Title: C-terminal Truncated HBx Reduces Doxorubicin Cytotoxicity via ABCB1 Upregulation in Huh-7 Hepatocellular Carcinoma Cells

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Running Title: Reduced doxorubicin cytotoxicity by HBx 1-120.

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Supplementary Materials and Methods

Materials

RPMI 1640 medium (Cat. No: LM011-01) and fetal bovine serum (FBS) were purchased from JBI (Daegu, Korea). TRIzol was purchased from QIAGEN (KJ Venlo, Netherlands). M-MLV reverse transcriptase was purchased from Promega (Madison, WI, USA). Reverse Transcriptase Premix (Cat. No: EBT-1515) was purchased from Elpis Biotech (Daejeon, Korea). The α-Tubulin, MDR1, and PARP antibodies, and HRP-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Maxime PCR Premix (Cat. No: 25025) was purchased from iNtRON (Daejeon, Korea). ProPREP lysis buffer was purchased from iNtRON. Rhodamine 123 was purchased from Invitrogen (Carlsbad, CA, USA). Doxorubicin (Cat. No: D1515) and R(+)-Verapamil monohydrochloride hydrate (Cat. No: V106) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The MDR1-specific inhibitor C-4 was purchased from Santa Cruz Biotechnology. The antibodies Mcl-1, procaspase-3, and cleaved-caspase-3 were purchased from Cell Signaling (Danvers, MA, USA). ON-TARGETplus SMARTpool (siABCB1, siControl) was purchased from Dharmacon (Livermore, CA, USA). The Annexin V-FITC Apoptosis Detection Kit (Cat. No: BMS500FI) was purchased from eBioscience (San Diego, CA, USA).

Cell culture and transfection

Huh-7 cells stably expressing HBx Full or C-terminal-truncated HBx were established as described previously (22).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol reagent in accordance with the manufacturer’s instructions. cDNA was prepared from 2 μg of total RNA using M-MLV reverse transcriptase
and Reverse Transcriptase Premix. The cDNA thus obtained was amplified by PCR in a 20 μL reaction volume containing 1× reaction buffer, dNTP mixture, I-Taq DNA polymerase, and primer pairs specific to each gene. The PCR primers used were as follows. β-actin: 5′-GTG GGG CGC CCC AGG CAC CAG GGC-3′ (forward) and 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ (reverse), and ABCB1, 5′-ACT GAG CCT GGA GGT GAA GA-3′ (forward) and 5′-CCT TCT CTG GCT TTG TCC AG-3′ (reverse). PCR was carried out with an initial denaturation at 95°C for 5 min, followed by 25 cycles for β-actin (denaturation 95°C, 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min). PCR conditions for the amplification of ABCB1/MDR1 were as follows. Initial denaturation at 95°C for 5 min, followed by 30 cycles (denaturation for 1 min at 95°C, annealing for 30 sec at 59°C, and extension for 1 min at 72°C). This was followed by a final extension step for 5 min at 72°C. PCR products were electrophoresed on 1% agarose gels and GelRed™ (Biotium; Hayward, CA, USA) staining was visualized using a UV-transilluminator.

**MTT assay**

Huh-7 stable cells were seeded at 20,000 cells/cm² in a 24-well plate (SPL) and incubated for 24 h in an incubator in an atmosphere of 5% CO₂. For the viability assay, the cells were treated with doxorubicin (2 μM) or verapamil (10 μM) for 48 h. MTT reagent (Sigma-Aldrich) was added directly to each well to a final concentration of 0.6 mg/mL. After 4 h, the medium was removed and formazan crystals formed in the cells were dissolved in dimethyl sulfoxide, the absorbance of the formazan solution was measured using an ELISA reader with a 540 nm filter. Each sample was assayed in triplicate.

**siRNA transfection**

Huh-7 stable cells were seeded at 20,000 cells/cm² in 6-well plates and incubated for 24 h.
The siABCB1/MDR1 and siCONTROL (ON-TARGETplus SMARTpool, Dharmacon) were mixed with transfection reagent (5× siRNA buffer, Dharmacon) according to the manufacturer's instructions. Cells were transfected with either ABCB1 siRNA (50 nM) or non-targeting control siRNA (50 nM) for cell viability, flow cytometry, and Western blot analyses.

**Rhodamine 123 retention assay**

Huh-7 stable cells were seeded on plates (20,000 cells/cm²) and incubated for 24 h. They were then treated with the MDR1 inhibitor verapamil (10 μM) for 1 h, then with 2 μM rhodamine 123 for 1 h. The cells were collected and washed with HBSS, resuspended in 1 mL HBSS at a density of 1 × 10⁶ cells/mL, and analyzed with a flow cytometer (FC500, Beckman Coulter, Fullerton, CA) to measure the fluorescence intensity of rhodamine 123 taken up by the cells (excitation wavelength = 488 nm, emission wavelength = 575 nm).

**Annexin-V/PI apoptosis assay**

Stable cells were seeded on plates and incubated for 24 h in the presence of doxorubicin (2 μM) and siABCB1 (50 nM). After 24 h, cells were collected and washed with 1× annexin binding buffer, resuspended in the same buffer, and treated with both annexin and PI for 25 min. The cells were then analyzed using a flow cytometer. Annexin and PI fluorescence were measured at 518 and 535 nm, respectively.

**Western blot analysis**

Huh-7 stable cells were seeded at 20,000 cells/cm² on 100 mm dish plates and incubated for 24 h. To detect apoptosis markers, the cells were treated with doxorubicin (2 μM), verapamil (10 μM), and siABCB1 (50 nM) for 48 h. To confirm that ABCB1 expression was blocked,
the cells were incubated with siControl and siABCB1 (50 nM) for 48 h. Cultured cells were rinsed twice with ice-cold PBS and the total protein was extracted using Pro-PREP protein lysis buffer according to the manufacturer’s instructions. The protein concentration of the lysis supernatant was determined using the BCA protein assay kit (Sigma; St. Louis, MO, USA). Equal amounts of cell lysate (30 μg of protein for detecting apoptosis markers and 20 μg of protein for detecting ABCB1) were separated by SDS-PAGE (8-10% for the detection of ABCB1, 12% for the detection of apoptosis markers). The separated proteins were transferred to Hybond-P PVDF membranes (Amersham Biosciences and Millipore; Temecula, CA, USA). The membranes were blocked with blocking solution (BIO-FACT; Daejeon, Korea) for 1 h at 25–27°C and probed overnight at 4°C with diluted primary antibody. The membranes were then washed and incubated with HRP-conjugated secondary antibody (1/2500). The signals were visualized using the Hisol™ ECL PLUS detection kit (BIO-FACT; Daejeon, Korea) and WesternBright™ ECL solution (Advansta; Menlo Park, CA, USA).

**In vivo experiments**

Female 6-week-old Balb/c-nu/nu mice were purchased from Lab Animal Co. Ltd (Seoul, Korea). The mice were housed in a specific-pathogen-free environment in the Animal Resource Facilities at Pusan National University and autoclaved food and water were provided *ad libitum*. All experimental procedures complied with the Pusan National University IACUC in accordance with institutional guidelines. To perform the subcutaneous tumor growth and resistance assay, 10 female nude mice were acclimated to the housing facility (5 mice/cage, 50% relative humidity, 12 h light/dark cycle) for two weeks. Matrigel mixed with Huh-7 stable cells (5 × 10⁶ cells in 200 μL Matrigel) was subcutaneously injected into the posterior thighs of the mice. Tumor volumes were measured periodically (thrice per week) using a digital caliper and calculated according to the equation, \( V (\text{mm}^3) = \frac{W \times L^2}{2} \),
where W and L were the width (longest diameter) and length (shortest diameter) of the tumor, respectively. When the tumors reached a volume of 50–100 mm$^3$, the mice were randomly assigned to control and experimental groups and doxorubicin treatment was initiated. Doxorubicin was intraperitoneally injected at a concentration of 5 mg/kg in 0.1 mL of PBS twice a week. The experiment was terminated when the tumor volume reached 2000 mm$^3$.

**Statistical analyses**

Data are presented as the mean ± standard deviation (SD). Statistical comparisons between the groups were performed using the Student’s t-test. *$P<0.05$ was considered statistically significant, and statistical analyses were performed using Microsoft Excel.