Subcellular daunorubicin distribution and its relation to multidrug resistance phenotype in drug-resistant cell line SMMC-7721/R

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AIM: To investigate the correlation between subcellular daunorubicin distribution and the multidrug resistance phenotype in drug-resistant cell line SMMC-7721/R.

METHODS: The multidrug resistant cell line SMMC-7721/R, a human hepatocellular carcinoma cell line, was established. Antisense oligonucleotides (AS-ODN) were used to obtain different multidrug resistance phenotypes by inhibiting the expression of mdr1 gene and/or multidrug resistance-related protein gene(mrp) using Lipofectamine as delivery agent. Expression of mdr1 and mrp genes was evaluated by RT-PCR and Western blotting. Intracellular daunorubicin (DNR) concentration was measured by flow cytometry. Subcellular DNR distribution was analyzed by confocal laser scanning microscopy. Adriamycin (ADM) and DNR sensitivity was examined by MTT method.

RESULTS: Low level expression of mdr1 and mrp mRNAs and no expression of P-Glycoprotein(P-gp) and multidrug resistance-related protein (P190) were detected in parental sensitive cell SMMC-7721/S, but over-expression of these two genes was observed in drug-resistant cell SMMC-7721/R. The expression of mdr1 and mrp genes in SMMC-7721/R cells was down-regulated to the level in the SMMC-7721/S cells by AS-ODN. Intracellular DNR concentration in SMMC-7721/S cells was 10 times higher than that in SMMC-7721/R cells. In SMMC7721/S cells intracellular DNR distributed evenly in the nucleus and cytoplasm, while in SMMC-7721/R cells DNR distributed in a punctate pattern in the cytoplasm and was reduced in the nucleus. DNR concentration in SMMC-7721/R cells co-transfected with AS-ODNs targeting to mdr1 and mrp mRNAs recovered to 25 percent of that in SMMC7721/Scells. Intracellular DNR distribution pattern in drug-resistant cells treated by AS-ODN was similar to drug-sensitive cell, and the cells resistance index (RI) to DNR and ADM decreased at most from 88.0 and 116.0 to 4.0 and 2.3, respectively. Co-Transfection of two AS-ODNs showed a stronger synergistic effect than separate transfection.

CONCLUSIONS: P-gp and P190 are two members mediating MDR in cell line SMMC7721/R. Intracellular drug concentration increase and subcellular distribution change are two important factors in multidrug resistance (MDR) formation. The second factor, drugs transport by P-gp and P190 from cell nucleus to organelle in cytoplasm, may play a more important role.

INTRODUCTION
Multidrug resistance (MDR) remains a significant obstacle for cancer chemotherapy. The MDR observed in many cell lines is most commonly accompanied with overexpression of one or both of the members of the ATP-binding cassette superfamily of transport proteins, P-glycoprotein (P-gp) and multidrug resistance-related protein(Mrp, P190)[1-7]. P-gp or P190 acts as an energy-dependent outward transport pump, removing drugs from the cytoplasm and from the plasma membrane, thereby decreasing intracellular drug accumulation[8-12].

Human hepatocellular carcinoma drug-resistant cell line SMMC7721/R showed a strong multidrug resistance to DNR and other anthracycline, and overexpression of P-gp and P190 was observed in this cell line. Previous studies suggested that subcellular drug distribution contributing to cells drug resistance may be mostly mediated by P-gp and/or P190 in many other cell lines[13-20]. But there is no direct evidence suggesting the role of these two pump proteins in MDR of SMMC7721/R. In order to understand MDR phenotype and mechanism in SMMC7721/R, based on previous studies of antisense technology related to mdr1 gene and mrp gene, we used laser scanning confocal microscopy to evaluate the intracellular distribution of DNR and then explored the correlation of intracellular drug(DNR) transportation and distribution with multidrug resistance phenotype.

MATERIALS AND METHODS
Cell lines and culture conditions
Human hepatocellular carcinoma cell line SMMC-7721 was provided by Cancer Research Institution, West China Hospital of Sichuan University. Drug-resistance cell line was established by the stepwise selection with increasing concentration of ADM as previously described[21]. The ADM gradually increased from 0.005 µg/ml to 0.1 µg/ml.
Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum in a 5% CO₂ atmosphere at 37°C.

**Materials**
Phosphorothioate antisense oligonucleotides (AS-ODN): targeting to mdr1 start codon region (AS-ODN/mdr1): 5’-CCA TCC CGA CTT GGC CCG CG-3’ [22], targeting to mdr1 coding region (AS-ODN/mrdr1): 5’-TGC TGT TCG TGC CCC CGG CG-3’ [22]. Control oligonucleotide (AS-ODN/nononsense) was a 20-mer nonsense phosphorothioate oligonucleotide. All oligonucleotides were synthesized by Life Technologies Inc., USA. Lipofectamine, TRIzol, RT-PCR kit, and primers were also purchased from Life Technologies Inc. The Western Blotting Kit was purchased from Boehringer Mannheim, Germany. Antibodies against mdr1/P-gp and mrp/P-gp were from Santa Cruz, USA. DNR was purchased from Daunorubicin distribution and multidrug resistance

**Treatment of cells with AS-ODNs**
The experimental protocols were similar to those previously described [22,24]. Briefly, cells (5 × 10⁵) were seeded in a 25 ml flask at 1 × 10⁵ cells/ml and grown to 75% confluence. Cells were transfected with 1.5 nmol of AS-ODN with 50 µl of Lipofectamine. Cells were harvested at different times after transfection for analysis.

**Detection of drug sensitivity of cells by MTT** [25,26]
Cells were exposed to drug at 37°C for 2 h, then were washed and seeded (50 000 cells/ml) in 96-well microplates for 72 h. MTT (20 µl, 2.5 mg/ml) was added to each well for 3 h. Medium was discarded and 150 µl of DMSO were added to each well. Optical densities were measured at 490 nm (A490). The tumor cells living ratio (TCL) was determined according to the formula: TCL= A490_experiment/A490_control × 100%. The 50% inhibitory concentration (IC₅₀) was calculated according to concentration-TCL curve. Resistance index (RI) was calculated using the formula: RI=IC₅₀drug resistant cells/IC₅₀parent cells.

**Measurement of mdr1 and mrp mRNAs by RT-PCR**
Primers Mrp: 5’-TGA AGG ACT TCG TGT CAG CC-3’, 5’-GTC CATGAT GGT GTT GAG CC-3’; mdr1: 5’-GGG TCC GAT ACA TGG TTT TCC-3’, 3’-TTC AGT GCG ATC TTC CCA GC-5’, 5’-microglobulin/βM: 5’-ACC CCC ACT GAA βM RNA transcripts were used as control for the amount of mRNA used. Total RNA from cells was extracted using TRIzol. The effect of AS-ODN was studied after 72 h pre-incubation with AS-ODN and Lipofectamine. Mrp, mdr1, and βM expression was used as control for the amount of RNA transcripts. An aliquot of each reaction mixture was then analyzed by electrophoresis on 2% agarose gel. Densitometry was performed using UVP gel image analysis system (BIO-RAD, USA). Results were expressed as the ratio of fluorescence intensity values between each experimental sample and control sample of SMMC7721/R cells without treatment.

**Results**

| Cells          | ICD (mg/L) | RI        |
|----------------|------------|-----------|
| DNR            | ADM        | DNR       | ADM       |
| SMMC7721/S     | 0.003±0.0006 | 0.004±0.0008 | 1.0       |
| SMMC7721/R     | 0.264±0.0094 | 0.463±0.0254 | 88.0       | 116.0      |
| AS-ODN/mdr1    | 0.094±0.0065 | 0.079±0.0034 | 31.4       | 19.8       |
| AS-ODN/mrp     | 0.072±0.0002 | 0.097±0.0009 | 24.0       | 24.3       |
| SMMC7721/R+    | 0.012±0.0001 | 0.099±0.0007 | 4.00       | 2.3        |
| AS-ODN/mdr1+mrp| 0.245±0.0110 | 0.451±0.0187 | 81.7       | 112.8      |
| AS-ODN/nononsense | 0.245±0.0110 | 0.451±0.0187 | 81.7       | 112.8      |

Values (IC₅₀) represent the mean ± standard deviation of at least three experiments and value (RI) represent the mean of three experiments. All values were calculated as described in MATERIALS AND METHODS. The concentration of each AS-ODN is 0.5 µmol/L and the treatment time is 72 hours.

**Observation of intracellular DNR distribution by confocal laser scanning microscopy (CLSM)**

The experimental procedures were similar to those previously described [22-24]. Cells (1 × 10⁵) were seeded to 960 mm² petri dish with a slide inside and incubated in a 5% CO₂ atmosphere at 37°C. After cells had reached 75% confluence, normal medium was replaced with serum-free RPMI 1640 medium and cells were incubated with DNR at 2 µg/ml for 1 h. The effect of AS-ODN was studied after 72 h pre-incubation with AS-ODN and Lipofectamine. After two washes with PBS and addition of drug free medium cells grown on slides were examined with CLSM (MRC-1024ES, BIO-RAD Inc., USA). Observation of intracellular drug distribution was observed using the 488 nm laser line for excitation and the filter that allows measurement of emitted light above 515 nm.

**Detection of intracellular DNR concentration by flow cytometry (FCM)**

Cells (1 × 10⁵) were seeded into 25 ml flask and grown to 95% confluence. They were then dissociated with pancreatin and suspended in serum-free medium. DNR was added to a final concentration of 2 µg/ml and incubated for 1 h at 37°C. After two washes with PBS each sample was divided into 3 tubes to be analyzed by FCM [22] (Elite ESP, Coulter Inc., USA). Results were expressed as the ratio of fluorescence intensity values between each experimental sample and control sample of SMMC7721/R cells without treatment.

**Table 1**

| Cells          | ICD (mg/L) | RI        |
|----------------|------------|-----------|
| DNR            | ADM        | DNR       | ADM       |
| SMMC7721/S     | 0.003±0.0006 | 0.004±0.0008 | 1.0       |
| SMMC7721/R     | 0.264±0.0094 | 0.463±0.0254 | 88.0       | 116.0      |
| AS-ODN/mdr1    | 0.094±0.0065 | 0.079±0.0034 | 31.4       | 19.8       |
| AS-ODN/mrp     | 0.072±0.0002 | 0.097±0.0009 | 24.0       | 24.3       |
| SMMC7721/R+    | 0.012±0.0001 | 0.099±0.0007 | 4.00       | 2.3        |
| AS-ODN/mdr1+mrp| 0.245±0.0110 | 0.451±0.0187 | 81.7       | 112.8      |
| AS-ODN/nononsense | 0.245±0.0110 | 0.451±0.0187 | 81.7       | 112.8      |
Expression of mdr1 and mrp gene

As shown by RT-PCR, the amplification products of mrp, mdr1, and β2M were 256bp, 168bp, and 120 bp, respectively. Overexpression of mrp and mdr1 mRNAs were detected in SMMC7721/R, but low level mRNA expression in SMMC7721/S was observed. The mRNA expression in SMMC7721/R cells treated with AS-ODN decreased to the level of SMMC7721/S (Figure 1).

No P-gp and P190 were detected in parental cells SMMC7721/S. Over-expression of P-gp and P190 was observed in drug resistant cells SMMC7721/R. Treatment of SMMC7721/R with AS-ODNs inhibited the expression of P-gp and P190 (Figure 2).

**Figure 1** Quantification of PCR. The ratio between the mdr1 or mrp and β2M gene is expressed as described in MATERIALS AND METHODS. (a. parental cell-SMMC7721/S; b. SMMC7721/R; c. SMMC7721/R incubated with 0.5 µmol/L AS-ODN for 24 hours)

**Figure 2** Expression of P-gp and P190 analyzed with Western blot. (a) immunoblotted with anit-P-gp antibody; (b) immunoblotted with anti-P190 antibody. 1. parental cell-SMMC7721/S; 2. SMMC7721/R treated by AS-ODN(0.5 µmol/L, 72 hours); 3. SMMC7721/R.

**Figure 3** Intracellular DNR concentrations in cells treated with antisense oligonucleotids. The concentration of each AS-ODN is 0.5 mmol/L and the treatment time is 72 hours. Data are the mean±standard deviation of three independent experiments. (1. SMMC7721/R; 2. SMMC7721/S; 3. SMMC7721/R treated with AS-ODN/ mdr1+mrp; 4. SMMC7721/R treated with AS-ODN/ mdr1; 5. SMMC7721/R treated by AS-ODN/ mrp; 6. SMMC7721/R treated with AS-ODN/ nonsense.)

**Figure 4** Intracellular DNR distribution in parental sensitive cells SMMC7721/S.

**Figure 5** Intracellular DNR distribution in drug-resistant cells SMMC7721/R.
Intracellular drug concentration results in increase of drug resistance in cells. Intracellular DNR concentration in SMMC7721/R decreased to 10 percent of that in SMMC7721/S and meanwhile resistance to ADM and DNR increased by 116 times and 88 times respectively than that of SMMC7721/S. Co-transfection of AS-ODN/mdr1 and AS-ODN/mrp only restored intracellular DNR concentration to 40 percent of that of SMMC7721/S. Inconsistently, the drug resistance to DNR was reduced from 88-fold to 4-fold and to ADM from 116-fold to 2.3-fold when compared with SMMC7721/S. These data indicate that some other factors must play a more important role in MDR mechanism besides the reduction of intracellular drug accumulation. The mechanism focused in recently is modified drug localization\(^{33-34}\). We used CSLM technology to explore this interesting point.

In our studies, we observed that in cell line SMMC7721/S DNR fluorescence distributed evenly in the nucleus and cytoplasm, while in cell line SMMC7721/R DNR distributed in a punctate pattern in the cytoplasm and was reduced in the nucleus. Transfection of AS-ODN changed the subcellular DNR distribution pattern in SMMC7721/S cells to that in SMMC7721/R cells. This observation indicates that P-gp or P\(^{\text{mrp}}\) not only pumps DNR out of cells, but also transports DNR from nuclear to cytoplasm and into some organelles such as Golgi apparatus. As P-gp or P\(^{\text{mrp}}\) probably locates in cell membrane, nuclear membrane, Golgi apparatus, or endoplasmic reticulum\(^{13,54-59}\), the actions of these two proteins may cause reduction of intracellular and intranuclear drug concentration and drug accumulation in some organelles. The combined effect may prevent the targeting of the drugs to nucleus, which reduces cell death even if the total amount of drugs inside the cells was not dropped significantly. Golgi apparatus is widely believed to be the organelle which holds the drugs\(^{54,59}\). Previous studies found that there were some differences in subcellular drug distribution pattern between different cell lines in which MDR phenotype was mediated by P-gp and by P\(^{\text{mrp}}\)\(^{49}\). However, we did not observe such phenomenon in the present study.

Our studies suggest that after the potent inhibition of P-gp or P\(^{\text{mrp}}\) expression by AS-ODN intracellular drug concentration was increased and subcellular drug distribution changed, which leads to the reversal of multidrug resistance in cell line SMMC7721/R. Therefore, we believe that over-expression of P-gp and/or P\(^{\text{mrp}}\) is an important mechanism in mediating MDR in cell line SMMC7721/R.

Although co-transfection of AS-ODNs targeting the two genes didn’t enhance the inhibitory effect on expression of P-gp or P\(^{\text{mrp}}\) when compared with separate transfection of either AS-ODN, the synergistic effects of the two AS-ODNs on reduction of intracellular or intranuclear drugs and on recovery of cell drug sensitivity were much more prominent than separate transfection. This finding indicates that MDR in SMMC7721/R is mediated at least by both P-gp and P\(^{\text{mrp}}\). The combination of MDR reversal methods against these proteins is effective in drug-resistant cells.

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