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Up-regulation of connexin 32 gene by 5-aza-2′-deoxycytidine enhances vinblastine-induced cytotoxicity in human renal carcinoma cells via the activation of JNK signalling

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ABSTRACT

Enforced expression of connexin (Cx) 32 gene, a member of gap junction gene family and a tumor suppressor gene in human renal cell carcinoma (RCC), enhanced vinblastine (VBL)-induced cytotoxicity on RCC cells, due to the suppression of multidrug resistance 1 (*MDR1*) gene product, P-glycoprotein (P-gp). Also, Cx32 gene in RCC is silenced by hypermethylation of CpG islands in a promoter region of the Cx gene. In this study, we investigated if a DNA demethylating agent, 5-aza-2′-deoxycytidine (5-Aza) could enhance susceptibility of RCC cells (Caki-1) to VBL. We found that 5-Aza treatment up-regulated Cx32 in Caki-1 cells, and the induction of the Cx led to the suppression of P-gp through inhibition of Src and subsequent activation of c-Jun NH_2-terminal kinase (JNK). Moreover, increased transcription activity of c-Jun by the JNK activation contributed to the down-regulation of MDR1, thus indicating a central role of JNK signalling to suppress P-gp level in 5-Aza-treated Caki-1 cells. Chemical sensitivity to VBL in Caki-1 cells was increased by 5-Aza pre-treatment, and this effect was abrogated by short interfering RNA (siRNA)-mediated knockdown of Cx32. Furthermore, co-treatment of 5-Aza or a P-gp inhibitor with VBL drastically enhanced JNK activation comparing to only VBL treatment in Caki-1 cells. These results suggest that the restoration of Cx32 by 5-Aza pre-treatment improves chemical tolerance on VBL in Caki-1 cells and that the JNK activation is a key factor to induce the effect.

1. Introduction

Renal cell carcinoma (RCC) has a very poor prognosis, due in large part to the fact that of nearly 30% of all patients with localized disease, 40% ultimately develop distant metastases following removal of the primary tumor, and available chemotherapeutic agents are ineffective against metastatic RCC [1,2]. For instance, vinblastine (VBL) is one of the few cytotoxic agents with reproducible activity in RCC, but the outcome of treatment of metastatic RCC with VBL alone is often disappointing [3]. Combination therapy of VBL with other agents such as interferon-γ is also ineffective against metastatic RCC [4]. P-glycoprotein (P-gp), the multidrug resistance 1 (*MDR1*) gene product appears to function as an energy-dependent transport pump capable of decreasing the intracellular concentration of a wide range of anticancer agents such as VBL, which confers a chemoresistant phenotype on cancer cells [5]. Since overexpression of P-gp has been found in nearly 80% of RCCs, the chemoresistance of RCC has been ascribed in large part to P-gp [6]. Thus, P-gp seems to be an attractive target to improve chemotherapy in metastatic RCC [7].

In general, it is considered that disruption of functions to maintain homeostasis in cellular society leads to the appearance of malignancies in the cells [8]. That is, the down-regulation of tumor suppressor genes keeping the homeostasis directly relates to the development of cancers. Among the tumor suppressor genes contributing to tissue homeostatis, down-regulation of connexin (*Cx*) genes, a member of gap junction (GJ) is associated with the development of cancers [9]. It has been well established that Cx acts as a tumor suppressor gene by keeping electrical and metabolic cell homeostatis via GJ-dependent transfer of small molecules less than 1500 Da among neighboring cells [9]. In addition to the GJ-dependent mechanism, the *Cx* gene exerts a tumor-suppressive effect in a GJ-independent manner [10]. In fact, we have recently demonstrated that Cx32 acts as a tumor

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suppressor gene against metastatic RCC in both GJ-dependent and GJ-independent manners [11]. As an important function of *Cx* genes in the GJ-independent mechanism, there is potentiating effect of Cx on anti-cancer agent-induced cytotoxicity in cancer cells [12]. Actually, we have reported that enforced expression of *Cx32* gene could enhance VBL-induced cytotoxicity against the RCC via the reduction of P-gp level in a GJIC-independent manner [13]. This report means that restoration of *Cx32* gene expression by an adequate approach is an effective procedure to heighten chemical sensitivity to VBL in RCC, based on the GJ-independent function.

Methylation of CpG islands in 5′ regions of tumor suppressor genes is known to inhibit transcription, leading to silencing of the corresponding genes. We have reported that Cx32 is down-regulated in RCC due to hypermethylation of CpG islands in the promoter region [14,15]. Also, we have confirmed that zebularine, a DNA demethylating agent, induced re-expression of the *Cx32* gene in RCC cells *in vitro* [16]. These reports led us to speculate that treatment with DNA demethylating agents can induce the restoration of Cx32-regulated events such as the decrease of P-gp level in RCC, due to a GJ-independent function. In this study, we examined whether a DNA demethylating agent, 5-aza-2′-deoxycytidine (5-Aza), which is frequently utilized in human clinical trials [17], could restore the expression of *Cx32* gene and increase chemical sensitivity to VBL in Caki-1 cells.

2. Materials and methods

2.1. Materials

All culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). VBL was obtained from Wako Pure Chemicals (Osaka, Japan). PP2 (a Src inhibitor) and PP3 (a negative control of PP2) were from Biomol (Plymouth Meter, PA, USA). Non-specific (NS) small interfering RNA (siRNA), HP validated siRNAs for Cx32 (cat# SI00003514) and c-Jun (cat# SI00300580), and HiPerfect Transfection Reagent were obtained from Qiagen Japan (Tokyo, Japan). PCR primers were purchased from Sigma Genosys (Hokkaido, Japan). Other chemicals were purchased from Sigma (St. Louis, MO, USA), unless otherwise noted. Antibody against Cx32 was obtained from Zymed (San Francisco, CA, USA). Antibody against P-gp was from Sigma. All other antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell culture and treatment

A representative human metastatic RCC cell line (Caki-1), obtained from ATCC (Manassas, VA, USA), was routinely maintained in McCoy's 5A medium supplemented with 10% foetal bovine serum and penicillin–streptomycin at 37 °C in an atmosphere of 5% CO_2. The cells were plated and treated with 5-aza-CdR (0.5 μM) for 48 h, subsequently other chemicals were added to the culture system, and the treatment was continued for each indicated time.

2.3. Cell viability assay

The cells were cultured on microtiter plates (3×10^4 cells/well) and treated with each chemical at the indicated doses for the indicated treatment periods. Cell viability was then determined using the Cell Proliferation Assay Kit with WST-1 reagent (Sigma), according to the manufacturer's protocol.

2.4. Apoptosis analysis

After the treatment described in figure legend, the cells were harvested by trypsinization, washed with PBS, re-suspended in 70% ethanol in PBS, and kept at 4 °C for at least 30 min. Before analysis, cells were washed again with PBS and resuspended and incubated for 30 min in PBS containing 0.05 mg/ml propidium iodide, 1 mM EDTA, 0.1% Triton X-100, and 1 mg/ml RNase A. The suspension was then passed through a nylon mesh filter, and the ratio of subG1 cell population in total cells was analyzed on a Becton Dickinson FACScan (Franklin Lakes, NJ, USA) to confirm the induction of apoptosis. Caspase 3 activity as an index of apoptosis was determined using Caspase-Glo 3/7 Assay Kit (Promega, Madison, MI, USA), according to the manufacturer's protocol.

2.5. P-gp functional assay

The functional activity of P-gp in the cells was performed using the Rhodamine 123 (Rh123) [18]. After the treatment described in figure legend, the culture medium was removed, Hanks' balanced salt solution (HBSS) containing 10 mM Rh123 was added to each well, and Rh123 was loaded to the cells by incubation for 6 h at 37 °C. After the incubation, the cells were washed with HBSS, subsequently lysed with 0.3 M NaOH and neutralized with 0.3 M HCl. Then, the accumulation of Rh123 in the lysate was measured on a fluorescence spectrophotometer (Hitachi, Ibaraki, Japan) at 492 nm excitation and 522 nm emission. Each value was normalized by cell viability in each group.

2.6. Reverse transcription-realtime polymerase chain reaction (RT-realtime PCR) and analysis

Total RNA was isolated by using ChargeSwitch® Total RNA Cell Kits (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized as previously described [19]. RT-realtime PCR was performed by using ABI Prism 7000 Sequence Detection System (Applied Biosystems Japan Ltd. Tokyo, Japan) and SYBR® Premix Ex Taq™ (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's instruction. The PCR reaction was carried out by using primers MDR1 (NCBI reference number 42741658): sense primer (nucleotides 2779–2798); antisense primer (nucleotides 2905–2924), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NCBI 7669491): sense primer (nucleotides174–193), antisense primer (nucleotides 313–332). The reaction was performed at 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, then 60 °C for 1 min. The abundances of amplified DNA were determined from the threshold cycle values and were normalized to the values for the control gene GAPDH to yield the relative abundance. The values of MDR1/GAPDH were normalized to those of control.

2.7. Immunoblot analysis

After each treatment, the cells were lysed in Cell Lysis/Extraction Reagent with Protease Inhibitor Cocktail, and Phosphatase Inhibitor Cocktail 1 and 2 (Sigma), and 15 μg protein extract from each sample was loaded onto 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes. The blots were incubated with each primary antibody. Each immunoreactive band was detected with an ECL system (Amersham, Buckinghamshire, UK) and the Cooled CCD camera-linked Cool Saver System (Atto, Osaka, Japan). Molecular sizing was performed with the Rainbow MW marker (Amersham). Protein concentration was determined by the DC Protein Assay System (Biorad, Hercules, CA, USA). In order to determine the activation or inactivation of each signal molecule, we checked level of each phosphorylated plus un-phosphorylated signal molecule (described as total in figures) in addition to that of each phosphorylated signal molecule (described as P in figures). Also, we confirmed equal loading of each sample, using β-actin as an internal standard.

2.8. Transfection of siRNA

Cx32 and c-Jun were down-regulated by short interfering RNAs (siRNAs) targeting Cx32 and c-Jun, respectively. For transfection, the cells were seeded in a 6-well plate (5×10^5 cells/plate) and transfected with HiPerfect Transfection Reagent according to the manufacturer's protocol. After the cells were pretreated with the siRNA for 24 h, combination treatment of 5-aza-CdR and the siRNA was further continued for 48 h. Then, knockdown of Cx32 and c-Jun by siRNA was confirmed by immunoblot analysis. The level of P-gp, and the activated status of c-Src and c-Jun were evaluated by immunoblot analysis. MDR1 mRNA level was determined by RT-realtime PCR. As a negative control, NSsiRNA was used.

2.9. c-Jun transcription activity assay

In order to evaluate c-Jun transcription activity in the cells, TransAM AP-1 c-Jun Transcription Factor Assay Kit (cat# 46096, Active Motif Japan, Tokyo, Japan) was utilized. The procedure was performed according to the manufacturer's instruction. In brief, nuclear extract form the cells (8×10^6 cells/sample) was prepared using Nuclear Extract Kit (cat# 40010, Active Motif Japan, Tokyo, Japan), according to the manufacturer's protocol.

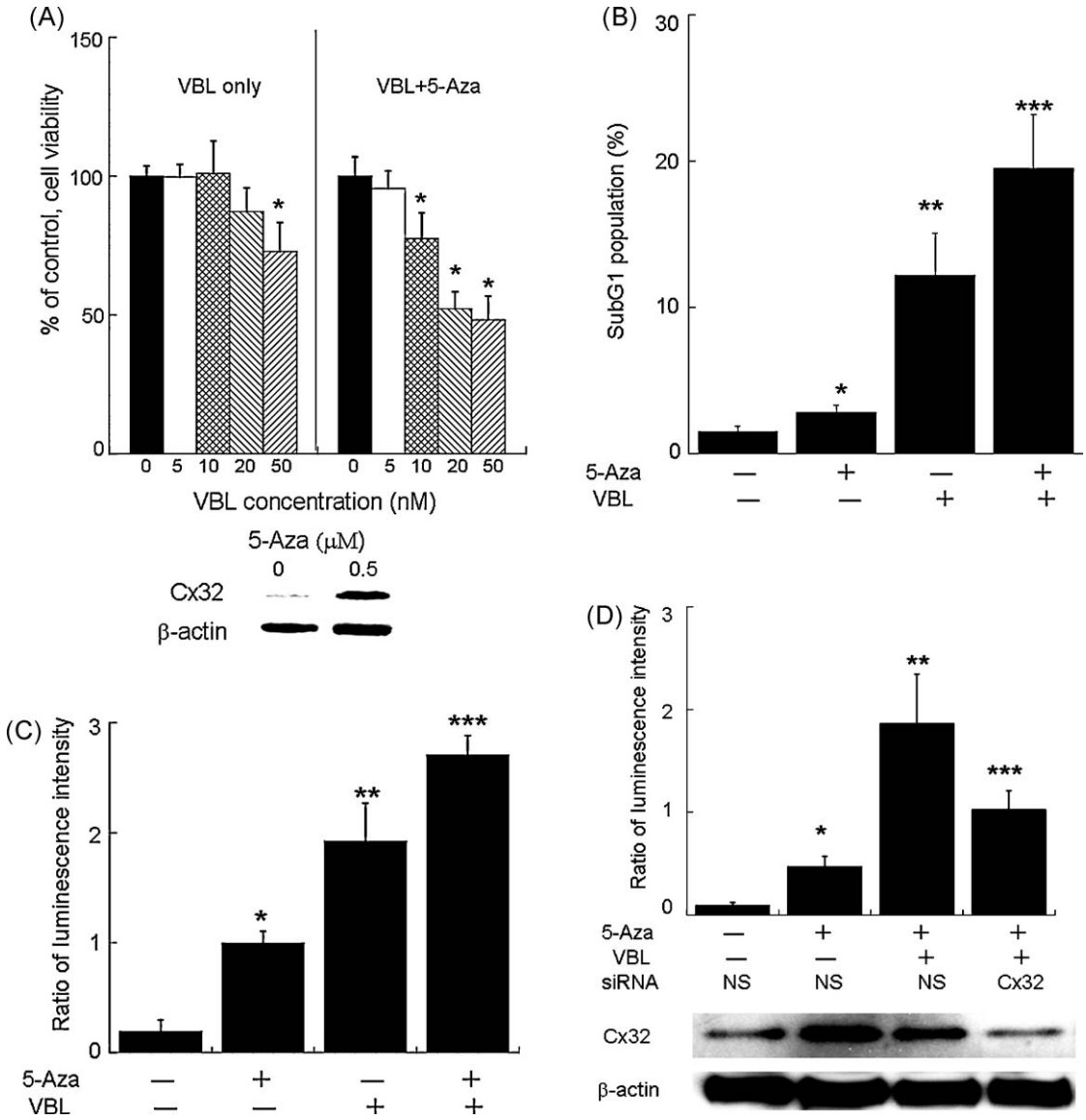

Fig. 1. Up-regulation of Cx32 by 5-Aza contributes to enhancement of VBL-induced cytotoxicity in Caki-1 cells. (A) Upper graph: Caki-1 cells were treated with 0.5 μM 5-Aza for 48 h and subsequently with VBL at indicated doses for 48 h. Then, cell viability was determined by WST-1 assay. The data were expressed as the mean ± S.D. of six samples. *Significant difference from control. Down panel: The cell extracts (5×10^5 cells/sample) were collected 48 h after treated with 0.5 μM 5-Aza. Immunoblot analyses were performed using antibodies against Cx32 and β-actin. This result was representative of two independent experiments. (B) The cells (1×10^6 cells/sample) were treated with 0.5 μM 5-Aza for 48 h and subsequently with 50 nM VBL as indicated for 48 h. After the treatment, subG1 population was determined by FACS analysis. The data were expressed as the mean ± S.D. of three samples. *Significant difference from control, **significant difference from control and 5-Aza treatment, and ***significant difference from other three groups. (C) The cells (1×10^4 cells/sample) were treated with 0.5 μM 5-Aza for 48 h and subsequently with 50 nM VBL at indicated doses for 24 h. After the treatment, caspase 3 activity was measured as mentioned in Section 2. The data were expressed as the mean ± S.D. of four samples. *Significant difference from control, **significant difference from control and 5-Aza treatment, and ***significant difference from other three groups. (D) Upper graph: After the treatment with siRNA and 5-Aza as described in Section 2, the cells were further treated with 50 μM VBL for 24 h. Then, caspase 3 activity was determined. The data were expressed as the mean ± S.D. of four samples. *Significant difference from control, **significant difference from control and 5-Aza treatment, and ***significant difference from other three groups. Lower panel: After the treatment with siRNA and 5-Aza described in Section 2, immunoblot analyses were performed using antibodies against Cx32 and β-actin. This result was representative of two independent experiments.

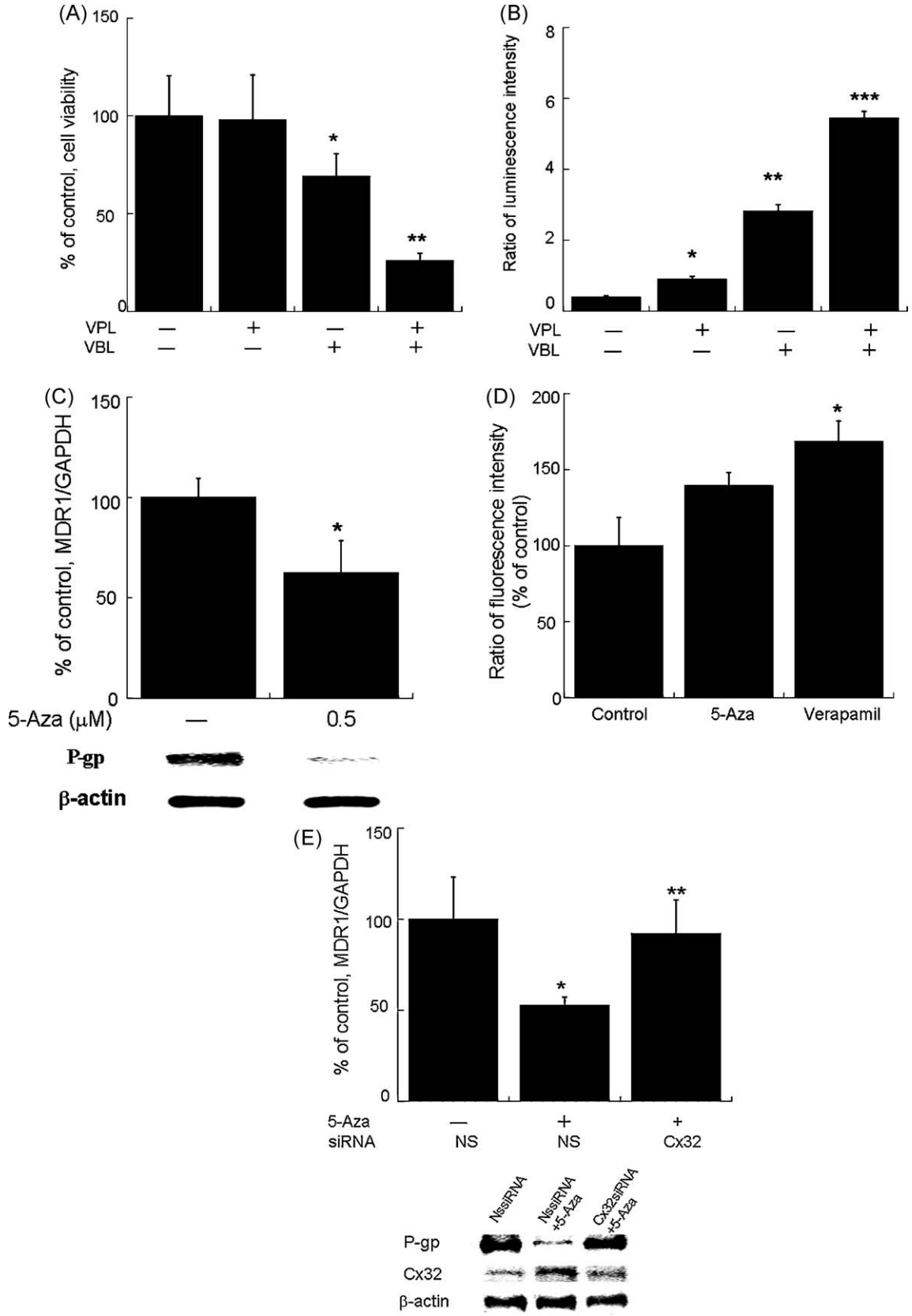

Fig. 2. Up-regulation of Cx32 by 5-Aza enhances VBL-induced cytotoxicity in Caki-1 cells via inhibition of P-gp function. (A) Caki-1 cells were treated with 50 nM VBL and 10 μM VPL for 48 h. Then, cell viability was determined by WST-1 assay. The data were expressed as the mean ± S.D. of six samples. *Significant difference from control and VPL treatment, and **significant difference from other three groups. (B) The cells (1×10^4 cells/sample) were treated with the same treatment described above. After the treatment, caspase 3 activity was measured as mentioned in Section 2. The data were expressed as the mean ± S.D. of four samples. *Significant difference from control, **significant difference from control and VPL treatment, and ***significant difference from other three groups. (C) Upper graph: The cells (3×10^5 cells/sample) were treated with 0.5 μM 5-Aza for 48 h, and subsequently MDR1 mRNA level was measured by RT-realtime PCR as described in Section 2. The data were expressed as the mean ± S.D. of four samples. *Significant difference

The extracts were used as sources to determine c-Jun transcription activity. The nuclear extract containing 10 μg protein was added to well in a 96-well plate on which has been immobilized an oligonucleotide that contains a TRE (5′-TGATCA-3′) and incubated for 1 h at room temperature. After washing, a phospho-c-Jun antibody was incubated with the plate for 1 h at room temperature. Subsequently washing and secondary antibody conjugated to horseradish peroxidise was incubated with the plate for 1 h at room temperature. After washing, developing solution was added to the well and incubated for 10 min. After the procedure, stopping solution was added to the well, and optical density at 450 nm with a reference wavelength of 655 nm was determined using a microplate reader (Tecan Japan, Kanagawa, Japan). Provided K-562 nuclear extract was used as a positive control.

2.10. Statistical analysis

Data are expressed as the mean ± SE, and analyzed by one-way analysis of variance followed by Dunnett's *t*-test, Tukey–Kramer test or Student's *t*-test. $P < 0.05$ was taken as significant difference.

3. Results

3.1. Effect of 5-Aza on VBL-induced cytotoxicity in Caki-1 cells

In order to estimate the usefulness of combination of VBL with 5-Aza as a cancer therapy against metastatic RCC, we evaluated the effect of 5-Aza on VBL-induced cytotoxicity in Caki-1 cells. Treatment with 5-Aza significantly potentiated the VBL-induced cytotoxicity and this effect showed a dose-dependency (Fig. 1a). On the other hand, VBL treatment alone showed a significant cytotoxicity only at the maximum dose (50 μM) (Fig. 1a). Also, we confirmed that the 5-Aza treatment up-regulated Cx32 protein level (Fig. 1a). Next, to evaluate if the enhancement of 5-Aza on the VBL-induced cytotoxicity could depend on the induction of apoptosis, we examined two representative markers of apoptosis. As shown in Fig. 1b, combination of 5-Aza with VBL significantly enhanced DNA fragmentation estimated as subG1 population compared to the VBL treatment alone. Furthermore, caspase 3 activity showed the same tendency (Fig. 1c). Finally, to confirm that 5-Aza-dependent restoration of Cx32 contributed to the enhancement of the VBL-induced cytotoxicity, we investigated whether knockdown of 5-Aza-induced Cx32 by siRNA could cancel the 5-Aza-dependent increase of caspase 3 activity. As shown in Fig. 1d, about 60% of the increased caspase 3 activity by 5-Aza was abolished under knockdown of Cx32 by siRNA treatment. These results suggest that the induction of Cx32 by 5-Aza mainly contributed to the enhancement of VBL-induced cytotoxicity in Caki-1 cells.

3.2. Effect of 5-Aza on P-gp in Caki-1 cells

As described in the introduction, resistance of RCC to VBL is closely associated with the level of P-gp [6], so we checked if the restoration of Cx32 by 5-Aza could affect the level of P-gp in Caki-1 cells. As shown in Fig. 2a, verapamil (VPL, a specific inhibitor against P-gp) significantly potentiated VBL-induced cytotoxicity in Caki-1 cells, and in addition, the inhibitor enhanced VBL-induced caspase 3 activation, Fig. 2b, indicating that p-gp related to resistance of the cells to VBL. Next, we estimated whether 5-Aza could inhibit P-gp function in Caki-1 cells. As shown in Fig. 2c, 5-Aza treatment significantly reduced MDR1 mRNA level as well as P-gp level. Also, the 5-Aza treatment suppressed P-gp function when evaluated as the accumulation level of Rh123 (Fig. 2d). Finally, to confirm that Cx32 mediated 5-Aza-dependent reduction of MDR-1 mRNA level and P-gp level, we examined if knockdown of Cx32 by siRNA could abrogate the 5-Aza-dependent reduction. Under the suppression of Cx32 by siRNA, 5-Aza-dependent down-regulation of MDR1 and P-gp was mostly cancelled (Fig. 2e). These results suggest that the 5-Aza treatment suppressed P-gp function, mainly via the induction of Cx32.

3.3. Effect of 5-Aza on signal pathway regulating MDR-1 mRNA level in Caki-1 cells

Of several factors regulating *MDR1* gene expression, Src and c-Jun N-terminal kinase (JNK) are key factors to regulate the expression [20,21]. Then, we evaluated if 5-Aza treatment could regulate MDR1 mRNA level due to the modulation of the two factors and their related signalling through the up-regulation of Cx32. As shown in Fig. 3a, PP2 (a Src inhibitor) significantly reduced MDR1 mRNA level in Caki-1 cells, and PP3 (a negative control of PP2) did not affect the mRNA level (data not shown), indicating that Src is a positive regulator necessary for the induction of *MDR1* gene. Also, as shown in Fig. 3b, the inactivation of Src by PP2 suppressed the activation of Akt, a main member of Src signalling. In conjunction with this inactivation of Akt, the inhibition of apoptosis signal-regulating kinase 1 (Ask1) was released when measured as dephosphorylated level at Ser83 in Ask1 [22]. In turn, the activation of Ask1 (phosphorylation at Thr845) was induced, leading to the activation of JNK signalling (Fig. 3b). Similarly, 5-Aza treatment induced the same event with PP2 treatment (Fig. 3c). Next, to confirm contribution of JNK signalling to the 5-Aza-dependent reduction of MDR1 mRNA level, we estimated the effect of SP600125 (a JNK inhibitor) on MDR1 mRNA level. As shown in Fig. 3d, SP600125 treatment mostly abrogated the suppression of MDR1 mRNA level by 5-Aza. Finally, we examined if Cx32 could mediate 5-Aza-dependent inactivation of Src signalling and activation of JNK signalling. As shown in Fig. 3e, under knockdown of Cx32 by siRNA, Src was re-activated, and phosphorylation of c-Jun (a phosphorylation target molecule by JNK) was partially abrogated. These results suggest that up-regulation of Cx32 by 5-Aza suppressed MDR1 mRNA level via the inactivation of Src signalling and the activation of JNK signalling in Caki-1 cells.

3.4. A role of c-Jun to regulate MDR-1 mRNA level in Caki-1 cells

Since it has been known that c-Jun acts as an important transcription factor to regulate MDR1 mRNA level [23], we examined a role of c-Jun in 5-Aza-induced suppression of MDR1 mRNA level. In connection with Fig. 3e, 5-Aza treatment significantly enhanced c-Jun transcription activity

from control. Lower panel: The cell extracts were collected 48 h after treated with 0.5 μM 5-Aza. Immunoblot analyses were performed using antibodies against P-gp and β-actin. This result was representative of two independent experiments. (D) After the cells (3.75×10^4 cells/sample) were treated with 0.5 μM 5-Aza or 10 μM VPL, Rh123 was loaded to the cells as described in Section 2. Then, the accumulation of Rh123 into the cells was determined from each fluorescence density. The data were expressed as the mean ± S.D. of six samples. *Significant difference from control. (E) Upper graph: After the treatment with siRNA and 5-Aza described in Section 2, MDR1 mRNA level was determined by RT-realtime PCR. The data were expressed as the mean ± S.D. of four samples. *Significant difference from control and **significant difference from 5-Aza treatment. Lower panel: After the treatment with siRNA and 5-Aza described in Section 2, immunoblot analyses were performed using antibodies against Cx32, P-gp and β-actin. This result was representative of two independent experiments.

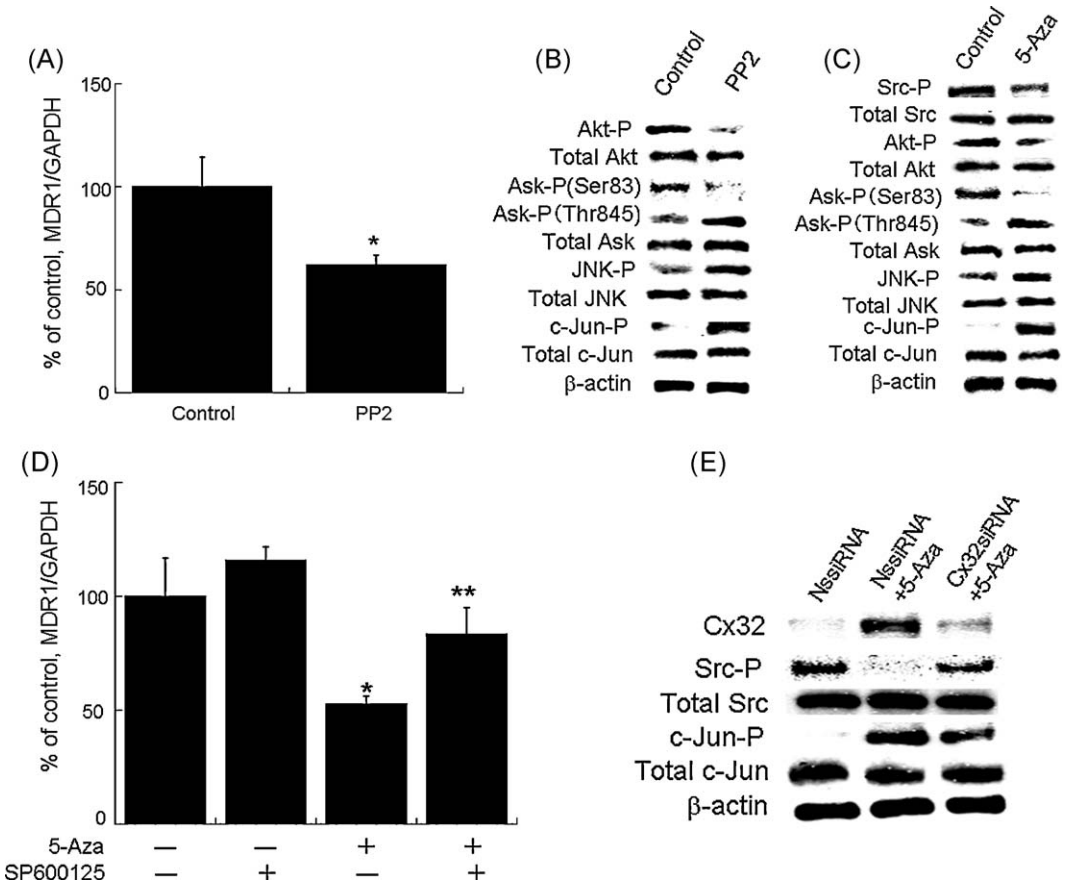

Fig. 3. Up-regulation of Cx32 by 5-Aza suppresses MDR1 mRNA level via the inactivation of Src signalling and activation of JNK signalling. (A) Caki-1 cells (3×10^5 cells/sample) were treated with 0.1 μM PP2 for 24 h, and subsequently MDR-1 mRNA level was measured by RT-realtime PCR as described in Section 2. The data were expressed as the mean ± S.D. of four samples. *Significant difference from control. (B) The cells (1×10^6 cells/sample) were treated with 0.1 μM PP2 for 24 h, and subsequently three samples in each treatment group were combined to prepare immunoblot samples. After that, immunoblot analyses were performed using antibodies against total Akt, phosphorylated-Akt (Akt-P), total-Ask, phosphorylated-Ask (Ask-P, Ser83 and Thr845), total JNK, phosphorylated-JNK (JNK-P), total c-Jun, phosphorylated-c-Jun (c-Jun-P), and β-actin. This result was representative of two independent experiments. (C) The cells (1×10^6 cells/sample) were treated with 0.5 μM 5-Aza for 36 h, and subsequently three samples in each treatment group were combined to prepare immunoblot samples. After that, immunoblot analyses were performed using antibodies against total Src, phosphorylated-Src (Src-P), total Akt, phosphorylated-Akt (Akt-P), total Askphosphorylated-Ask (Ask-P, Ser83 and Thr845), total JNK, phosphorylated-JNK (JNK-P), total c-Jun, phosphorylated-c-Jun (c-Jun-P), and β-actin. This result was representative of two independent experiments. (D) The cells (3×10^5 cells/sample) were treated with 0.5 μM 5-Aza for 24 h and subsequently treated with 0.1 μM SP600125 for 24 h. After the treatment, MDR1 mRNA level was measured by RT-realtime PCR as described in Section 2. The data were expressed as the mean ± S.D. of four samples. *Significant difference from control and SP600125 treatment and **significant difference from other three groups. (E) After the treatment with siRNA and 5-Aza as described in Section 2, immunoblot analyses were performed using antibodies against Cx32, total Src, Src-P, total c-Jun, c-Jun-P, and β-actin. This result was representative of two independent experiments.

compared to non-treatment, and as expected, knockdown of Cx32 by siRNA mostly abrogated the 5-Aza-induced increase of the transcription activity in Caki-1 cells (Fig. 4a). Furthermore, knockdown of c-Jun by siRNA significantly recovered 5-Aza-decreased MDR1 mRNA level (Fig. 4b). These results indicate that the 5-Aza-dependent induction of Cx32 suppressed MDR-1 mRNA level via the transactivation of c-Jun in Caki-1 cells.

3.5. A role of JNK on VBL-induced cytotoxicity in 5-Aza-treated Caki-1 cells

It has been reported that JNK activation plays a central role in VBL-induced apoptosis in cancer cells [24], so it can be hypothesized that 5-Aza-induced down-regulation of P-gp further enhances VBL-induced JNK activation and that the enhancement of the JNK activation is absolutely required for the enhancement of VBL-induced cytotoxicity in 5-Aza-treated Caki-1 cells. Thus, we examined this possibility. As shown in Fig. 5, VPL markedly enhanced VBL-induced JNK activation. As well, the 5-Aza treatment showed a similar tendency. Taken together these results support the above hypothesis.

4. Discussion

In our previous studies, we have reported that enforced expression of Cx32, a member of GJ protein family, reduces several malignant phenotypes of primary and metastatic RCC cell lines [11,25]. Also, we have shown that the expression of Cx32 abrogates chemoresistance (resistance against VBL) observed in metastatic RCC cells. These reports suggest that combination of VBL and Cx32-mediated tumor suppressive functions are promising as a new therapy against metastatic RCC. However, in order to establish the above combination strategy for clinical usage, an effective pharmacological procedure to promote expression of Cx32 in metastatic RCC is absolutely required. We have already reported that loss of Cx32 function in RCC depends on methylation of the promoter regions [15]. Additionally, loss of Cx function based on mutations or deletion of DNA has been found to be a rare event in cancers [26]. These reports mean that demethylation of the promoter regions by DNA methyltransferase inhibitors is effective to gain Cx function in cancers. In fact, we have observed that two DNA methyltransferase inhibitors (decitabine and zebularine) can restore the expression of Cx32 gene in RCC cells *in vivo* as well as *in vitro* [16]. Thus, the approach to induce re-expression of Cx32 gene by DNA methyl-

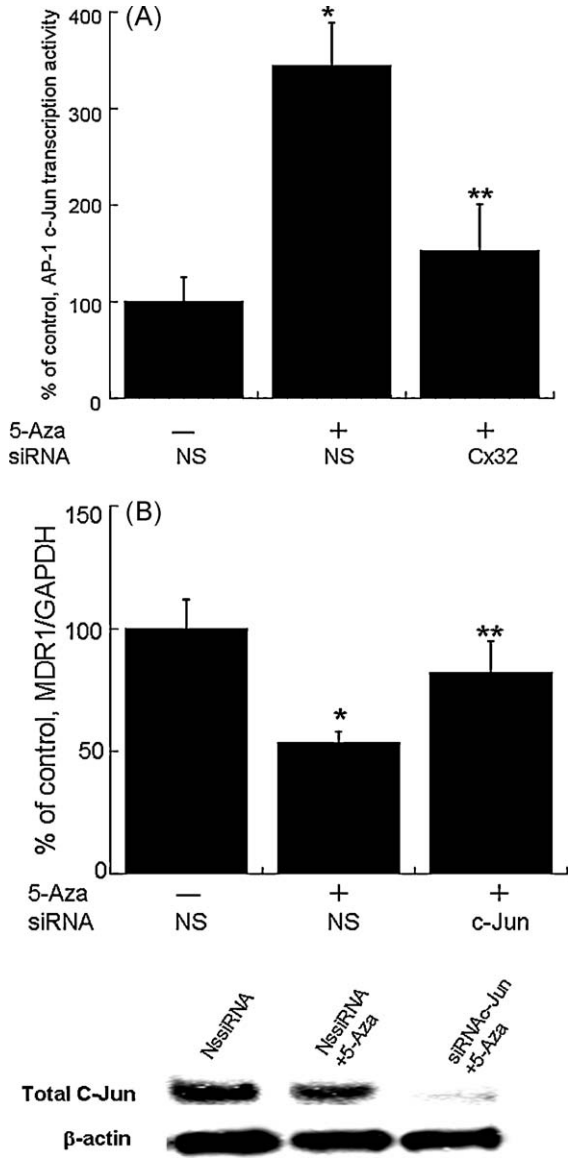

Fig. 4. Up-regulation of Cx32 by 5-Aza suppresses MDR1 mRNA level via the activation of c-Jun transcription activity. (A) After the treatment with siRNA and 5-Aza as described in Section 2, c-Jun transcription activity was determined as described in Section 2. The data were expressed as the mean ± S.D. of four samples. *Significant difference from control and **significant difference from 5-Aza treatment. (B) Upper panel: After the treatment with siRNA and 5-Aza as described in Section 2, MDR1 mRNA level was determined by RT-realtime PCR. The data were expressed as the mean ± S.D. of four samples. *Significant difference from control and **significant difference from control and 5-Aza treatment. Lower panel: After the treatment with siRNA and 5-Aza described in Section 2, immunoblot analyses were performed using antibodies against total c-Jun and β-actin. This result was representative of two independent experiments.

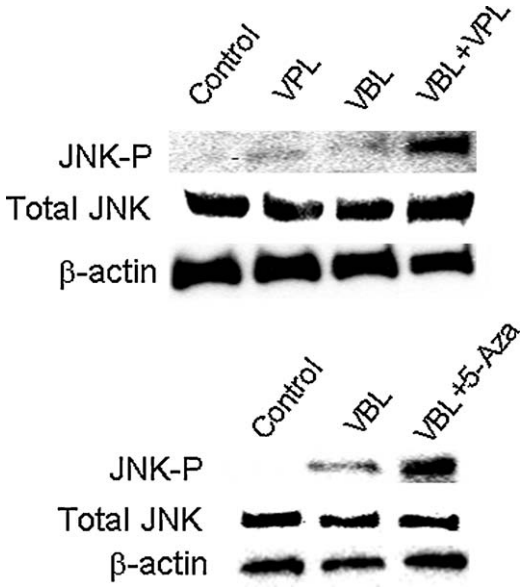

Fig. 5. JNK activation contributes to enhancing effect of 5-Aza on VBL-induced cytotoxicity in Caki-1 cells. Upper panel: Caki-1 cells (1×10^6 cells/sample) were treated with 50 nM VBL and 10 μM VPL for 12 h, and subsequently immunoblot analyses were performed using antibodies against total c-Jun, c-Jun-P and β-actin. This result was representative of two independent experiments. Lower panel: The cells were treated with 0.5 μM 5-Aza for 48 h and subsequently with VBL for 12 h. Then, immunoblot analyses were performed using antibodies against total c-Jun, c-Jun-P and β-actin. This result was representative of two independent experiments.

transferase inhibitors may lead to establishment of the combination strategy for clinical usage in metastatic RCC. In this context, the present study was undertaken to clarify this possibility.

In this study, we confirmed that 5-Aza treatment enhanced cytotoxic effect of VBL on Caki-1 cells and that the restoration of Cx32 by 5-Aza closely related to the enhancing effect of 5-Aza using siRNA for Cx32. These results indicated that the combination of VBL and 5-Aza could be effective to regulate metastatic RCC. In order to clarify the mechanism, we examined what critical event was related to the enhancing effect of Cx32. Since it has been well established that P-gp, an adenosine triphosphate-driven efflux pump for VBL, is a critical factor to determine resistance of metastatic RCC against this chemotherapeutic agent [6,27], we estimated the contribution of this molecule to the enhancing effect of VBL in Caki-1 cells. This study showed that P-gp level was significantly reduced by 5-Aza treatment. As mentioned above, over-expression of P-gp closely relates to the appearance of resistance in cancer cells against VBL, so the reduction of P-gp level by 5-Aza may contribute to the enhancement of VBL-induced cytotoxicity. Also, knockout of 5-Aza-induced Cx32 by siRNA restored the P-gp level. These results suggests that the restoration of Cx32 by 5-Aza potentiates the VBL-induced cytotoxicity in Caki-1 cells via the suppression of P-gp.

With respect to factors determining the level of P-gp, we can speculate that Src signalling is a key molecule in Caki-1 cells, from the following issues. We have found that enforced expression of Cx32 suppressed Src activation and that the inactivation of Src by the Cx mainly contributes to the tumor suppressive effects in Caki-1 cells [11]. Also, previous reports have suggested that Src indirectly regulates the level of P-gp [20,28,29]. These reports show a possibility that 5-Aza-induced expression of Cx32 could reduce the level of P-gp via the inactivation of Src signalling. In fact, we observed that 5-Aza treatment reduced the P-gp level and that the decreased P-gp level was mostly recovered under knockdown of Cx32 by siRNA. Additionally, PP2 (an inhibitor against Src) significantly suppressed MDR-1 mRNA level. These results completely support the above possibility.

Of molecules located in the downstream of Src signalling, it is well known that hypoxia-inducible factor 1α (HIF1α) acts as a positive transcription factor for *MDR1* gene induction [28,29]. However, we could not detect HIF1α in Caki-1 cells in our previous study. This means that HIF1α is not associated with the regulation of MDR1 in Caki-1 cells. Of the molecules located in the downstream of Src, other than HIF1α, we can pick up JNK as a candidate regulating the expression of MDR1 due to the following results. These are, (1) the inactivation of Src can induce the inactivation of Akt [30]; (2) the inactivation of Akt leads to release the suppression of Ask1 activity [22]; (3) the increase of Ask1 activity causes the activation of JNK [31]; and (4) the enhancement of JNK activity contributes to the reduction of P-gp level [32]. Actually, we observed that 5-Aza

treatment as well as PP2 induced the inactivation of Akt and activation of Ask1/JNK. Also, we confirmed that the induction of *MDR1* gene by 5-Aza was mostly cancelled by the inhibition of JNK activity using a JNK inhibitor, SP600125. Overall, it seems that JNK can negatively regulate the level of P-gp in Caki-1 cells.

How might P-glycoprotein expression be down-regulated by elevated JNK? It has been reported that the promoter of the *MDR1* gene possesses a negative binding site of AP-1 (c-Jun/c-Fos, etc., [32]). This study has shown that adenoviral JNK increased the AP-1 binding activity of the *MDR1* gene in the cells. It is therefore very likely that enhanced JNK activity promoted the phosphorylation of c-Jun, which in turn stimulated c-Jun/c-Fos binding to the AP-1 element of the *MDR1* gene, thereby leading to the repression of MDR1 mRNA expression and ultimately the repression of P-gp protein expression. Similarly, our present study showed that 5-Aza treatment induced the activation of c-Jun transcription activity via the up-regulation of Cx32 and that the knockout of c-Jun by siRNA increased MDR1 mRNA level in Caki-1 cells. Since we confirmed that the change of MDR1 mRNA level closely related to that of P-gp protein level in 5-Aza-treated Caki-1 cells, the above results suggest that the induction of Cx32 by 5-Aza ultimately suppresses P-gp level via the activation of c-Jun transcription activity in Caki-1 cells.

It has been reported that the activation of JNK-dependent apoptotic signalling mainly contributes to VBL-induced cytotoxicity in several types of cancer [24]. As mentioned, pre-treatment of 5-Aza before VBL suppressed P-gp function via Cx32-mediated activation of JNK signalling, finally leading to the enhancement of the VBL-induced cytotoxicity in Caki-1 cells. Taking together this report with the present result, we can speculate a possible scheme how the restoration of Cx32 by 5-Aza pre-treatment improves chemical tolerance on VBL in Caki-1 cells as follows. That is, the restoration of Cx32 by 5-Aza pre-treatment induces the inactivation of Src signalling and activation of JNK signalling, subsequently suppresses P-gp function via JNK-dependent increase of c-Jun transcription activity. As a consequence, the reduction of P-gp level finally enhances VBL-induced apoptosis due to further activation of JNK. Actually, we observed that combination of VBL with 5-Aza or VPL enhanced activation status of JNK comparing to VBL treatment alone. Thus, it can be speculated that amplification of JNK signalling by combination of VBL with 5-Aza finally improves cancer therapy against metastatic RCC. This speculation warrants *in vivo* study to examine whether this combination strategy can have clinical potential. Also, in order to further establish the usefulness of the combination strategy in RCC therapy, we should confirm the effectiveness of this strategy in other RCC cell lines.

Conflicts of interest

None.

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