Octahedral Pt(IV) complex K101 induces apoptosis via ERK1/2 activation and the p53 pathway in human colon cancer cells

Young-Ee Kwon[a] and Kuk-Hwan Kim[b]

Recently, the synthesized octahedral Pt(IV) compound *trans,cis*-Pt(acetato)$_2$Cl$_2$(1,4-butanediamine), K101, showed potent anti-tumor activity *in vitro* and *in vivo*. For the further investigation of K101-induced anti-cancer activity, we tested cytotoxicity against various cancer cell lines and performed the histoculture drug response assay (HDRA) against human colorectal tumor tissues *in vitro*. We investigated the signaling pathway of K101-induced apoptosis via expression of p53 and ERK1/2 in the human colon cell line HCT116. The cytotoxicity and the three-dimensional HDRA of K101 were evaluated using the MTT assay. To study the K101-induced apoptosis pathway, we performed FACS analysis and immunoblotting of p53, p21^{WAF-1}, Bax, Fas and ERK1/2 in HCT116 cells treated with or without K101. The cytotoxic IC$_{50}$ values of K101 ranged from 1.15 to 2.38 μmol/l, compared to cisplatin ranging from 2.13 to 13.1 μmol/l. Among several cancer cell lines, K101 showed greater potency than cisplatin in colon cancer cell lines. In the HDRA, K101 showed 80.0–91.4% efficacy rates compared with 48.6% for cisplatin against colorectal cancer patient tissues. In the signaling pathway, the expression of p53 and phospho-ERK1/2 was increased in a time-dependent manner by treatment with K101 in the HCT116 cells. When K101 was treated with MEK inhibitor U0126, the cell death rate was increased. The octahedral Pt(IV) complex K101 could be an attractive candidate as a chemotherapeutic agent against colon cancer. ERK1/2 activation and the p53 pathway may play significant functions in mediating K101-induced apoptosis in human colon cancer cells. *Anti-Cancer Drugs* 17:553–558 © 2006 Lippincott Williams & Wilkins.

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Introduction

Cisplatin (*cis*-diamminedichloroplatinum) (Fig. 1) [1] is widely used to treat various types of human cancer such as ovarian, testicular, head and neck, bladder, and lung cancer [2–4]. However, its spectrum of anti-tumor activity is narrow and its use in the clinic has been limited by its undesirable side-effects, including nephrotoxicity, ototoxicity, neurotoxicity, nausea, vomiting and myelosuppression [5,6]. Consequently, interest in this field led to the development of cisplatin analogs, such as carboplatin and oxaliplatin, further improving the efficacy of platinum-based chemotherapy and extending the scope of its clinical application [7,8]. However, the incidence of drug resistance, acquired or intrinsic, continues to limit the effectiveness of platinum-based chemotherapy. Significantly, the prototype Pt(IV) complex, satraplatin (JM216), entered phase III clinical trials in 2001 as an orally active anti-cancer drug [9]. It was reported that Pt(IV) complexes with axial ligands have more potent and broader anti-tumor activity than cisplatin, and their mechanism may be different from that of cisplatin [10]. The generally accepted mode of action of cisplatin arises from its binding to DNA, inhibiting replication of DNA and transcription of RNA, causing DNA damage and killing cancer cells mainly by apoptosis [7,11]. It has been reported that various platinum complexes induce apoptosis in cancer cells [12–14]. Thus, cell signaling pathway studies about the platinum complex are essential for analyzing mechanisms of drug-induced cancer cell death and it has been suggested that p53 is required for programmed cell death [15]. The general role of p53 is to affect cell cycle arrest, DNA repair and apoptosis via the regulation of many downstream proteins containing p21, Bcl-2, etc. [16]. The p53 tumor-suppressor gene may play an important role in the cellular response to DNA damage in mammalian cells [17]. ERK is known as a regulator of cell proliferation, differentiation, survival and apoptosis [18]. It has been reported that ERK is an activator or a mediator in cell survival or apoptosis by cisplatin. Among the signaling pathways, the p53 and ERK pathways are known to play an important role in cisplatin-induced

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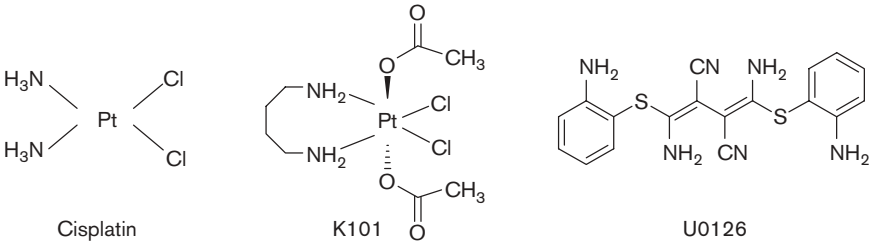
Fig. 1



Chemical structures of cisplatin, K101 and MEK inhibitor U0126.

apoptosis [19]. In our previous study, we demonstrated the potent in-vitro and in-vivo activity of a series of (1,4-butanediamine)Pt(IV) complexes with axial acetato, trifluoroacetato ligands [20]. We report here the results of in-vitro cytotoxicity, histoculture drug response assay (HDRA) efficacy, FACS analysis and the cell signaling pathway of the *trans,cis*-Pt(IV)(acetato)$_2$Cl$_2$(1,4-butanediamine), K101 (Fig. 1).

Materials and methods

Reagents

Cisplatin and MTT were purchased from Sigma (St Louis, Missouri, USA). The new Pt(IV) complex K101 was synthesized at STC Life Science Center, Seoul, Korea. K101 and cisplatin were dissolved in DMSO, adding PBS immediately before use, and then sterilized through a 0.22-μm disk filter. The final concentration of DMSO in cell culture media was controlled to be below 0.1%. Anti-p53 (DO-1), anti-p21 $^{WAF-1}$ (C-19), anti-Bax (B-9) and anti-Fas (B-10) were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Anti-ERK and anti-phospho-ERK rabbit polyclonal antibodies and secondary antibody were purchased from New England Biolabs (Beverly, Massachusetts, USA). MEK inhibitor U0126 was purchased from Calbiochem (San Diego, California, USA).

Cell lines and culture conditions

Cancer cell lines for human leukemia (HL-60), colon cancer (HCT116, HCT15), lung cancer (A549) and central nervous system (CNS) cancer (XF498), and mouse leukemia (L1210) were obtained from the Korean Cell Line Bank, Daejon, Korea. These cell lines were grown in RPMI 1640 or DMEM (Gibco/BRL, Gaithersburg, Massachusetts, USA) media supplemented with 10% heat-inactivated FCS (Gibco/BRL), penicillin (100 U/ml) and streptomycin (100 μg/ml) in a highly humidified atmosphere of 5% CO_2 at 37°C.

Cytotoxicity

Cytotoxicity was determined by using the MTT colorimetric assay [21]. Each cell line was put into 96-well plates (1×10^4/ well) and then 10^{-4} to 10^{-8} mol/l of K101 was added to each well. After cultivating cancer cell lines at 37°C and 5% CO_2 for 72 h, MTT reagent (5 mg/ml) was added at 50 μl/well and an additional cultivation was performed at 37°C and 5% CO_2 for 4 h. Then, 100 μl/well of DMSO was added and the plates were shaken on a plate shaker for 10 min. The optical densities of the wells were read using an ELISA plate reader (Bio-Tek, Winooski, Vermont, USA) at 570 nm. For calibration, a blank test was performed on the same 96-well plate under the same conditions. The experiment was performed on more than three wells under the same conditions with different concentrations and the same experiment was repeated over 3 times.

Three-dimensional histoculture drug response assay (HDRA)

Specimens

The tissue samples from colorectal cancer patients were supplied by The Korean Cancer Center Hospital. Surgical or biopsied specimens were transported in HBSS (Gibco) to the MetaBio Laboratory, Seoul, Korea where the assay was conducted.

HDRA with the MTT end point

The cancerous portion of the specimens were scissor-minced into pieces approximately 1 mm in diameter, which were then placed on each of the prepared collagen sponge gel (Health Design, Rochester, New York, USA) surfaces in 24-well plates. The plates were incubated for 3 days at 37°C with the drugs dissolved in RPMI 1640 medium containing 20% FCS in a humidified atmosphere of 95% air/5% CO_2. The concentrations of the drugs were 10 μg/ml for cisplatin and 10 and 20 μg/ml for K101. After histoculture, 900 μl HBSS containing 0.1 μg/ml collagenase (type I; Sigma) and 100 μl MTT solution, dissolved in 4 mg/ml PBS, were added to each culture well and incubated for 2 h. After extraction with DMSO, the absorbance of the solution in each well was read at 540 nm. The inhibition rate (%) = [(1 – mean absorbance of treated tumor/g)/(mean absorbance of control tumor/g)] × 100. When the inhibition rate was increased 30%,

the chemosensitivity of tumors to drugs was scored as positive.

Flow cytometry

For FACS analysis, HCT116 cells were cultured in presence of 1 and 10 μmol/l K101 at 37°C and 5% CO_2 for 48 h. The cells were detached using 0.25% trypsin and 1 mmol/l EDTA in PBS, and washed twice in flow cytometry buffer containing PBS (pH 7.4), 1% FBS and 0.01% sodium azide. Then, 1×10^6 cells were resuspended in flow cytometry buffer. After washing, the cells were stained using the Annexin-V–FITC apoptosis detection kit [including propidium iodide (PI); Sigma] and placed in the dark overnight. The Annexin-V and PI fluorescence of individual nuclei were measured using a FACS calibration flow cytometer (Becton Dickinson, Mountain View, California, USA). The viable cells were not stained by both of PI and Annexin-V – the early apoptosis cells were stained by Annexin-V and the primarily cells in late-stage apoptosis were stained by PI only.

Immunoblot analysis

HCT116 cells were grown in 60-mm culture dishes for 72 h to 70–80% confluency, followed by a 24-h treatment with each concentration of K101. Cells were pretreated with MEK inhibitor U0126 (20 μmol/l) for 1 h. Cells (5×10^6 cells) were then washed with PBS and lysed in 500 μl of ice-cold cell lysis buffer (20 mmol/l Tris, pH 7.4, with 120 mmol/l NaCl, 25 mmol/l β-glycerophosphate, 2 mmol/l EDTA, Triton X-100, 10% glycerol, 1 mmol/l DTT, 1 mmol/l PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin). The lysates were centrifuged at 10 000 *g* for 1 min at 4°C. Each supernatant was added to an equal volume of sample buffer (200 mmol/l Tris–HCl, pH 6.7, containing 5% SDS, 20% glycerol, 0.2 mg/ml BPB and 10% β-mercaptoethanol) and then boiled for 3 min. The protein (30 μg/lane) was separated by SDS–PAGE using 10% polyacrylamide gels and subsequently transferred to PVDF membranes (Amersham, Pharmacia Biotech, USA). The membranes were blocked with 5% non-fat milk, washed and incubated with primary antibodies in 0.5% non-fat milk. The membranes were incubated with peroxidase-conjugated secondary antibody. After extensive washing, specific bands were detected by using enhanced chemiluminescence (Amersham), according to the manufacturer's protocol.

Results

In-vitro cytotoxicity against various cancer cell lines

Anti-cancer activities of K101 were evaluated *in vitro* using various cancer cell lines such as human leukemia (HL-60), mouse leukemia (L1210), human colon cancer (HCT116, HCT15), human lung cancer (A549) and human CNS cancer (XF498). Anti-cancer activities (IC_{50}) of cisplatin and K101 against each cancer cell line are shown in Table 1. The IC_{50} values of K101 and cisplatin were calculated from the dose–survival curves obtained from the MTT assay. Among several cancer cell lines, K101 showed greater potency than cisplatin in colon cancer cell lines.

Efficacy of K101 against colorectal cancer tissue using three-dimensional HDRA

When the inhibition rate was 30% or more, the chemosensitivity of tumors to drugs was scored as positive. Table 2 gives the overall in-vitro chemosensitivity determined by the HDRA with the MTT endpoint for each colorectal cancer patient tissue. K101 (20 μg/ml) showed a 94.4% efficacy rate against female colorectal cancer patients with a total efficacy rate of 91.4%.

Flow cytometry for detecting apoptosis

In order to detect apoptosis of human colon cancer cells by K101, FACS analysis was performed using HCT116 treated with K101. The scattering of 1 and 10 μmol/l K101-treated cells in Fig. 2 shows that extracellular binding of Annexin-V was increased as uptake of PI. This result showed that K101-induced cell death was caused by apoptosis in human colon cancer.

Immunoblot analysis for K101-induced apoptosis-mediating proteins

The effects of K101 on p53, ERK1/2, $p21^{WAF-1}$, Bax and Fas signaling were analyzed by immunoblotting, and are illustrated in Fig. 3. A dose-dependent increase in p53

Table 1 IC_{50} values of cisplatin and K101 against various cancer cell lines

Cell lines	IC_{50}(μmol/l)	
	Cisplatin	K101
HL-60	2.13	1.15
L1210	2.53	1.23
HCT116	13.10	1.95
HCT15	7.98	1.75
A549	4.63	2.38
XF498	2.13	1.15

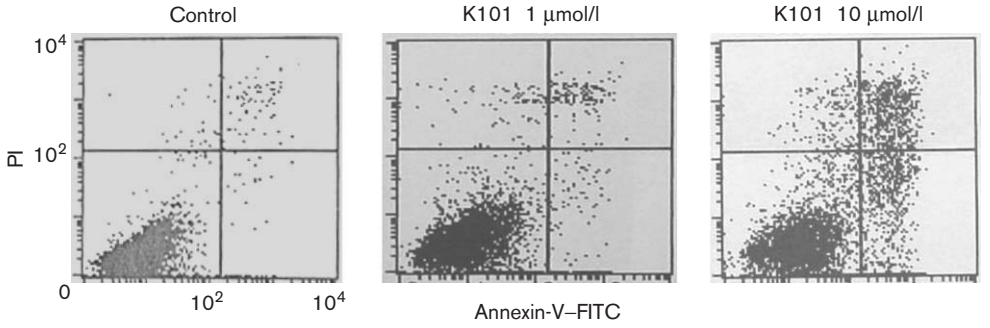
MTT assay was performed in three independent experiments, each consisting of at least triplicates. IC_{50} values were calculated using the quantal dose–response (Probits computer program edited by R.J. Tallarida *et al.*).

Table 2 In-vitro chemosensitivity determined by the HDRA for colorectal cancer patient tissues

Drugs	Concentration (μg/ml)	Patients		Total
		Male	Female	
Cisplatin	10	7/17[a]	10/18	17/35
		41.2%[b]	55.6%	48.6%
K101	10	14/17	14/18	28/35
		82.4%	77.8%	80.0%
K101	20	15/17	17/18	32/35
		88.2%	94.4%	91.4%

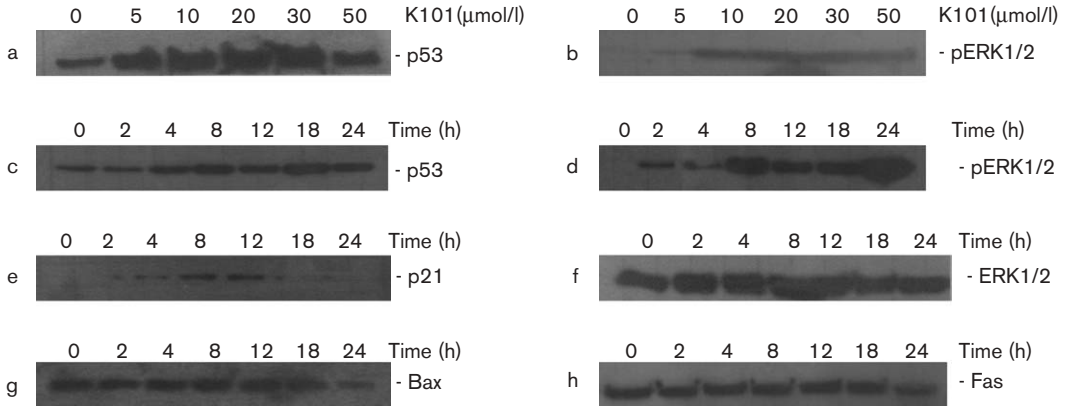
Treatment tissues are 35 cases from colorectal cancer patients. When the inhibition rate is 30% increased, the chemosensitivity of tumors to drugs is scored as positive.
[a]Data are shown as number of sensitive cases/number of evaluable cases.
[b]Percent values represent efficacy rate against colorectal cancer.

Fig. 2



Detection of apoptosis by flow cytometry. Apoptosis of human colon cells (HCT116) was determined by flow cytometry. It was measured by flow cytometric analysis with Annexin-V and PI staining. As a result, apoptosis was observed during exposure to indicated doses of K101.

Fig. 3



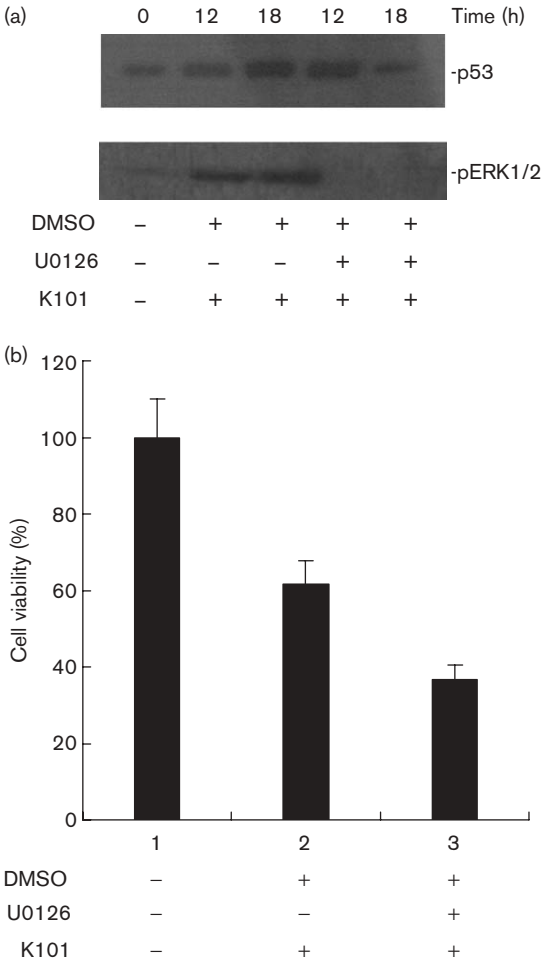
Immunoblot analysis of p53, ERK, p21, Bax and Fas in human colon cell line in response to K101. (a–h) K101 induces p53, p21 and phospho-ERK activation of HCT116 cells in a dose- and a time-dependent manner. HCT116 cells were treated either with different doses of K101 for 24 h (a and b) or 30 μmol/l K101 for the indicated times (c–h).

protein accumulation after K101-induced DNA damage was observed in HCT116 cells (Fig. 3a). Phospho-ERK1/2 was induced from 10 μmol/l K101 (Fig. 3b). The peak level of p53 accumulation occurred with 30 μmol/l K101, therefore this concentration was used. The ERK1/2 activation increased gradually from 2 to 24 h and expression of p53 increased from 4 h (Fig. 3c and d). In the case of $p21^{WAF-1}$, it was induced from 8 to 12 h after K101 treatment (Fig. 3e) and non-phosphorylated-ERK1/2 showed no significant change in expression (Fig. 3f). The expression levels of Bax and Fas, which are well known as other key proteins of apoptosis, showed no significant changes by K101 treatment (Fig. 3g and h). These results suggested that K101-induced apoptosis could be associated with ERK1/2 and p53 signal pathways, and that Bax and Fas signaling may not be connected with that. K101-induced ERK1/2 phosphorylation and p53 accumulation were inhibited by MEK1 inhibitor U0126 (Fig. 4a). The effects of MEK inhibitor U0126 against K101-treated colon cancer cells are illustrated in Fig. 4(b). When both U0126 and K101 were used in HCT116 cells, ERK1/2 activation was inhibited completely, p53 accumulation was inhibited incompletely and the cell survival rate (%) was significantly decreased in comparison to K101 treatment alone. Cell survival rates of U0126-treated cells, K101-treated cells and K101 + U0126-treated cells were 96.4, 61.6 and 36.8%, respectively, compared with control cells as determined by MTT assay.

Discussion

The new octahedral Pt(IV) complex K101 was recently synthesized as one of the new chemotherapeutics. So far, it has shown anti-cancer activity *in vitro* and *in vivo*, and suitable pharmaceutical properties such as good yield, simple synthesis and stability in aqueous solution [20]. The cytotoxicity of K101 was tested against human cancer cell lines HL-60, HCT116, HCT15, A549 and

Fig. 4



(a) MEK inhibitor blocks K101-induced p53 ERK activation in HCT116 cells. HCT116 cells pretreated with DMSO (control) or U0126 (20 μmol/l) for 1 h prior to a 24-h exposure to K101 (30 μmol/l) for the indicated times. (b) MEK inhibitor increases K101-induced apoptosis in HCT116 cells. The HCT116 cells were pretreated with U0126 (20 μmol/l) or DMSO (0.1%) for 1 h prior to a 2-day exposure to K101 30 μmol/l. U0126 is a MEK inhibitor. The cytotoxicity test was performed with a MTS method in three independent experiments, each consisting of at least triplicates. Column 1, control line; column 2, DMSO and K101; column 3, U0126 and K101. The results represent the cell viability (%).

XF498, and murine leukemia L1210. IC_{50} values of K101 and cisplatin are shown in Table 1. K101 has the better cytotoxicity, which is about 2–7 times higher than that of cisplatin. In particular, it was more potent in killing human colon cancer cell lines than cisplatin. Cancers are highly individual in their response to chemotherapy. Many attempts to predict *in vitro* tumor cell response to drugs have largely failed.

A new in-vitro technology, the HDRA, has solved these problems [22]. The HDRA, developed by Hoffman *et al.* is a modernized version of tumor fragment culture [23], based on the collagen sponge–gel matrix, three-dimensional culture system of Leighton [24]. The HDRA result is known to closely represent the drug response of the tumor patient, and the correlation of the results between HDRA and clinical drug response is 92% [25]. Our HDRA results indicate that the in-vitro efficacy rates of K101 at 10 and 20 μg/ml and cisplatin at 10 μg/ml in HDRA were 80.0, 91.4 and 48.6%, respectively, against colorectal cancer patient tissues (Table 2). Clinically used platinum agents containing cisplatin have shown limits in their efficacy against several cancers such as colon and breast cancer. However, the new octahedral Pt(IV) complex K101 showed excellent efficacy against colorectal cancer patient tissues *in vitro*.

The p53 tumor-suppressor protein is activated and stabilized in response to various kinds of cellular stress, including DNA damage [26]. It has been reported that cisplatin induces ERK1/2 activation (in ovarian carcinoma cell lines) and the p53 response to DNA damage is influenced by cisplatin-induced ERK1/2 activation [27]. To compare the signaling pathway of K101-induced apoptosis with that of cisplatin-induced apoptosis, we confirmed apoptosis using FACS analysis, and then the expression of various proteins such as p53, $p21^{WAF-1}$, ERK1/2, Bax and Fas was analyzed by immunoblotting. In cisplatin-induced apoptosis, it has been reported that p53 is a key mediator in the cellular response to DNA damage [28–30]. In this study, p53 was increased in a dose- and time-dependent manner. Therefore, p53 is likely to function as an important regulator of K101-induced apoptosis against colon cancer HCT116 cells. The increased accumulation of p53 was accompanied by increased $p21^{WAF-1}$ expression. A downstream target of activated p53, $p21^{WAF-1}$ was increased 8–12 h after K101 treatment. A decrease in $p21^{WAF-1}$ expression after 12 h could be caused by caspase activation during K101-induced apoptosis, as suggested by previous cisplatin-relating studies [31–33]. ERK is well known as a major protein kinase that serves to integrate various extracellular signals, resulting in the regulation of cell proliferation, differentiation, survival and apoptosis [18,34]. Cisplatin treatment of ovarian carcinoma cells or HeLa cells has been reported to result in the induction of ERK activity [19,27]. Furthermore, inhibition of cisplatin-induced ERK activity by the MEK inhibitor PD98059 resulted in enhanced cytotoxicity in response to cisplatin treatment. Thus, the combination of cisplatin with MAPK inhibitors warrants further investigation for potential clinical benefits [27]. In this study, phosphorylated ERK1/2 was expressed in a time-dependent manner by 30 μmol/l K101 treatment, but the expression of inactivated ERK1/2 showed no changes over the course of time. Other apoptosis regulatory proteins, Bax and Fas, showed no change in their expression following K101 treatment. Inhibition of ERK1/2 activation by U0126, a MEK inhibitor, in K101-treated cells resulted in a significant decrease in ERK1/2

activation and p53 accumulation. K101 (30 μmol/l) and U0126 (20 μmol/l)-treated cells showed more enhanced cytotoxicity than K101 treatment alone. Therefore, ERK1/2 activation may be linked to the p53 signaling pathway. Recently, it has been reported that ERK1/2 as a protein kinase targets the phosphorylation of p53 after cisplatin-induced DNA damage and that the ERK1/2 pathway is one of the upstream mediators of p53 DNA damage response after cisplatin exposure in human ovarian cancer cells [27].

In conclusion, the octahedral Pt(IV) complex K101 has more potent anti-tumor activity than the Pt(II) complex cisplatin against human colorectal cancer by the three-dimensional HDRA *in vitro*. We have identified using FACS analysis that K101-induced cell death is apoptosis. This study provides evidence that ERK1/2 phosphorylation, p53 and $p21^{WAF-1}$ could be related to human colon cancer cells, and that ERK1/2 pathway is one of the upstream mediators of p53 DNA damage by K101-induced apoptosis. Inhibition of K101-induced ERK1/2 activity by the MEK inhibitor U0126 resulted in enhanced cytotoxicity in response to K101-treated human colon cancer cells.

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