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| | Journal Name | | | Manuscript No. | | | | | | Author Received: | No. of pages: 4 | PE: Priya M |

*Journal of Viral Hepatitis,* 2012 doi:10.1111/jvh.12028

# Dissimilar expression of multidrug resistance *mdr1* and *bcrp* by the replication of hepatitis C virus: role of the nonstructural 5A protein

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*Received July 2012; accepted for publication October 2012*

SUMMARY. Multidrug resistance associated with the overexpression of ATP-dependent binding cassette (ABC) proteins is widely accepted as an important cause of treatment failure in patients with neoplastic or infectious diseases. Some of them play also a pivotal role in detoxification processes. Herein, we investigated the effect of hepatitis C virus (HCV) replication and nonstructural 5A (NS5A) protein on the expression and functional activity of two ABC transport proteins: MDR1 and BCRP. RT-quantitative real-time polymerase chain reaction (qPCR) was carried out for *mdr1* and *bcrp* mRNAs in both Huh7 cells expressing NS5A and Huh7.5 cells containing either full-length- or subgenomic-HCV replicon systems. The functional activity of these pumps was studied by performing a dye efflux assay with $DiOC_2$ and Rhodamine 123. A dose-dependent down-regulation of *mdr1* expression was documented in Huh7 cells expressing the NS5A protein, as well as in both replicon systems. In contrast, a significant increase of *bcrp* expression in both systems was recorded, which were in full agreement with the dye efflux assay results. These results warrant further *in vivo* studies in HCV patients with cholestasis and/or patients that are refractive to the pharmacotherapy due to the activity of these pumps.

*Keywords:* hepatitis C virus, NS5A, MDR1, BCRP.

## INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma, with an estimated 170 million people infected worldwide. The inability to culture HCV *in vitro* has severely limited meaningful definitive studies on pathogenesis, therapeutics and vaccines. The development of hepatoma-derived cell lines bearing autonomously replicating HCV RNA (replicons) overcame this restriction. The HCV nonstructural 5A (NS5A) phosphoprotein has been extensively studied due to its ability to subvert the antiviral response, to regulate the HCV replication and to modulate host intracellular signalling pathways [1].

The cells' ability to acquire resistance to pharmaceuticals is termed multidrug resistance (MDR), and it is often mediated by overexpression of ATP-binding cassette (ABC) transporters. At least two of them are widely associated with MDR: MDR1 (P-gp or ABCB1) and BCRP (ABCG2). MDR genes are induced during oxidative stress, thereby playing a critical role in detoxification processes [2]. In addition, several of these pumps exhibit high expression in severe liver diseases. In this regard, Qadri *et al.* [3] demonstrated the up-regulation of MRP2 by NS5A. In contrast, Ros *et al.* [4] have shown no significant difference for MRP2 in HCV-infected patients. Besides, controversial results have been reported regarding MDR1 in HCV-infected patients [4,5]. At present, BCRP expression in the context of HCV infection is unknown.

The aim of this study was to investigate the effect of the HCV replication and – in particular – the role of NS5A on the expression and functional activity of MDR1 and BCRP.

## MATERIALS AND METHODS

The full-length (FL) *ns5a* gene of HCV (genotype 1b) was amplified by reverse transcriptase-polymerase chain reaction

Abbreviations: ABC, ATP-dependent binding cassette; FL, full-length; HCV, hepatitis C virus; MDR, multidrug resistance; NS5A, nonstructural 5A; q-PCR, quantitative real-time polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction.

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(RT-PCR) and cloned into the expression vector pT-Rex-DEST31 (Gateway; Life Technologies Corp, CA, USA).

The Huh7.5 cells and HCV replicon cell lines Con1/FL-Neo (I) and Con1/SG-Neo (I) [Huh7.5 cells containing the FL and the subgenomic (SG) genotype 1b HCV replicon, BB7, respectively] were generously donated by Dr. Charles M. Rice (Rockefeller University, NY, USA).

Huh7 cells were transfected using Lipofectamine 2000 (Life Technologies Corp) with either the recombinant vector p-NS5A (NS5A-Huh7 cells) or a control vector (p-Ctrl). Cells were harvested by trypsinization at 18, 30 and 42 h post-transfection.

Western blot analysis was performed with monoclonal antibodies for NS5A (Biodesign, ME, USA), $\beta$-actin (Sigma-Aldrich, MO, USA), MDR1 and BCRP (Alexis Corp, Lausen, Switzerland). The amount of each protein was normalized to $\beta$-actin expression (Scion Image Program; NIH, MD, USA).

Cellular RNA was isolated using a commercial reagent (TRIzol®; Life Technologies Corp) and was reverse transcribed using ~~MMLV reverse transcriptase~~ (Life Technologies Corp) and random primers (Promega, WI, USA).

Comparative quantitative real-time polymerase chain reaction (qPCR) was performed with an iQ5 Real-Time PCR Detection System (Bio-Rad, CA, USA) with iQ SYBR Green Supermix (Bio-Rad) and 250 nM gene-specific primers (sequences available upon request). $\beta$-Actin and 18S were used as reference genes for normalization. The initial amount of each sample template was determined as a relative expression, as compared to one of the samples chosen as reference which is considered the '1× sample'. Relative quantification was performed using the $\Delta\Delta C_T$ method following the manufacturer's instructions.

Results reported in the *in vitro* experiments are the mean ± SD expression of at least four independent assays. All data were independently analysed in duplicate using both the statistical package R Development Core Team (http://www.R-project.org) and the Tadpole program (Cambridge University, UK).

The drug efflux assay was performed using Multidrug Resistance Direct Dye Efflux Assay (Chemicon®, CA, USA) based on the differential affinity of the transporters to $DiOC_2$ and Rhodamine 123 (Rho123), the former being a highly specific substrate of MDR1 (although weakly transported by BCRP), while the latter is effluxed by MDR1 but not by BCRP. To discriminate the BCRP from MDR1 activities, vinblastine was incorporated in each experiment. The assay was performed following the manufacturer's instructions. Single-cell fluorescence was quantified using a flow cytometer, FACScan (Becton Dickinson, NJ, USA).

## RESULTS

An increasing NS5A expression was recorded by Western blot in NS5A-Huh7 cells, reaching a plateau at 30–42 h post-transfection, due to which the subsequent experiments were carried out at 18 and 30 h post-transfection. The transfection efficiency was 35–40% as established by two independent methodologies: (i) immunofluorescence staining with a monoclonal anti-NS5A antibody and (ii) flow cytometry of green fluorescent protein-transfected Huh7 cells.

Dissimilar results were obtained when mRNA levels of *mdr1* and *bcrp* were studied by qPCR in NS5A-Huh7 cells. After 18 h of Huh7 transfection – when the viral protein expression was lower – there was no significant variation in the expression of *mdr1* and *bcrp* genes (1.17 ± 0.22 and 1.02 ± 0.16, respectively). However, at 30 h post-transfection – when the viral protein expression was higher – a statistically significant decrease of *mdr1* mRNA level (0.64 ± 0.06) was detected, whereas *bcrp* expression (0.78 ± 0.21) remained within control levels (Fig. 1a). In agreement, a statistically significant decrease of *mdr1* mRNA level was detected in both Huh7.5 FL and SG cells (0.48 ± 0.05 and 0.75 ± 0.02). In contrast, a statistically significant increase of *bcrp* mRNA level was documented in both HCV replicon systems. These increments were greater with HCV-FL replicon (8.76 ± 0.98) than with HCV-SG (5.33 ± 0.55; Fig. 1b).

MDR1 and BCRP protein levels recorded by Western blot strictly mirrored those of their respective mRNAs (figure available upon request). The highest level of protein expression was documented for BCRP in the HCV-FL replicon system.

No significant variation in the ability to release $DiOC_2$ and Rho123 was observed in NS5A-Huh7 cells at 18 and 30 h post-transfection. However, a slight decrease in the activity of MDR1 and a significant increase in the activity of BCRP were documented in both Huh7.5-FL and Huh7.5-SG replicon systems (Fig. 1c).

## DISCUSSION

Hepatic uptake and biliary excretion of organic anions are mediated by hepatobiliary transport systems. A reduction in transporter expression and/or function may cause or maintain cholestasis and jaundice [6]. In contrast, overexpression of MDR1 and BCRP was reported to mediate MDR.

Herein, we investigated the effect of the HCV replication and – in particular – the NS5A protein expression on the *mdr1* and *bcrp* mRNA, as well as protein levels and efflux activity of both of the corresponding pumps.

We demonstrated a significant decrease in *mdr1* mRNA levels in the context of two HCV replicon systems, as well as in NS5A-Huh7 cells. Accordingly, we postulate that *mdr1* expression levels are strongly influenced by NS5A. Furthermore, a synergistic effect of other HCV proteins (structural and/or nonstructural) is suggested. In agreement with our results, Hinoshita *et al.* [5] have also

LOW RESOLUTION FIG

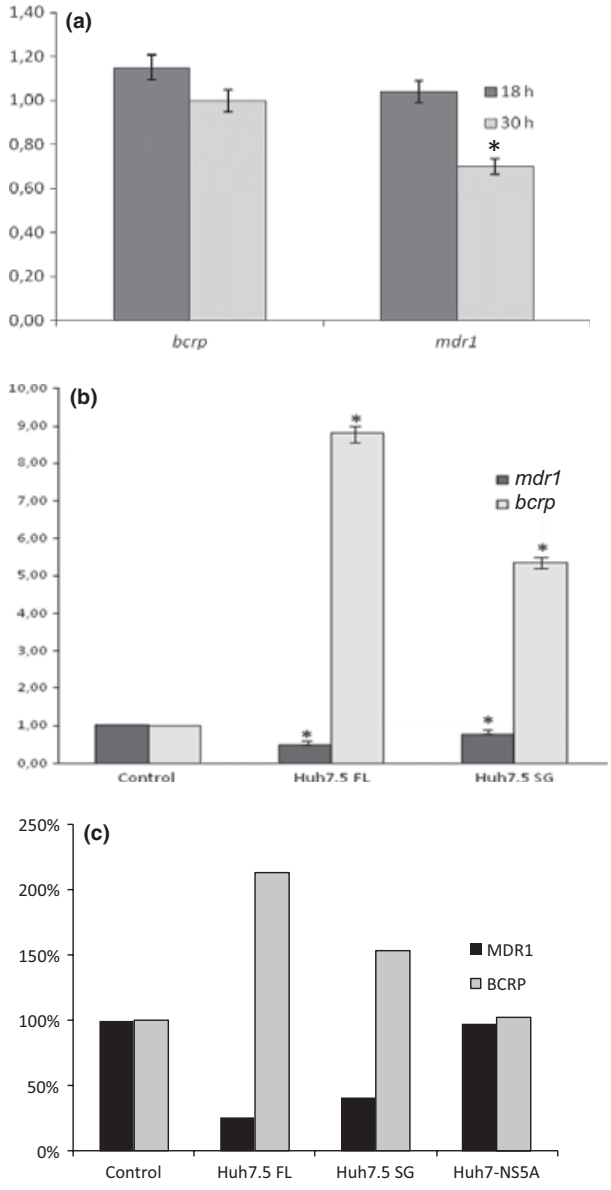


**Fig. 1** Relative quantification of *bcrp* and *mdr1* mRNA by RT-qPCR. (a) *Bcrp* and *mdr1* mRNA levels in Huh7 cells expressing nonstructural 5A (NS5A) protein at different elapsed time post-transfection (18 and 30 h); the control sample (*NS5A.ctrl-Huh7* cells) is considered the '1× sample'. (b) *Mdr1* and *bcrp* mRNA levels in Huh7.5 cells (Control) and in Huh7.5-FL and Huh7.5-SG replicon systems. $*P < 0.05$. (c) MDR1 and BCRP functional activity in Huh7.5 cells (Control), Huh7.5-FL, Huh7.5-SG and Huh7 cells expressing NS5A protein at 30 h post-transfection – (NS5A-Huh7). A 100% of activity was arbitrarily assigned to the value observed with the corresponding control cells for each experiment.

demonstrated a decreased expression of MDR1 in human livers with HCV infection. In contrast, Ros *et al.* [4] have reported an increased expression of this transporter in the regenerating bile ductules from HCV-infected livers. We hypothesize that the altered expression of ABC transporters in patients might be associated with the HCV replication, and/or to the development of liver disease resulting from the virus–cell interaction.

On the other hand, when we analysed the expression levels of *bcrp*, dissimilar results were obtained between HCV replicon systems and the NS5A-Huh7 cells. A significant increase of *bcrp* mRNA was documented in both Huh7.5 cells containing the HCV-FL and the HCV-SG replicon systems, as compared with Huh7.5 cells. However, *bcrp* mRNA levels were not affected by the sole NS5A expression, even at 30 h post-transfection. These results might suggest that the up-regulation of *bcrp* gene expression is mediated by HCV structural and nonstructural proteins and that the mere expression of NS5A is not sufficient to mediate this effect. However, an eventual compensatory increase of BCRP associated with the decreased MDR1 expression cannot be formerly ruled out with the experiments performed herein. BCRP is recognized for its important role in the absorption, tissue distribution and elimination of drugs; however, to the best of our knowledge, no information exists about the effect of HCV replication on this pump expression [7].

The effect of HCV-FL and HCV-SG on both MDR1 and BCRP pump activities strictly mirrored those obtained with qPCR and Western blot for the corresponding mRNAs and proteins. Unexpectedly, when NS5A-Huh7 cells were analysed, no significant changes could be recorded in MDR1 activity, possibly due to a lower sensitivity of the Die Efflux Assay to detect the 30–40% of cells expressing NS5A protein in our transient transfection experiments.

To our knowledge, this is the first report describing the action of HCV replication on the expression of MDR1 and BCRP, pointing to a pivotal role by the NS5A protein. The decrease in the expression of liver detoxification-related genes might produce the toxic bile accumulation and then encourage cholestasis. This infrequent course is observed in 2–5% of HCV-infected patients leading to a considerable hepatocellular injury, reportedly proposed to be associated with a direct cytopathic effect [8]. On the other hand, the up-regulation of MDR genes such as BCRP by HCV replication might justify the low efficacy of antitumoral- or antiviral-therapy recorded in some HCV-infected patients.

## ACKNOWLEDGEMENTS

This work was supported by grants from Universidad de Buenos Aires (UBACyT 20020100101063 and 20020090200189). The financial help of an in house grant from Fondazione Italiana Fegato is acknowledged. The authors are indebted to Ana María Andreetta for technical support and to Victoria Illas for enhancing the readability of this manuscript.

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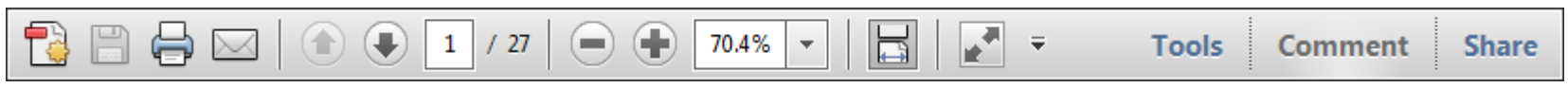


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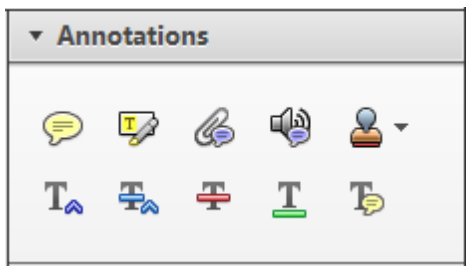


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Strikes a line through text and opens up a text box where replacement text can be entered.

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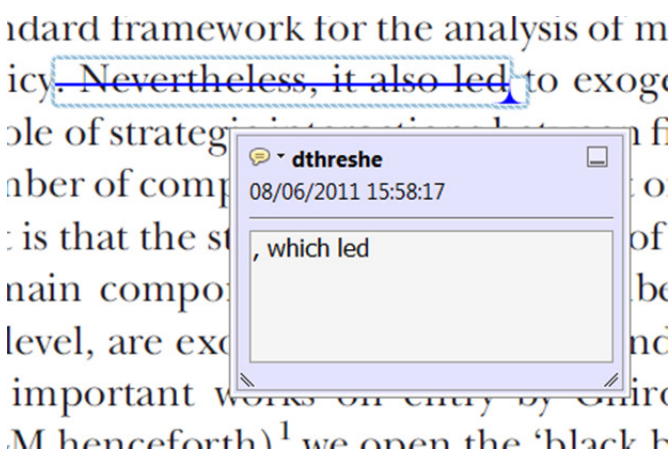


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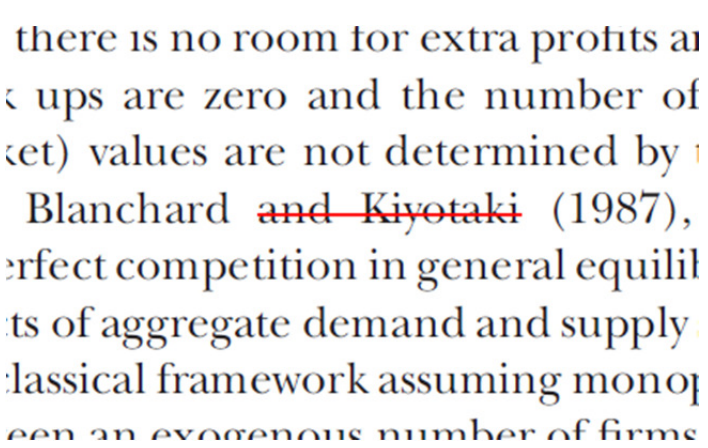

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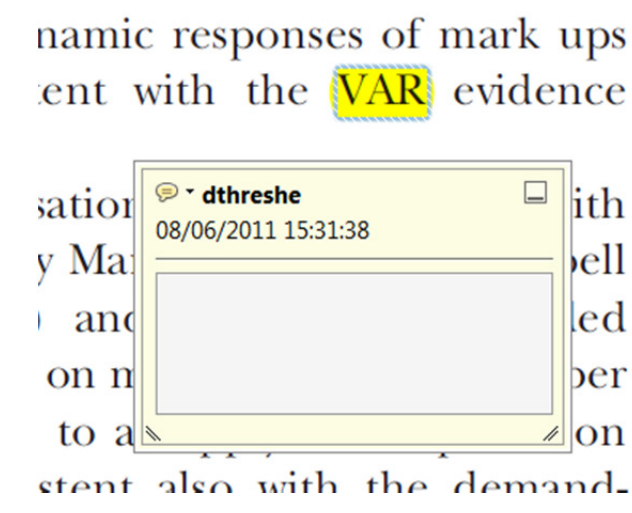


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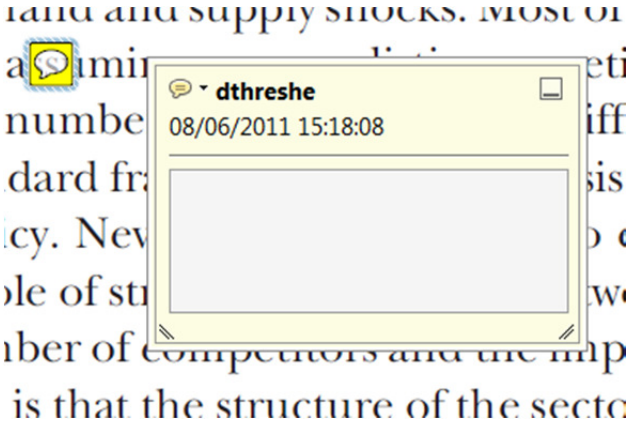

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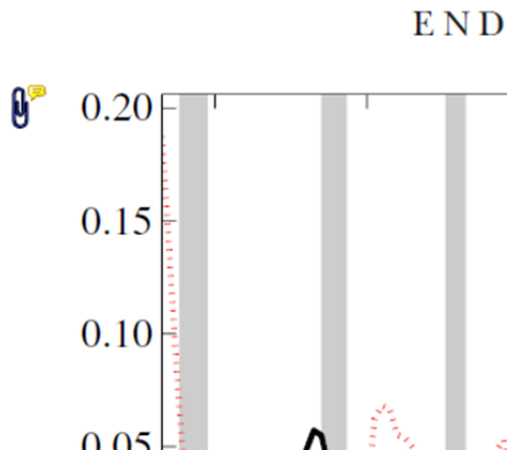


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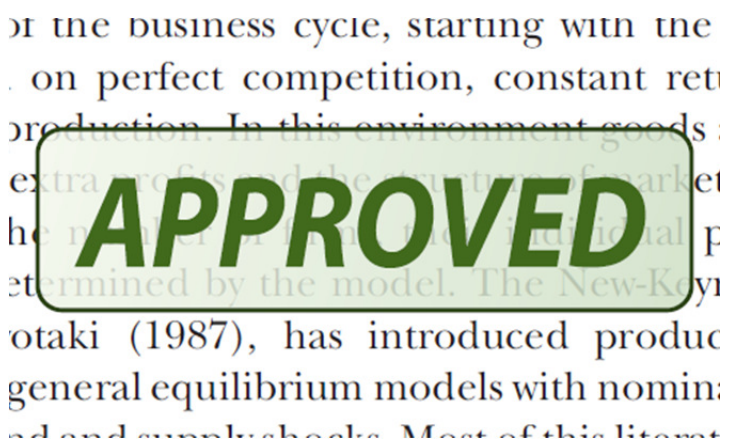


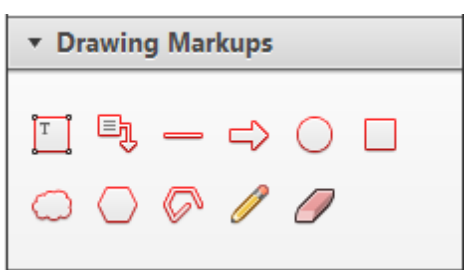


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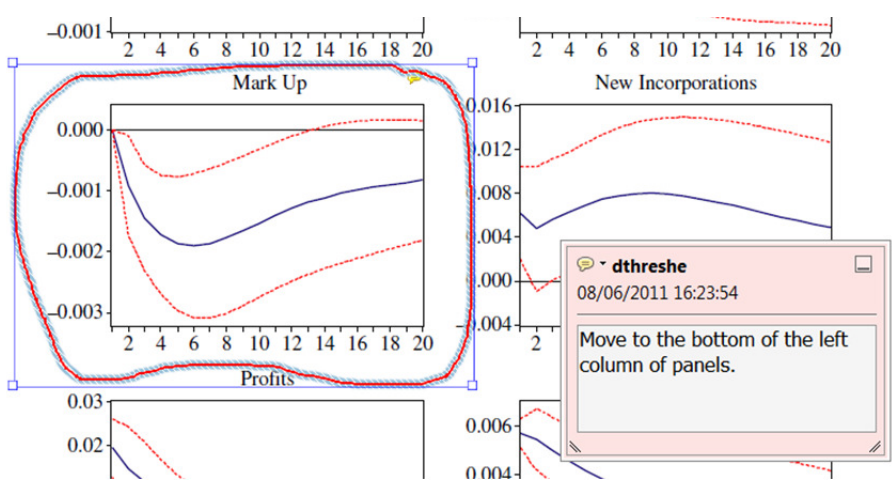
- Double click on the shape and type any text in the red box that appears.

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