Characterization and resistance mechanisms of cisplatin-resistant human hepatocellular carcinoma cell line

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ABSTRACT

الأهداف: دراسة الخصائص الحيوية وآلية المقاومة لخط خلية السرطان الغدي الكبدي الخلوي المقاوم (HCC) لعقار سيسبلاتين.

الطريقة: أجريت هذه الدراسة بقسم علم الأدوية بجامعة تشونقكينق الطبية – تشونقكينق – الصين، خلال الفترة ما بين أبريل 2005م وحتى نوفمبر 2007م. تم إنشاء خط خلية السرطان الكبدي الخلوي الغدي المقاوم (QGY/CDDP) (HCC) بواسطة زيادة تركيز عقار سيسبلاتين (CDDP) وتلقي العقار بصورة متقطعة. تم اكتشاف الحساسية الكيميائية للدواء بواسطة طريقة 3-4,5-dimethylthiazol-2yl-2,5- diphenyltetrazolium bromide - MTT تم تحديد وقت ازدواج الخلية بواسطة حساب وتحليل دورة الخلية التي أجريت بواسطة تيار طريقة تعداد الخلايا (FCM). تم اكتشاف تراكم البلاتين داخل الخلية بواسطة قياس امتصاص الطيف الذري وتم تحليل ظهور P–glycoprotein P–gp، وجلوتاثيون إس–ترانسفيرس – الفا (GST–π) بواسطة طريقة (FCM).

النتائج: تم إنشاء خط خلية (QGY/CDDP) بعد ثلاثة أشهر مع مقاومة مستقرة لـ(CDDP) والتعرض للمقاومة العرضية لعوامل عديدة من الأدوية الكيميائية. مقارنة مع خط خلية الوالدين، ووقت ازدواج الخلية لفترة (QGY/CDDP): وانخفاض تناسب الخلية في S و G_2/M–phase، والزيادة في طور G_0/G_1. في خلايا (QGY/CDDP)، انخفض تراكم البلاتين داخل الخلية وزيادة في ظهور (GST–π)، ولكن بقي الظهور P–gp مستقراً.

خاتمة: أظهر خط خلية (QGY/CDDP) النمط الظاهري للمقاومة النمطية والمستقرة وخصائص الخلايا المقاومة. إن ميكانيكية المقاومة لـ(CDDP) قد تكون متوسطة (غير مباشرة) بواسطة تخفيض تراكم البلاتين داخل الخلية وارتفاع ظهور (GST–π)، ولكن ذلك لا يتصاحب مع ظهور P–gp.

Objectives: To study the biological characteristics and resistant mechanisms of the cisplatin-resistant human hepatocellular carcinoma (HCC) cell line.

Methods: The study took place in the Department of Pharmacology, Chongqing Medical University, Chongqing, China, between April 2005 and November 2007. A resistant HCC cell line (QGY/CDDP) was established by stepwise increasing cisplatin (CDDP) concentration and intermittent administration. Drug-chemo sensitivity was detected by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell doubling time was determined by cell counting, and cell cycle analysis was performed by flow cytometric (FCM) assay. Intracellular platinum accumulation was detected by atomic absorption spectrometry and the expression of P-glycoprotein (P-gp) and glutathione S-transferase-π (GST-π) were analyzed by FCM assay.

Results: QGY/CDDP cell line was established after 3 months with stable resistance to CDDP and exhibited cross-resistance to many other chemotherapeutic agents. Compared with parental cell line, cell doubling time of QGY/CDDP prolonged; and the cell proportion decreased in S and G_2/M-phase and increased in G_0/G_1–phase. In QGY/CDDP cells, intracellular platinum accumulation decreased and GST-π expression increased, but P-gp expression kept stable.

Conclusion: QGY/CDDP cell line shows the typical and stable resistant phenotype and characteristics of resistant cells. Its mechanisms of resistance to CDDP may be mediated by reduced accumulation of intracellular platinum and higher GST-π expression, but it is not associated with P-gp expression.

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Cisplatin (cis-diamminedichloroplatimun [CDDP]) is one of the most important drugs in the front line treatment of surgically resected malignant tumor. The anti-tumor activity of CDDP is attributed to the formation of a variety of platinum-DNA adducts, including mono-adducts, and intrastrand and interstrand cross-links.[1] As one consequence of DNA platination, the cell cycle is arrested in order to allow the cell to repair the damage. If repair fails, apoptosis is induced by activation of various pathways.[2] However, it is well known that tumor cells gain acquired or intrinsic resistance to treatment by this anticancer reagent and overcoming clinical resistance has become one of the most important goals in therapy.[3] Several mechanisms of resistance have been identified so far including reduced drug accumulation,[4] increased detoxification by glutathione (GSH)[5] or metallothionein,[6] increased repair of DNA damage by the nucleotide excision repair pathway,[7] increased tolerance to the Pt-DNA damage,[8] and reduced apoptotic response.[9] In spite of extensive efforts using genetic and proteomic approaches, the mechanism underlying CDDP resistance remains unclear. The most frequent type of primary liver cancer is hepatocellular carcinoma (HCC), which is a tumor easily acquiring resistance to drug therapy.[10] In the case of HCC, CDDP administration has been shown to be more effective than other anticancer agents and in combination with drugs such as 5-flurouracil (5-FU) induces additive and synergistic results.[11,12] Studies on platinum complexes resistance to HCC are relatively fewer. Cell lines derived from HCC, such as QGY with a passage number of 20 from the original HCC stock have been shown intrinsically sensitivity to chemotherapeutic drugs including CDDP and can be used as a model to investigate the effects of different drugs in liver cancer cells.[13] In order to investigate the characteristics and mechanisms of resistance to CDDP in liver cancer, and to develop methods of overcoming resistance, we established a CDDP-resistant cell line *in vitro* by stepwise increasing CDDP concentration and intermittent treatment from HCC cell line QGY. Then, we analyzed the biological characteristics of the CDDP -sensitive and -resistant cell lines, and investigate the resistant mechanisms to CDDP.

Methods. ***Cell culture.*** Human HCC cell line QGY was provided by Institute for Viral Hepatitis, Chongqing University of Medical Sciences, China. These cells were grown as monolayer in RPMI-1640 medium (Gibco/BRL, Maryland, USA) containing 10% fetal bovine serum, penicillin (100U/ml) and streptomycin (100µg/ml) in a humidified atmosphere of 5% carbon dioxide (CO_2) at 37ºC. The culture medium was renewed every 3 days.

Establishment of the CDDP-resistant cell line, QGY/CDDP. The resistant cell line, which was named QGY/CDDP, was established by intermittent exposure to CDDP and stepwise increasing inducting concentration. The parental QGY cell line was exposed to concentration of CDDP from 0.1 µg/ml to a final 0.5 µg/ml during a 3-month period. Exposure was continuous for 48 hours, drug was then removed, and cells were exposed again when they had regained their normal growth properties. Typically, the concentration was increased by 0.05 µg/ml with 3 exposures at each concentration. Once the resistant phenotype developed, the QGY/CDDP cell line was maintained by continuous exposure to 0.1 µg/ml CDDP.

Detection of drug chemosensitivity by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were diluted with culture medium to the seeding density, suspended in 96-well culture plates (200 µl, containing 10^4 cells, per well) and treated continuously with 20 µl of various concentration of the chemotherapeutic agents. After incubation at 37ºC in a highly humidified incubator with 5% CO_2 for 68 hours, 20 µl of MTT (5 mg/ml) were added to each well and the plates were further incubated for 4 hours. Medium was discarded, and 200 µl of dimethyl sulfoxide were added to each well to solubilize the intracellular formazan crystals. The optical densities were measured at 570 nm by a microplate reader Model E2550 (Bio-Rad, Tokyo, Japan). Each experiment was repeated 3 times. The 50% inhibitory concentration (IC_{50}) was calculated using non-linear regression (sigmoidal dose response, variable slope; GraphPad Prism™ analysis software package, GraphPad Software, San Diego, USA). Resistance index (RI) was calculated using the formula: RI=IC_{50} (resistant cells)/IC50 (parent cells).

Growth curves and doubling time. Cells were seeded into 15ml culture flasks at a density of $5x10^4$ cells/ml and incubated in 5% CO_2 incubator. Then, the numbers of parental and resistant cells were counted every 24 hours for 7 days. Each experiment was performed in triplicate. Growth curves were drawn according to the data and the doubling time (TD) was calculated from the formula:[14] $TD = Txlog2/ (logN_t-logN_0)$, T stands for cell culture time (hours); N_t stands for cell number after T hours and N_0 stands for initial cell number.

Determination of cell cycle distribution by flow cytometry (FCM). Parental and resistant cells ($2x10^6$) were collected by trypsinization and centrifugation, washed with phosphate buffered saline (PBS) and fixed in 70% ethanol at 4ºC for one hour. The fixed cells were washed twice in PBS, stained with propidium iodide at 4ºC for 30 minutes, and analyzed by FCM.

Cytogenetic analysis. Exponentially growing cells were treated with 0.05 µg/ml colchicines for 2 hours

at 37ºC. Cells were then harvested by trypsinization and swollen in a hypotonic solution of 0.075mol/L potassium chloride (KCl) for 20 minutes at 37ºC. Cells were then fixed with a mixture of one part of ice-cold glacial acetic: 3 parts methanol and dropped onto slides and stained with 5% Giemsa for 10 minuntes, and 100 metaphase spreads were counted for each cell line.

Platinum accumulation. Parental and resistant cells during the exponential growth phase were incubated for 2 hours with CDDP (100 μg/ml). The cells were then harvested, washed 3 times with PBS and counted. $1x10^7$cells were digested in nitric acid at 80ºC for 5 hours, then total intracellular platinum content was determined by inductance coupling plasma atomic eradiate spectrophotometer (Model PE-400, USA). All results were obtained from at least 3 independent experiments and the data were expressed as mean±SD.

Detection of P-gp and GST-π expression by FCM. Parental and resistant cells ($2×10^6$) were collected by trypsinization and centrifugation, washed with PBS and fixed in 0.25% paraformaldehyde for 15 minutes and in 70% ethanol at 4ºC for one hour. The fixed cells were washed twsice in PBS, incubated with mouse monoclonal antibodies against P-gp and GST-π (Sigma, St. Louis, USA) at 4ºC for one hour, then stained with fluorescein isothiocyannate (FITC)-labelled goat anti-mouse IgG at 4ºC for 30 minutes, and analyzed by FCM.

Statistical analysis. All statistical calculations were performed using the SAS software package. Statistical analysis was performed by t-test and x^2 test. P<0.05 was considered statistically significant.

Results. ***Establishment of QGY/CDDP and multidrug resistant (MDR) phenotype.*** QGY/CDDP was obtained by intermittent exposing QGY cells to CDDP in stepwise increments of concentration raging from 0.1 to 0.5μg/ml over 3 months. As shown in **Table 1**, the parental cells QGY were highly sensitive to CDDP. However, the drug resistant cells QGY/CDDP were 10.81-fold resistant to CDDP when compared with QGY cells. Moreover, QGY/CDDP cells were observed the resistance to 5-FU, vincristine (VCR), mitomycin C (MMC), epirubicin (EPI) and hydroxycamptothecine (HPCT) and verified its MDR phenotype.

Biological characteristics of QGY/CDDP. From the growth curves (**Figure 1**), we found QGY/CDDP

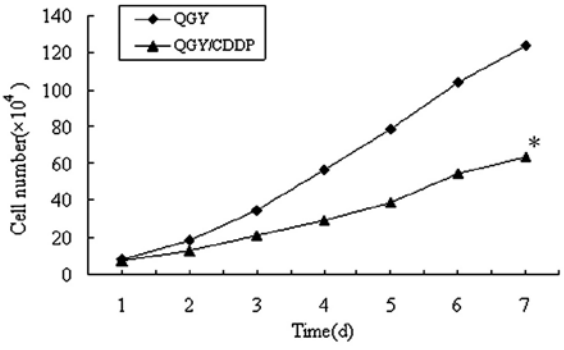

Figure 1 - Growth curves of QGY and QGY/CDDP cells. Cells were seeded into 15ml culture flasks at a density of $5×10^4$ cells/ml and incubated in 5% CO_2 incubator. Then the number of parental and resistant cell line was counted every 24 hours for 7 days. Data are expressed as mean ± SD from 3 experiments. *p<0.05 compared with QGY.

Table 1 - Drug chemosensitivity of QGY and QGY/CDDP cells.

Drugs	IC_{50} (μg/ml)		Resistance index
	QGY	QGY/CDDP	
CDDP	0.836	9.038 (6.472*, 8.946†)	10.81 (7.74*, 10.70†)
5-FU	3.290	26.375	8.02
VCR	0.625	4.965	7.94
MMC	0.177	0.894	5.05
EPI	0.432	1.644	3.81
HCPT	8.419	27.243	3.25

Cells were treated with various concentrations of the chemotherapeutic agents for 68 hours. Quantification of the cell proliferation ability was based on the OD values in destained MTT. Each experiment was repeated 3 times. IC_{50} (50% inhibitory concentration) was calculated using GraphPad Prism™ analysis software. Resistance index (RI) was calculated using the formula: IC_{50} (resistant cells) over IC_{50} (parent cells). *the IC_{50} and RI of the cells grown in CDDP-free medium for 3 months; †the IC_{50} and RI of the cells frozen for 3 months. CDDP - cisplatin, 5-FU - 5-flurouracil, VCR - vincristine, MMC - mitomycin C, EPI - epirubicin, HPCT - hydroxycamptothecine.

Table 2 - Cell cycle distributions of QGY and QGY/CDDP cells.

Cell line	Cell cycle (%)		
	G_0/G_1	S	G_2/M
QGY	56.98	37.35	5.67
QGY/CDDP*	65.11	32.03	2.86

Cells (2×10^6) were collected by trypsinization and centrifugation, fixed in 70% ethanol for one hour, stained with propidium iodide for 30 minutes, the distribution of cell cycle was analyzed by flow cytometry. Statistical analysis was performed by χ^2 test.
*p<0.01 compared with QGY.

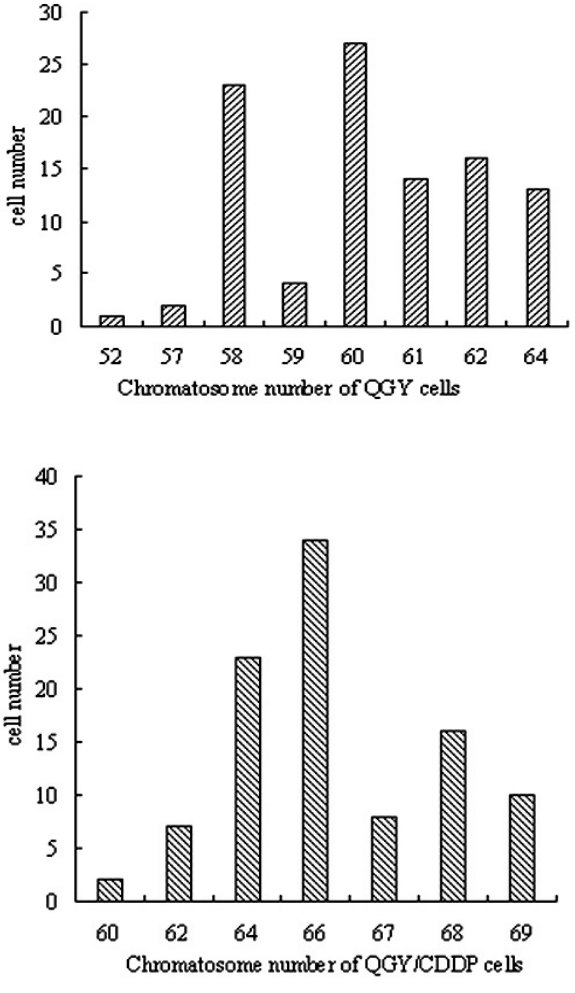

Figure 2 - Chromatosome numbers distribution of QGY and QGY/CDDP cells. Cells were treated with 0.05 μg/ml colchicines for 2 hours at 37°C. Cells were then harvested and stained with 5% Giemsa. Chromatosome numbers of 100 metaphase spreads were counted for each cell line.

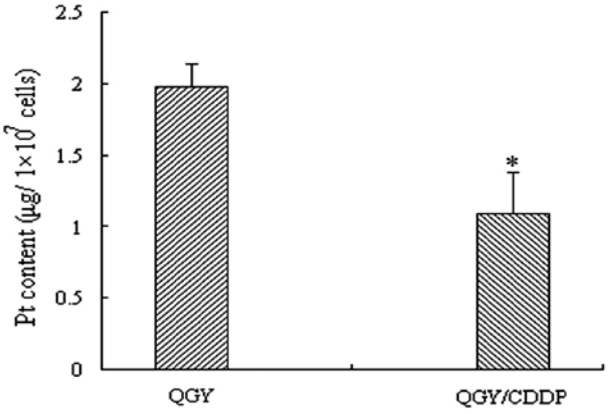

Figure 3 - Platinum intracellular content in the QGY and QGY/CDDP cell lines. Cells were incubated for 2 hours with CDDP (100 μg/ml). Then $1x10^7$cells were digested in nitric acid at 80°C for 5 hours, then total intracellular platinum (Pt) content were determined by inductance coupling plasma atomic eradiate spectrophotometer. Data are mean±SD of values obtained from 3 experiments. Statistical analysis was performed by t test.*p<0.01 compared with QGY.

cells grew slower than QGY cells. The TD of QGY cells was 36.286±1.489 hours and QGY/CDDP was 45.73±1.687 hours. The result showed that the TD of QGY/CDDP prolonged 9 hours contrasted to parental cell line (p<0.01). In addition, cell cycle distributions of the 2 cell lines showed in **Table 2** indicated the percentage of QGY/CDDP cells in G_0/G_1 phase was higher than that of the parental QGY line, whereas the S and G_2/M fraction was reduced. Cytogenetic analysis revealed a range of chromosome number of 52-64 for QGY and 60-69 for QGY/CDDP. They were hypo-triploid (**Figure 2**).

Platinum intracellular accumulation. The cellular platinum content in QGY cells after CDDP treatment was 1.976±0.1605 μg/10^7 and QGY/CDDP 1.096±0.2861 μg/10^7. **Figure 3** shows that the resistant cell line QGY/CDDP had a significantly reduced intracellular platinum accumulation (p<0.01).

P-glycoprotein (P-gp) and glutathione-S-transferase π (GST-π) expression analysis. The parent and resistant cell lines were compared for the expression of P-gp and GST-π using flow cytometry. The P-gp expressions were not detected in QGY and QGY/CDDP cells, but GST-π expression in resistant cells QGY/CDDP showed a higher positive rate of 62.3% compared with 0.2% in parent QGY cells.

Discussion. Multidrug resistance is a common problem in cancer chemotherapy. Establishing an ideal MDR cell model from a parental carcinoma cell line by exposing to certain anticancer agent *in vitro* is the key to study characteristics and mechanisms of

drug resistance. In the clinic, patients are treated with intermittent chemotherapy and develop drug resistance. Therefore, in terms of the method of induction, the drug resistance induced by intermittent exposure to anticancer agents may have more clinical relevance than that produced by continuous exposure. In order to simulate the resistance confronted in the clinic, we established an *in vitro* resistant model of the QGY HCC cells by intermittent exposing to CDDP for 3 months. The degree of resistance in QGY/CDDP cells was 10.81-fold to the inducing drug CDDP and remained stable after frozen and resuscitated. When we cultured QGY/CDDP cells in the CDDP-free medium for 3 months, the resistance level in QGY/CDDP, which was remained, 70% verified the stability of the resistant phenotype. Simultaneously, the variant degree of resistance to other anticancer drugs (especially to 5-FU and VCR) with no structural or functional similarity to CDDP was observed in QGY/CDDP. This result verified that the resistant cell line induced by CDDP showed its MDR phenotype. Thus, QGY/CDDP cell line might be served as an ideal model for studying the mechanisms of resistance to CDDP due to its MDR phenotype and stable resistance.

Compared with the parental cells, we found the size of QGY/CDDP increased, some cells distorted and huge cells were formed. The formation of huge cells suggests that the cells were damaged and the cell division was decreased. This phenomenon also emerged in other resistant cell line.[15] Under electron microscope, the changes of morphology of QGY/CDDP cells including abnormality of nucleus, emergence of false inclusion body, decreasing and shortening of microvillus showed metabolism and division of these resistant cells were weakened. The altered growth kinetics in QGY/CDDP (namely higher G_0/G_1 and lower S phase percentage, and a longer doubling time) indicates that QGY/CDDP had an increased proportion of non-proliferating cells and a decreased cell cycle progression rate. Those changes may be partly responsible for the reduced cytotoxicities of many anticancer agents to QGY/CDDP. These results of different growth rates of the resistant cells and their parental cells were also reported in some studies about resistant cells.[16,17] Moreover, we found that chromosome number of the QGY/CDDP cell line increased. This may be due to the cells containing more chromosome number were adapted to CDDP and generated survival advantage, or drug action prompted cells to produce adaptive change. A major mechanism of resistance to CDDP is a decreased effective concentration of drug in the cell. Reduction in CDDP concentration of 20-70% has been observed in cell lines resistant to CDDP.[18] In this study, a significantly decrease in total cellular platinum accumulation compared to the parent cell line QGY has been found in the resistant cell line QGY/CDDP ($p<0.01$). This result confirmed that reduced drug accumulation is one of the important mechanisms involved in CDDP resistance. However, the mechanism of reduced accumulation remains uncertain. It could result either from reduced uptake or active efflux, or both. Cisplatin is generally believed to enter the cells through passive diffusion and the uptake of CDDP is not saturable. The accumulation of CDDP is not inhibited significantly by excess amounts of analogues of CDDP, such as transplatin and carboplatin.[19] In addition, Gately and Howell[20] proposed a model of CDDP accumulation that accommodates most of the existing observations. In this model, 50% of the initial rate of uptake is due to passive diffusion and the remaining 50% is by facilitated diffusion through an unidentified gated channel. This suggests that CDDP transported into carcinoma cells must be effused by an active efflux pump and enhanced active efflux of CDDP have been observed in some CDDP-resistant cells now.[21,22] P-glycoprotein, the product of the MDR1 gene, is a 170-kDa ATP-binding cassette (ABC) membrane transporter. It is thought to be an energy-dependent multidrug efflux transporter that rapidly extrudes many types of hydrophobic chemotherapeutic agents from resistant cells and thus reduces the cellular accumulation of drug.[23] However, there is a dispute on the relationship of P-gp and resistant mechanisms of CDDP and numerous studies have shown that P-gp is not a major factor responsible for MDR in CDDP resistant mechanisms.[24,25] We examined the expression of this protein in cultured CDDP sensitive cell line and CDDP resistant cell line by flow cytometry, found no correlation between expression of P-gp and CDDP resistance. That is to say CDDP is not a substrate for P-gp. Whether other pumps such as multi-drug resistance associated protein (MRP) and lung resistance related protein (LRP) may play a role in CDDP resistance remains for further study.

Glutathione-S-transferase π is a member of a family of isozymes-GSTs (which are divided into 6 classes: α, μ, ω, π, θ, and ζ) that plays an important role in the detoxification of many xenobiotic substances through conjugation to GSH and elevated levels of GSTs have been associated with the development of resistance toward chemotherapy agents.[26] High levels of GST-π have been reported in a number of resistant cell lines induced by various chemotherapeutic agents that are substrates GSTs and can be directly inactivated through catalytic conjugation to GSH through thioether bond formation.[16,21] Many anticancer drugs that decompose to produce electrophilic species can be detoxified via GSH metabolism and reduced anticancer activity. In our study, we also found that the expression of GST-π in QGY/CDDP cell line was significantly higher than

in QGY cell line. These results suggest that GST-π is involved in resistance mechanisms of QGY/CDDP cell line.

In summary, CDDP resistance mechanisms are involved in many factors. We have now shown that the primary factors resulting in QGY/CDDP resistance to CDDP were the reduction of intracellular platinum accumulation and higher expression of GST-π without the involvement of P-gp. However, the exact mechanism of the reduced intracellular platinum accumulation is not clear. Further study whether other pumps such as MRP and LRP participate in the enhanced active efflux of CDDP. In addition, our study is limited in 2 aspects of the resistance mechanisms to CDDP and lack the entirety. Thus, additional investigation of other resistance mechanisms such as DNA repair enzymes and anti-apoptotic proteins is waited to carry out.

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