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Mitochondria-mediated and p53-associated apoptosis induced in human cancer cells by a novel selenophene derivative, D-501036

Her-Shyong Shiah [a], *Wan-Shu Lee* [a], *Shin-Hun Juang* [b,d], *Pao-Chiung Hong* [c], *Chia-Chi Lung* [a], *Ching-Jer Chang* [e], *Kai-Ming Chou* [f], *Jang-Yang Chang* [a,g,*]

[a] *Institute of Cancer Research, National Health Research Institutes, Taipei, Taiwan*
[b] *Department of Pharmacology, Development Center for Biotechnology, Taipei, Taiwan*
[c] *Department of Medicinal Chemistry, Development Center for Biotechnology, Taipei, Taiwan*
[d] *School of Pharmacy, China Medical University and the Department of Medical Research, China Medical University Hospital, Taichung, Taiwan*
[e] *Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN, USA*
[f] *Department of Cell Biology and Neuroscience, University of South Alabama, AL, USA*
[g] *Division of Hematology/Oncology, Department of Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan*

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ABSTRACT

D-501036 [2,5-bis(5-hydroxymethyl-2-selenienyl)-3-hydroxymethyl-*N*-methylpyrrol], a novel selenophene derivative, is a highly potent cytotoxic agent with broad spectrum antitumor activity. The present study was undertaken to explore the mechanism(s) through which D-501036 exerts its action mode on the cancer cell death. D-501036 was found to suppress the growth of KB and $HepG_2$ cells in an irreversible manner. The results of annexin-V assays and PARP cleavage studies were consistent with the D-501036-induced apoptosis. Findings provided a strong support for the induction of mitochondria-mediated apoptosis by this drug. The examination of two canonical pathways of initiation caspases, those for caspases -8 and -9, revealed that caspase-9 protein and the activities of caspases -9 and -3 were increased in a dose- and time-dependent manner. The concentrations of Fas/Fas-L and procaspase-8 and the activity of caspase-8 were not altered. Furthermore, the mitochondrial membrane potential permeability and the release of cytochrome *c* to the cytosol were both increased by D-501036. The concentrations of the pro-apoptotic protein Bax and translocation of Bax from the cytosol to the mitochondria were increased in response to D-501036, whereas the concentrations of the anti-apoptotic protein Bcl-2 were decreased. Two DNA damage-related pro-apoptotic proteins, Puma and Noxa, were upregulated in a dose- and time-dependent manner. These pro-apoptotic and anti-apoptotic proteins are downstream effectors of p53. Accordingly, the phosphorylated and total forms of p53 were induced and p53 was translocated from the cytosol to the mitochondria in response to D-501036 treatment. Collectively, we conclude that D-501036 induces cellular apoptosis through the p53-associated mitochondrial pathway.

* *Corresponding author at*: Institute of Cancer Research, National Health Research Institutes, 7F, 161, Sec. 6, Min-Chiuan East Road, Taipei 114, Taiwan. Tel.: +886 2 26534401x25110; fax: +886 2 27929654.
E-mail address: jychang@nhri.org.tw (J.-Y. Chang).
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1. Introduction

Plants, herbs and other living organisms are valuable sources of anticancer drugs. Examples of useful antitumor drugs obtained from natural sources include camptothecins, epipodophyllotoxins, taxanes and vinca alkaloids, each of which possesses a unique mechanism of action. Polythiophenes represent another group of natural products with the potential antitumor activity. Certain polythiophenes and their analogs, the polyselenophenes, exhibit the potent growth-inhibitory activity against the tumor cells in the panel of the NCI screening program.[1] D-501036 [2,5-bis(5-hydroxymethyl-2-selenienyl)-3-hydroxymethyl-*N*-methylpyrrol] is a novel selenophene derivative found to exert a substantial antitumor activity both in vivo and in vitro [1]. D-501036 is highly toxic to cancer cells but spares normal cells. The drug is also active against tumor cell lines that are resistant to other anticancer drugs as a consequence of overexpression of P-glycoprotein (MDR1). The DNA damage induced by D-501036 is essential to its antitumor activity [1].

The most preponderant form of programmed cell death is apoptosis, which is also known as type 1 cell death [2]. Apoptosis is characterized by the activation of a specific family of cysteine proteases, the caspases, followed by the caspase-mediated specific morphological changes including cell shrinkage, chromatin condensation, and disintegration of the cell into small fragments that can be engulfed by neighboring cells without inciting inflammation. Two canonical pathways of initiation caspases, directed by caspases -8 and -9, are recognized which are mediated by two distinct sets of signals [3]. The cell surface signal-mediated apoptosis occurs mainly through activation of death receptors (DR) including the tumor necrosis factor-α receptor 1 (TNFR1), Fas (also known as CD95 and APO-1), and DR4 and DR5 [4]. Upon activation of the death receptor by the appropriate ligand, a death-inducing signaling complex (DISC) is formed, resulting in binding of the protein termed Fas-associated death domain (FADD) to the pro-domain of procaspase-8 and subsequent autocatalytic liberation of active caspase-8 [5,6]. By contrast, the stress-induced mitochondria-mediated apoptosis is initiated by the release of cytochrome *c* to the cytosol [7]. This event is followed by the ATP-dependent formation of the apoptosome complex composed of cytochrome *c*, apoptotic protease-activating factor 1 and caspase-9. Activation of the effector caspases and induction of cell death are final events common to both canonical pathways.

Mutation of p53, a classical tumor suppressor, is frequently associated with oncogenesis. Cellular functions modulated by the p53 protein include DNA synthesis, DNA repair, cell cycle arrest, gene transcription, senescence and apoptosis. The gene encoding p53 is thought to be highly conserved based on the comparison of phenotypes from *Caenorhabditis elegans*, *Drosophila*, and transgenic mice. The most conserved function of the p53 protein is the tumor suppression through the induction of apoptosis [8–13]. Elevated expression of pro-apoptotic genes with promoters containing the p53 responsive element represents one mechanism whereby p53-dependent apoptosis is induced [14]. Intracellular concentrations of p53 are robustly increased by stresses such as DNA damage, ionizing radiation, UV radiation, hypoxia, heat shock, growth factor withdrawal, oncogene activation and exposure to cytotoxic agents [14,15]. The effectors of the p53-mediated apoptosis in response to DNA damage include Noxa and Puma [16–18]. Additionally, the translocation of a specific polymorphic form of p53 from the cytosol to the mitochondria is associated with the induction of apoptosis [19]. In response to the genotoxic insult, both transcription-dependent and transcription-independent cell death may be modulated concurrently by p53 with the resultant amplification of the apoptotic signal.

The present study was conducted to elucidate the mechanisms through which the drug D-501036 causes cancer cell death. Death was found to be attributable to the induction of apoptosis mediated by the canonical mitochondrial pathway and through activation of p53.

2. Materials and methods

2.1. Materials

The chemical structure of D-501036 is presented in Fig. 1. The compound was synthesized in the laboratory of Dr. Ching-Jer Chang (Purdue University) and shown by the high-pressure liquid chromatography (HPLC) and the mass spectroscopy to be at least 98% pure. A 20 mM stock solution of D-501036 was prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C. The final concentration of DMSO in D-501036-containing medium was upmost to 0.025%, which concentration did not cause cytotoxic effects on cancer cells. The concentration of DMSO equivalently used in D-501036-treated cells was administrated as the solvent control throughout the all experiments. Antibodies against caspases -8 and -9, Puma, and p53 phosphorylated at Ser15 (p-p53) were purchased from Cell Signaling Technology Inc. (Beverly, MA). Horseradish peroxidase-conjugated secondary antibody and antibodies against Fas (CD95 and APO-1), Bcl-2, Mcl-1, Bak, Noxa, and p53 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against Fas ligand (Fas-L) was obtained from Oncogene Research Products (Boston, MA), PARP and Bax from Trevigen (Gaithersburg, MD), cytochrome *c* from BD Pharmigen (San Jose, CA), and α-tubulin from Sigma Chemical (St. Louis, MO). Western blot chemiluminescent reagent was purchased from Perkin-Elmer Life Sciences (Boston, MA). Cell

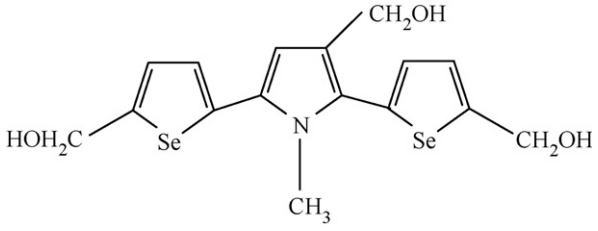

Fig. 1 – Chemical structure of D-501036.

[1] A protocol to run COMPARE analysis can be accessed from the NSC numbers at the following website: http://dtp.nci.nih.gov/dtpstandard/dwindex/index.jsp.

culture reagents were obtained from Gibco-BRL Life Technologies (Gaithersburg, MD). All other chemicals were obtained from E. Merck Co. (Darmstadt, Germany) or Sigma Chemical and were of standard analytic grade or higher.

2.2. Cell lines and cell culture

KB cells, derived from a human cervical cancer, and $HepG_2$ cells, derived from a human hepatocellular carcinoma, were obtained from the American Type Culture Collection (Rockville, MD) and propagated in RPMI-1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cultures were maintained in a humidified incubator at 37 °C in the presence of 5% CO_2. The doubling times of KB and $HepG_2$ cells were around 24 and 36 h, respectively.

2.3. Growth inhibition and clonogenic assays

For the growth-inhibition assay, KB and $HepG_2$ cells were seeded at a density of 10^4/well in 24-well plates. After 24 h, cells were exposed to various concentrations of D-501036 for three-generation times. The methylene blue dye assay was then used to measure the extent of cell growth [20]. Briefly, the cells were fixed and stained with 0.5% methylene blue in 50% ethanol for 1 h. After washing and drying the fixed/stained preparations, the dye was dissolved by 1% N-lauroyl sarcosine at room temperature. The concentration of the dissolved dye was determined by the signal intensity at the wavelength of 590 nm. In the clonogenic assay, cells (5×10^4/well) were plated in six-well plates [21]. After 24 h, cells were exposed to the varying concentrations of drug for one-generation time. Cultures were subjected to trypsinization followed by the determination of cell number. One hundred viable cells were then plated in triplicate into six-well plates, cultured for 7–10 days, and fixed and stained with 0.5% methylene blue in 50% ethanol for 1 h. After washing and drying the fixed/stained preparations, colonies were counted and cloning efficiency was determined for each drug concentration.

2.4. Flow cytometric analysis

For the measurements of annexin-V–PI binding, the Annexin-V-FLUOS Staining Kit (Roche Applied Science, Indianapolis, IN) was used according to the manufacturer's instructions. Briefly, cells were trypsinized in phosphate buffered saline (PBS), collected by centrifugation, and resuspended in 100 ml of Annexin-V-FLUOS labeling solution. After incubation at room temperature for 15 min in the dark, cells were analyzed by the flow cytometry (FACSVantage, Becton Dickinson Labware, Franklin Lakes, NJ). Several controls were used to optimize instrument settings and to determine gating for the Windows-based platform. Annexin-V and PI double-negative cells were defined as live cells, annexin-V-positive and PI-negative cells as early apoptotic cells, and annexin-V and PI double-positive cells as late apoptotic and necrotic cells. The annexin-V–PI binding assay was determined at least three times.

The proportion of retention of the dye JC-1 by variously treated cells was used as a measure of the mitochondrial potential transition (MPT; $\Delta\psi_m$) [22]. Cells were treated with D-501036 at concentrations and treatment durations indicated in the text and legends. Ten minutes prior to the conclusion of each treatment, cells were labeled in vitro with 10 μg/ml JC-1 and incubated at 37 °C according to the instructions of the manufacturer. After removal of the medium and rinsing of the dish with cold PBS, cells were harvested and suspended in PBS. The measurement of the amounts of JC-1 retained by 10,000 cells per sample was performed with a flow cytometer (FACSVantage, Becton Dickinson Labware). JC-1 is a cationic dye that indicates mitochondrial leakage by a shift in its fluorescence emission from red (~590 nm) to green (~525 nm). A decrease of red fluorescence or an increase of green fluorescence suggests that the MPT is increased. The determination of MPT was performed at least three times.

2.5. Determination of caspase activities

The activities of caspases -3, -8 and -9 were determined using Caspase Fluorometric Assay kits (R&D System Inc., Minneapolis, MN) or the CaspACE Assay System-Fluorometric (Promega Corporation, Madison, WI). The cleavage of the appropriate specific fluorogenic peptide substrate was measured according to the instructions of the manufacturer. Briefly, 3×10^6 cells were collected and subjected to hypotonic lysis, and lysates were clarified by centrifugation. The equal amounts of extract were incubated with 50 μM of fluorescent substrate. After 1 h of incubation at 37 °C, the fluorescence of the cleaved substrate was determined using the Victor 1420 Multilable Counter (Wallac, Turku, Finland). For calculations of the caspase activity, the fluorescence values for untreated samples were subtracted from those of drug-treated samples. The caspase activity was determined at least three times.

2.6. Western blot analysis

KB and $HepG_2$ cells were treated with the indicated concentrations of D-501036, followed by extraction of proteins. A lysis buffer containing 50 mM Tris–HCl (pH 7.6), 25 mM NaCl, 0.5% Triton X-100, and 2 mM dithiothreitol (DTT) was used to extract the cytosolic proteins. The extraction buffer was supplemented on the day of use with 1× cocktail protease inhibitors (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium fluoride, and 1 mM sodium orthovanadate. Proteins (20–50 μg) were subjected to electrophoresis through the distinct percentages of SDS polyacrylamide gel, followed by transfer of proteins onto the polyvinylidene difluoride membranes. The membranes were blocked with a buffer containing 5% non-fat milk in PBST (1× PBS, 0.2% Tween 20) at room temperature for 1 h, and subsequently incubated in the same buffer containing various primary antibodies at 1–2 μg/ml. Membranes were then incubated with anti-rabbit and/or anti-mouse antibodies conjugated with a horseradish peroxidase at room temperature for 1 h. The proteins of interest were detected using the chemiluminescence method (Perkin-Elmer Life Sciences) followed by autoradiography.

2.7. Isolation of cytosolic and mitochondrial proteins

To isolate the cytosolic and mitochondrial proteins, cells treated with varying concentrations of D-501036 were

collected and suspended in mitochondria isolation buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM DTT, 0.1 mM PMSF, 10 μM leupeptin) in 250 mM sucrose [23]. After an incubation on ice for 3 min, cells were disrupted with a glass homogenizer. Homogenates were centrifuged twice at 2500 × *g* at 4 °C to remove unbroken cells and nuclei. The mitochondria were harvested by centrifugation at 12,000 × *g* for 90 min. The supernatants were removed and passed through a 0.2 μm filter to obtain the cytosolic proteins. The fractionated proteins were analyzed by Western blotting.

2.8. Statistical analysis

All data are expressed as means with standard deviations. The Student's *t*-test was employed to compare the mean of each group with that of the control group. Differences with *P*-values of <0.05 at both tails were considered statistically significant. All statistical analyses were performed using the software of SPSS for Windows Release 11.5 (SPSS, Chicago, IL).

3. Results

3.1. Growth and clonogenicity of KB and $HepG_2$ cells treated with D-501036

D-501036-dependent growth inhibition was determined by comparing the percentage of viable drug-treated cells with that of untreated controls under comparable incubation conditions. The IC_{50} value was defined as the concentration of drug that inhibited cell growth by 50%. D-501036 exerted a dose-dependent inhibition of growth for both KB and $HepG_2$ cells (Table 1). The IC_{50} values were 470 ± 23 and 2.0 ± 0.2 nM for KB and $HepG_2$ cells, respectively. The capacity of D-501036 to inhibit the colony formation was also examined. To this end, the percentage of visible colonies of drug-treated cells was compared with that of untreated controls under comparable incubation conditions. The LC_{50} value was defined as the drug concentration that inhibited colony formation by 50%. A D-501036 concentration-dependent loss of KB and $HepG_2$ cell colony formation was observed. The LC_{50} values were 210 ± 59 and 1.7 ± 0.1 nM for KB and $HepG_2$ cells, respectively (Table 1). The ratios of LC_{50} to IC_{50} were 0.45 and 0.85 for KB and $HepG_2$ cells, respectively. These findings are fully consistent with an irreversible inhibition of growth in response to this drug.

Table 1 – Cytotoxic effect of D-501036 on KB and HepG2 cells

Cell line	KB	$HepG_2$
Growth inhibition assay (IC_{50}, nM)	470 ± 23	2.0 ± 0.2
Clonogenic assay (LC_{50}, nM)	210 ± 59	1.7 ± 0.1
Ratio of LC_{50}/IC_{50}	0.45	0.85

Results are expressed as mean ± S.D. IC_{50}, the concentration of drug that inhibited cell growth 50%; LC_{50}, the concentration of drug that inhibited colony forming ability 50%.

3.2. Induction of apoptosis by D-501036

To ascertain whether the observed irreversible growth inhibition was associated with apoptosis, D-501036-treated KB and $HepG_2$ cells were subjected to the annexin V–PI binding assay and analyzed by flow cytometry. An increase in annexin V-positive and PI-negative cells from 1.8 ± 0.3% for non-treated controls to 2.5 ± 0.3, 4.1 ± 0.5 and 5.8 ± 0.4% for D-501036-treated KB cells at drug concentrations of 1×, 4× and 10× the IC_{50} dose, respectively, was observed after 24 h (Fig. 2A). Similar findings were obtained for the other cell type examined in this study. An increase in apoptotic $HepG_2$ cells from 1.9 ± 0.2% for non-treated controls to 2.3 ± 0.4 and 5.7 ± 0.5% for D-501036-treated cells at the drug concentrations of 4× and 10× the IC_{50} dose, respectively, was observed after 15 h (Fig. 2B). To confirm the induction of apoptosis by D-501036, the cleavage of PARP, another early marker of apoptosis [24], was examined by Western blotting. As shown in Fig. 2C, PARP was cleaved in a dose- and time-dependent manner in KB cells treated with D-501036 for 24 h (Fig. 2C, upper panel) and at the drug concentration of 1× the IC_{50} dose, respectively (Fig. 2C, lower panel).

3.3. Apoptosis induced by D-501036 is associated with the activations of caspase-3 and caspase-9, but not with activation of caspase-8

PARP is an endogenous substrate of caspase-3, a protease subject to activation by a variety of apoptotic pathways [24,25]. It was therefore of interest to determine the effect of D-501036 on the caspase-3 activity. When KB cells were exposed to increases in D-501036 between 1× and 10× the IC_{50} concentration, the caspase-3 activity was increased in a dose-dependent and time-dependent manner between 24 and 36 h of incubation (Fig. 3A). Experiments were then conducted to ascertain which of the canonical pathways of initiation caspases was associated with the observed activation of caspase-3. In parallel with the increase in caspase-3 activity, the activity of caspase-9 was increased in a dose-dependent and time-dependent manner between 24 and 36 h of exposure to D-501036 (Fig. 3B), whereas no alteration of caspase-8 activity was noted (data not shown). Western blot analyses were performed to determine whether the concentrations of procaspase-9 and caspase-9 were altered in drug-treated cells (Fig. 3C). After 24–36 h of treatment at the drug concentration of 10× the IC_{50} dose, the level of procaspase-9 was observed to decline in parallel with an increase in the level of caspase-9. These results support the hypothesis that apoptosis in response to D-501036 is associated with the mitochondrial pathway, as opposed to the death receptor pathway, of activation.

3.4. Alteration of mitochondrial membrane potential and release of cytochrome c to the cytosol in response to treatment with D-501036

Additional evidence supporting the involvement of the mitochondrial pathway as opposed to the death receptor pathway in the induction of apoptosis by D-501036 was sought. The mitochondrial membrane potential permeability

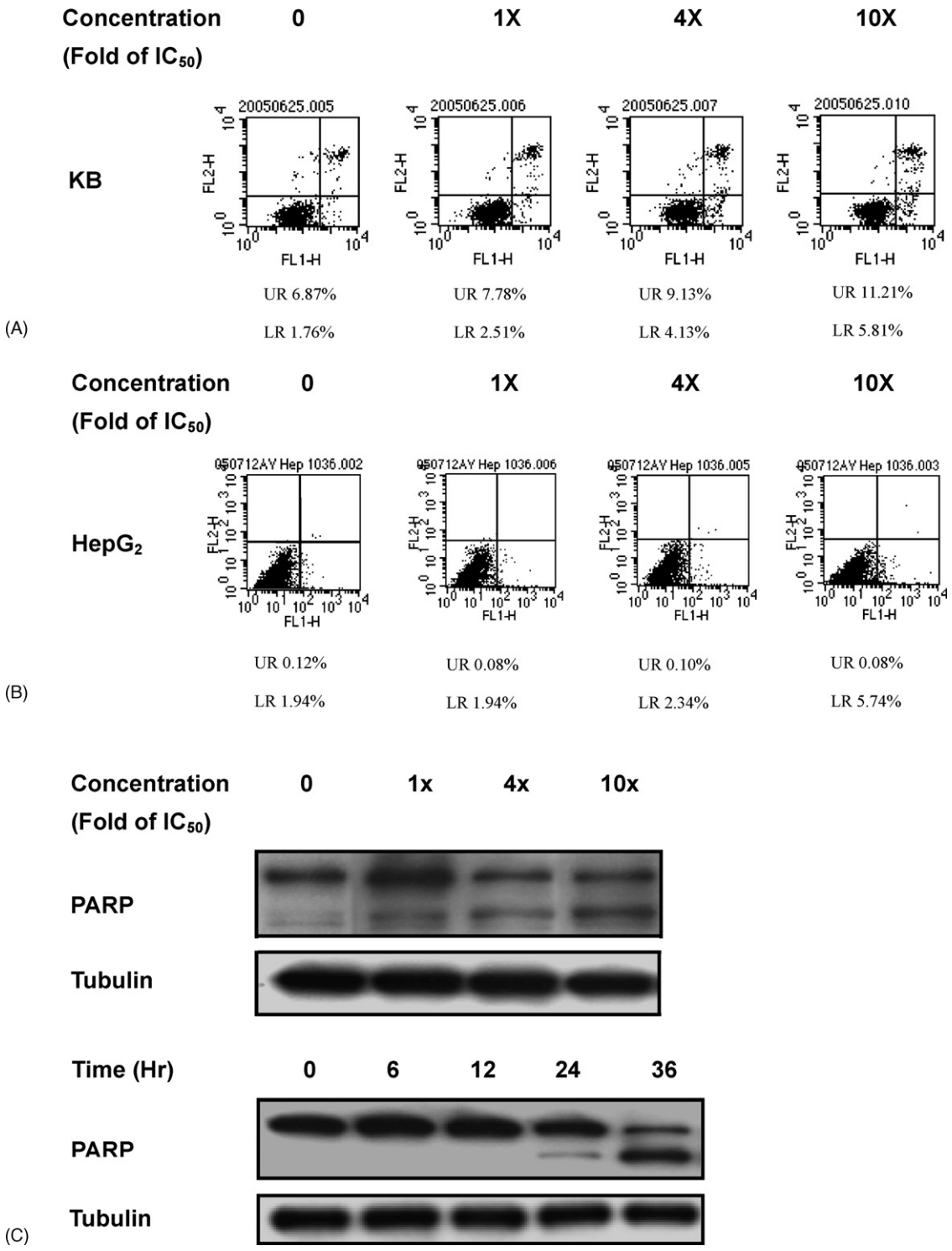

Fig. 2 – Apoptotic effect of D-501036 on KB and HepG$_2$ cell lines. (A) KB and (B) HepG$_2$ cells were treated with the indicated concentrations of D-501036 for 24 and 15 h, respectively. Apoptosis indicated by annexin-V positivity were analyzed by flow cytometry. The X- and Y-axis represent annexin-V and PI staining, respectively. UR (annexin-V and PI double positive staining) and LR (positive annexin-V but negative PI staining) represent the upper and lower right quadrants, respectively. (C) KB cells were treated with the indicated concentrations and for the indicated time. The PARP cleavage was examined by Western blot analysis.

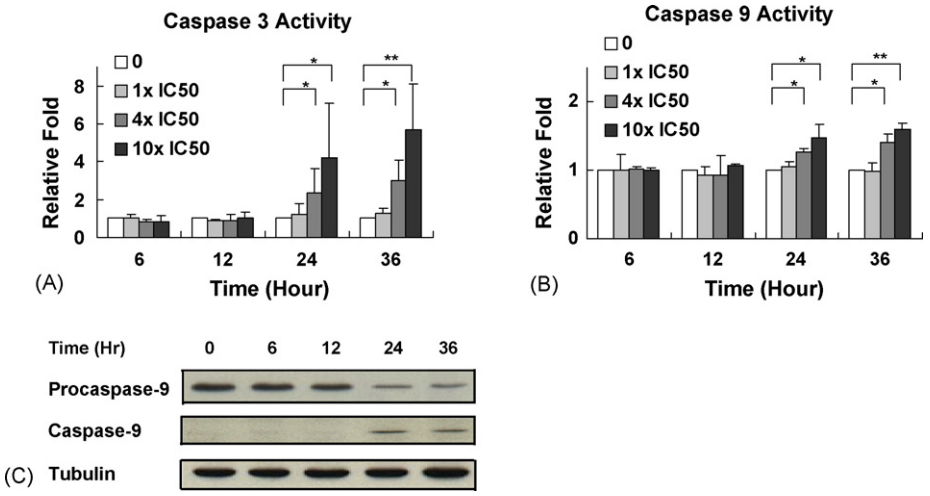

Fig. 3 – Effect of D-501036 on caspase activity in KB cell line. Cells were treated with the indicated concentrations of D-501036 for the indicated time. The caspase activities were examined by flow cytometry analysis using fluorogenic peptide substrates for (A) caspase-3 and (B) caspase-9. The results are expressed by means of triplicate analysis and the bars indicated the standard deviations. * and ** are significantly different from the control at $P < 0.05$ and 0.01, respectively. (C) The protein levels of procaspase-9 and caspase-9 were examined by Western blot analysis.

of drug-treated and control preparations was measured and compared. JC-1 dye was used to measure the MPT, which a decrease in the ratio of the signal intensity of red (~590 nm) to green (~525 nm) indicated an increase in the MPT. As shown in Fig. 4A, the MPT was increased in a dose- and time-dependent manner in drug-treated KB cells. As compared with that of control preparations, the MPT of treated preparations was observed to increase modestly at times as early as 6 h at concentrations of 1×, 4× and 10× the IC_{50} concentration of D-501036. More prominent increases were observed at 12 and 24 h of exposure to the drug, and the most prominent increase was observed after 24 h of exposure to D-501036 at 10× the IC_{50} concentration. Concentrations of cytochrome *c* protein present in the mitochondria and cytosol of drug-treated and non-treated preparations were also determined. Cytochrome *c* content was increased in both mitochondrial and cytosolic fractions after 24 h of exposure to D-501036, indicating both up-regulation of cytochrome *c* and its release to the cytosol from the mitochondria (Fig. 4B). Fas/Fas-L concentrations, which increase upon activation of the death receptor pathway, were measured in cells treated with similar concentrations of D-501036. However, no significant changes in the levels of Fas/Fas-L were observed (data not shown). These results confirm that induction of apoptosis by D-501036 is mediated by the mitochondrial pathway.

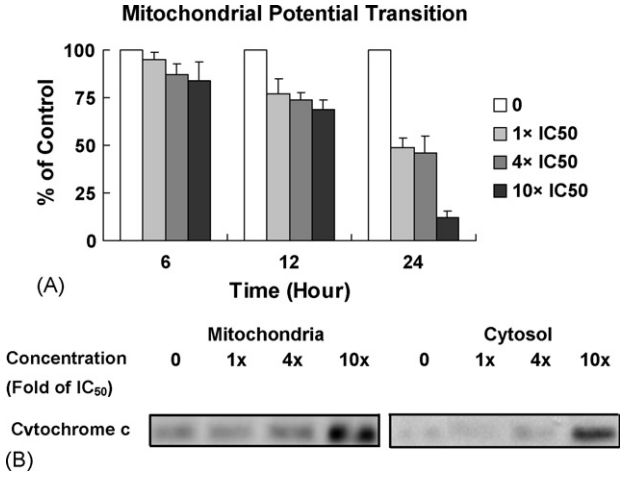

Fig. 4 – Effect of D-501036 on mitochondrial potential transition, expression and translocation of cytochrome *c*. Cells were treated with the indicated concentrations of D-501036 for the indicated time. (A) Mitochondrial potential transition was detected using JC-1 assay. The column indicated the ratio of the signal intensity of red (~590 nm) to green (~525 nm), after normalization with the value of the controls. The results are expressed by means of triplicate analysis and the bars indicated the standard deviations. (B) Cytochrome *c* was fractionated into cytosolic and mitochondrial portions, and then was determined using Western blot analysis.

3.5. *Decreased expression of anti-apoptotic proteins and increased expression of pro-apoptotic proteins in D-501036-treated tumor cells*

Experiments were performed to explore the potential role of the Bcl-2 family of proteins in the effects of D-501036. It was of particular interest to ascertain whether the expression of anti-apoptotic and pro-apoptotic proteins was altered in the drug-treated preparations. The expression of the anti-apoptotic protein Bcl-2 in KB cells was decreased in a drug concentration-dependent manner after 24 h of treatment (Fig. 5A). Also, the expression of the pro-apoptotic protein Bax in KB cells was substantially increased and in a drug concentration-dependent manner (Fig. 5B). The expression of other pro-apoptotic proteins in drug-treated cells was also examined. Bak expression was increased only slightly and no change in

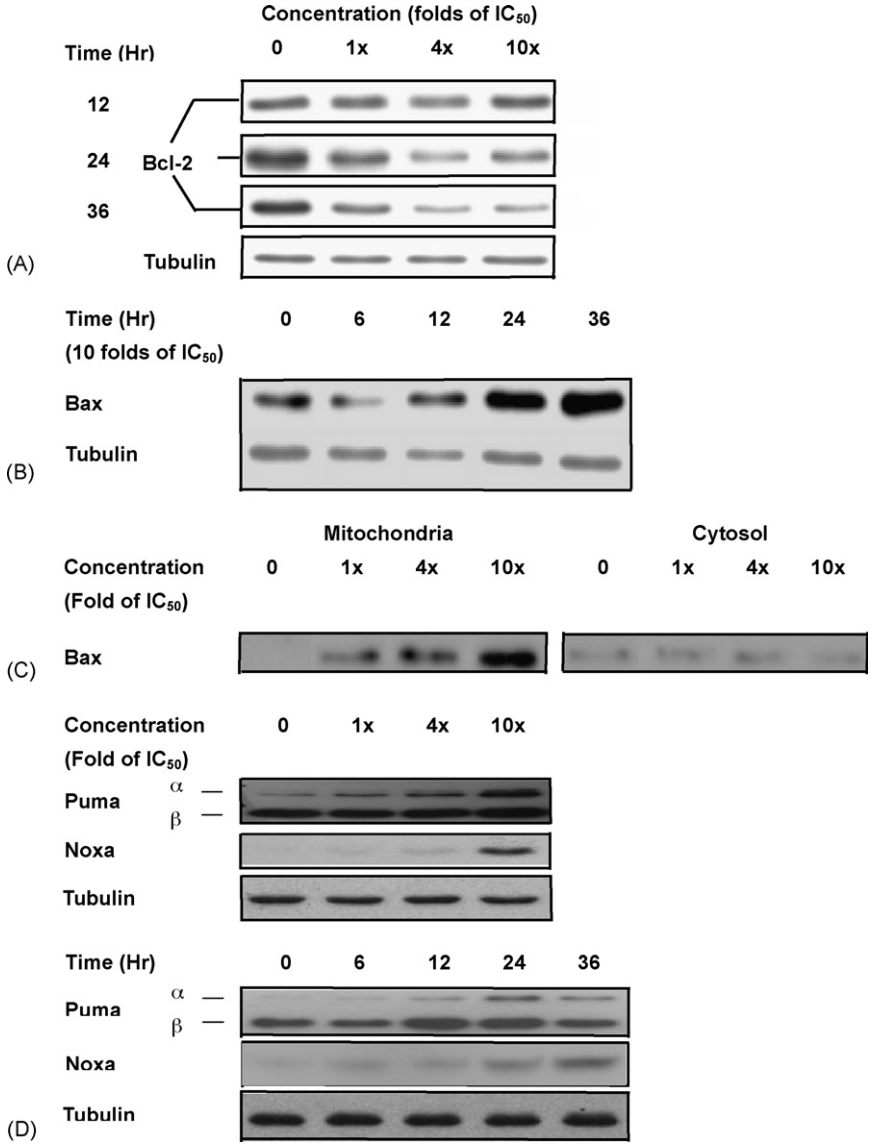

Fig. 5 – Effect of D-501036 on Bcl-2 family proteins. (A) Cells were treated with the indicated concentrations of D-501036 for the indicated times. The protein level of Bcl-2 was examined by Western blot analysis. (B) Cells were treated with 10-fold IC_{50} of D-501036 for the indicated time. The protein level of Bax was examined by Western blot analysis. (C) After treatment with D-501036, whole cells were fractionated into cytosolic and mitochondrial portions. The Bax level of each portion was examined by Western blot analysis. (D) Cells were treated with the indicated concentrations of D-501036 (upper panel) and with the indicated times (lower panel), the levels of Puma and Noxa were examined by Western blot analysis.

Mcl-1 expression was observed (data not shown). Since Bax exerts its apoptotic effects through an action at the mitochondria, experiments were performed to determine whether Bax was translocated from the cytosol to the mitochondria in response to the drug. The concentration of Bax in mitochondrial fractions of KB cells was found to increase in response to D-501036 treatment (Fig. 5C). Similar findings were observed with $HepG_2$ cells (data not shown). The expression of the BH3-only pro-apoptotic proteins, Noxa and Puma, was also examined. As shown in Fig. 5D, the expression of Noxa and of the α-form of Puma in KB cells treated with D-501036 was found to increase in a dose- and time-dependent manner in KB cells treated with D-501036 for 24 h (Fig. 5D, upper panel) and at the drug concentration of 4× the IC_{50} dose (Fig. 5D, lower panel). Taken together, these findings strongly favor the proposal that the expression of pro-apoptotic proteins is increased concurrently with a decrease in the expression of anti-apoptotic proteins in tumor cells exposed to D-501036.

3.6. *p53 is associated with D-501036-induced apoptosis*

Bax, Puma and Noxa are downstream pro-apoptotic proteins induced by p53 whereas the anti-apoptotic protein, Bcl-2, is suppressed by p53 [26–30]. Furthermore, the phosphorylation of p53 at Ser15 has been linked with both induction and suppression of the apoptosis-related proteins and with the

translocation of p53 from the cytosol to the mitochondria [28,31]. Experiments were therefore performed to measure the expression of p53 protein, its phosphorylation state, and the extent of its translocation from the cytosol to the mitochondria in response to treatment with D-501036. As shown in Fig. 6A, total p53 expression was increased in a time-dependent manner in drug-treated KB cells, and the active form of p53 increased in parallel with total p53 (Fig. 6A). Additionally, p53 was found to be translocated from the cytosol to the mitochondria in response to the drug treatment for 12 h (Fig. 6B). These findings strongly implicate p53 in the induction of apoptosis by D-501036.

4. Discussion

An extensive body of evidence now exists in support of the role of tumor cell apoptosis in the beneficial effects of anti-cancer drugs. Although individual apoptotic agents may not prove effective in the treatment of cancer, combinations of agents that induce apoptosis through distinct pathways and in a synergistic fashion frequently prove highly effective [32]. For example, the induction of cancer cell deaths by the tumor necrosis factor-related apoptosis-inducing ligand and the histone deacetylase inhibitor are through two distinct canonical pathways [4,33]. Both are ineffective to induce apoptosis when they are applied as single agents. However, the synergistic effect on the apoptosis can be observed in both agents used in combination [34]. The present study was therefore undertaken to identify the mechanisms through which D-501036 induces apoptosis in cultured tumor cells. Initially, D-501036 was observed to have potent cytotoxic actions on both KB and $HepG_2$ cells. The ratio of inhibition of clonogenicity to inhibition of growth was less than 1 for both cell lines, indicating that the cytotoxicity of D-501036 was irreversible. This finding prompted an examination of the possibility that apoptosis was responsible for the antitumor actions of the drug.

Two apoptosis-related indicators were examined. The percentage of cells displaying the annexin V-positive/PI-negative pattern was increased after D-501036 treatment in a dose-dependent manner. A second early apoptosis marker, the cleaved form of PARP, was also increased by D-501036 in a dose- and time-dependent manner. Both results were fully consistent with the induction of apoptosis by D-501036. To identify the canonical apoptotic pathway activated in response to the drug, the roles of caspases -3, -8 and -9 were explored. Functional assays for the caspases were performed. The activity of caspase-3 was found to be elevated, consistent with an increase in expression of cleaved PARP, a product of caspase-3 activity. In addition, the underlying initiator for the increased caspase-3 activity was found to be caspase-9, rather than caspase-8. It was therefore proposed that mitochondria play a more important

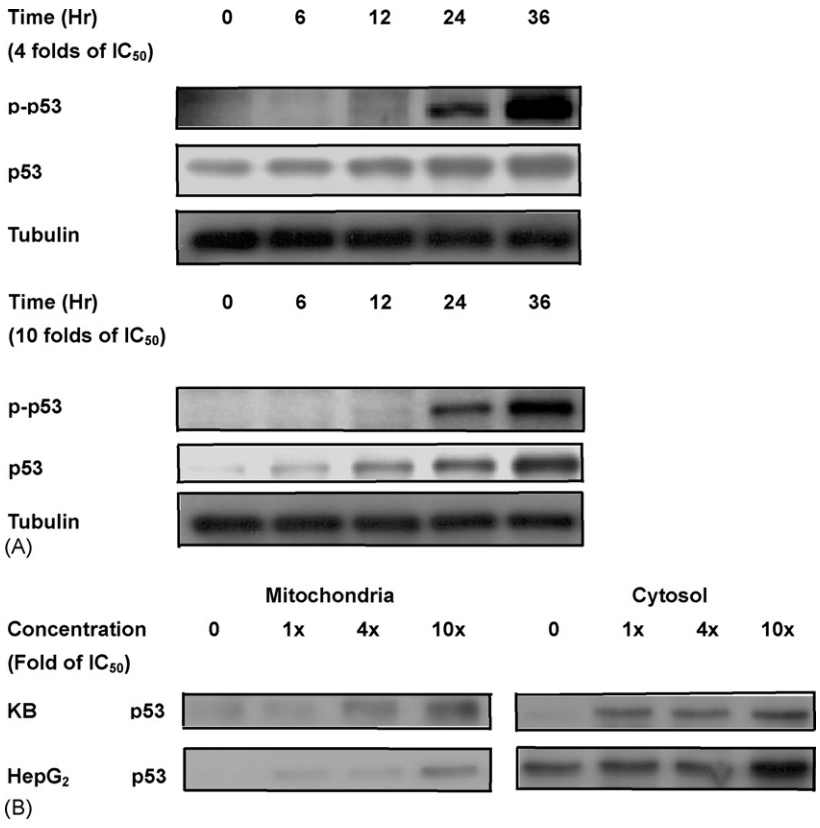

Fig. 6 – Effect of D-501036 on the tumor suppressor protein, p53. Cells were treated with the indicated concentrations of D-501036 for the indicated time. (A) The protein levels of total p53 and phosphorylated p53 at Ser15 were examined by Western blot analysis. (B) Cells treated with D-501036 were fractionated into cytosolic and mitochondrial portions. The total p53 were examined by Western blot analysis.

role than death receptors in the apoptosis induced by D-501036. This proposal was confirmed by the finding that expression of Fas and Fas-L did not change significantly. Moreover, the mitochondrial membrane permeability was increased and cytochrome *c* was released into cytosol after D-501036 treatment. Surprisingly, the amount of mitochondrial cytochrome *c* was also increased after D-501036 treatment. An increase in mitochondrial cytochrome *c* during apoptosis due to other causes has been observed by other investigators [35,36]. The proposal [37] that the expression of cytochrome *c* and the expression of Bcl-2 are regulated in an inverse manner may explain these observations. Alternatively, an increase mitochondrial cytochrome *c* may represent an attempt by the apoptotic cell to increase anti-oxidant activity within the organelle [38]. Regardless of the mechanism whereby mitochondrial cytochrome *c* is increased, the findings of the present report indicate that D-501036 is a cytotoxic, rather than cytostatic, antitumor drug and that the cell death induced by D-501036 is attributable to activation of the canonical mitochondrial pathway.

The mitochondria-mediated apoptosis is precisely regulated by the proteins that comprise a large group termed the Bcl-2 family [39]. In the present study, the mitochondrial permeability was found to be increased by D-501036 through the alterations in expression and localization of Bcl-2 family proteins. These alterations included downregulation of Bcl-2, upregulation of Bax, translocation of Bax to mitochondria, and upregulation of two BH3-only proteins, Puma and Noxa. These various Bcl-2 family proteins are known to be subject to regulation by p53 [26–30]. For example, Bcl-2 is transcriptionally downregulated by p53 [26], Bax is directly upregulated and activated by p53 [26,27], sequestered Bax is released by p53 [28,40], and both Puma and Noxa are transcriptionally upregulated by p53 in response to DNA damage [29,30]. p53 that is phosphorylated at Ser15 is associated with p53 stabilization [41], is not exported from the nucleus [41], and is known to induce apoptosis [42]. To correlate D-501036-induced apoptosis with p53, therefore, both phosphorylated and total p53 contents were measured. Studies revealed that total p53 content of drug-treated cells was upregulated with time and that phosphorylated p53 was subsequently upregulated. Furthermore, several lines of evidence have been previously provided that support (a) the translocation of p53 from the cytosol to the mitochondria after phosphorylation of this protein at Ser15 and (b) the importance of this translocation to apoptosis [28,31]. In the present study, a substantial fraction of p53 was translocated to the mitochondria in response to D-501036 treatment (Fig. 6B). Transcription-dependent and transcription-independent forms of cell death are both modulated by p53. Since D-501036 induces a powerful apoptotic signal, it is therefore conceivable that both forms of cell death are essential to D-501036-dependent apoptosis. Findings of the present study are consistent with an association between p53 upregulation and mitochondria-mediated apoptosis induced by D-501036. However, the possibility that D-501036 can induce apoptosis when p53 is not functional, cannot be ruled out.

In summary, D-501036 has significant potential as an anticancer agent. The antitumor activity of D501036 is attributable to induction of apoptosis through activation of the p53-related mitochondrion-stress pathway. The mechanism by which this agent induces the tumor cell death provides a rationale for its use in combination with other chemotherapeutic agents that induce apoptosis through distinct pathways.

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