The combination of glycyrrhizin and lamivudine can reverse the cisplatin resistance in hepatocellular carcinoma cells through inhibition of multidrug resistance-associated proteins

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Abstract. Cisplatin is commonly used as a chemotherapeutic agent for hepatocellular carcinoma (HCC). However, it cannot satisfactorily improve the survival rate for patients with advanced HCC due to intrinsic or acquired drug resistance caused by multidrug resistance-associated proteins (MRPs). To clarify whether or not glycyrrhizin and lamivudine have modulator effects on HCC treated with cisplatin, we established a cisplatin-resistant Huh7 HCC cell line and analyzed the mRNA expression of MRPs in the resistant cells. The resistant cells showed 14.1-fold higher resistance to cisplatin, and they expressed higher levels of MRP2 (6.29-fold), MRP3 (3.2-fold), MRP4 (11.3-fold) and MRP5 (3.39-fold) mRNAs than the wild-type cells by using real-time PCR. However, MRP1, MDR1 and GST-π mRNA were not induced. Compared with the treatment of the resistant cells with cisplatin only, co-treatment with cisplatin and glycyrrhizin or lamivudine significantly decreased the cell viability to 76.8% and 79.5%, respectively. Co-treatment with cisplatin and both glycyrrhizin and lamivudine further decreased the cell viability to 65.1%. Intracellular concentration of cisplatin in the resistant cells decreased to 36.4% of that of the wild-type cells while it increased to 47.7% or 48.4% when glycyrrhizin or lamivudine were added separately, or 60% when they were added together. Our findings indicate the following: i) high expression of MRP2, MRP3, MRP4 and MRP5 decreases cisplatin accumulation in cisplatin-resistant HCC cells and contributes to cisplatin resistance; ii) glycyrrhizin and/or lamivudine accumulate cisplatin in resistant cells by inhibiting the cisplatin efflux from the cells; and iii) glycyrrhizin and lamivudine both act as modulators and have the effect of reversing cisplatin resistance, and co-treatment with glycyrrhizin and lamivudine enhances modulator activity in reversing the cisplatin resistance.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, especially in Asia and Africa (1), and infection of hepatitis B virus (HBV) is a high risk factor of HCC. The treatment of HCC remains unsatisfactory because chemotherapy, either as a single agent or by a multidrug regimen, does not prolong life in most cases (2) and is often impeded by a marked drug resistance in the patient. Patients with HCC were classified into two groups depending on response to chemotherapy: the first group had an intrinsic drug resistance and members were insensitive to drug treatment at the onset of therapy, and the second group acquired drug resistance after the first treatment with a chemotherapeutic agent. This multidrug resistance (MDR) can be caused by expression of plasma membrane transporters belonging to the MDR/P-glycoprotein family. These transporters mediate ATP-dependent efflux of chemotherapeutic drugs across the plasma membrane (3). As MDR1 mRNA is spontaneously expressed in HCC (4), inhibitors of MDR1 can theoretically modulate drug effects. However, inhibitors of MDR1, such as verapamil, have not improved the efficacy of chemotherapy on HCC (5).

Studies on cell lines derived from HCC indicated that the MDR phenotype is attributable not only to expression of the MDR1 gene but also to other mechanisms (6). Multidrug resistance-associated proteins (MRPs), which are distinct from MDR/P-glycoproteins, are integral membrane glycoproteins, some of which confer resistance to chemotherapeutic agents (7). The first cloned member of this family is MRP1 (ABCC1), which was demonstrated to confer resistance to a number of drugs, including doxorubicin, vincristine, and etoposide (8). Since the cloning of MRP1, eight new MRP proteins have been cloned (9). MRP1 is expressed in many different cell types, although not in significant amounts in human hepatocytes (10). In hepatocytes, additional MRP isoforms, MRP2 (ABCC2), MRP3 (ABCC3), MRP4 (ABCC4) and MRP5 (ABCC5) have been identified (7). MRP2, which carries organic anions into

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the bile, is known as the canalicular multispecific organic anion transporter (cMOAT) (11). MRP2 confers resistance to several chemotherapeutic agents including anthracycline, vinca alkaloids, etoposide, camptotesine, and methotrexate (12). MRP3 is also an organic anion transporter that can transport glucuronide conjugates, glutathione-S-conjugates, and monoanionic bile salts (13). MRP4 and MRP5 confer a unique drug resistance phenotype and transport representatives of a different class of amphipathic conjugates in that both transporters have been implicated in resistance to and transport of purine and nucleotide analogues. Unlike MRP1 and MRP2, MRP4 and MRP5 do not confer resistance against anthracyclines, vinca alkaloids or epipodophyllotoxins. MRP4 and MRP5, however, induce resistance to purine analogues and other nucleoside-based antiviral drugs (14,15).

Cisplatin is commonly used as a chemotherapeutic agent for HCC although it cannot satisfactorily improve the survival rate for the patients with advanced HCC due to development of drug resistance (16). In lung cancer, decreased accumulation of cisplatin was observed in cisplatin-resistant cell lines from non-small cell lung cancer cells, and the amount of intracellular cisplatin showed a correlation with the sensitivity to cisplatin (17). In cases of lung cancer, MRP2 has been reported to be involved in the development of cisplatin resistance. Human MRP2 mRNA is upregulated by 4- to 6-fold in cisplatin-resistant cell lines derived from various human tumors, which resulted in decreased drug accumulation (18). Furthermore, Koike *et al* transfected an expression vector containing MRP2 antisense cDNA into the human hepatoma cell line, and showed that the transfectants displayed an increased sensitivity to cisplatin (19). In addition to MRP2, MRP4 and MRP5 were overexpressed in cisplatin-resistant small lung cancer cells in both cell lines (20) and *in vivo* (21).

Glycyrrhizin, extracted from the roots of *Glycyrrhizae globa*, is widely administered to chronic liver disease patients in Japan because of its anti-inflammatory effects on the hepatocytes (22). Glycyrrhizin in its glucuronate form is a substrate of MRP2 (11). Lamivudine, which was first used to treat human immunodeficiency virus (HIV) infection and was approved for the treatment of HBV infection in 1999 (23), is a nucleotide analogue and substrate of MRP4 and MRP5 (14,15).

In the present study, to clarify whether glycyrrhizin and lamivudine have modulator effects on HCC treated with cisplatin or not, we established a cisplatin-resistant hepatocellular carcinoma cell line and analyzed mRNA expression of MRPs in the resistant cells.

Materials and methods

Cell lines and culture. The human hepatoma cell line, Huh7, was established from a well-differentiated hepatocellular carcinoma (24). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 5% fetal calf serum and 1% penicillin-streptomycin mixed solution (10000 U PCs and 10 mg SM/ml in 0.85% NaCl, Nacalai tesque Inc., Kyoto, Japan), and maintained at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. Approximately $1.0x10^6$ viable cells were seeded in a 100-mm dish, and the medium was replaced with 7 ml of fresh medium

after 24 h. After 72 h, the cells were sub-cultivated by 0.5 g/l trypsin/0.53 mmol/l EDTA mixed-solution (Nacalai tesque Inc.), and seeded to another 100-mm dish.

Establishment of the resistance of the cells to cisplatin. A low concentration (10 ng/ml) of cisplatin (Sigma-Aldrich, St. Louis, USA) was added to the medium. The cells that survived after 72 h of exposure to cisplatin were sub-cultivated and seeded in another plate. Cells were judged to have acquired resistance when they were able to maintain logarithmic growth upon addition of cisplatin to the culture solution. The culture solution was sub-cultivated and left unexposed to further cisplatin for approximately two weeks before verifying resistance. Further resistance was established by gradually raising the concentration of cisplatin in the culture solution (25). For the present study, cells were cultured to acquire a target resistance of 5 μg/ml cisplatin.

Cytotoxicity of cisplatin. Cytotoxicity of cisplatin was measured by Alamar blue™ assay (Trek diagnostic systems Ltd., West Sussex, UK), which is a modified method of MTT assay (26). All assays were performed in triplicate. Wild-type Huh7 and the cisplatin-resistant Huh7 cell line were seeded onto a 96-well plate at a cell density of $5x10^2$/well, and exposed to cisplatin after 24 h upon exchanging the medium. Alamar blue™ assay was performed after 24, 48, 72, 96, and 120 h to obtain the proliferation curves for both the wild-type cells and the resistant cells. Cytotoxicity of cisplatin was measured by IC_{50}, the concentration corresponding to 50% survival.

RNA isolation and cDNA synthesis. Total RNAs were extracted from each cell line (the wild-type cells and the resistant cells) 72 h after adding glycyrrhizin and lamivudine by an RNAqueous-4PCR kit (Ambion Inc., TX, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total RNA by reverse transcription with a SuperScript™ first-strand synthesis system kit (Invitrogen, CA, USA), according to the manufacturer's instructions.

Polymerase chain reaction (PCR). PCR was performed by a thermal cycler (i-Cycler; Bio-Rad Lab., Japan). The PCR primers and each annealing temperature were as follows: MDR1, a 255-bp product was generated using 5'-TCACCTT CGTCAGCTACTTCGG-3' (forward primer) and 5'-CAGGA GGTCACAGCCGACTTTAAAC-3' (reverse primer) at an annealing temperature of 60˚C; GST-π, a 250-bp product was generated using 5'-CTCACTCAAAGCCTCCTG-3' (forward primer) and 5'-CTGCCTTCACATAGTCATCC-3' (reverse primer) at an annealing temperature of 60˚C; MRP1, a 294-bp product was generated using 5'-CGGAAACCATCCACGACC CTAATC-3' (forward primer) and 5'-ACCTCCTCATTCGCA TCCACCTTG-3' (reverse primer) at an annealing temperature of 60˚C; MRP2, a 755-bp product was generated using 5'-AT GGCAGTGAAGAAGAAGACGATGAC-3' (forward primer) and 5'-GACGATGATGGTGAAGACAGGAGTGG-3' (reverse primer) at an annealing temperature of 65˚C; MRP3, a 752-bp product was generated using 5'-AACTATGCCCCCGATG AGGACCAA-3' (forward primer) and 5'-AGAGTGGGAGA TGGCGTTGAAGAAG-3' (reverse primer) at an annealing temperature of 65˚C; MRP4, a 696-bp product was generated

using 5'-CGGGCATACAAAGCAGAAGAGAGG-3' (forward primer) and 5'-CAGTTCCTCATCCGTGTGCTCCTT-3' (reverse primer) at an annealing temperature of 68˚C; and MRP5, a 401-bp product was generated using 5'-ATCAAGC AAGGAAGCGGGAACACC-3' (forward primer) and 5'-GC CACAAGGAACCACGGGAAGACT-3' (reverse primer) at an annealing temperature of 65˚C. Primers were designed based on the reported sequences (27,28). The ß-actin gene was used as the internal control. For a typical PCR run 1 μl of the synthesized cDNA in a total volume of 50 μl reaction mixture containing 1 unit rTaq DNA polymerase (Toyobo, Osaka, Japan), 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 500 nM each of forward and reverse primers, was denatured at 95˚C for 30 sec, annealed for 30 sec, and extended at 72˚C for 30 sec for 40 cycles. PCR products were verified by eletrophoretic analysis in 2% agarose gel.

Real-time PCR. Quantitative real-time PCR was performed with iQ™ SYBR-Green Supermix (Bio-Rad Lab., Japan) using a total volume of 50 μl reaction mixture containing 1 μl of the synthesized cDNA. Real-time PCR was carried out using the i-Cycler iQ detection system (Bio-Rad Lab., Japan). The cycling conditions were the same as for normal PCR. The melt curve program was performed immediately after completion of PCR by increasing the temperature by 0.5˚C from 55˚C to 90˚C.

Cytotoxicity of cisplatin co-administered with glycerrhizin and/or lamivudine as modulators. Lamivudine (2'3'-dideoxy-3'-thiacytidine, 3TC), was purchased from GlaxoSmithkline (Tokyo, Japan) and glycyrrhizin was kindly donated by Minophagen Pharm. (Tokyo, Japan). Each cell line was divided into five groups: i) medium only (no medication), ii) cisplatin only (addition of 5 μg/ml cisplatin), iii) cisplatin and glycyrrhizin (addition of 5 and 100 μg/ml, respectively), iv) cisplatin and lamivudine (addition of 5 and 1 μg/ml, respectively), and v) cisplatin, glycyrrhizin and lamivudine (addition of 5, 100, and 1 μg/ml respectively). The cell-lines were seeded onto a 96-well plate at a cell density of $5x10^2$/ well. After 24 h, the medium was exchanged with 200 μl of medicated medium. Following medium exchange, the cell-lines were cultured for 96 h before the number of cells was examined by Alamar blue assay, performed in triplicate. Cytotoxicity was measured as cell viability relative to viability in the cisplatin-only group.

Examination of the intracellular concentration of cisplatin. Seventy-two hours after adding the modulator agents, 5.0-$7.0x10^6$ cells were collected from each cell line. The concen-tration of each medicine was the same as that of above. The cells were washed twice by PBS(-), then dispersed in 50 μl PBS(-), and sonicated (Bioruptor, Cosmo Bio Co.Ltd, Tokyo Japan). The amount of cisplatin per cell in each group was examined by measuring the concentration of cisplatin in cell-crushed liquids. Measurement of cisplatin was performed with atomic absorption spectrophotometry which sets platinum to the marker (29).

Statistical analysis. Statistical significance was established using the paired two-tailed Student's t-test.
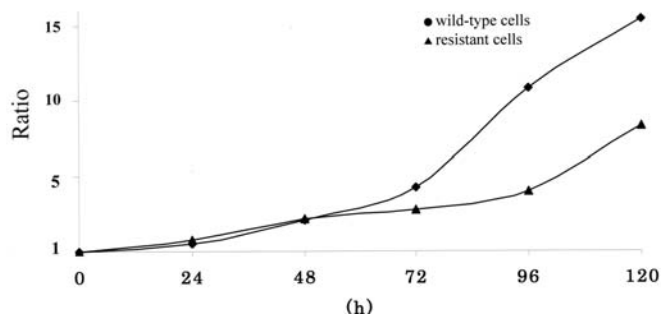

Figure 1. Proliferation curve of the wild-type cells and the resistant cells. The ratio with the number of cells at the start of cultivation is plotted.
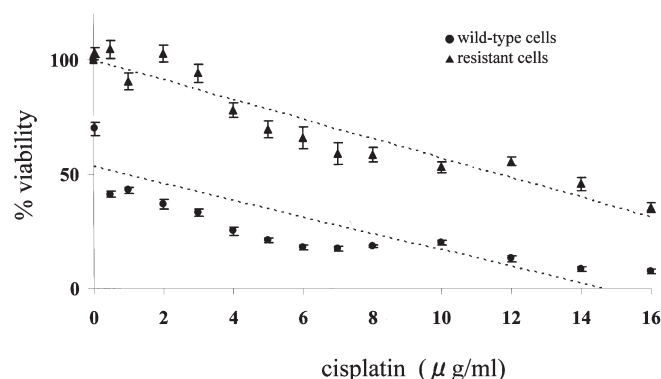

Figure 2. The cytotoxic effects of cisplatin on wild-type cells and resistant cells. The IC_{50} of each cell line was calculated by drawing the approximation straight line (dotted line) which was created using the method of least-squares. Each point represents the mean ± SE (n=3).

Results

Establishment of the cisplatin-resistant cell line. Logarithmic growth of the wild-type cells and the resistant cells was measured for 120 h following inoculation. Proliferation potency between the wild-type cells and the resistant cells was comparable after approximately 48 h of growth, after which point the wild-type cells were superior in their proliferation potential, as demonstrated by cell concentrations double that of the resistant cells (Fig. 1). After 72 h, the IC_{50} value of cisplatin for the wild-type cells was 0.82 μg/ml and 11.6 μg/ml for the resistant cells (Fig. 2). The resistant cells showed a 14.1-fold increased resistance to cisplatin. Compared with cisplatin-free control groups, the % viability of the wild-type cells treated with 5 μg/ml of cisplatin decreased in a time-dependent manner to 52.6±3.79, 39.6±3.24, 29.0±2.97, and 24.0±3.57% at 48, 72, 96 and 120 h, respectively. On the other hand, the % viability remained unchanged for the resistant cells over the course of the culture (Fig. 3).

PCR of the MRP family in wild-type and resistant cells. RT-PCR products for MDR1, GST-π, MRP1, MRP2, MRP3, MRP4 and MRP5 are shown for both the wild-type and resistant cells in Fig. 4. Both cell lines showed similar expr-ession of the MDR1, GST-π, MRP1, MRP2, MRP3, MRP4 and MRP5 genes. Real-time PCR results confirmed that the mRNA expression of MDR1, GST-π and MRP1 remained
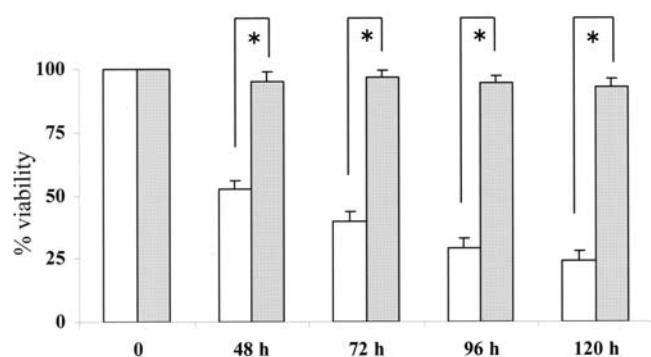
Figure 3. Suppression of the proliferation of wild-type and resistant cells by cisplatin. Each bar represents the mean ± SE (n=8). Empty bars, wild-type cells x cisplatin; filled bars, resistant cells x cisplatin. *P<0.01.

Figure 5. Quantitative real-time PCR analysis of the mRNA expression of the MRP family in wild-type and resistant cells. The ratio of mRNA of the MRP family between the wild-type and resistant cells was examined by using real-time PCR. Each result represents the average value of 3 to 9 trials. Empty bars, wild-type cells; filled bars, resistant cells.
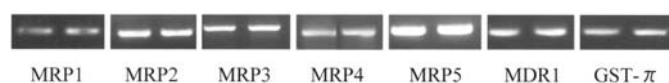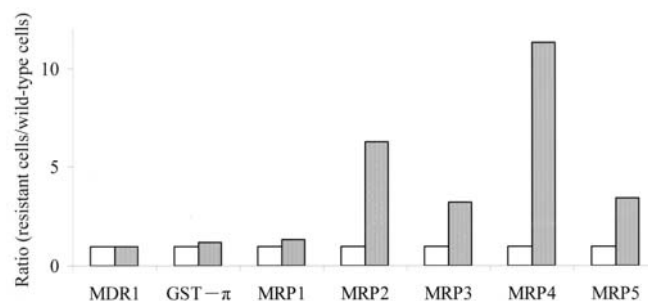

Figure 4. Electrophoretic analysis by reverse transcription-polymerase chain reaction of MRP transcripts expressed in wild-type and resistant cells. Left lane, wild-type cells; right lane, resistant cells.
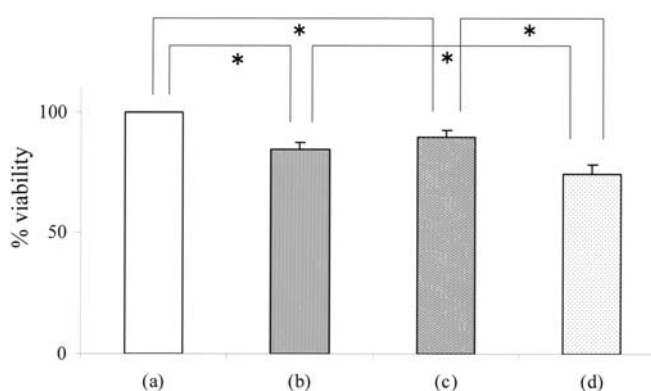

Figure 6. Modulatory effects of glycyrrhizin and lamivudine on the cisplatin-induced cytotoxicity to the resistant cells. (a) Addition of cisplatin only. (b) Addition of cisplatin and glycyrrhizin. (c) Addition of cisplatin and lamivudine. (d) Addition of cisplatin, glycyrrhizin and lamivudine. Each bar represents the mean ± SE (n=8). *P<0.01.

unchanged between the wild-type cells and resistant cells (Fig. 5). However, the mRNA expression of MRP2, MRP3, MRP4 and MRP5 was significantly different between the two cell lines (P<0.01). The resistant cells showed up-regulation of MRP2 (6.29-fold), MRP3 (3.2-fold), MRP4 (11.3-fold) and MRP5 (3.39-fold), as compared to the wild-type cells.

Modulatory effects of glycyrrhizin and lamivudine on the cisplatin-induced cytotoxicity to the resistant cells. We first confirmed the absence of cytotoxic effects from glycyrrhizin or lamivudine treatment alone, on both the wild-type cells and the resistant cells. Compared with the drug-free group, the % viability of cells in the glycyrrhizin (100 μg/ml) addition group and the lamivudine (1 μg/ml) addition group was 97% and 99% at 96 h, respectively (data not shown).

Next, we investigated the cytotoxic effects of combined cisplatin and glycyrrhizin or lamivudine treatment on the resistant cells, expressed as a relative ratio (%) of viability for resistant cells treated with cisplatin alone. Co-treatment with cisplatin and glycyrrhizin decreased the relative viability to 76.8±3.39% at 96 h (Fig.6). Similarly, the % viability for co-treatment with cisplatin and lamivudine diminished to 79.5±3.17% (P<0.01). Moreover, co-treatment with cisplatin, glycyrrhizin and lamivudine led to a marked decrease in relative viability to 65.1±3.46% (P<0.01). These results indicate that glycyrrhizin and lamivudine co-operatively decrease the viability, as compared to treatment with glycyrrhizin or lamivudine alone.

Measurement of the intracellular cisplatin concentration of resistant cells. The intracellular cisplatin concentration of the resistant cells was significantly decreased to 36.4±4.97% of that of the wild-type cells (Fig. 7). However, intracellular cisplatin concentrations of the resistant cells increased to 47.7±0.36% by addition of glycyrrhizin and to 48.4±0.78%
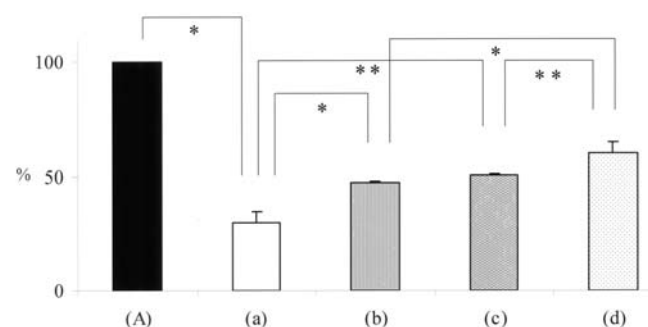

Figure 7. Comparison of the intracellular cisplatin concentration of the resistant cells. (A) Intracellular cisplatin concentration when adding cisplatin to the wild-type cells was 100%. (a) Addition of cisplatin only. (b) Addition of cisplatin and glycyrrhizin. (c) Addition of cisplatin and lamivudine. (d) Addition of cisplatin, glycyrrhizin and lamivudine. Each bar represents the mean ± SD (n=3). *P<0.01, **P<0.05.

by addition of lamivudine. Furthermore, the concentration increased to 62.0±4.8% by co-administering glycyrrhizin and lamivudine. These findings suggest that glycyrrhizin and lamivudine co-operatively increase intracellular cisplatin concentration in the resistant cells treated with cisplatin as the modulator.

Discussion

We have established a cisplatin-resistant Huh7 hepatocellular carcinoma cell line. The resistant cells showed a 14.1-fold increased resistance to cisplatin, as compared to wild-type cells (Fig. 2). The resistant cells also expressed higher levels of MRP2, MRP3, MRP4 and MRP5 mRNAs with 6.29-, 3.2-, 11.3- and 3.39-fold increases as measured by real-time PCR (Fig. 5). However, MRP1, MDR1 and GST-π mRNAs were not induced.

Although the precise mechanism remains unknown, it appears that cisplatin resistance is multifactorial, as characterized by decreased cisplatin accumulation across the plasma membrane, increased intracellular detoxification, and increased DNA repair ability from DNA damage (30-32).

Expression of the MDR1 gene has been associated with resistance to adriamycin and vincristine, but not to cisplatin (33,34). However, expression of the MDR1 gene was shown to be upregulated by cisplatin in myelogenous leukemia cells (35) and HCC cell lines including Huh7 cells (28). In HCC, MRP1 is unlikely to be involved in the MDR phenotype, because MRP1 mRNA expression is very low in the liver (10). In the present study, MDR1 and MRP1 mRNAs were not significantly upregulated in cisplatin-resistant Huh7 cells (Fig. 5), suggesting that MDR1 and MRP1 are not involved in the resistance to cisplatin in the resistant cell line.

The mechanism of cellular detoxification of many harmful xenobiotics involves intracellular conjugation of the xenobiotic-reactive center with glutathione, glucuronide, or sulfate (36). These conjugations generally render the xeno-biotics less chemically reactive and, hence, less toxic to the cell. Glutathione conjugation reactions, catalyzed by the many isozymes of glutathione S-transferase (GST), are particularly important for detoxification of electrophilic xenobiotics, which includes many carcinogens and cytotoxic drugs (37). The GST-π gene, which encodes an isozyme of GST, conjugates glutathione to toxic compounds for detoxification (38). Cisplatin is also detoxicated by GST, however GST-π mRNA levels were not induced in the cisplatin-resistant Huh7 cells in the present study (Fig 5). These findings suggest that glutathione conjugation reaction is a minor determinant of the cisplatin resistance in the resistant Huh7 cells.

MRP2, which localizes to hepatocyte canalicular membranes, is an organic anion transporter (11). MRP2 is a major transporter of bilirubin, glucuronides, and other organic anions, from the liver into the biliary tract. It is also able to transport anticancer agents, including cisplatin. Human MRP2 mRNA expression has been shown to be 4- to 6-fold higher in cisplatin-resistant cell lines derived from various human tumors exhibiting decreased drug accumulation (18). Down-regulation of MRP2 mRNA via MRP2 antisense cDNA enhances their sensitivity to cisplatin (19). In the present study, MRP2 mRNA was induced 6.29-fold in the resistant cells when compared to wild-type cells. Our data also suggests that increased expression of MRP2 correlates with cisplatin resistance in the cisplatin-resistant Huh7 cells, in agreement with previous reports.

MRP3 is also an organic anion transporter capable of transporting anticancer agents and is localized to the hepatocyte basolateral membranes. It has been reported that cisplatin slightly increases MRP3 mRNA expression in HepG2 cells (~1.5-fold) and antisense RNA of MRP3 increases cellular sensitivity to cisplatin (39). However, overexpression of MRP3 cDNA results in resistance to etoposide and teniposide, but not to other drugs affected by multidrug resistance (40,41). Several studies have also tried to link MRP3 expression in cancer cell lines and patient-derived tumor samples to resistance against anticancer agents. The results in cancer cell lines were either negative (10,42) or the resistance spectrum found did not fit the known drug resistance spectrum associated with MRP3 (43). Based on the low levels of resistance found in MRP3 cDNA transfectant cells and the narrow spectrum of anticancer agents to which MRP3 mediates resistance, it is speculated that MRP3 plays a limited role in clinically relevant drug resistance. In the present study, we showed that MRP3 mRNA expression was increased in the resistant cells by 3.2-fold. Our data suggests that elevated MRP3 levels correlate with cisplatin resistance in the cisplatin-resistant Huh7 cells.

Within the MRP subfamily, MRP4 and MRP5 are unique. All lack the third N-terminal transmembrane domains (TMDs) which are known as TMD0 but retain the hydrophilic linker, known as the L0 linker (9). MRP4 and MRP5 share <40% similarity with the other MRP proteins. Both proteins also share <40% similarity with each other. Nevertheless, MRP4 and MRP5 are much more similar to the other MRPs than to other members of the MRP family. MRP4 is localized to the basolateral membrane in human, rat, and mouse hepatocytes (44) and is widely expressed, with mRNA levels ranging from very high in the prostate to barely detectable in the liver (45). MRP4 is also present in many human cancer cell lines (10). MRP4 was induced in a cisplatin-resistant small lung cancer cell line. This induction has been associated with resistance to nucleoside analogues, such as azidothymidine (AZT), 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) (14). The resistance is due to decreased intracellular drug concentration as a result of decreased drug efflux. Norris et al (46) have suggested a correlation between increased MRP4 levels and tumor prognosis in patients with primary neuroblastoma. The authors attribute this correlation to the ability of MRP4 to transport irinotecan and its active metabolite SN38. We showed that MRP4 mRNA was expressed at a higher level in the resistant cells (11.3-fold). These findings suggest that MRP4 expression correlates with cisplatin resistance in Huh7 cells.

MRP5 is ubiquitously expressed in human systemic organs, with extremely high levels observed in skeletal muscle, brain, and heart (10). MRP5 is thought to be localized in the basolateral membrane of most tissues, and functions to efflux conjugated compounds from the cells (47). When MRP5 is transfected into drug-sensitive cells, it confers resistance to antifolate drugs such as methotrexate and to nucleoside-based drugs such as 6-MT, 6-TG, PMEA, AZT, cytosine arabinoside, 5-fluorouracil, and gemcitabine (15,48). MRP5-expressed inside-out membrane vesicles demonstrated the direct transport of the monophosphate metabolites of nucleoside-based drugs, such as 5-fluorouracil and 6-thioguanine (48,49). In vivo, up-regulation of the MRP5 gene has been observed as a result of exposure to cisplatin in lung cancers (21). Pratt et al (48) found a three-fold increase in resistance to cisplatin and oxaliplatin in their MRP5 transfectants, as well as a two-fold increase in resistance to doxorubicin. However, no cisplatin or doxorubicin

resistance was observed by McAleer *et al* (49) and Wijnholds *et al* (15). Wijnholds *et al* conversely found some resistance to etoposide/teniposide that was not identified elsewhere (15). It is unlikely that MRP5 will obviously contribute to MDR or platin drug resistance in the clinic. In the present study, we showed that MRP5 mRNA expression increased 3.39-fold in the resistant cells, suggesting a correlation with cisplatin resistance in Huh7 cells.

Several investigators have reported that decreased accumulation of cisplatin was observed in cisplatin-resistant cell lines (20). Decreased accumulation of cisplatin was observed in cisplatin-resistant cell lines from non-small cell lung cancer (NSCLC), and a good correlation was found between the amount of intracellular platinum and the sensitivity to cisplatin in lung cancer cell lines. This evidence suggests that intracellular accumulation is a major determinant of cisplatin resistance in NSCLC (50). It was demonstrated that MRP2 and MRP3 mRNA expression was at least 10-fold higher than MRP1 mRNA expression in HCC cells. MRP2 and MRP3 were localized to the plasma membrane of these carcinoma cells, whereas MRP1 was expressed only on the intracellular membranes of some HCCs. Both MRP2 and MRP3 may thus contribute to the chemoresistance (51). Other groups reported that MRP2 acts as an important transporter in chemoresistance, with mRNA and protein expression levels generally maintained or even increased in human HCC (52). However, the expression levels of MRP4 and MRP5 in HCC have remained unclear. In the present study, we first showed that the cisplatin-resistant HCC cells expressed higher levels of MRP2, MRP3, MRP4 and MRP5 mRNA than the wild-type cells by using real-time PCR (Fig. 5). Also, the intracellular cisplatin concentration of the resistant cells was significantly decreased, compared with the wild-type cells (Fig.7). Our results suggest that the high expression of MRP2, MRP3, MRP4 and MRP5 decreases the cisplatin accumulation in the resistant cells and contributes to the cisplatin resistance.

Next, the modulatory effects of glycyrrhizin and lamivudine on the cisplatin-induced cytotoxicity were examined in the resistant cells (Fig. 6). Compared with the cisplatin treatment alone, cell viability following co-treatment with cisplatin and glycyrrhizin decreased to 76.8%. Similarly, co-treatment with cisplatin and lamivudine reduced cell viability to 79.5%. Furthermore, co-treatment with cisplatin, glycyrrhizin and lamivudine significantly lowered the viability of the resistant cells to 65.1%. These findings suggest that glycyrrhizin and lamivudine significantly reverse the effect of cisplatin resistance by acting as modulators, and that co-treatment with glycyrrhizin and lamivudine results in more potent modulator activity.

We also showed that the intracellular cisplatin concentration of the resistant cells was significantly decreased to 36.4% with cisplatin alone and increased to 47.7% by addition of glycyrrhizin compared to wild-type cells (Fig. 7). Although glycyrrhizin does not have antiviral properties, it primarily acts as an anti-inflammatory or cytoprotective drug. It improves mortality and liver function in patients with sub-acute hepatic failure, chronic hepatitis, and cirrhosis with activity. Glycyrrhizin may prevent the development of hepatocellular carcinoma in patients with chronic hepatitis C (53).

Glycyrrhizin is a glucuronate form, and is also a substrate of MRP2 and MRP3 (54). Our results suggest that glycyrrhizin could increase the intracellular concentration of cisplatin in the resistant cells. We also demonstrated that the intracellular concentration of cisplatin in the resistant cells was increased to 48.4% of that of the wild-type cells by addition of lamivudine (Fig. 7). Lamivudine, a nucleoside analogue which directly inhibits HBV DNA polymerase, was first developed as a reverse transcriptase inhibitor for use in HIV infection (55). The efficacy of lamivudine as an anti-HBV agent has been confirmed using randomized controlled trials in a wide variety of clinical situations. It has been shown to reduce HBV DNA levels and serum transaminases, and improve histological indices in patients with both HBeAg-positive and -negative diseases (56). Lamivudine could increase the concentration of cisplatin in the resistant cells because it is a nucleoside analog and the substrate of MRP4 and MRP5, which are nucleoside analog pumps. Moreover, co-treatment with glycyrrhizin and lamivudine increased the intracellular concentration of cisplatin in the resistant cells to 62.0% of that of the wild-type cells (Fig. 7). These findings suggest that glycyrrhizin is a competitive substrate for MRP2 and MRP3 and lamivudine is a competitive substrate for MRP4 and MRP5. Therefore, glycyrrhizin and lamivudine act as modulators to reverse the effects of cisplatin resistance by inhibiting cisplatin efflux from the resistant HCC cells.

Pharmacological reversal of the MRP family by some compounds has been demonstrated in cell or tissue culture, however there is minimal evidence of their therapeutic effectiveness in clinical trials or against solid tumors in animals. To date, there are no potent modulators of cisplatin resistance available in the clinic (9,11). One main reason for this is that the concentration of modulators in serum required for reversal cannot be achieved. Transcatheter arterial chemoembolization (TACE) improves the survival of patients with unresectable HCC and has become the standard treatment (57). TACE can deliver high concentrations of cancer chemotherapeutic agents directly to the HCC via the hepatic arterial route. Glycyrrhizin and lamivudine are well known and widely used as therapeutic agents for chronic liver diseases. TACE with cisplatin, co-administered with glycyrrhizin and lamivudine via the hepatic artery, can be performed safely and may improve the survival of patients with advanced HCC, though further studies are necessary.

In conclusion, our findings indicate that elevated expression of MRP2, MRP3, MRP4 and MRP5 decreases the cisplatin accumulation in the cisplatin-resistant HCC cells and contributes to the resistance, and the combination of glycyrrhizin and lamivudine inhibit the cisplatin efflux from the HCC cells, acting as modulators to reverse cisplatin resistance.

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