RAPID COMMUNICATION Jpn. J. Cancer Res. **92**, 597–602, June 2001

Reversal of Cisplatin Resistance by the 1,4-Benzothiazepine Derivative, JTV-519

Takashi Nakamura,[1] Fumiaki Koizumi,[1] Noboru Kaneko,[3] Tomohide Tamura,[2] Fumiko Chiwaki,[1,3] Yasuhiro Koh,[1] Shigeru Akutagawa,[1] Nagahiro Saijo[2] and Kazuto Nishio[1,4]

[1]Pharmacology Division, National Cancer Center Research Institute, [2]Medical Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045 and [3]Department of Cardiology and Pneumology, Dokkyo University School of Medicine, 880 Kitakobayashi, Mibu, Tochigi 321-0293

The 1,4-benzothiazepine derivative JTV-519 is a new type of calcium ion channel modulator. We examined the modulatory effect of JTV-519 on the antitumor activity of several platinum compounds (cisplatin, carboplatin, and nedaplatin) in a human cancer cell line resistant to cisplatin (PC-14/CDDP) *in vitro*. PC-14/CDDP cells showed 8-fold resistance to cisplatin compared with the parental PC-14 cells as determined by dye formation [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT] assay. In PC-14/CDDP, but not PC-14 cells, augmentation of cytotoxicity was observed when a nontoxic concentration (10 μM) of JTV-519 was combined with the platinum compounds. Increased intracellular cisplatin accumulation was observed in PC-14/CDDP cells in the presence of JTV-519 as measured by atomic absorption assay. Therefore, increased cisplatin accumulation was considered to be a possible mechanism underlying the reversing effect of JTV-519 on cisplatin resistance. These results suggest that JTV-519 is a potent agent reversing cisplatin resistance.

Key words: Cisplatin resistance — JTV-519 — Lung cancer — Benzothiazepine

Platinum analogues such as cisplatin, carboplatin, and nedaplatin are key anticancer agents used in the chemotherapy of solid tumors. Resistance to platinum compounds is a major obstacle to the successful treatment of cancer. Cellular resistance to the platinum compounds was investigated by characterizing cisplatin-resistant cells.[1)] In order to clarify the mechanisms of cisplatin resistance, we have established a cisplatin-resistant human lung cancer cell line (PC-14/CDDP) from PC-14 cells, and have characterized this line.[1–4)] Decreased intracellular accumulation of cisplatin is a major resistance mechanism in PC-14/CDDP,[3)] and Na-ATPase-linked active influx[5)] and active efflux by the glutathione-S conjugate export pump (GS-X pump) have been suggested to be involved in the resistance.[6,7)] On the other hand, P-glycoprotein (encoded by the *MDR-1* gene) and multidrug resistance protein 1 (MRP1) do not actively export cisplatin, and cisplatin resistance is, therefore, not reversed by calcium channel modulators such as verapamil.[2,3)] In addition, we previously demonstrated that cisplatin-resistant cell lines including PC-14/CDDP showed cross-resistance to other platinum agents such as carboplatin and nedaplatin.[4)] Decreased intracellular accumulation, increased detoxification, and increased DNA-repair ability are major mechanisms of resistance. Thus, the mechanisms of cisplatin resistance are considered to be multifactorial.[1)] Some agents reversing cisplatin resistance have been developed.[8)] We have previously reported that amphotericin B reversed cisplatin resistance.[3)] However, no agent reversing cisplatin resistance has yet been approved for clinical use.

JTV-519 (K201) is a novel 1,4-benzothiazepine derivative.[9–15)] Its chemical formula is 4-[3-(4-benzylpiperidin-1-yl)propionyl]-7-methoxy-2,3,4,5-tetrahydro-1,4-benzothiazepine monohydrochloride (Fig. 1). JTV-519 reportedly suppresses intracellular calcium overload.[9,12)] The unique character of JTV-519 led us to speculate that it would affect drug resistance, because some calcium antagonists including verapamil have been demonstrated to reverse the resistant phenotype.[8)] JTV-519 is under clinical evaluation for use in treating myocardial infarction, suggesting the possibility of clinical application of JTV-519 to cancer chemotherapy. Herein, we describe the activity of JTV-519 as a novel agent that reverses cisplatin resistance.

JTV-519 was obtained from Japan Tobacco Inc. Central Pharmaceutical Research Institute (Osaka). Cisplatin and carboplatin were obtained from Bristol Myers Squibb (Tokyo). Nedaplatin was obtained from Shionogi Co. (Osaka). The PC-14 cell line derived from a human non-small cell lung cancer was donated by Prof. Hayata, Tokyo Medical University (Tokyo). A cisplatin-resistant cell line, PC-14/CDDP, was derived from PC-14 by stepwise dose escalation of cisplatin.[16)] This subline showed approximately 8-fold resistance to the growth-inhibitory effect of cisplatin as determined by 3-(4,5-dimethylthiazol-2-yl)-

[4] To whom all correspondence should be addressed.
E-mail: knishio@gan2.res.ncc.go.jp

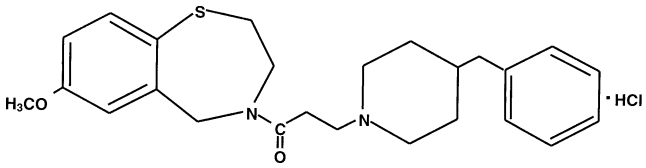

Fig. 1. Chemical structure of JTV-519.

2,5-diphenyltetrazolium bromide (MTT) assay, and the resistant phenotype has been stable for more than 1 year. The cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum under a humidified atmosphere of 5% CO_2 and air at 37°C.

In order to determine the growth-inhibitory effects of platinum compounds, we utilized the tetrazolium dye assay developed by Mosmann, with some modifications.[17, 18] Briefly, 180 μl aliquots of exponentially growing cell suspension containing 10^3 PC-14 and PC-14/CDDP were seeded in 96-well microtiter plates (Becton Dickinson & Co., Lincoln Park, NJ), incubated for about 12 h, and then 20 μl of the drug solution at the indicated concentrations was added. After 72 h exposure to the drug, 20 μl of a MTT (Sigma Chemical Co., St. Louis, MO) solution (5 mg/ml in phosphate-buffered saline, PBS) was added to each well, and the plates were incubated for a further 4 h, followed by centrifugation at 800*g* for 15 min. The medium was then removed, 200 μl of dimethylsulfoxide was added to each well, and the optical density of each resulting solution was measured at 562 and 630 nm using DeltaSoft II (BioMetallics Inc., Princeton, NJ) for a Bio Kinetics Reader EL 340 (Bio-Tech Instruments Inc., Winooski, NJ). Each experiment included 6 replicate wells for each drug concentration and more than three independent experiments were carried out. Wells containing only RPMI-fetal bovine serum (FBS) and MTT were used as controls. The IC_{50} was defined as the drug concentration required for a 50% reduction in the optical density for each test: optical density=[(mean absorbance of 6 wells containing the drug−mean absorbance of 6 control wells)/(mean absorbance of 6 drug-free wells−mean absorbance of 6 control wells)]×100 at each drug concentration. The sensitization (enhancement) was defined as: IC_{50} of cisplatin with JTV-519 in the cells/IC_{50} of cisplatin in the cells.

To confirm the data obtained from the MTT assay, we performed colonogenic assays with PC-14 and PC-14/CDDP cells. The colony assay performed in this study was a modification of Hamberger-Salmon's double agar method.[19] In brief, cells cultured in RPMI 1640 supplemented with 10% FBS were harvested, and cell viability was checked by trypan blue exclusion. One milliliter of cell suspension (1×10^4 cells) in 0.3% agar in RPMI 1640 containing 10% fetal calf serum was seeded onto 6-well plates underlayered with 0.5% agar containing enriched McCoy's 5A medium (Gibco, Tokyo) in the presence of each concentration of CDDP and JTV-519. The plates were incubated at 37°C in the presence of 5% CO_2. Starting on the 7th day of incubation, the numbers of colonies were counted daily. Colonies larger than 60 μm were counted with a CP-2000 automatic colony counter (Shiraimatsu, Tokyo). All assays were performed in triplicate and the value obtained in each experiment represented the mean number of colonies in triplicate assays. The colony survival percentage was calculated as: (number of colonies in a test well/number of colonies in a drug-free well)×100. The IC_{50} was defined as the drug concentration required for a 50% reduction in the number of colonies in each test. The cellular platinum contents were measured by atomic absorption spectrophotometry as described previously.[2, 20] Cells (2×10^6/ml) were seeded into 150×15 mm tissue culture dishes, preincubated for 3 h, then incubated with 0.1 or 0.3 m*M* cisplatin for 3 h at 37°C. These cisplatin concentrations were chosen to obtain measurable platinum levels in this experimental setting. The incubated cells were collected by centrifugation and washed 3 times with PBS, and then the cell pellets were measured with an atomic absorption spectrophotometer, Spectra AA-40 (Varian Instruments, Palo Alto, CA), in the laboratories of Shionogi Biolab (Osaka). The data are expressed as mean±SD and differences between values were analyzed using the unpaired Student's *t* test. Differences for which $P<0.05$ were considered statistically significant.

To determine whether JTV-519 modulates cisplatin resistance, we examined the growth-inhibitory effect of cisplatin with or without JTV-519 on PC-14/CDDP cells by MTT assay. PC-14/CDDP showed 7.8-fold resistance to cisplatin as compared with PC-14 cells (Fig. 2A) and cross-resistance to carboplatin (×3.8) and nedaplatin (×3.6). JTV-519 (<15 μM) alone showed no growth-inhibitory effect on PC-14 or PC-14/CDDP cells. We exposed PC-14 and PC-14/CDDP cells to cisplatin and JTV-519 concurrently for 72 h. JTV-519 enhanced the cisplatin-sensitivity of PC-14/CDDP cells. The modulatory effect of JTV-519 was unremarkable in PC-14 cells. JTV-519 (10 μM) completely reversed the cisplatin resistance of PC-14/CDDP cells. JTV-519 also reversed the resistance of PC-14/CDDP, but not PC-14, cells to carboplatin and nedaplatin (Fig. 2A). The *in vitro* results are summarized in Table I. The reversing effect of JTV-519 on cisplatin resistance was dose-dependent (Fig. 2B). Other schedules of cisplatin and JTV-519 were examined by MTT assay. Pretreatment (for 24 h and 3 h) and posttreatment (for 24 h) with JTV-519 also enhanced the cisplatin sensitivity of PC-14/CDDP cells (data not shown). The modulatory effect of JTV-519 on PC-14/CDDP cells was

confirmed by colony formation assay (Fig. 2C). JTV-519 (5 μM) partially reversed cisplatin resistance in PC-14/CDDP in this assay.

To elucidate the mechanisms by which JTV-519 reverses cisplatin resistance, intracellular cisplatin accumulation was examined in the presence of JTV-519. The intracellular platinum concentration in PC-14/CDDP cells was one-fifth of that in PC-14 cells when the cells were exposed to cisplatin alone (Fig. 3), as reported previously.[20] In the presence of JTV-519 (10 μM), intracellular platinum was significantly increased in PC-14 and PC-14/CDDP cells. In particular, a more than 3-fold increase was observed in PC-14/CDDP cells in the presence of JTV-519.

In the present study, we demonstrated that JTV-519 sensitized cells to cisplatin and other platinum analogues, such as carboplatin and nedaplatin, in PC-14/CDDP cells (Table I). Furthermore, the sensitizing effect of JTV-519 was observed not only *in vitro*. We previously reported that amphotericin B has sensitizing effects on cisplatin resistance.[3] However, application of amphotericin B is limited in clinical settings, because of its synergistic nephrotoxicity in combination with cisplatin. JTV-519 is now under clinical evaluation for use in the treatment of cardiovascular disease. *In vivo* studies of JTV-519 and cisplatin have shown no obvious toxicity in mice (data not shown). The reversing effect of JTV-519 in PC-14 and PC-14/CDDP tumors is now being evaluated *in vivo*. Our preliminarily experiment demonstrated that co-administration of JTV-519 and cisplatin decreased PC-14/CDDP tumor size and the minimum T/C. These findings suggest that JTV-519 reverses the cisplatin resistance of PC-14/CDDP

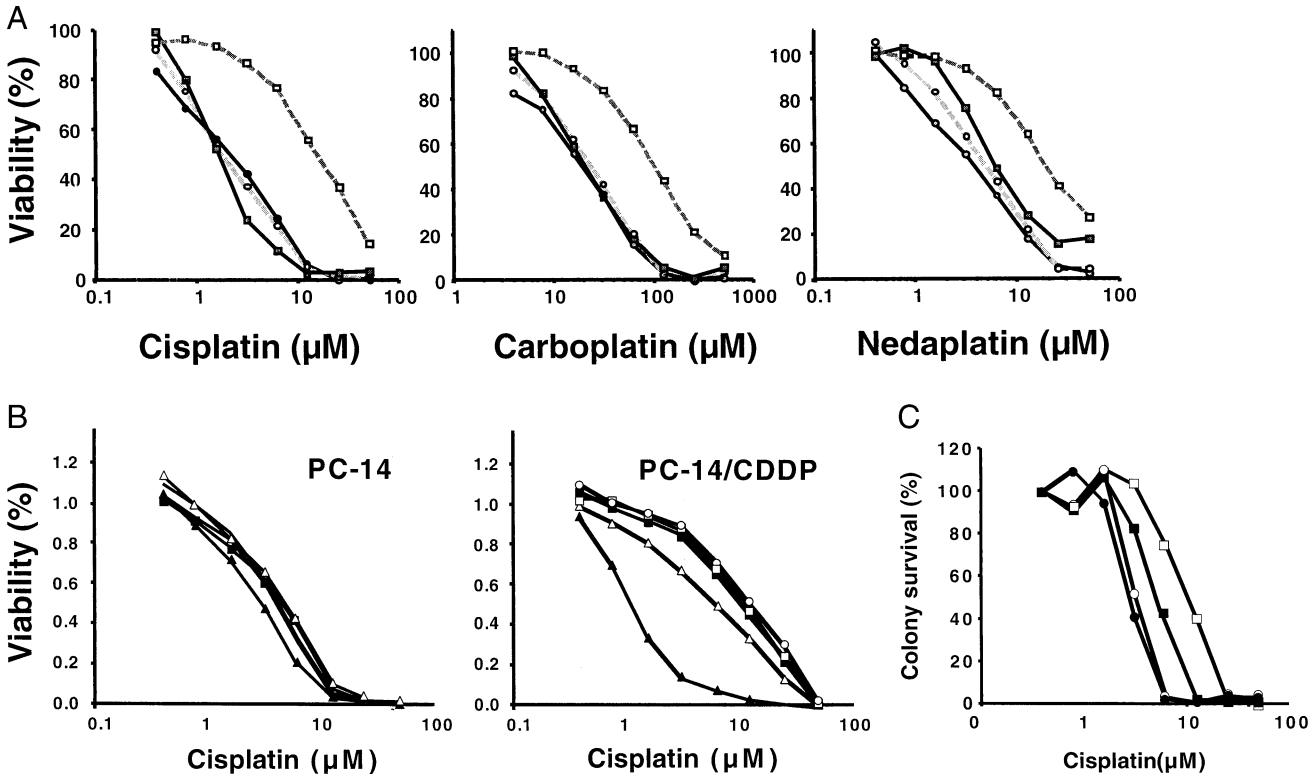

Fig. 2. Effect of JTV-519 on the platinum sensitivity of PC-14 and PC-14/CDDP cells. (A) Growth inhibition curves of PC-14 and PC-14/CDDP cells by cisplatin, carboplatin, and nedaplatin in the presence of 10 μM JTV-519 as determined by MTT assay. Open squares with dashed lines, PC-14/CDDP cells treated with platinum alone; closed squares with solid lines, PC-14/CDDP cells treated with platinum and JTV-519 (10 μM); open circles with dashed lines, PC-14 cells treated with platinum alone; closed circles with solid lines, PC-14 cells treated with platinum and JTV-519 (10 μM). (B) Dose-dependent effects of JTV-519 on the cisplatin sensitivity in PC-14 and PC-14/CDDP cells. Growth-inhibition curves were obtained by MTT assay. Several concentrations of JTV-519 (▲ 10 μM, △ 5 μM, ■ 3 μM, □ 1 μM, ○ 0 μM) were administered concurrently with cisplatin. (C) Effects of JTV-519 on cisplatin sensitivity of PC-14 and PC-14/CDDP cells as determined by colony formation assay. PC-14 and PC-14/CDDP cells were exposed to cisplatin with or without 5 μM JTV-519. Open squares, PC-14/CDDP cells exposed to platinum alone; closed squares, PC-14/CDDP exposed to cisplatin and JTV-519 (5 μM); open circles, PC-14 exposed to platinum alone; closed circles, PC-14 exposed to platinum and JTV-519 (5 μM).

Table I. Effects of JTV-519 on Sensitivity to Cisplatin and Its Analogues in PC-14 and PC-14/CDDP Cells Determined by MTT Assay

Compound		IC_{50} value (μg/ml)	
		PC-14 (Enhancement)	PC-14/CDDP (Enhancement)
Cisplatin	with JTV-519 (10 μM)	1.77±0.89[a] (0.73)[b]	2.16±1.12 (0.12)[c]
	without JTV-519	2.40±1.05	18.6±2.83
Carboplatin	with JTV-519	29.5±11.3 (0.80)	26.1±7.8 (0.18)
	without JTV-519	37.1±10.8	143.0±39.1
Nedaplatin	with JTV-519	4.36±1.53 (0.61)	4.16±1.28 (0.16)
	without JTV-519	7.10±1.53	25.9±9.12

a) IC_{50} value of each platinum analogue. Results are presented as mean±SD of more than 3 independent experiments.
b) Enhancement=IC_{50} of compound with JTV-519/IC_{50} of compound alone in PC-14 cells.
c) Enhancement=IC_{50} of compound with JTV-519/IC_{50} of compound alone in PC-14/CDDP cells.

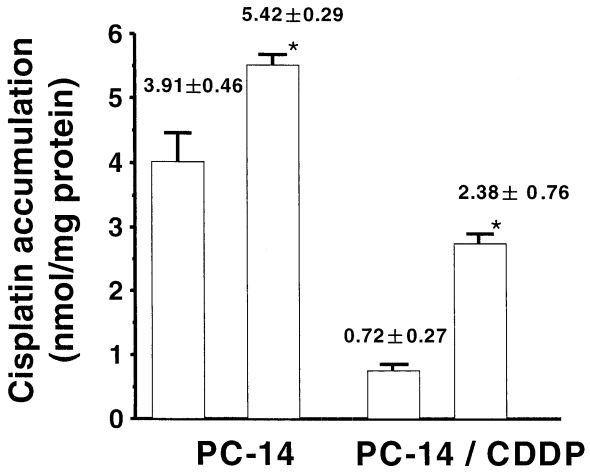

Fig. 3. Effects of JTV-519 on intracellular cisplatin accumulation in PC-14 and PC-14/CDDP cells. The intracellular platinum concentrations were determined 3 h after exposure to cisplatin alone (left) or to cisplatin in the presence of 10 μM JTV-519 (right) as determined by atomic absorption assay. $*P<0.05$ vs. control; one way analysis of variance followed by Scheffé's test.

tumors *in vivo*. In view of these observations, we expect JTV-519 combined with cisplatin to be clinically beneficial in patients with cisplatin-resistant tumors.

In this study we have demonstrated that an increase in cisplatin accumulation induced by JTV-519 sensitized the cells to cisplatin. However, the magnitude of the JTV-519-induced increase in cisplatin accumulation was too small to account fully for the sensitizing effects of JTV-519. For example, the sensitivity of PC-14/CDDP cells to cisplatin was modulated so as to reach approximately the same level as observed in PC-14 cells (Fig. 2). Nevertheless, the presence of JTV-519 (10 μM) increased sensitivity to cisplatin by approximately 3-fold as compared with controls not treated with JTV-519, and the increased level was still only approximately 60% of that observed with the parental PC-14 cells (Fig. 2). These findings indicate that the mechanism of the sensitizing effect of JTV-519 is more complex than merely increased drug accumulation. JTV-519 has been demonstrated to protect against intracellular calcium ion overloading[9, 12, 13] and binding to annexin V.[21, 22] These observations suggest that one or more of the processes in the pathway from intracellular accumulation of cisplatin to intrastrand cross-link formation[20, 23, 24] may be augmented or accelerated by JTV-519-induced changes in the intracellular environment, which result from the loss of potassium and other cytoplasmic constituents.

In PC-14 cells, the platinum sensitivity (Fig. 2) and intracellular accumulation of platinum compounds (Fig. 3) were increased by JTV-519, although the magnitudes were relatively small compared with those in PC-14/CDDP cells. Therefore, we speculate that JTV-519 can reverse intrinsic resistance to cisplatin.

We failed to find a reversing effect of JTV-519 on cisplatin resistance in another cell line (H69/CDDP),[23] suggesting that the reversing effect of JTV-519 is specific to the cisplatin resistance in PC-14/CDDP cells. Although the mechanism of the cisplatin resistance in PC-14/CDDP remains unclear, increased GS-X pump activity (related to glutathione biosynthesis) contributes to the active efflux of cisplatin and to resistance.[6, 7] Indeed, PC-14/CDDP cells show increased GS-X pump activity measured with leukotriene C4 as a substrate, whereas no increased expression of mRNA of *MRP* family genes was observed in PC-14/CDDP as compared with PC-14 cells (data not shown). At the same time, decreased active influx of cisplatin was also observed in PC-14/CDDP cells. The mechanisms involved in the case of H69/CDDP were different from those in PC-14/CDDP cells. Increased intracellular detoxification mediated by increased metallothionein was observed in H69/CDDP cells as compared with parental

H69 cells.[23] Increased DNA repair ability was also observed in H69/CDDP cells. However, there was no difference in the intracellular accumulation of cisplatin between H69 and H69/CDDP cells, suggesting that the mechanism of cisplatin resistance in H69/CDDP cells is different from that in PC-14/CDDP cells.[23] Therefore, it is likely that the process involved in the active efflux of cisplatin is related to the reversing effect by JTV-519 on cisplatin resistance in PC-14/CDDP cells.

To elucidate the mechanisms of action of JTV-519, we are now evaluating the effects of JTV-519 on the above processes. In addition, a preliminary *in vivo* experiment has demonstrated that JTV-519 augments the cisplatin-induced antitumor effect on transplanted PC-14/CDDP tumors. More precise preclinical evaluations of the efficacy of the combined administration and determination of the optimum timing of administration, as well as optimum dosage, and toxicity, are essential. In conclusion, JTV-519 reverses a certain type of cisplatin resistance in a human lung cancer cell line at a non-toxic dose. JTV-519 is a candidate lead compound for clinical cisplatin resistance-reversing agents.

This research was supported by the 2nd Term Comprehensive 10-Year Strategy for Cancer Control.

(Received February 23, 2001/Revised April 24, 2001/Accepted April 26, 2001)

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