Screening of deoxyribozyme with high reversal efficiency against multidrug resistance in breast carcinoma cells

Peng Gao a, *, Jun-Min Wei b, Peng-Yu Li b, Cui-Juan Zhang a, Wen-Cheng Jian b, Yu-Hua Zhang a, Ai-Yan Xing a, Geng-Yin Zhou a, *

a Department of Pathology, Shandong University, School of Medicine, Jinan, China
b Qilu Hospital, Shandong University, Jinan, China

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Abstract

Specific inhibition of P-glycoprotein (Pgp) expression, which is encoded by multidrug resistance gene-1 (MDR1), is considered a well-respected strategy to overcome multidrug resistance (MDR). Deoxyribozymes (DRz) are catalytic nucleic acids that could cleave a target RNA in sequence-specific manner. However, it is difficult to select an effective target site for DRz in living cells. In this study, target sites of DRz were screened according to MDR1 mRNA secondary structure by RNA structure analysis software. Twelve target sites on the surface of MDR1 mRNA were selected. Accordingly, 12 DRzs were synthesized and their suppression effect on the MDR phenotype in breast cancer cells was confirmed. The results showed that 4 (DRz 2, 3, 4, 9) of the 12 DRzs could, in a dose-dependent response, significantly suppress MDR1 mRNA expression and restore chemosensitivity in breast cancer cells with MDR phenotype. This was especially true of DRz 3, which targets the 141 site purine-pyrimidine dinucleotide. Compared with antisense oligonucleotide or anti-miR-27a inhibitor, DRz 3 was more efficient in suppressing MDR1 mRNA and Pgp protein expression or inhibiting Pgp function. The chemosensitivity assay also proved DRz 3 to be the best one to reverse the MDR phenotype. The present study suggests that screening targets of DRzs according to MDR1 mRNA secondary structure could be a useful method to obtain workable ones. We provide evidence that DRzs (DRz 2, 3, 4, 9) are highly efficient at reversing the MDR phenotype in breast carcinoma cells and restoring chemosensitivity.

Keywords: breast cancer • multidrug resistance • chemosensitivity • deoxyribozyme

Introduction

Chemotherapy is important in systematic treatment of patients with breast cancers after surgery. However, multidrug resistance (MDR) constitute a major obstacle for successful chemotherapy [1]. MDR means tumours can exhibit a cross-resistant phenotype against a variety of antineoplastic drugs that differ widely with structures and mechanisms of action [2]. One of underlying mechanisms for MDR involves overexpression of membrane-spanning P-glycoprotein (Pgp), which is encoded by multidrug resistance gene-1 (MDR1). Pgp acts as a drug efflux pump and exports chemotherapeutic agents from cancer cells [3]. In cancers including breast cancer, overexpression of Pgp has been found to be correlated with poor outcome for patients with chemotherapy [4]. Although classic MDR modulators such as verapamil (VRP) and cyclosporin A have been found to be able to reverse MDR in vitro, their clinical applications are limited because of their innate toxicities [5]. Successful reversal of drug resistance is still awaiting new therapeutic strategies such as gene therapy and immunity therapy [6].

*Correspondence to: Peng GAO, M.D.,
Department of Pathology,
Shandong University, School of Medicine,
Jinan Wen Hua Xi Road 44, Jinan 250012, China.
Tel.: 86-531-88382574
Fax: 86-531-88383168
E-mail: gaopeng@sdu.edu.cn

Geng-Yin ZHOU, M.D.,
Department of Pathology, Shandong University,
School of Medicine, Jinan Wen Hua Xi Road 44,
Jinan 250012, China.
Tel.: 86-531-88382045
Fax: 86-531-88383168
E-mail: zhougy@sdu.edu.cn

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An alternative procedure to restore chemosensitivity in tumour cells is by inhibiting translation of MDR1 mRNA to Pgp through gene therapy. Inhibition of Pgp-mediated drug extrusion may allow chemosensitivity of cancer cells to anti-neoplastic drugs and result in successful treatment of MDR cells. Antisense oligonucleotides (ASODN) and hammerhead ribozymes for specific inhibition of Pgp expression in some malignant tumours have been well established [7, 8]. However, ribozymes are RNA molecules that are unstable in cell medium and are easily degradable [9], thus making them inconvenient for experimental use. Compared with ribozymes, deoxyribozyme (DRz), especially the 10–23 type DRz, is composed entirely of DNA and is more stable in vitro [10]. The 10–23 DRz was derived from an in vitro selection technique using a combinatorial library of DNA sequences. Consisting of a conserved catalytic domain of 15 nt and two substrate-binding arms of variable length and sequence, they bind and cleave target RNA with its only substrate requirement being a purine-pyrimidine (R-Y, R = A or G; Y = U or C) dinucleotide.

Many reports showed that DRzs inhibited gene expression of viral RNAs [11] as well as mRNAs of oncogenes or receptors such as BCR-ABL fusion gene [12]. DRzs can recognize and cleave target RNA containing R-Y dinucleotide easily in a chemical system. However, it is difficult to select an effective target site for DRz or to predict the cleavage activity of individual DRz in living cells. Before being cleaved by DRz, the mRNA target site must be accessible for combination [13]. As target mRNA has a secondary structure in living cells and the R-Y dinucleotides inside this secondary structure are hard to access and therefore combine [14], the R-Y dinucleotides on the surface of mRNA are more likely to be effective targets for DRz. In this study, we used a computer RNA structure analysis program (m-fold 3.2) to search for effective target sites of DRz against MDR1 mRNA and confirmed their presence in breast cancer cells.

miRNAs (miR) are short single-stranded RNAs consisting of 20 to 25 nucleotides. They are able to bind complementary sequences in 3′-untranslated regions (3′-UTR) of target genes to induce mRNA degradation, suppress translation, or both [15]. Zhou et al. showed that miR-27a expression was increased in human ovarian MDR cancer cells (5.1-fold) and cervix MDR cancer cells (3.8-fold) compared with their respective parental cells [16]. Furthermore, transfection of ovarian MDR cells with antagonists of miR-27a could reduce MDR1 mRNA level and decrease Pgp expression. However, the roles of miRNAs in cancer cells are tissue- and tumour specific. For example, miR-155 acts as an oncogene in leukaemias and lymphomas, whereas it has suppressive functions in endocrine tumours [17]. Whether inhibition of miR-27a in breast MDR cancer cells could reverse their MDR phenotype has not been clarified.

In the present study, DRz targets in MDR1 mRNA were screened, using a RNA secondary structure analysis program. Twelve DRzs targeting to the R-Y dinucleotide on the surface of MDR1 mRNA's secondary structure were synthesized. We verified their suppressive function in breast cancer cells with MDR phenotype. Furthermore, the reversal efficiency of DRzs, ASODN and anti-miR-27a inhibitor against MDR phenotype was compared.

### Materials and methods

#### Cell culture

The parental breast carcinoma cell lines MCF-7 and MDA-MB-231, both of which are sensitive to adriamycin, were obtained from the American National Cancer Institute. The MDR subline cells MCF-7/ADM and MDA/ADR were generated by step-wise selection of parental cells in increasing concentrations of adramycin [18].

#### Screening of DRz

By using a computer RNA secondary structure analysis program (m-fold 3.2, web site: http://www.bioinfo.rpi.edu/applications/mfold/doc/mfold-3.2.html) and referencing a related study [19], the 5′-region of MDR1 mRNA was analysed. The secondary structure of 5′-region of MDR1 mRNA is shown in Figure S1. Twelve R-Y dinucleotides were identified on the surface of the MDR1 mRNA secondary structure (location shown in Table 1 and Fig. S1). Phosphorothioate DRzs targeting to these 12 dinucleotides (the sequence shown in Table 1) were synthesized.

#### Synthesis of ASODN and anti-miR-27a inhibitor

Phosphorothioate ASODN targeting translation initiation codon AUG was synthesized using the sequence shown in Table 2. The sequences of the controls including unspecific DRz and ASODN are also shown in Table 2. The unspecific ASODN is a fragment of random oligonucleotides. The unspecific DRz contains a catalytic core sequence, but the binding arms are randomly chosen sequences. Anti-miR-27a inhibitor and miR inhibitor negative controls were purchased from Ambion (Austin, TX, USA). The transfection reagent control (mock control) was also used.

| DRz | R-Y dinucleotide location in MDR1 mRNA | Sequence of substrate-binding arms |
|-----|--------------------------------------|----------------------------------|
| DRz 1 | 85 | 5′-AAGUCCGA GU AUCUUCUU-3′ |
| DRz 2 | 99 | 5′-CUUCAA AU UUUCGGUC-3′ |
| DRz 3 | 141 | 5′-AGGUGGG AU GAUUUGUG-3′ |
| DRz 4 | 160 | 5′-GGGGCGCA AU GGAGAGC-3′ |
| DRz 5 | 171 | 5′-AUGAGAGA GC AAAGAGA-3′ |
| DRz 6 | 265 | 5′-GUUUCGCU AU UCAAAUG-3′ |
| DRz 7 | 287 | 5′-GACAAGUU GU AUUGUGUG-3′ |
| DRz 8 | 302 | 5′-UGGUGGAG AC UUUGGGCC-3′ |
| DRz 9 | 501 | 5′-ACAGUGGA AU UGGUGUGG-3′ |
| DRz 10 | 541 | 5′-UAGGUUUC AU UUGUGUGC-3′ |
| DRz 11 | 601 | 5′-UUACUGCU AU AAUAGCUC-3′ |
| DRz 12 | 650 | 5′-GUUGGGGA GC UUAACACC-3′ |

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The sequences of MDR1 mRNA from −14 to +10 are presented with GUC target and UG target showed in italics. The ASODN is complementary to (−11 to +5) of MDR1 mRNA sequence. The binding arms of DRz is complementary to (−14 to −7) and (−3 to +5) of MDR1 mRNA sequence.

Transfection of cancer cells with MDR phenotype

X-tremeGENE (Roche, Penzberg, Germany) was used to increase the uptake of nucleic acids according to the method provided by the company. Briefly, cells in exponential phase of growth were plated in six-well plates at a density of 2 × 10^5 cell/well. After 24 hrs, the cells were transfected with the complex consisting of X-tremeGENE and nucleic acids in culture medium with no serum. Twelve hours later, the medium was replaced with normal culture medium.

Detection of MDR1 mRNA by RT-qPCR

Total RNA was extracted from cells and quantified with the Quant-iT RiboGreen RNA Assay Kit (Invitrogen, Carlsbad, CA, USA). cDNA was prepared from 10 ng RNA sample by reverse transcription and quantitative PCR amplification was performed over 40 cycles with primers and probes as follows: MDR1, forward primer, 5'-AGGGGAGGCGGGAUUGGACUGA-3' (from −14 to +10); reverse primer, 5'-TGCACTGAGACATTGGACT-3' and probe, 5'-GCCAGGGCTGGTGTTCTCCATA-3'. β-actin was used as the control set. All reactions were run in duplicate. Threshold Cycle (CT) data were collected and average ΔCT of each group was calculated as following: ΔCT = average CT_{Treated} – average CT_{Control}. ΔCT was defined as relative MDR1 mRNA expression level (2^−ΔΔCT) and used for analysis. The difference of relative MDR1 mRNA expression between MDR cancer cells and the treated groups was calculating using the 2^−ΔΔCT method (ΔΔCT = ΔCT of mock control group – ΔCT of treated group), which means the fold change for MDR1 mRNA expression in mock control compared to that in the treated group.

Quantitative analysis of Pgp by flow cytometry

The amount of Pgp was analysed quantitatively by fluorescence-activated cell sorting as previously reported [20].

Table 2 The sequence of DRz 3, ASODN and unspecific DRz or ASODN

| Nucleotides       | Sequence                                      |
|-------------------|-----------------------------------------------|
| MDR1 mRNA        | 5'-AGGGGAGGCGGGAUUGGACUGA-3' (from −14 to +10) |
| DR 3             | 5'-CAAGATCCA CCGGACCT -3'                     |
| Unspecific DRz   | 5'-GCCGTACG CTTAGGACT -3'                    |
| ASODN            | 5'-TCAAGATCCATCCCGA-3'                       |
| Unspecific ASODN | 5'-GCACTAGTAAGCTGTG-3'                       |

Determination of intracellular Rhodamine (Rh123) retention

The cells were seeded in 6-well plates, cultured for 12 hrs and then were incubated with 200 ng/ml Rh123 at 37°C for 1 hr. After washed, the cells were cultured in Rh123-free culture medium at 37°C for 30 min. and harvested for measurement of Rh123 efflux. The sample was determined mean fluorescence intensity (FI) by flow cytometry using a 530-nm-long band-pass filter. All analyses were performed in triplicate in three separate experiments and the results were expressed as the mean FI, which reflected cellular content of the dye retained.

Chemosensitivity assay in the treated cells

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) assay was used to detect chemosensitivity in vitro [21]. Briefly, Adriamycin (Doxorubicin), Vinblastine or Hydroxyurea was delivered into the cells, respectively, at various concentrations and incubated for another 48 hrs in a humidified 5% CO2 atmosphere. The absorbance of each well was measured using a Bio-Rad microplate reader (Hercules, CA, USA) at 540 nm. The absorbance of untreated controls was taken as 100% survival, and the percentage inhibition was calculated as cell survival rate (%) = 100 – (T/B) / (U – B) and growth inhibition (%) = 100 – cell survival rate (%), where T (treated) is the absorbance of drug-treated cells, U (untreated) is the absorbance of untreated cells and B (blank) is the absorbance in the absence of both drug and MTT. Relative drug resistance was assessed by determining the IC50, which is defined as the concentration of cytotoxic drug causing 50% inhibition of cell growth. The value of relative drug resistance was determined by comparing the IC50 of treated cells relative to untreated MCF-7 cells. The reversal fold was calculated by comparing of IC50 for anti-cancer drugs (Adriamycin or Vinblastine) to IC50 for anti-cancer drugs plus DRzs in breast MDR cells. All analyses were performed in triplicate in three separate experiments.

Statistical analysis

The difference expression of MDR1 mRNA was analysed with the one-way ANOVA and Student’s t-test. A P-value less than 0.05 was considered to be statistically significant. Statistical analysis was done with Prism 5 software (GraphPad Software, San Diego, CA, USA).

Results

DRz 3 was proved to be the best DRz to reverse MDR phenotype in breast cancer cells.

Thirty-six hours after transfection with DRzs at a concentration of 5 μg/ml, significant down-regulation of MDR1 mRNA expression was observed in four treated MCF-7/ADM groups (DRz 2, 3, 4, 9), with fold changes (MDR cells/treated cells) of 3.16, 5.35, 3.72 and 2.23, respectively (Table 3). DRz 10, 12 could also decrease MDR1 mRNA expression at some degree with fold changes of 1.85, 1.73, 2.23, respectively (Table 3). DRz 2, 3 could also decrease MDR1 mRNA expression at some degree with fold changes of 1.85, 1.73, 2.23, respectively (Table 3). DRz 10, 12 could also decrease MDR1 mRNA expression at some degree with fold changes of 1.85, 1.73, 2.23, respectively (Table 3). DRz 10, 12 could also decrease MDR1 mRNA expression at some degree with fold changes of 1.85, 1.73, 2.23, respectively (Table 3).
Table 3  Fold change Of MDR1 mRNA and chemosensitivity assay in MCF-7/ADM cells transfected with DRzs

| Group  | Fold change Of MDR1 mRNA | IC50 for Adriamycin | Relative drug resistance (reversal fold) | IC50 for Vinblastine | Relative drug resistance (reversal fold) |
|--------|--------------------------|---------------------|----------------------------------------|---------------------|----------------------------------------|
| DRz 1  | 1.21                     | 12.20 ± 1.37        | 110.9                                  | 234 ± 16.5          | 254.1                                  |
| DRz 2  | 3.16                     | 3.22 ± 0.63         | 29.3 (3.83)                            | 39 ± 15.7           | 42.4 (7.22)                            |
| DRz 3  | 5.35                     | 0.61 ± 0.23         | 5.5 (20.4)                             | 6.5 ± 2.3           | 7.1 (43.1)                             |
| DRz 4  | 3.72                     | 1.56 ± 0.67         | 14.2 (7.89)                            | 29 ± 13.5           | 31.3 (9.78)                            |
| DRz 5  | 1.13                     | 13.10 ± 2.03        | 119                                    | 256 ± 21.7          | 278.3                                  |
| DRz 6  | 1.85                     | 8.83 ± 0.33         | 80.3                                   | 146.3 ± 8.3         | 157.8                                  |
| DRz 7  | 1.31                     | 11.43 ± 0.77        | 103.9                                  | 215 ± 23.4          | 233.7                                  |
| DRz 8  | 1.73                     | 9.15 ± 0.75         | 83.2                                   | 169 ± 13.2          | 183.7                                  |
| DRz 9  | 2.23                     | 5.21 ± 0.62         | 47.5 (2.35)                            | 119 ± 25.2          | 129.3 (2.37)                           |
| DRz 10 | 1.49                     | 10.82 ± 1.12        | 98.4                                   | 192 ± 18.8          | 208.7                                  |
| DRz 11 | 1.33                     | 11.02 ± 1.27        | 108.2                                  | 209 ± 25.3          | 227.2                                  |
| DRz 12 | 1.23                     | 11.36 ± 0.53        | 103.3                                  | 183.9 ± 9.3         | 198.9                                  |
| Mock control | 1.06          | 13.86 ± 1.32        | 112.3                                  | 289 ± 8.7           | 306                                    |
| U D    | 1.05                     | 13.29 ± 1.12        | 111.2                                  | 267 ± 15.5          | 286                                    |
| MCF-7  | NA                       | 0.11 ± 0.07         | 1                                      | 0.92 ± 0.15         | 1                                      |

Fold-change of MDR1 mRNA expression, which means relative MDR1 mRNA expression in mock control compared to that in the treated group, was calculated. The groups of DRz 2, 3, 4, 9 showed fold changes of 3.16, 5.35, 3.72 and 2.23, respectively. The value of relative drug resistance was the IC50 of the treated cells relative to MCF-7. The reversal fold was calculated by comparing of IC50 for anti-cancer drugs to IC50 for anti-cancer drugs (Adriamycin or Vinblastine) plus DRzs. DRz 3 was proved to be the best one at inhibiting MDR1 mRNA expression and reversing the MDR phenotype, which showed a 20.4-fold reduction in drug resistance for Adriamycin and a 43.1-fold reduction in drug resistance for Vinblastine. U D: Unspecific DRz; NA: not applicable.

to be the best one at reversing the MDR phenotype in MCF-7/ADM (Table 3) and MDA/ADR cells (Table S1). Therefore, DRz 3 was chosen to compare its suppression function and reversal efficiency with ASODN and anti-miR-27a inhibitor at different concentrations.

Cell toxicity

MTT assay showed that DRz 3, ASODN and anti-miR-27a inhibitor almost had no cell toxicity at concentrations of 10 μg/ml or below (Table S2). Compared with VRP, which is a classic chemical modulator, DRz 3 had similar reversal efficiency with less toxicity (Table 4).

MDR1 mRNA expression in transfected cells

The MCF-7/ADM cells were treated with DRz 3, ASODN or anti-miR-27a inhibitor at concentrations ranging from 0.5 μg/ml to 10 μg/ml and the MDR1 fold change was determined 36 hrs after transfection. The results showed that anti-miR-27a inhibitor and ASODN could only suppress the expression of MDR1 mRNA significantly at concentration of 5 μg/ml or above. However, DRz 3 could suppress it at concentrations of 0.5 μg/ml (Fig. 1A) or above, in a dose-dependent response. No changes were observed in the unspecific control groups.

In fact, the suppressive effect of DRz 3 was the best one among all the nucleic acids. For example, at concentration of 5 μg/ml, significant decrease of MDR1 mRNA expression was observed in DRz 3 group 36 hrs after transfection, with a fold change of 5.35. However, for the ASODN and anti-miR-27a inhibitor group, there was a reduction of MDR1 mRNA, with fold changes of 2.12 and 2.76, respectively.

Next, MCF-7/ADM cells were transfected with DRz 3, ASODN or anti-miR-27a inhibitor, respectively, at concentration of 5 μg/ml and observed continuously every 12 hrs up to 72 hrs. The results showed that the suppressive effect of DRz 3 was rapid and could degrade MDR1 mRNA 12 hrs after transfection, with the best suppression efficiency appearing 36 hrs after transfection. The effect gradually became reduced 60 hrs after transfection in DRz group and anti-miR-27a inhibitor group or 48 hrs in ASODN (Fig. 1B).

Expression of Pgp in transfected cells

Flow cytometric analysis of surface Pgp expression was carried out with representative figures shown in Figure 2. Both the P-gp-specific antibody and the IgG control were used at a concentration of 1 μg/1 × 10^6 cells. Decreased FI in the transfectedants reflects
Table 4 Evaluation of cytotoxicity and chemosensitivity in cells treated with DRz 3 and VRP

| Group                  | Cytotoxicity | IC50 for Adriamycin (µM) | Relative drug resistance (reversal fold) | IC50 for Vinblastine (nM) | Relative drug resistance (reversal fold) |
|------------------------|--------------|--------------------------|----------------------------------------|---------------------------|----------------------------------------|
| MCF-7                  | NA           | 0.11 ± 0.03              | 1                                      | 0.92 ± 0.13               | 1                                      |
| MCF-7/ADM + Mock control | NA           | 12.50 ± 0.18             | 114                                    | 286 ± 6.9                 | 311                                    |
| MCF-7/ADM + DRz 3      | 95.1 ± 2.7   | 0.61 ± 0.09              | 5.5 (20.4-fold)                        | 6.5 ± 2.2                 | 7.1 (49.4-fold)                        |
| MCF-7/ADM+ VRP (5 µM)  | 91.0 ± 3.5   | 1.52 ± 0.15              | 13.8 (8.3-fold)                        | 16.3 ± 4.3                | 17.7 (17.6-fold)                       |
| MCF-7/ADM+ VRP (10 µM) | 81.6 ± 5.9   | 0.86 ± 0.11              | 7.8 (14.6-fold)                        | 8.9 ± 2.6                 | 9.7 (32.1-fold)                        |
| MCF-7/ADM+ VRP (20 µM) | 75.9 ± 7.6   | 0.59 ± 0.08              | 5.4 (21.1-fold)                        | 5.8 ± 1.1                 | 6.3 (49.4-fold)                        |

The value of relative drug resistance was the IC50 of the treated cells relative to MCF-7. The reversal fold was calculated by comparing of IC50 for anti-cancer drugs to IC50 for anti-cancer drugs (Adriamycin or Vinblastine) plus reversal agents. DRz 3 had similar reversal efficiency with VRP at concentration of 20 µM. All analyses were performed in triplicate in three separate experiments.

Table 5 Evaluation of chemosensitivity in the transfected MCF-7/ADM cells

| Group                  | IC50 for Adriamycin (µM) | Relative drug resistance (reversal fold) | IC50 for Vinblastine (nM) | Relative drug resistance (reversal fold) | IC50 for Hydroxyurea (µM) | Relative drug resistance (reversal fold) |
|------------------------|--------------------------|----------------------------------------|----------------------------|----------------------------------------|----------------------------|----------------------------------------|
| MCF-7                  | 0.12 ± 0.08              | 1                                      | 0.95 ± 0.16                | 1                                      | 152 ± 16                   | 1                                      |
| MCF-7/ADM              | 13.20 ± 0.27             | 110                                    | 286 ± 5.7                  | 301                                    | 186 ± 15.7                 | 1.2                                    |
| MCF-7/ADM + DRz 3      | 0.62 ± 0.23              | 5.2 (20.4-fold)                        | 6.3 ± 2.2                  | 6.6 (43.1-fold)                        | 166.3 ± 22.2               | 1.1                                    |
| MCF/ADM + ASODN        | 1.65 ± 0.56              | 13.8 (8-fold)                          | 30.5 ± 6.3                 | 32.1 (9.4-fold)                        | 163.5 ± 13.3               | 1                                      |
| MCF/ADM + anti-miR27   | 1.41 ± 0.29              | 11.8 (11.2-fold)                       | 25.7 ± 5.1                 | 29.2 (13.1-fold)                       | 16.8 ± 23.1                | 1.1                                    |

The value of relative drug resistance was the IC50 of the treated cells relative to MCF-7. The reversal fold was calculated by comparing of IC50 for anti-cancer drugs to IC50 for anti-cancer drugs (Adriamycin or Vinblastine) plus reversal agents. DRz 3 had similar reversal efficiency with VRP at concentration of 20 µM. All analyses were performed in triplicate in three separate experiments.

loss of Pgp expression. DRz 3 could inhibit Pgp expression even at 0.5 µg/ml. Compared with the other groups, the DRz 3 group had significantly decreased Fl and reduced Pgp expression 36 hrs after transfection, both in MCF-7/ADM cells (Fig. 3A, P < 0.05) and MDA/ADR cells (Fig. S2A, P < 0.05). During the continuous observations of DRz at 5 µg/ml, the inhibitory effect of DRz 3 on Pgp expression appeared 12 hrs after transfection and reached its climax 36 hrs after transfection, with better and longer inhibition efficiency than that for the other two groups (Fig. 3B and Fig. S2B, P < 0.05).

Analysis of Pgp function in transfected cells

Rh123 is specific substrate transported by Pgp. Increased intracellular Rh123 retention in treated cells as compared to the mock control indicated that the efflux function of Pgp was inhibited. This experiment was conducted 36 hrs after transfection with DRz 3, ASODN or anti-miR-27a inhibitor at 5 µg/ml. Rh123 retention showed that intracellular Rh123 in cells treated with DRz 3 was significantly higher than that of the other two groups both in MCF-7/ADM cells (Fig. 4, P < 0.05) and MDA/ADR cells (Fig. S3, P < 0.05).

Evaluation of chemosensitivity in transfected cells

The experiment was conducted 36 hrs after transfection with DRz 3, ASODN or anti-miR-27a inhibitor at 5 µg/ml. IC50 of the treated MCF-7/ADM cells for Adriamycin and Vinblastin are shown in Table 5. Compared with the ASODN or anti-miR-27a inhibitor group, a significant reduction in drug resistance to Adriamycin and Vinblastin was found in the DRz 3 group, which showed a 20.4-fold reduction in drug resistance to Adriamycin and a 43.1-fold reduction in drug resistance to Vinblastin.
43.1-fold reduction in drug resistance to Vinblastin. The reversal efficiency of DRz 3 is better than the other two groups. The chemosensitivity to Hydroxyurea, which is not transported by Pgp, was not affected in the transfected cells (Table 5). Moreover, DRz 3 proved to have the advantage for reversing the MDR phenotype in MDA/ADR cells over ASODN or the anti-miR-27a inhibitor (Table S3).

**Discussion**

Being composed of DNA, DRz is relatively easy to synthesize and handle and holds great promise for diagnostic and therapeutic applications. DRz can degrade target RNA at R-Y dinucleotides easily in isolated chemical systems. However, the secondary structure of target RNA in living cells could protect...
the target sites and make them inaccessible to DRz. Therefore, although there are lots of R-Y dinucleotides in MDR1 mRNA, only a few are suitable to serve as target choices. Cairns et al. showed that more than 90% of DRz against R-Y dinucleotides selected randomly in Human Papilloma Virus (HPV)-16 mRNA were proved unworkable [22]. The suppression effect of DRz depends on its accessibility to the target site of MDR1 mRNA. We obtained the secondary structure of the 5'/H11032 region of MDR1 mRNA by using RNA structure analysis software (m-fold 3.2) and subsequently 12 R-Y dinucleotides on the surface of MDR1 mRNA were selected as targets. We suggested that the 12 surface target sites were more suitable for cleavage by DRz.

Twelve DRzs were synthesized accordingly and were chemically modified with phosphorothioate bonds in three nucleotides of 3' and 5' ends of DRz to improve properties like enhanced biostability and high target affinity [23]. After transfection into breast cancer cells with a MDR phenotype, 4 (DRz 2, 3, 4, 9) of the 12 DRzs were proved to reduce MDR1 mRNA expression significantly. Another two DRzs (DRz 10, 12) could also decrease MDR1 mRNA expression at some degree (P < 0.05), whereas the other 6 DRzs had no significant effect. DRz 3, which targets to the 141 site R-Y dinucleotide of MDR1 mRNA, proved to be more efficient at degrading MDR1 mRNA and restoring chemosensitivity of MDR cells than the other 11 DRzs. The results showed that DRzs could vary significantly in their abilities to suppression MDR1 mRNA expression and suggest that screening targets of DRzs according to MDR1 mRNA secondary structure could be more useful to obtain workable ones than random selection.

Furthermore, DRz 3 was transfected into the MDR cells at different concentrations, in a comparison with ASODN and anti-miR-27a inhibitor. The results showed that DRz 3 at 0.5 g/ml could effectively suppress expression of MDR1 mRNA and inhibit synthesis of Pgp, with a 10-fold reduction in concentration as compared to that of ASODN and anti-miR-27a inhibitor (5 g/ml). At the same concentration, the suppressive effect of DRz 3 was significantly better and longer than that of ASODN and anti-miR-27a inhibitor. The unspecific controls, including unspecific ASODN, DRz or miR inhibitor negative control, had no effect on MDR1 mRNA expression.

During the continuous observation period, DRz 3 showed a more rapid and longer effect than ASODN or anti-miR-27a inhibitor. The best suppressive effect of DRz 3 appeared 36 hrs after transfection, reducing gradually 60 hrs after transfection because of degradation by nucleases in the cell cytoplasm [24].

Fig. 4 Rh123 retention showed that intracellular Rh123 in cells treated with DRz 3 was significantly higher than that of the other two groups in MCF-7/ADM cells (*P < 0.05; **P < 0.01), suggesting the efflux function of Pgp in DRz 3 group was inhibited more significantly.
MDR cells more effectively than ASODN and anti-miR-27a inhibitor.

The antagonim of miR-27a was proved to suppress MDR1 mRNA expression and inhibit Pgp synthesize in ovarian cancer cell line A2780 DX5 with an indirect and unidentified pathway [16]. Our results also showed that anti-miR-27a inhibitor could suppress MDR1 mRNA and Pgp expression at 5 μg/ml both in MCF-7/ADM and MDA/ADR cells. However, it was less effective than that of DRz 3.

The results of MTT assay showed that the IC50 of cells treated with DRz 3 decreased significantly 36 hrs after transfection. A 20.4-fold reduction in drug resistance for Adriamycin and a 43.1-fold reduction in drug resistance for Vinblatine was found in the cells treated with DRz 3 at 5 μg/ml. These data suggest that DRz 3 can restore the chemosensitivity to Adriamycin and Vinblatine in cells with a MDR phenotype. Moreover, its reversal efficiency is better than ASODN and anti-miR-27a inhibitor. The chemosensitivity to Hydroxyurea, which is not transported by Pgp, was not effected in the transfected cells, thus confirming the specific inhibition of Pgp expression by DRz 3. Compared with a classic chemosensitizer such as VRP, DRz 3 has similar reversal efficiency with almost no toxicity.

In summary, the present study suggests that screening target sites of DRz according to the secondary structure of MDR1 mRNA could be a useful method to obtain effective sites for cleavage. We conclude that DRz 3, targeting 141 site R-Y dinucleotide of MDR1 mRNA, reverses the MDR phenotype in breast carcinoma cells with high efficiency and restores their chemosensitivity. Its reversal efficiency is better than 11 other DRzs, ASODN or anti-miR-27a inhibitor.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 The secondary structure of 5'-region of MDR1 mRNA is shown and the twelve R-Y dinucleotides on the surface of the MDR1 mRNA secondary structure were selected as targets for DRzs.

Fig. S2 (A) Anti-miR-27a inhibitor and ASODN could only suppress Pgp expression at concentration of 5 μg/ml or above (P < 0.05). However, DRz 3 could suppress it at concentrations of 0.5 μg/ml or above, in a dose-dependent response in MDA/ADR cells. (B) During the continuous observations of DRz at 5 μg/ml, the inhibitory effect of DRz 3 on Pgp expression was better and longer than that for the other two groups in MDA/ADR cells (P < 0.05).

Fig. S3 Rh123 retention showed that intracellular Rh123 in cells treated with DRz 3 was significantly higher than that of the other two groups in MDA-7/ADR cells.

Table S1 Fold change of MDR1 mRNA and chemosensitivity assay in MDA/ADR cells transfected with DRzs

Table S2 Cell toxicity of DRz 3, ASODN, ribozyme and anti-miR-27a inhibitor

Table S3 Evaluation of chemosensitivity in the transfected MDA/ADR cells

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