Modulation of BCRP mediated atypical multidrug resistance phenotype by RNA interference*

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Multidrug resistance (MDR) in human cancers is one of the major causes of failure of chemotherapy. The emergence of breast cancer resistance protein (BCRP), a member of the ABC transporter family, has necessitated the development of antagonists. To overcome the BCRP-mediated atypical multidrug drug resistance, two small interfering RNA constructs (RNAi) targeting two different regions of BCRP mRNA were designed to inhibit the atypical MDR expression by transfecting them into MCF-7/MX100 cell lines. The multidrug resistance index to mitoxantrone and the intensity of mitoxantrone fluorescence of MCF-7/MX100 decreased after transfected by pSUPER-BCRP-A and pSUPER-BCRP-B respectively; the BCRP mRNA level and the BCRP protein level of MCF-7/MX100 decreased after treated with pSUPER-BCRPs. The two constructed RNAi plasmids could reverse the atypical mutidrug resistance mediated by BCRP, but neither can reversed it completely, this may due to low transfection efficiency and transient transfection.

Key words: BCRP, RNA interference, atypical MDR, breast cancer

Clinical MDR of malignancies to many antineoplastic agents, a common phenomenon in cancer patients, is the major obstacle in the successful treatment of cancer. Although the mechanistic basis for this phenomenon is complex, the overexpression of ABC transporters is often associated with this phenotype. Various members of the protein superfamily of ABC transporters [15] have been associated with MDR of human cancers when overexpressed [10], including P-gp, the MRP and its homologues MRP2 and MRP3. A relatively new member of the ABC-transporter family is BCRP [7], a 72 kD ABC half-transporter consisting of 655 amino acid residues, which probably homodimerize to form an active transport complex [3]. BCRP is overexpressed in a variety of human MDR cancer cell lines, exhibiting an atypical MDR or a non-Pgp-mediated MDR phenotype. Elevated expression of BCRP results in resistance of various cancer cell lines to antitumor drugs, including mitoxantrone (MX), topotecan, daunorubicin, doxorubicin and bisantrene [26].

Reversal of MDR is a major goal in the clinical management of cancer. Pharmacological inhibition of the ABC transporters can be expected to resensitize cells to the action of antitumor agents. Inhibitors of P-gp [27] and MRP [17] have been reported, and P-gp reversal agents are being tested in the clinic [2, 27]. Recently, a few substances have shown BCRP-inhibiting effects: the Aspergillus fumigatus secondary metabolite FTC [24]; its derivatives demethoxy-FTC [29], Ko132, Ko134 and Ko143 [1]; the so-called second-generation MDR modulator GF120918 [6]; the quinazoline-based HER family tyrosine kinase inhibitor CI1033 [8]; experimental camptothecin analogues [19, 23]; estrogens like estrone and 17β-estradiol [12], and the fungal secondary metabolite TPS-A, a diketopiperazine [30]. An alternative procedure to circumvent BCRP-mediated MDR in cancer cells is to prevent the biosynthesis of BCRP by selectively blocking the expression of the BCRP-specific mRNA. Thus, in previous studies, antisense oligonucleotides [13] and hammerhead ribozymes [14] were designed and successfully applied to decrease the expression level of BCRP mRNA.

A novel means for specific inhibition of a gene of interest

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is the use of small interfering RNA (siRNA). Chemically synthesized 21-nt siRNA duplexes against two regions of the P-gp-encoding mRNA have been designed for disruption of P-gp-mediated drug extrusion in a specific manner and resensitization of gastrointestinal tumor cells to treatment with the antineoplastic agent daunorubicin [20]. The siRNA molecules can now be synthesized in mammalian cells from a plasmid DNA under the control of a RNA polymerase III promoter [22]. In this study, vectors expressing shRNA duplexes against two regions of the BCRP-encoding mRNA have been transfected into BCRP mediated MDR cell lines for disruption of BCRP-mediated drug extrusion in a specific manner.

Material and methods

Cell lines and culture conditions. The MCF-7 MX8 cells were selected in a single step and maintained in 8 ng/ml mitoxantrone. The MCF-7 MX100 sublines were independently selected in our laboratory in a stepwise manner [25]. MCF-7 cells and resistant sublines were maintained in RPMI 1640 medium (Gibico); and both were complemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Resistant cell lines were maintained in the mioxantrone at concentration of 8 ng/ml, all cells were kept at 37 °C in 5% CO_2.

Cloning of BCRP target sequences and construction of pSUPER-BCRP vector. The siRNA target sequences were selected according to the recommendation by others (http://www.ambion.com). The chemically synthesized 59 nt oligonucleotides (Sangon) as complementary oligonucleotides encoding human BCRP specific siRNA were:

5'GATCCCCTGACCTGAAGGCATTTACTTAGAGCAG TAAATGCCTTCAGGTCATTTTTGAA3'

5'AGCTTTCAAAAATGACCTGAAGGCATTTACTGCT CTAAGTAAATGCCTTCAGGTCAGGG 3'

5'GATCCCCGGTTGGAACTCAGTTTATCTAGAGCGAT AAACTGAGTTCCAACCTTTTTGAA 3'

5'AGCTTTCAAAAAGGTTGGAACTCAGTTTATCGCT CTAGATAAACTGAGTTCCAACCGGG 3'

with BglII/HindIII sites at the end harboring two 19bp regions for siRNA. which was 78–96 and 531–549 bases downstream of the first nucleotides of the start codon of BCRP (GenBank accession number 004827). These oligonucleotides were annealed after phosphorylation of their 5' terminate, and then subcloned into the BglII/HindIII sites of pSUPER [4] to generate pSUPER-BCRP constructs, named pSUPER-BCRP-A and pSUPER-BCRP-B, respectively. Constructs which could be cut by Bg1II but not HindIII were positive clones (Fig. 1, 2), further identification was performed by sequencing.

Transfection of RNAi constructs. Transient transfection of pSUPER-BCRPSs was carried out using Lipofectamine^{TM}2000 (Invitrogen). 250 μl of DMEM medium without serum and pSUPER-BCRPs or control pSUPER 4 μg per well were preincubated for 5–10 minutes at room temperature. During the time for this incubation, 250 μl of DMEM medium without serum were mixed with 10 μl Lipofectamine^{TM}2000. The two mixtures were mixed and incubated for 20 minutes at room temperature for complex formation and then the cells were transfected according to manufacturer's protocol.

Cytotoxicity assay. One week after transfection, the cytotoxicity assays were performed using the sulforhodamine B method previously described [28]. Briefly, cells were plated in flat-bottom 96-well plates at a density of $2x10^4$ cells per well and allowed to attach for 24 h at 37 °C in 5% CO_2. Mitoxantrone at various concentrations was allowed to incubate at 37 °C in 5% CO_2 for 48, 96 h. After incubation, the

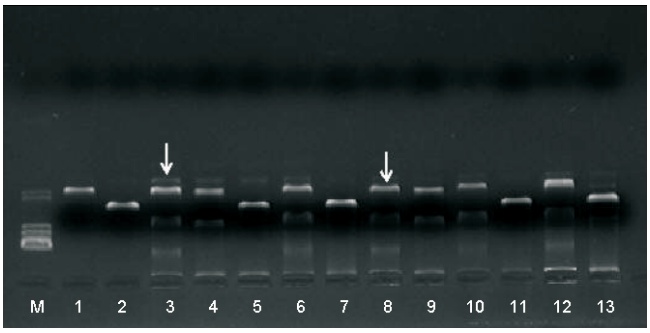

Figure 1. The chemically synthesized 59 nt oligonucleotides are cloned into the unique BglII and HindIII sites. Upon ligation, BglII site is destroyed. The constructs which could not be cut with BglII but HindIII are the positive clones. The arrows show two positive clones.

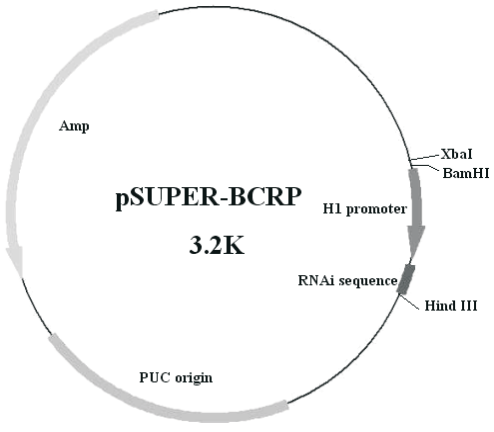

Figure 2. Physical map of pSUPER-BCRP constructs. The chemically synthesized stem-loop producing oligonucleotide was subcloned into pSUPER between BglII/HindIII. Polymerase III-dependent H1-RNA promoter drives the expression of 19 bp stem and 6 nt loop RNA which is processed into functional siRNA by cellular enzymes.

cells were fixed in 50% TCA and stained with sulforhodamine B solution (0.4% sulforhodamine B w/v in 1% acetic acid). Optical densities were read on a Bio-Rad plate reader at an absorbance of 540 nm. Each concentration was tested in triplicate and controls were done in replicates of eight.

Efflux assays. One week after transfection, the efflux assays were performed based on those previously described with minor modifications [18]. Suspensions of log phase cells were transferred to 96-well plates, the cells were resuspended in complete medium alone (phenol red-free RPMI 1640 with 10% FCS), or complete medium containing the 20 μM mitoxantrone incubated at 37 °C in 5% CO_2 for 30 min. Cells in complete medium alone yielded the Blank histogram which is a measure of cell autofluorescence, while cells in complete medium with a fluorescent compound generated the Control. After the 30 min incubation period, the cells were washed with ice-cold complete medium and either placed on ice in the dark, allowed to incubate for 1 additional hour at 37 °C in 5% CO_2. The Efflux histogram was generated from cells which were incubated 30 min with 20 μM mitoxantrone and then allowed to efflux for 1 h in complete medium alone; The cells were then washed in cold PBS and placed on ice. A FACSCalibur flow cytometer equipped with a 635 nm red diode laser and 670 nm bandpass filter was used to detect mitoxantrone fluorescence [25]. At least 10000 events were collected. The mean channel number for each histogram was used as the measure of fluorescence for calculation of efflux values.

Quantitative Real-time RT-PCR. 24 hs, 48 hs, 1 week and 3 weeks after transfection, the mRNA levels of BCRP were measured by real-time RT-PCR using an ABI PRISM 7000 sequence detector system (Applied Biosystems, Foster City, CA). Total RNA was isolated from 6-well plates using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. In addition, the mRNA levels of internal control gene, PBGD, was measured and used to normalize the mRNA levels of the drug resistance genes. The sequences of primers were designed according to FANEYTE et al [9] (Tab. 1). The constituents of each PCR (25 μl) were 5 μl of template RNA (200 ng/reaction) or dH_2O, 2.5 μl $MgCl_2$ (6 mM), 2x (forward and reverse) 1 μl of primer 10 μM each (sangon), 12.5 μl of 2x QuantiTect SYBR Green PCR (P.E. Applied Biosystems), and 3 μl of dH_2O. To compare the expression levels among different tumor samples, the relative expression level of the resistance genes was calculated using the comparative CT method and compared with a calibrator. Accordingly, C_T = (mean of BCRP CT) – (the mean of PBGD C_T). The C_T value is defined as the C_T value of the BCRP for MCF-7MX100 for a calibrator ($C_T = C_{T(BCRP)} - C_{T(calibrator)}$). The relative gene expression in a particular sample is then given by the following: relative amount of target = $2 - C_T$ value [5].

Table 1. Sequences of primer set

BCRP	74-bp amplicon
Forward primer	5'-CACAAC CATTGCATCTTGGC-3'
Reverse primer	5'-GCTGCA AAGCCGTAAATCCA-3'
PBGD	84-bp amplicon
Forward primer	5'- ACGATCCCGAGACTCTGCTTC-3'
Reverse primer	5'- GCACGGCTACTGGCACACT -3'

Immunofluorescence. One week after transfection, cells were seeded on sterile 11x22 mm coverslips at $4x10^4$ cells per slide then washed three times with iced PBS and fixed immediately with cold acetone for 4–5 minutes, blocked with 5% normal goat serum for 30 min the primary antibody (1:50; 60 min) before incubated with BCRP, antibody was applied. Fifty minutes later, a rabbit anti mouse IgG labeled with FITC secondary antibody was applied and slides were incubated for 30 minutes.

Western blot analysis. For detection of BCRP, cellular protein extracts were prepared as described before [16]. Samples of 30 μg cellular proteins were diluted with sample buffer and separated on 4% stacking and 7.5% resolving SDS-PAGE gels. Separated proteins were transferred to a 0.2 μm cellulose nitrate membrane (Schleicher and Schuell, Dassel, Germany). To avoid unspecific binding, filters were incubated in 5% nonfat dry milk, 0.05% Tween-20 in TBS overnight. Subsequently, filters were incubated with anti-BCRP (5D3 clone R and D system) diluted 1:2000 in the same solution for 2 h and, afterwards, with horseradish peroxidase-conjugated rabbit antimouse IgG (1:10000). As control for equivalent protein loading, filters were simultaneously incubated with a mouse MAb directed against β-actin (Chemicon, Temecula, CA) diluted 1:1000. The protein-antibody complexes were visualized by chemoluminescence (ECL system, Amersham, Buckinghamshire, UK).

Results

Reversal of the drug resistance phenotype by siRNA. The siRNA-mediated reversal of the multidrug-resistant phenotype was assessed by comparison of IC_{50} values determined by cell proliferation assay in pSUPER-BCRPs treated tumors and controls (Tab. 2). Cytotoxicity experiments were performed 48 h after transfection with pSUPER-BCRPs. Both pSUPER-BCRP-A and pSUPER-BCRP-B decreased the degree of mitoxantrone resistance from 1208-fold to 592-fold (decrease to 49% of the initial value, i.e. 51% reversal; $p<0.001$) and 541-fold (decrease to 45% of the initial value, i.e. 55% reversal; $p<0.001$) in MCF-7MX100 cells. pSUPER-BCRP-B show a slightly enhanced chemosensitizing activity compared to pSUPER-BCRP-A.

Interdiction of the BCRP mediated mitoxantrone efflux by siRNAs. To provide confirmation to the cytotoxicity data presented above, mitoxantrone efflux assay was performed. After an accumulation and efflux period, mitoxantrone fluores-

Table 2. IC_{50} values for mitoxantrone with or without transfected with pSUPER-BCRP constructs

Cell lines	Without pSUPER-BCRP		pSUPER		pSUPER-BCRP- A		pSUPER-BCRP- B	
	IC_{50}(MXμM)	RR	IC_{50}(MXμM)	RR	IC_{50}(MXμM)	RR	IC_{50}(MXμM)	RR
MCF-7	0.013±0.006	1	0.012±0.09	0.94	0.017±0.098	1.0.3	0.017±0.072	1.03
MCF-7/MX100	15.704±2.953	1208	16.585±3.019	1275	7.695±1..93	592	7.033±1.54	541

cence was quantitated by flow cytometric analysis. The results are depicted in Figure 3. Both pSUPER-BCRP-A and pSUPER-BCRP-B can inhibit mitoxantrone efflux from BCRP expressing cells obviously, the intensity of mitoxantrone fluorescence decreased from 142.7 to 70.4 and 87.3 in pSUPER-BCRP-A and pSUPER-BCRP-B treated groups respectively. Consistent with the result of Cytotoxicity assay, the effects of effluxing mitoxantrone in pSUPER-BCRP-B treated group was more obviously than that in pSUPER-BCRP-A treated group.

Decrease of the BCRP mRNA expression by siRNAs.

BCRP mRNA expression estimated by real-time RT-PCR were assessed in a panel of cells lines, the results of these analyses are summarized in Figure 4. The BCRP mRNA level of MCF-7/MX100 was regarded as 1. Both siRNA constructs targeting different regions of BCRP mRNA and could decrease BCRP mRNA level 24 hours after transfection, the peak of mRNA reduction was reached after 3 days using MDR-A siRNA, but 3 weeks later the BCRP mRNA level began to increase gradually (Fig. 4).

Knockdown of BCRP protein levels by RNAi. Immunofluorescence and Western blot experiments demonstrated that both siRNA constructs decreased the cellular BCRP content in MCFf-7/MX100 cells. This reduction of transmembrane transport protein concentration was related to the decrease of BCRP mRNA level.

The BCRP protein was stained by using FITC immunofluorescence, and the fluorescent intensity of MCF-7/ /MX100 transfected with pSUPER-BCRPs is much less than that of MCF-7/MX100 (Fig 5). This result is consistent with the change of BCRP mRNA level. The Western blot analyses showed that BCRP is a 72kD transmembrane transport protein, both RNAi constructs can knockdown the expression of BCRP obviously, but in none of the cases the cellular BCRP content could be reduced completely (Fig. 6).

Discussion

The drug resistance-mediating ABC transporter BCRP is upregulated in various drug-resistant cancer cell lines that were established by exposure to mitoxantrone, topotecan, doxorubicin or SN-38 and is upregulated in various drug-resistant cancer cell lines and tumors. Many experimental endeavors have been tried to overcome the BCRP mediated atypical MDR. The mechanism of RNAi-triggered mRNA

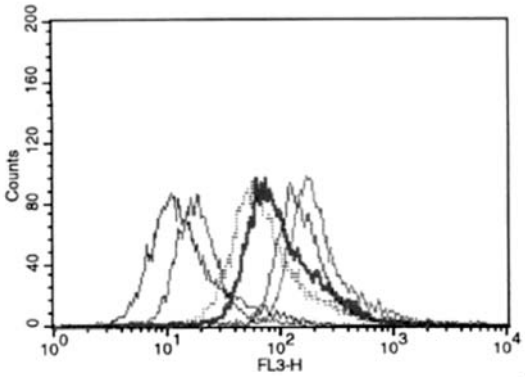

Figure 3. The lines from left to right stand for the fluorescence intensity of mitoxantrone in MCF-7/MX100 cells without mitoxantrone; MCF-7/MX100 cells, MCF-7/MX100 cells treated with pSUPER--BCRP-A, pSUPER-BCRP-B and pSUPER for an hour's efflux; and MCF-7/MX100 cells without efflux.

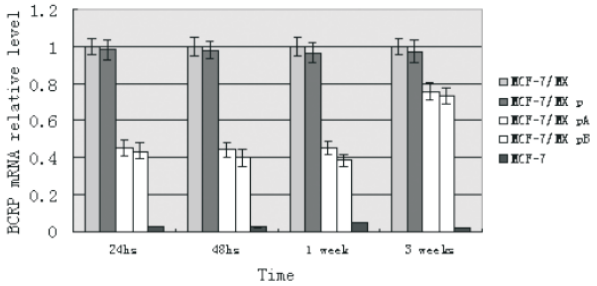

Figure 4. The BCRP mRNA relative level of MCF-7/MX is regarded as 1, then the BCRP mRNA relative level of MCF-7/MX treated with pSUPER-BCRP-A and pSUPER-BCRP-B was 0.45 and 0.38, the BCRP mRNA relative level of MCF-7 is 0.023.

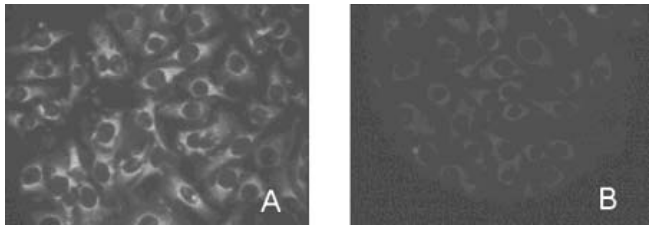

Figure 5. Results of BCRP staining with 5D3 clone
A: MCF-7/MX100 B: MCF-7/MX100 transfected with pSUPER-BCRPs, the intensity of MCF-7/MX100 transfected with pSUPER-BCRPs is much less than that of MCF-7/MX100.

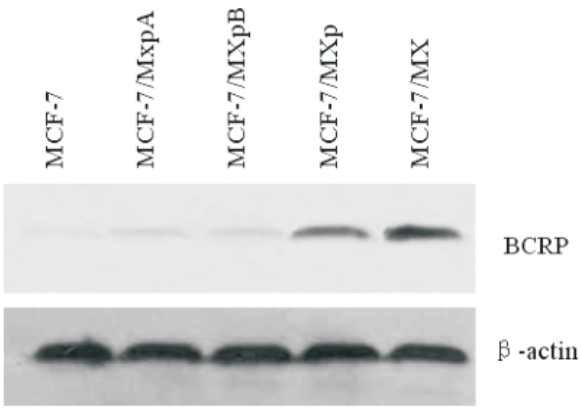

Figure 6. BCRP is a 72kD transmembrane transport protein, the Western blot analyses showed that both constructs can knockdown the expression of BCRP, but none of the cases the cellular BCRP content could be reduced completely.

destruction represents a powerful tool for the application of gene therapy of cancer, since drug resistance-mediating molecules are the potential targets for such a gene therapeutic strategy. Chemically synthesized 21-nt siRNA duplexes against two regions of the P-gp-encoding mRNA have been designed for disruption of P-gp-mediated drug extrusion in a specific manner and resensitization of gastrointestinal tumor cells to treatment with the antineoplastic agent daunorubicin [20].

In the present study, the modulation of the BCRP mediated atypical MDR phenotype was demonstrated by RNAi triggered by transfecting pSUPER-BCRP vectors producing shRNA targeting for BCRP-encoding mRNA into BCRP expressing cell lines. Both pSUPER-BCRP constructs showed a pronounced BCRP gene-silencing activity in BCRP expressing cells MCF-7/MX100 at the mRNA level determined by real-time PCR. Moreover, cytotoxicity assays with mitoxantrone and efflux assay of mitoxantrone demonstrated that they are all able to inhibit the mitoxantrone transporter effectively. The atypical MDR phenotype induced by BCRP could be reversed 51% and 55% in MCF-7/MX100 by pSUPER-BCRP-A and pSUPER-BCRP-B respectively. In addition, although the effect of instantaneous transfection using siRNA-containing expression vector on the multidrug resistant cell lines begin to weaken due to mitosis, it can last at least more than three weeks, longer than that of chemically synthesized siRNAs. Moreover, in the clinical situation, three weeks may be enough for a period of treatment and a two-fold or three-fold increased resistance level is already sufficient to inhibit a successful antineoplastic drug-based cancer therapy. Thus, for the potential treatment of cancer patients, even a transient application of anti-BCRP mRNA siRNA constructs might be an effective tool for the reversal of drug resistance.

A future challenge is to determine these requirements for effective siRNAs, which is likely to be determined by the accessibility of its target sequence in the intended substrate, in addition to the sequences that form the stems of the hairpin siRNAs, the loop size and the sequences at the base of the loop might also have a role in determining siRNA [4]. In this experiment, the chemosensitizing effects of both pSUPER--BCRP constructs in inhibiting BCRP are similar to each other, from 51% reversal to 55% reversal in MCF-7/MX100, this give evidence that the secondary structure of siRNA target mRNAs does not appear to have a strong effect on gene silencing [11].

In the experiment, the mixture of the two pSUPER-BCRP constructs had been used together to target for BCRP mRNA and to observer whether it has a more powerful effect on reversing this atypical MDR mediated by BCRP, contrary to what was expected, the chemosensitizing effect of these pSUPER-BCRP constructs mixture in inhibiting BCRP is even no more than that of pSUPER-BCRP-A. A vector expressing more than one shRNA targeting for different regions of BCRP gene will be constructed, to observe whether several shRNAs targeting for different regions of BCRP gene in the same cell can cooperate with each other.

In summary, RNAi targeting BCRP deliveried by pSUPER can degrade BCRP mRNA specially and resensitizes BCRP expressing cells to mitoxantrone cytotoxicity. RNAi is of interest as a new tool for in vitro studies as well as in functional assays of BCRP-dependent drug efflux activity for individual tailoring of regiments with potential therapeutic use. However both RNAi targeting BCRP constructs can not reverse BCRP mediated MDR completely, this may due to low transfection efficiency and transient transfection, reach the transfection efficiency of plasmids with EGFP and CMV promoter on MCF-7/MX100 is no more than 70%. More pronounced MDR-modulating effects of siRNA constructs could be expected by stable transfection of the multidrug-resistant cell lines using siRNA-containing expression vectors, such as adenovirus [32] and lentiviral [2] vector which are useful for more efficient and stable gene suppression in human cells, and a stable expression of small interfering RNAs targeting MDR1 can reverse multidrug resistance completely [31]. In addition, BCRP can be expressed and play an important role in normal tissue, and how to disrupt the replication of BCRP in multidrug resistant tumor cells selectively seems to be another difficult problem.

References

[1] ALLEN JD, VAN LOEVEZIJN A, LAKHAI JM,VAN DER VALK M, VAN TELLINGEN O et al. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. Mol Cancer Ther 2002; 1: 417–425.

[2] ATADJA P, WATANABE T, XU H, COHEN D. PSC-833, a frontier in modulation of P-glycoprotein mediated multidrug resistance. Cancer Metastasis Rev 1998; 17: 163–168.

[3] BORST P, OUDE ELFERINK R. Mammalian ABC transporters in health and disease. Annu Rev Biochem 2002; 71: 537–592.

[4] BRUMMELKAMP TR, BERNARDS R, AGAMI R. A system for stable expression of short interfering RNAs in mammalian cells. Science 2002; 296: 550–553.

[5] BURGER H, FOEKENS JA, LOOK MP, MEIER-VAN GELDER ME, KLIJN JG et al. RNA expression of breast cancer resistance protein, lung resistance-related protein, multidrug resistance-associated proteins 1 and 2, and multidrug resistance gene 1 in breast cancer: correlation with chemotherapeutic response. Clin Cancer Res 2003; 9: 827–836.

[6] DE BRUIN M, MIYAKE K, LITMAN T, ROBEY R, BATES SE. Reversal of resistance by GF120918 in cell lines expressing the ABC half-transporter, MXR. Cancer Lett 1999; 146: 117–126.

[7] DOYLE LA, YANG W, ABRUZZO LV, KROGMANN T, GAO Y et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci U S A 1998; 95: 15665–15670.

[8] ERLICHMAN C, BOERNER SA, HALLGREN CG, SPIEKER R, WANG XY et al. The HER tyrosine kinase inhibitor CI1033 enhances cytotoxicity of 7-ethyl-10-hydroxycamptothecin and topotecan by inhibiting breast cancer resistance protein-mediated drug efflux. Cancer Res 2001; 61: 739–748.

[9] FANEYTE IF, KRISTEL PM, MALIEPAARD M, SCHEFFER GL, SCHEPER RJ et al. Expression of the breast cancer resistance protein in breast cancer. Clin Cancer Res 2002; 8: 1068–1074.

[10] GOTTESMAN MM, FOJO T, BATES SE. Multidrug resistance in cancer: role of ATP-depending transporters. Nat Rev Cancer 2002; 2: 48–58.

[11] HARBORTH J, ELBASHIR SM, VANDENBURGH K, MANNINGA H, SCARINGE SA et al. Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. Antisense Nucleic Acid Drug Dev 2003; 13: 83–105.

[12] IMAI Y, TSUKAHARA S, ISHIKAWA E, TSURUO T, SUGIMOTO I. Estrone and 17beta-estradiol reverse breast cancer resistance protein-mediated multidrug resistance. Jpn J Cancer Res 2002; 93: 231–235.

[13] JIA P, WU SB, XU Q, WU MF, GAO QL et al. Antisense oligonucleotide reverses topotecan-resistant ovarian cancer cells. Ai Zheng 2003; 22: 1296–1300.

[14] KOWALSKI P, STEIN U, SCHEFFER GL, LAGE H. Modulation of the atypical multidrug-resistant phenotype by a hammerhead ribozyme directed against the ABC transporter BCRP/MXR/ABCG2. Cancer Gene Ther 2002; 9: 579–586.

[15] LAGE H. ABC-transporters: implications on drug resistance from microorganisms to human cancers. Int J Antimicrob Agents 2003; 22: 188–199.

[16] LAGE H, PERLITZ C, ABELE R. Enhanced expression of human ABC-transporter tap is associated with cellular resistance to mitoxantrone. FEBS Lett 2001; 503: 179–184.

[17] LAUTIER D, CANITROT Y, DEELEY RG, COLE SP. Multidrug resistance mediated by the multidrug resistance protein (MRP) gene. Biochem Pharmacol 1996; 52: 967–977.

[18] LEE JS, PAULL K, ALVAREZ M, HOSE C, MONKS A et al. Rhodamine efflux patterns predict P-glycoprotein substrates in the National Cancer Institute drug screen. Mol Pharmacol 1994; 46: 627–638.

[19] MALIEPAARD M, VAN GASTELEN MA, TOHGO A, HAUSHHER4 FH, VAN WAARDENBURGH RC et al. Cir-cumvention of breast cancer resistance protein (BCRP)-mediated resistance to camptothecins in vitro using non-substrate drugs or the BCRP inhibitor GF120918. Clin Cancer Res 2001; 7: 935–941.

[20] NIETH C, PRIEBSCH A, STEGE A, LAGE H. Modulation of the classical multidrug resistance (MDR) phenotype by RNA interference (RNAi). FEBS Lett 2003; 545: 144–150.

[21] NISHITSUJI H, IKEDA T, MIYOSHI H, OHASHI T, KANNAGI M, MASUDA T. Expression of small hairpin RNA by lentivirus-based vector confers efficient and stable gene-suppression of HIV-1 on human cells including primary non-dividing cells. Microbes Infect 2004; 6: 76–85.

[22] PAUL CP, GOOD PD, WINER I, ENGELKE DR. Effective expression of small interfering RNA in human cells. Nat Biotechnol 2002; 20: 505–508.

[23] PEREGO P, DE CESARE M, DE ISABELLA P, CARENINI N, BEGGIOLIN G. A novel 7-modi.ed camptothecin analog overcomes breast cancer resistance protein-associated resistance in a mitoxantrone-selected colon carcinoma cell line. Cancer Res 2001; 61: 6034–6037.

[24] RABINDRAN SK, ROSS DD, DOYLE LA, YANG W, GREENBERGER LM. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. Cancer Res 2000; 60: 47–50.

[25] ROBEY RW, HONJO Y, VAN DE LAAR A, MIYAKE K, REGIS JT et al. A functional assay for detection of the mitoxantrone resistance protein, MXR (ABCG2). Biochim Biophys Acta 2001; 1512: 171–182.

[26] ROSS DD, YANG W, ABRUZZO LV, DALTON WS, SCHNEIDER E et al. Atypical multidrug resistance: breast cancer resistance protein messenger RNA expression in mitoxantrone-selected cell lines. J Natl Cancer Inst 1999; 91: 429–433.

[27] SIKIC, BI. Pharmacologic approaches to reversing multidrug resistance. Semin Hematol 1997; 34: 40–47.

[28] SKEHAN P, STORENG R, SCUDIERO D, MONKS A, MCMAHON J et al. New colorimetric cytotoxicity assay for anticancer--drug screening. J Natl Cancer Inst 1990; 82: 1107–1112.

[29] VAN LOEVEZIJN A, ALLEN JD, SCHINKEL AH, KOOMEN GJ. Inhibition of BCRP-mediated drug efflux by fumitremorgin--type indolyl diketopiperazines. Bioorg Med Chem Lett 2001; 11: 29–32.

[30] WOEHLECKE H, OSADA H, HERRMANN A, LAGE H. Reversal of breast cancer resistance protein-mediated drug resistance by tryprostatin A. Int J Cancer 2003; 107: 721–728.

[31] YAGUE E, HIGGINS CF, RAGUZ S. Complete reversal of multidrug resistance by stable expression of small interfering RNAs targeting MDR1. Gene Ther 2004; 11: 1170–1174.

[32] ZHAO LJ, JIAN H, ZHU H. Specific gene inhibition by adenovirus-mediated expression of small interfering RNA. Gene 2003; 316: 137–141.