vitro* proof of concept that gentamicin can partially rescue a PFIC2-causing BSEP nonsense mutation by inducing a pharmacological RT. We also show that additional treatment with 4-PB could further increase the TC transport activity of the resulting

full-length protein by increasing its apical expression. Application of targeted pharmacotherapy based on PTC suppression could also be considered in other genetic liver diseases such as PFIC3 which involves another canalicular ABC transporter.(11)

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FIGURE LEGENDS

FIG. 1. Induction of readthrough of rat and human *ABCB11* nonsense mutations in NIH3T3 cells. Oligonucleotide sequences containing the nonsense mutations of BSEP/Bsep: p.Y354X, p.R415X, p.R470X, p.R1057X, p.R1090X and p.E/K1302X were cloned in a dual gene reporter system between lacZ and luciferase sequences and transfected in NIH3T3 cells. Readthrough efficiencies were estimated 24 hours after treatment with G418 (200 µg/mL), gentamicin (800 µg/mL), PTC124 (15 µM) or PBS (control). The readthrough efficiency was expressed as the luciferase/β-galactosidase ratio of the tested construct normalized to an in-frame control sequence that did not contain stop codon. Basal and induced readthrough of rat (A) and human (B) oligonucleotide sequences are expressed in percentage (mean +/- SEM, n=3 to 7 independent experiments) of the in-frame control sequence. * $p < 0.05$; ** $p < 0.01$ *versus* control.

FIG. 2. Full-length Bsep protein expression after gentamicin-induced readthrough in HEK293 cells. HEK293 cells were transfected with plasmids containing Bsep-GFP nonsense mutants. Twenty-four hours after treatment with gentamicin or PBS (control), cells were immunostained using anti-GFP antibody. Hoechst was used to stain nuclei. (A) Immunodetection of Bsep-GFP mutants by epifluorescence microscopy. Bar: 10 µm. (B) Percentage of cells expressing Bsep-GFP (the number of cells with a detectable degree of GFP was expressed as a percentage of the number of total cells identified using a Hoechst staining, mean +/- SEM, n=4 to 9 independent experiments). * $p < 0.05$ and ** $p < 0.01$ *versus* control.

FIG. 3. Immunodetection studies of Bsep^{R1090X} in HEK293 cells. HEK293 cells were transfected with plasmids containing Bsep-GFP (wt and R1090X). Cells transfected with Bsep^{R1090X}-GFP were treated for 24 hours with gentamicin or PBS (control). (A) Cells were immunostained using anti-GFP (green) and anti-occludine (red) antibodies and examined by confocal microscopy. Images show the projection of 2 to 3 slides of 0.3 µm; Bar: 10 µm. (B) Cells were lysed and analyzed by immunoblotting using anti-GFP and anti-actin antibodies. Each lane was loaded with 30 µg of proteins.

FIG 4. Effects of gentamicin combined or not with 4-PB, UDCA and incubation at 27°C on Bsep^{R1090X} expression in Can 10 cells. Can 10 cells were transfected with Bsep^{R1090X}-GFP and treated for 24 hours with gentamicin (800-1200 µg/mL) or PBS (control). Cells were immunostained using anti-GFP (green) and anti-ZO-1 (red) antibodies. (A) Immunodetection of Bsep-GFP by confocal microscopy. Images show the projection of 2 to 3 slides of 0.3 µm; Bar: 10 µm. (B) Percentage of cells expressing GFP was assessed as described in the material and methods section. ** $p < 0.01$ and *** $p < 0.001$, *versus* control. (C) Can 10 cells were transfected with Bsep^{R1090X}-GFP and treated for 18 hours with gentamicin. Then, cells were co-treated with 4-PB (1 mM) and/or UDCA (50 µM) or corresponding vehicles or incubated at 27°C for 24 additional hours. Cells were immunostained using anti-GFP and anti-ZO-1 antibodies and analyzed using epifluorescence microscopy. Percentage of cells expressing Bsep^{R1090X}-GFP in the cytoplasm (in black) and at the canalicular membrane (in gray) was assessed and expressed as means +/- SEM of 3 to 5 independent experiments. GFP positive cells: * $p < 0.02$ and ** $p < 0.005$ *versus* control 37°C. Canalicular GFP positive cells: # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.005$ *versus* control 37°C. Canalicular GFP positive cells: § $p < 0.05$ and §§ $p < 0.02$ *versus* gentamicin 37°C. 4-PB, 4-phenylbutyrate; UDCA, ursodeoxycholic acid.

FIG. 5. Characterization and functional study of Bsep^{R1090X} in MDCK cells. (A) MDCK clones stably expressing Bsep-GFP (wt or R1090X) and/or Ntcp-cMyc were lysed and analyzed by immunoblotting using anti-GFP and anti-cMyc antibodies. Each lane was loaded with 30 µg of proteins. (B) Vectorial transport of [^{3}H]TC in MDCK clones expressing Bsep^{wt} Ntcp or Bsep^{R1090X} Ntcp was assessed in monolayer system. Results (means ± SEM of 3 to 6 independent experiments) were normalized to total protein concentration in each insert membrane and

expressed as the amount (pmol) of [^{3}H]TC/mg of protein. **** $p < 0.0001$ *versus* Bsep^{wt} Ntcp. wt, wild-type.

FIG. 6. Effect of gentamicin alone or in combination with 4-PB on Bsep^{R1090X} expression and activity in MDCK cells. (A) MDCK clones stably expressing Bsep^{R1090X}-GFP and Ntcp-cMyc were treated with gentamicin or PBS (control) and examined by confocal microscopy. *Bottom*, *center* and *right* panels show *x-z*, *x-y* and *y-z* plane images, respectively. Bar: 10 µm. (B) MDCK clones stably expressing Bsep^{R1090X}-GFP and Ntcp-cMyc were treated with gentamicin alone or in combination with 4-PB or grown at 27°C and transcellular transport of [^{3}H]TC was measured. Results (means +/- SEM of 3 to 6 independent experiments) were expressed as the amount of [^{3}H]TC, normalized to protein amount in each insert. * $p < 0.05$ ** $p < 0.01$ *versus* non-treated cells; and # $p < 0.05$. 4-PB, 4-phenylbutyrate; TC, taurocholate.

TABLE 1. Main characteristics of the six PFIC2 patients carrying the six nonsense mutations of *ABCB11* studied *in vitro*.

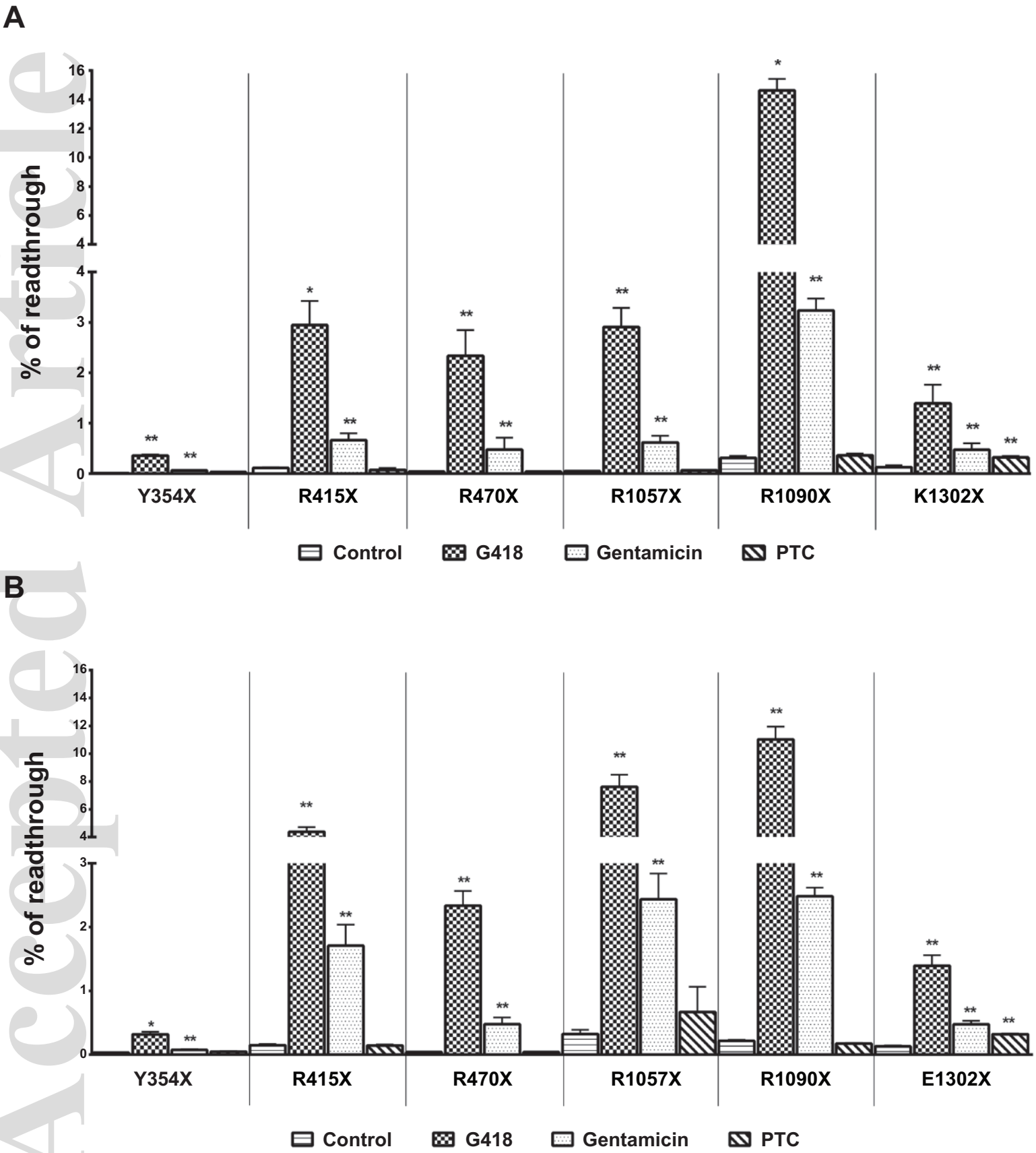
Patients	Mutations in *ABCB11*		Onset	BSEP IHC	Biliary [BA]	Evolution
1*	c.1062 T>A/ c.1062 T>A	p.Y354X/ p.Y354X	neonatal	neg	0.02mM	LT
2*	c.1243 C>T/ c.1243C>T	p.R415X/ p.R415X	neonatal	neg	NA	LT (dysplasia
3	c.1408 C>T/ c.1308+2 T>A	p.R470X/ p.(?)	neonatal	neg	0.19 mM	LT, HCC
4	c.3169 C>T/ c.1388C>T	p.R1057X/ p.T463I	9 months	faint	1 mM	not listed for
5*	c.3268 C>T/ c.3268 C>T	p.R1090X/ p.R1090X	neonatal	neg	0.02 mM	LT, alloI
6	c.3904 G>T/ c.3904 G>T	p.K1302X/ p.K1302X	neonatal	NA	NA	LT

*, clinical data previously reported (see reference 1). Abbreviations: [BA], bile acid concentration; alloI, post liver transplantation anti-BSEP alloimmunisation; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; LT, liver transplanted; NA, not available; neg, negative

TABLE 2. Basal readthrough levels in NIH3T3 cells, of oligonucleotide sequences containing the stop mutations of *ABCB11*.

Species	wt codon	Mutation	Oligonucleotides with PTC	Basal Readthrough (%)	SEM
Human	TAT	Y354X	GAAGGAGAA**TAA**ACACCAGGA	0.031	0.008
Rat	TAT	Y354X	GAAGAAGAG**TAA**ACACCAGGG	0.012	0.006
Human	CGA	R415X	AAGTTGGAT**TGA**ATCAAGGGT	0.149	0.016
Rat	CGA	R415X	AAGCTAGAC**TGA**ATCAAGGGT	0.111	0.014
Human	AGA	R470X¤	CTCATTCAG**TGA**TTCTATGAC	0.041	0.004
Rat	AGA	R470X¤	CTCATTCAG**TGA**TTCTATGAC	0.041	0.004
Human	CGA**§**	R1057X	CTGCTGGAC**TGA**CAACCCCCA	0.322	0.067
Rat	CGG**§**	R1057X	CTGCTAGAT**TGA**AAACCTCCA	0.047	0.005
Human	CGA	R1090X	TATCCTTCT**TGA**CCTGACTCG	0.214	0.019
Rat	CGA	R1090X	TATCCTTCT**TGA**CCCGATATT	0.312	0.039
Human	GAA**§**	E1302X*****¤	ACCCATGAA**TAA**CTGATGGCC	0.128	0.016
Rat	AAA**§**	K1302X*****¤	ACCCATGAA**TAA**CTGATGGCC	0.128	0.016
	TQ : in frame control		GCAGGAACACAACAGCAATTACAG	100	

The nonsense mutations are in bold. Nucleotides in position n-1 and n+1 of the stop codon are highlighted in grey. Basal level readthrough of each sequence is expressed in percentage (mean +/- standard error of mean (SEM), n=5 to 6 independent experiments) of the control sequence. PTC, premature termination codon. §, indicates that human and rat wild-type (wt) codons are different. *, indicates different amino acids between human and rat wild-type protein. ¤, indicates that human and rat sequences including the nonsense mutation are the same



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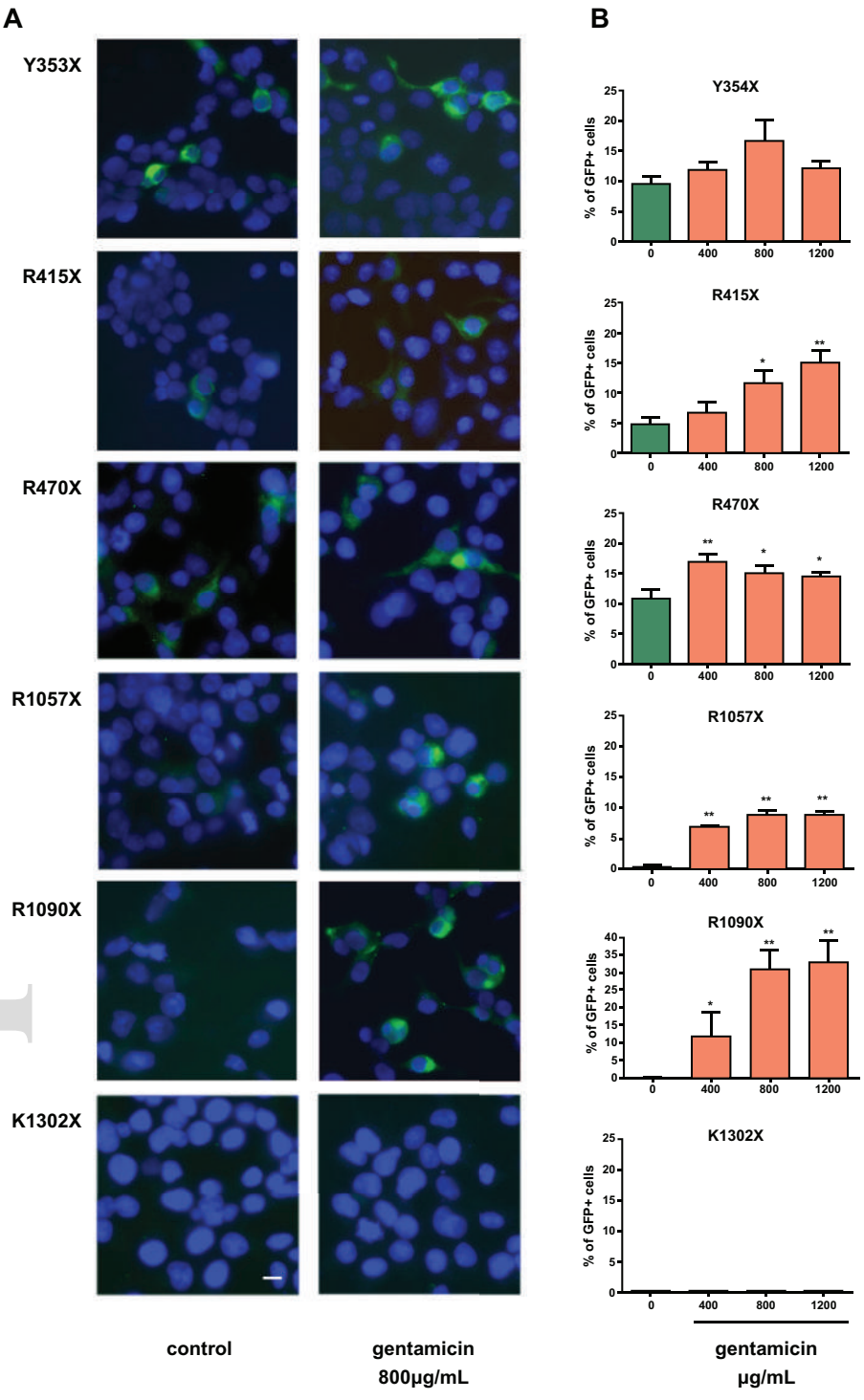

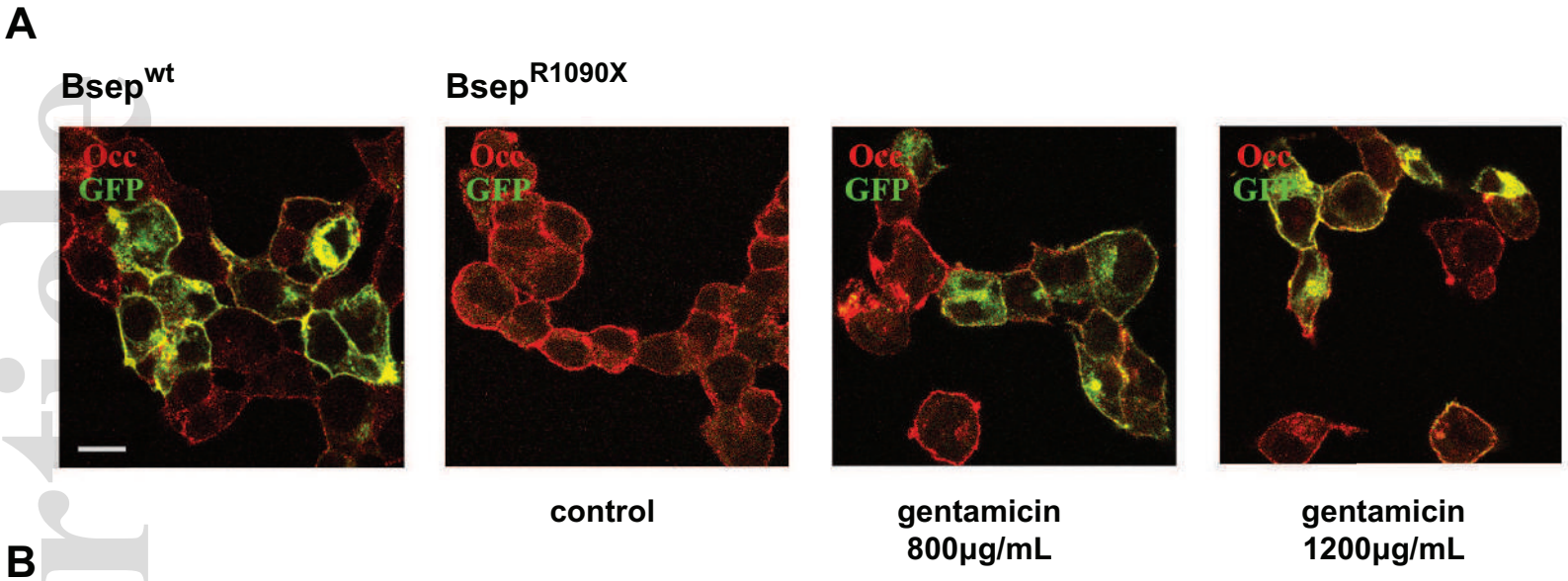

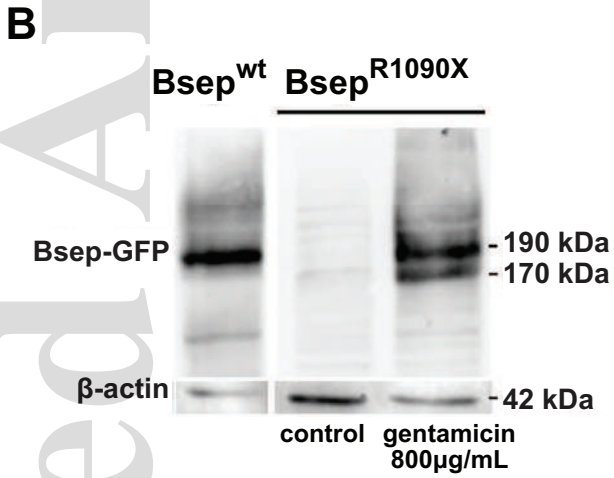

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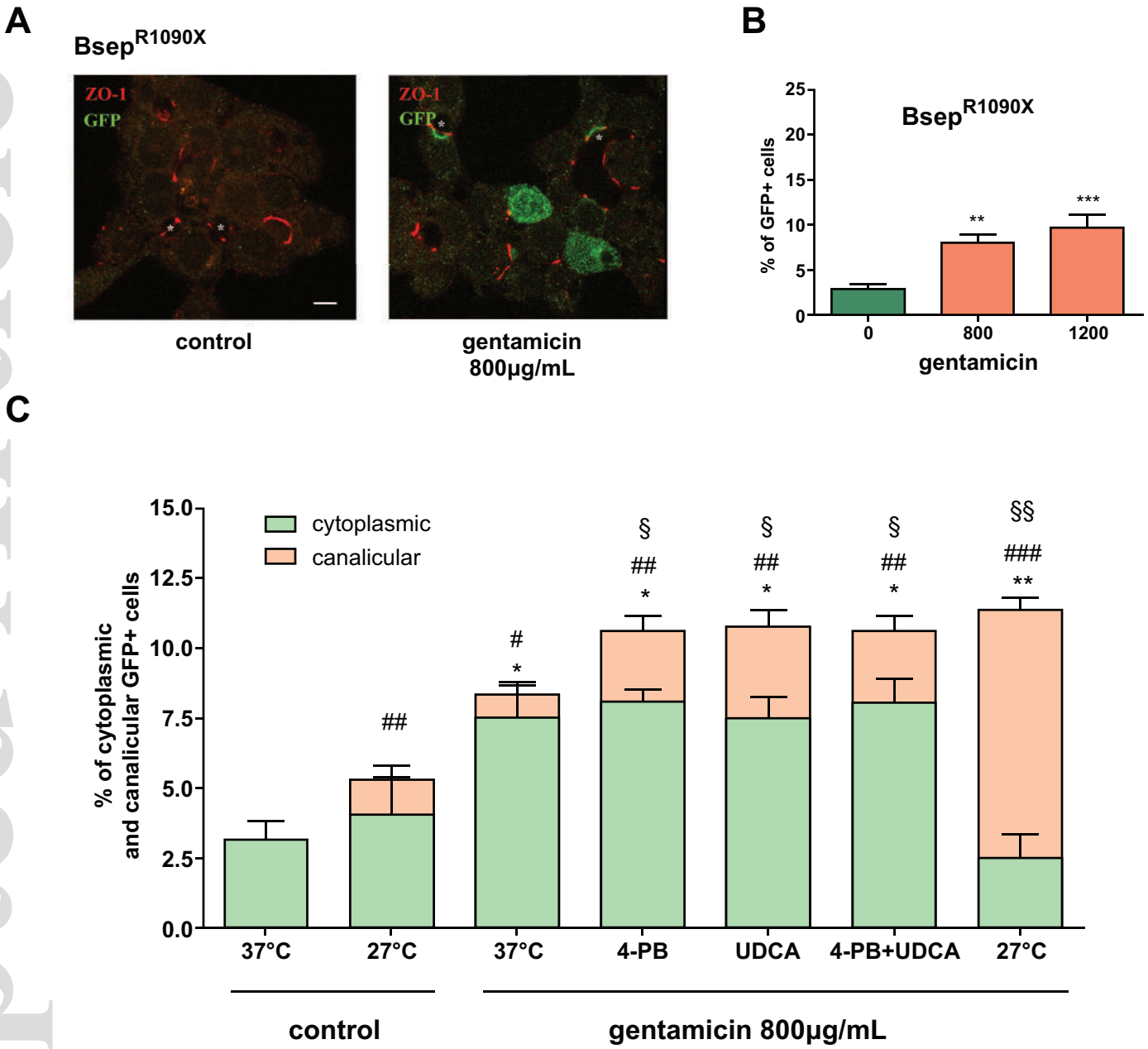

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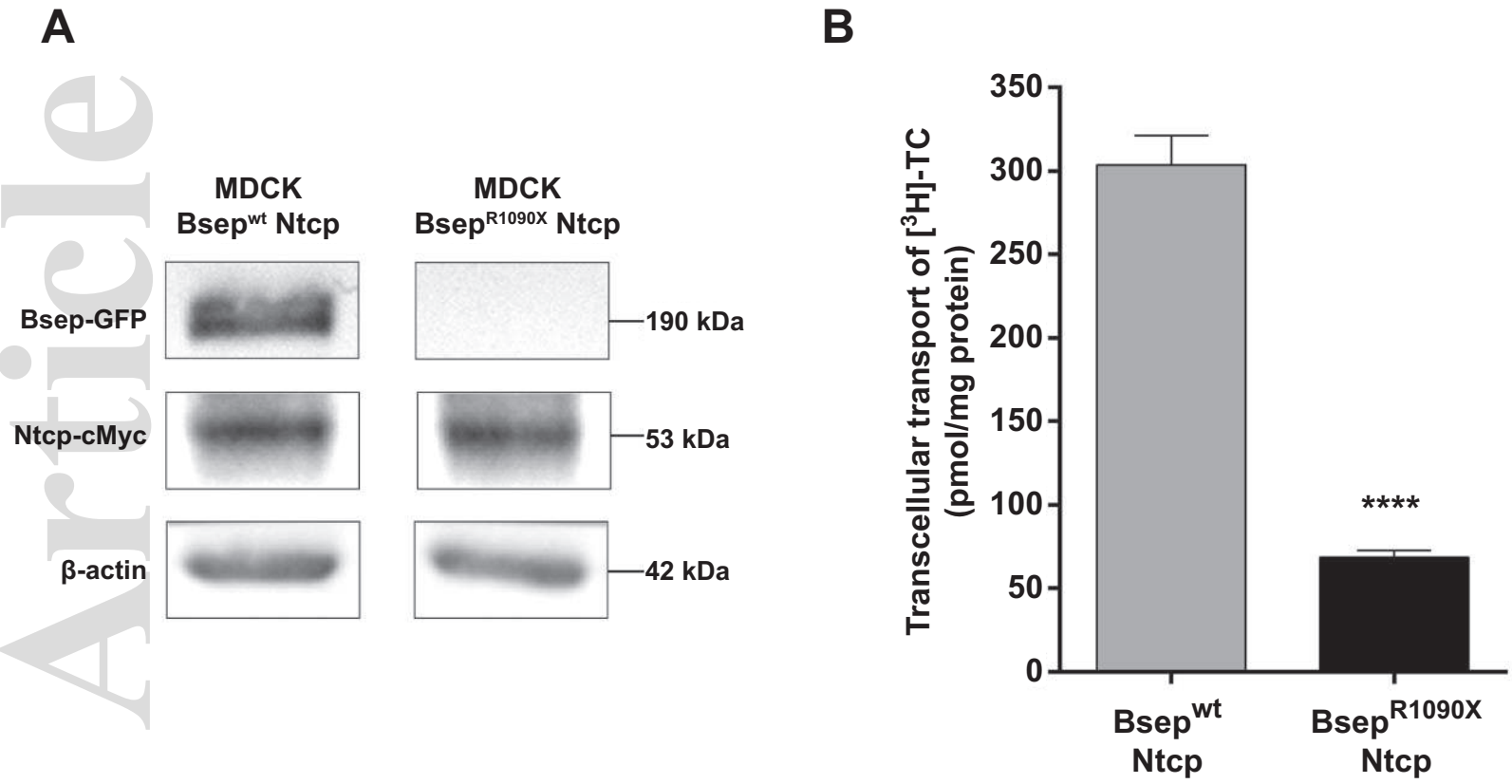

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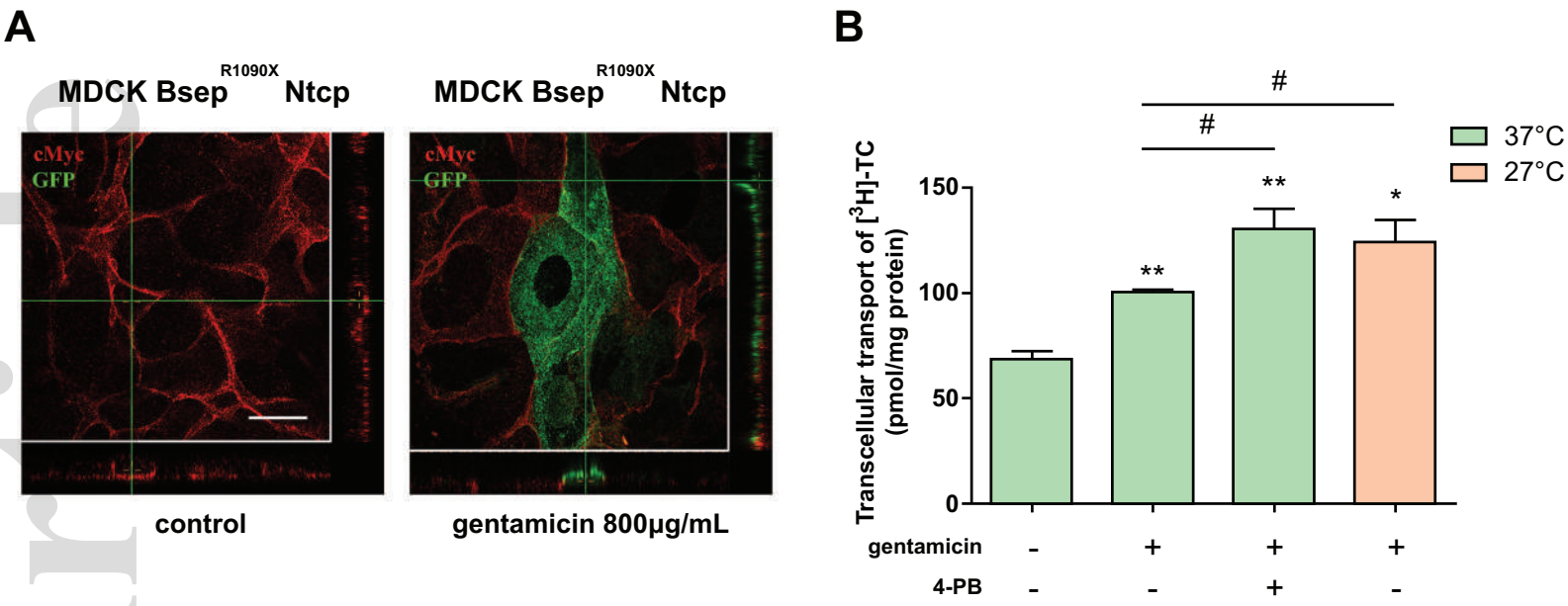

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