Reversal of MDR1/P-glycoprotein-mediated multidrug resistance by RNA interference

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Abstract. Simultaneous resistance of cancer cells to multiple cytotoxic drugs, multidrug resistance (MDR), is the major limitation to the successful chemotherapeutic treatment of disseminated neoplasms. The “classical” MDR phenotype results from decreased drug accumulation mediated by the adenosine triphosphate binding cassette (ABC)-transporter P-glycoprotein (MDR1/P-gp, ABCB1), the product of the human MDR1 gene. Inhibition of the drug extrusion activity of MDR1/P-gp by low-molecular weight pharmacologically active compounds as a method to reverse MDR in cancer patients has been studied extensively, but the clinical results have generally been disappointing. Thus, experimental therapeutic strategies to overcome MDR were developed. These strategies included gene therapeutic approaches with antisense oligonucleotides (ODNs) or ribozymes, and, most recently, the application of the RNA interference (RNAi) technology. RNAi is a physiological double stranded RNA-triggered mechanism resulting in gene-silencing in a sequence-specific manner. Transient RNAi can be attained by application of small interfering RNAs (siRNAs), whereas a stable RNAi-mediated gene-silencing can be achieved by transfection of mammalian cells with short hairpin RNA (shRNA) encoding expression vectors. Both techniques were applied to overcome MDR1/P-gp-mediated MDR in different in vitro models. In this mini review, the utilization of RNAi technology as a potential gene therapeutic tool for reversal of MDR will be discussed. © 2005 Elsevier B.V. All rights reserved.

Keywords: Drug resistance; Gene therapy; RNAi; MDR1/P-glycoprotein; ABCB1

1. Introduction

Drug resistance is the major reason why antineoplastic drug treatment modalities of human malignancies may fail. Human cancer cells can exhibit a cross-resistant phenotype against several unrelated antineoplastic drugs that differ widely with respect to molecular
structure and target specificity. This phenomenon has been termed multidrug resistance (MDR) [1]. The “classical” MDR phenotype is characterized by a typical cross-resistance pattern against structurally unrelated anticancer agents including vinca alkaloids, anthracyclines, or taxanes, and the reversibility by the calcium channel inhibitor verapamil and cyclosporin A derivatives. The underlying mechanism conferring this MDR phenotype is the cellular overproduction of the MDR1 gene encoded 170-kDa, membrane-spanning P-glycoprotein (MDR1/P-gp, P-170, PGY1, MDR1, ABCB1) [2], member of the superfamily of ABC (adenosine triphosphate binding cassette)-transporters [3]. An inhibition of MDR1/P-gp-mediated drug extrusion results in a re-sensitization of tumor cells to treatment with antineoplastic agents, and therewith, may allow a successful drug treatment of the multidrug-resistant cancer cells.

Low molecular weight pharmacologically active compounds, designated as MDR modulators or chemosensitizers, may circumvent the “classical” MDR phenotype by inhibiting the efflux pump activity of MDR1/P-gp [4–6]. An obstacle in applying classical MDR modulators arises from their commonly occurring intrinsic toxicity at doses necessary to be active, e.g. heart failure, hypotension, hyperbilirubinemia, and immunosuppression by cyclosporin A. Moreover, improved so-called second generation MDR modulators were demonstrated to induce enhanced activity of liver enzymes of the family of cytochrome P450 mixed-function oxidases resulting in an increased pharmaco-kinetic turn over of the applied anticancer drugs. Additionally, tumor cells can acquire resistance against the applied chemosensitizers, a so-called tertiary resistance. Consequently, it is necessary to develop alternative, less toxic and more efficient strategies to overcome MDR. Such an alternative procedure to circumvent MDR1/P-gp-mediated MDR in cancer cells is to prevent the biosynthesis of MDR1/P-gp by selectively blocking the expression the MDR1/P-gp-specific MDR1 mRNA by gene therapeutic technologies. This approach is aimed at increasing the efficiency and specificity of chemosensitization of multidrug-resistant cancer cells while at the same time reducing toxicity and undesirable side effects. Thus, in previous studies, antisense oligonucleotides (ODN) [7–9], or hammerhead ribozymes [10–12] were applied to modulate MDR1/P-gp-dependent MDR by decreasing the expression level of the MDR1/P-gp encoding mRNA. Recently, the RNA interference (RNAi) technology was introduced for specific gene expression.

Table 1
Studies on reversal of MDR1/P-gp-mediated MDR by RNAi

<table>
<thead>
<tr>
<th>Studies</th>
<th>Cell lines</th>
<th>Tumor origin</th>
<th>Duration of RNAi</th>
<th>Promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nieth et al. [25]</td>
<td>EPG85-257 RDB</td>
<td>Gastric carcinoma</td>
<td>Transient</td>
<td>-</td>
</tr>
<tr>
<td>Nieth et al. [25]</td>
<td>EPPP 85-181RDB</td>
<td>Pancreatic carcinoma</td>
<td>Transient</td>
<td>-</td>
</tr>
<tr>
<td>Wu et al. [26]</td>
<td>MCF-7/AdrR</td>
<td>Breast cancer</td>
<td>Transient</td>
<td>-</td>
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<tr>
<td>Wu et al. [26]; Arora et al. [27]</td>
<td>MCF-7/BC-19</td>
<td>Breast cancer</td>
<td>Transient</td>
<td>-</td>
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<tr>
<td>Wu et al. [26]; Arora et al. [27]</td>
<td>A2780Dx5</td>
<td>Ovarian carcinoma</td>
<td>Transient</td>
<td>-</td>
</tr>
<tr>
<td>Duan et al. [28]</td>
<td>SKOV-3TR</td>
<td>Ovarian carcinoma</td>
<td>Transient and stable</td>
<td>U6</td>
</tr>
<tr>
<td>Duan et al. [28]</td>
<td>OVCAR8TR</td>
<td>Ovarian carcinoma</td>
<td>Transient and stable</td>
<td>U6</td>
</tr>
<tr>
<td>Peng et al. [29], Peng et al. [38]</td>
<td>K562/A02</td>
<td>CML</td>
<td>Transient</td>
<td>-</td>
</tr>
<tr>
<td>Stege et al. [31]</td>
<td>EPG85-257 RDB</td>
<td>Gastric carcinoma</td>
<td>Stable</td>
<td>H1</td>
</tr>
<tr>
<td>Xu et al., [30]</td>
<td>NCI/ADR-RIES</td>
<td>Breast cancer</td>
<td>Transient and stable</td>
<td>U6</td>
</tr>
<tr>
<td>Yagie et al. [32]</td>
<td>KD30</td>
<td>CML</td>
<td>Stable</td>
<td>H1</td>
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</tbody>
</table>
suppression into biomedical research and applied for modulation of the “classical” MDR phenotype in different in vitro models (Table 1). In this mini overview, the application of the RNAi technology to overcome MDR in human cancers will be discussed.

2. RNA interference (RNAi) technology

Currently, the use of siRNAs as a means for specific inhibition of a gene of interest is under extensive investigation [13]. These between 21 and 25 nucleotides (nt) long, double-stranded RNA (dsRNA) molecules can direct degradation of eukaryotic mRNAs in a sequence-specific manner. This ubiquitous mechanism of gene regulation in plants and animals was designated as RNA interference (RNAi) [14]. Physiologically, the first step of RNAi is initiated by the processing of long regulatory dsRNA into small interfering RNA (siRNA) by the dsRNA-specific RNase III enzyme Dicer. By interaction of these siRNAs with the protein interaction domain PAZ (PIWI-Argonaute-Zwille/Pinhead) [15] of members of the Argonaute or PPD (PAZ/PIWI domain) protein family, the siRNAs are incorporated into a multiprotein RNA-inducing silencing complex (RISC). For incorporation of siRNAs into RISC, it is necessary that siRNAs are phosphorylated at the 5' end [16]. RISC becomes activated by unwinding the siRNA duplex. The antisense RNA strand guides RISC to the homologous sequence on the target mRNA for endoribonucleolytic digestion. The cleavage of the target mRNA occurs at a single site, 10 nt from the 5' phosphate of the antisense strand of the unwound siRNA molecule [17]. Due to the loss of the 7-methylguanine cap structure of the 3' cleavage product and the loss of the poly(A)-tail of the 5' cleavage product, the digested target mRNA is no longer protected against endogenous RNases and can be degraded (Fig. 1).

2.1. Exogenous delivery of siRNA molecules

The requirement for Dicer in maturation of siRNAs can be bypassed by introducing synthetic 21-nt siRNA duplexes that inhibit expression of transfected and endogenous genes in a variety of mammalian cells [18]. However, a major disadvantage of this approach is the transient gene expression-inhibiting effect of chemically synthesized siRNAs. Thus, the development of more effective and stable gene-silencing RNAi-mediating systems is of huge interest.

2.2. Endogenous expression of shRNAs

For stable long termed RNAi effects, expression vectors have been developed. These approaches use plasmid or viral expression vectors containing an expression cassette for the production of siRNA-like transcripts; that are, synthesis of shRNAs [19]; or as alternative, expression cassettes driving the production of sense and antisense strands separately, whereby the two strands hybridize inside the cell to form functional active siRNA [20,21].

Although it would be possible to apply RNA-polymerase II-driven promoters for expression of such short transcripts, in these gene-silencing expression vectors, commonly RNA polymerase III-specific promoters are used, i.e. the H1-RNA promoter [19], physiologically, this promoter drives the expression of a gene encoding H1-RNA, the RNA component of the human RNase P; U6-RNA promoter [22,23] physiologically
Fig. 1. RNAi pathways used for suppression of MDR1 gene expression: biological active anti-MDR1 RNAi-mediating molecules were chemically synthesized as siRNAs, or they were synthesized from expression vectors as short hairpin RNA (shRNA) which will be cleaved into siRNAs by the endogenous cellular RNase III enzyme Dicer. Expression vectors and siRNAs are transfected into cells directly using cationic lipid formulations. The expression cassettes commonly used RNA-polymerase III promoter-driven systems, i.e. H1-RNA or U6-RNA promoter. The pharmacological active siRNAs are recruited by members of the Argonaute family via their PAZ domain to form a ribonucleotide protein complex (RNP). The siRNAs are unwound before activation of RISC (RNA-induced silencing complex) and the strand complementary to the MDR1 target mRNA is incorporated into RISC. Beside other proteins, RISC contains an endonuclease that cleaves only the MDR1 target mRNA within the hybridized region. P, RNA-polymerase III promoter; T5, RNA-polymerase III termination signal.

driving the expression of the U6 small nuclear RNA which plays a crucial role in the processing of premature RNA; or the tRNA promoter tRNA_{VAL} [24]. The RNA-polymerase III-depending promoters have a defined start of transcription and a termination signal consisting of five consecutive thymidines (T5). Therewith, it can be used to direct the synthesis of small RNA molecules of interest lacking a poly-adenosin tail. Cleavage of the RNA transcript at the termination site is after the second uridine. Thus, the H1-RNA promoter produced small RNA is similar to the ends of chemically synthesized siRNAs.
containing two 3’ overhanging thymidines or uridines. The sequence of interest consists of a 19-nt sequence homologous to the target mRNA, linked with a 3–9-nt spacer sequence to the reverse complement of the same 19-nt target-specific sequence. The synthesized RNA transcript folds back to its complementary strand to form a 19 base pair shRNA molecule, which is then processed by Dicer to a corresponding siRNA molecule and passed into the RNAi pathway (Fig. 1). For suppression of MDR1 gene expression, H1-RNA promoter as well as U6-RNA promoter-depending expression cassettes were designed (Table 1). For better comparability of siRNA- and shRNA-mediated RNAi, the features of siRNAs and shRNAs are summarized in Table 2.

### 3. RNAi-mediated inhibition of MDR1/P-glycoprotein

Transient siRNA-mediated down regulation of the MDR1/P-gp expression resulted in relatively moderate inhibition in different cell models [25–30]. Since these studies used different experimental techniques, different protocols of proliferation assays [25,27–30] or clonogenic assays [26], to determine the extent of the MDR reversal, the efficiencies of the different transient RNAi approaches are not directly comparable. However, the most pronounced transient MDR reversal of nearly 90% was achieved in the pancreatic carcinoma-derived cell line EPP85-181RDB [25]. These data indicate that the efficacy of the RNAi effect may be siRNA sequence-dependent as well as cell line-dependent.

Moreover, some stable anti-MDR1/P-gp shRNA expression vectors were used to modulate the MDR phenotype [28,30–32]. In one of these studies, the stable RNAi effect was similar or less effective than a transient approach in the same cell model, the ovarian carcinoma-derived cell line SKOV-3 TR [28]. A more pronounced efficacy of a stable RNAi approach than a transient RNAi strategy was achieved in the breast carcinoma cell line NCI/ADR-RES [30], but also in this system the reversal of the multidrug-resistant phenotype was not complete. In both studies U6-RNA promoter-driven shRNA expression vectors were used. In contrast, two other investigations used H1-RNA promoter-depending shRNA expression cassettes [31,32]. In both studies, the multidrug-resistant phenotype of the gastric carcinoma cell line EPG85-257 RDB or the chronic myelogenous leukemia (CML) K562-derived cell line KD30 was completely reversed by shRNA-mediated RNAi.

### 4. Comparison of RNAi and alternative RNA technologies to inhibit MDR1/P-glycoprotein

Controversial data are available whether RNAi or other RNA technology-based gene-silencing technologies provide a higher gene-inhibitory specificity and potency. For
example, Bertrand et al. [33] reported that siRNAs appear to be quantitatively more efficient and its effect is lasting for a longer time in cell culture than antisense ODNs, or Yokota et al. [34] showed that siRNAs much more efficiently suppressed gene expression than ribozymes. In contrast to these studies, Vickers et al. [35] reported that the potency, maximal effectiveness, duration of action, and sequence specificity of antisense ODNs and siRNAs were found to be comparable. In the case of transient MDR 1/P-gp inhibition, it was demonstrated that several anti-MDR 1/P-gp siRNAs were clearly more potent than phosphorothioate antisense ODNs [30]. However, using methoxyethoxy- or hexitol-based antisense compounds, the same authors achieved MDR1/P-gp inhibition paralleling their siRNA effects [30]. For stable reversal of MDR1/P-gp-mediated MDR, a ribozyme-based [10] as well as a RNAi-based approach [31,32] demonstrated a complete knock down of MDR1 gene expression. Thus, for suppression of MDR1/P-gp, antisense derivatives and ribozymes may offer effectiveness comparable with that of RNAi.

5. Challenges for RNAi-mediated MDR1/P-gp suppression

Beside “delivery”, the general obstacle of cancer gene therapy, additional problems for clinical MDR1/P-gp inhibition by anti-MDR1/P-gp shRNA expression vectors may arise by the physiological expression of this ABC-transporter in several epithelial and endothelial cells. For example, MDR1/P-gp is strongly expressed in the luminal membranes of the endothelium of blood vessels in the brain [36] suggesting that this efflux pump plays an important role in the blood–brain barrier and therewith is crucial for limiting the potential neurotoxicity of many anticancer drugs. Thus, in the clinical situation it may be necessary that the application of anti-MDR1/P-gp RNAi approaches should be restricted to MDR1/P-gp-expressing multidrug-resistant cancer cells. One promising strategy for cancer cell-limited delivery of anti-MDR1 siRNAs would be the development of vector systems specific for multidrug-resistant cancer cells. Recently, a “replication-defective” E1A-mutant adenoviral vector that efficiently and selectively replicates in “classical” multidrug-resistant cells has been described [37]. Such a virus may provide the basis for the development of novel vectors for the specific gene therapeutic treatment of multidrug-resistant cancer cells.

6. Conclusion

RNAi technology has an enormous potential for the development of gene therapeutic strategies against neoplastic cells. Taken together, the argued studies demonstrated that RNAi may be a promising strategy to reverse MDR of human cancer cells. However, in clinics anti-MDR1/P-gp strategies may show the highest efficacy in combination therapies with conventional chemotherapeutic regimens. In future experimental endeavors, evaluation of RNAi-based reversal of MDR in mouse models will provide information to what extent this gene therapeutic approach will really become translated into clinical practice.

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References


Discussion

De Boer
How do you think you can choose the optimal siRNA for your pancreatic cells? Are there specific rules?

Lage
It is possible to modulate the structure of the siRNA molecule. For example, you can use different sizes and sequences of the loop structures and this may have some influence on the efficiency. On the other hand, you can use another promoter: up to now we don’t know why it doesn’t work. I saw data from a group who presented mutation analysis of the siRNA molecules and independent of all rules, you obtain any effect you can imagine. Mutation can eliminate gene-silencing effects, it can down-regulate, may have no influence or can increase them.

Galla
I have a question with respect to the in vivo system. If I understand it correctly, you bring the RNAi with this jet technique into the tumour. That means you reach only part of the tumour and then you have, let’s say, something like 30–50% transfected cells. Which proportion of the tumour do you really reach with that technique?

Lage
In these experiments we did not measure it exactly, but we reach about 60% of the tumour cells.
Sugiyama
If you locally inject the siRNA, it may not reach all the tumour cells because of the slow diffusion of the siRNA. To overcome the multidrug resistance, almost all the tumour cells should have the siRNA. So then, of course it may be better for you to administer siRNA using a specific drug delivery system. In my laboratory we have been trying to do that kind of thing, but even in the hepatocyte there is inefficient transfection of the siRNA, and with our technique we get, at most, only 50% of the hepatocytes transfected by siRNA. In my group, to knock down some transporters in some tissues we are now using the transgenic approach to make knock-down animals. It is good for experimental purposes, but it’s not good for therapy purposes. How are you going to overcome that kind of issue in vivo?

Lage
You are right. This is an experimental model and delivery is a problem for all gene therapeutic applications and that was not our question to deliver. We wanted to demonstrate that this system could work and that’s all. You’re right; this is not the best method.

Whittle
What do you think is underlying the biological variability in response of the pancreatic and gastric tumour cells? One is usually out and the other is not, why is that? The tissue specificity for the action of the RNAi?

Lage
I don’t know. Perhaps pancreatic cells have a much more increased level of cellular RNAases.

Whittle
Did you take several gastric cell lines and several pancreatic cell lines to see if the effect was consistent through both tumour types?

Lage
It was one cell line and we analysed several clones, in each case about 50 clones. In the pancreatic model we did not find any clone with any effect whereas in the gastric carcinoma cells we found about 1/3 with complete down-regulation, 1/3 with moderate down-regulation and 1/3 without any effect.

Van Tellingen
If I would listen to our clinicians in our institute, reversing MDR1/P-glycoprotein with small chemical drugs has been largely unsuccessful in therapy. Now you’re doing something the same but in a different way. What exactly would you expect to achieve with that? In my view, even though most of the MDR inhibitors, such as varspodar or evapodar, may not have the ideal properties. I would have expected some kind of effect in those tumours if P-glycoprotein was indeed the reason why tumours would become resistant and that is exactly what you are trying. I hear our clinicians say, it’s old wine in new sacks. What is your comment on this?
First, this is an experimental model to study such effects. Second, the clinical application of MDR modulators failed due to different reasons. One of these reasons is that the patients were not diagnosed exactly, if they expressed MDR1 or not, and you won’t have any effect, if the tumour expresses, for example, BCRP or modulates apoptotic pathways and is completely independent of MDR1. On the other hand, a vision could be to combine such strategies with other strategies. For example, in collaboration with a group in Munich we developed an oncolytic adenovirus that specifically replicates in multidrug resistant cells that express the MDR1/P-glycoprotein, and perhaps it would be a strategy to combine such oncolytic virus with anti MDR1 siRNAs in chemotherapy.

I think it is very true as you state that most studies have been performed in not very well characterised patients. In that case, of course, it would be very logical also to try to test this in diseases where some efficacy has been shown, for example leukemia-like tumours. I have another question. Do you envisage the use of this type of work for increasing drug uptake into the brain? Because one of my concerns, I think everybody’s concern, would be you don’t have much control over the time that you’re actually opening up the blood–brain barrier, at least for the P-glycoprotein part of it, and we don’t want the barrier opened for all the time. What do you think about that?

If you use such a strategy perhaps it would be a good idea to use different kinds of promoters, for example you can use inducible promoters, so you can perform time-depending inhibition of the ABC transporter. This approach is working, we have constructed such vectors with tetracycline-inducible siRNA expression but these experiments have just begun.

Could you say when you would to choose the ribozyme approach and when the RNAi approach?

From my point of view, both technologies show the same potential and the same problems.