Delivery of short hairpin RNAs by transkingdom RNA interference modulates the classical ABCB1-mediated multidrug-resistant phenotype of cancer cells

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Delivery of RNA interference (RNAi)-mediating agents to target cells is one of the major obstacles for the development of RNAi-based therapies. One strategy to overcome this barrier is transkingdom RNAi (tkRNAi). This technology uses non-pathogenic bacteria to produce and deliver therapeutic short hairpin RNA (shRNA) into target cells to induce RNAi. In this study, the tkRNAi approach was used for modulation of the “classical” ABCB1-mediated multidrug resistance (MDR) in human cancer cells. Subsequent to treatment with anti-ABCB1 shRNA expression vector bearing E. coli, MDR cancer cells (EPG85 257RDB) showed 45% less ABCB1 mRNA expression. ABCB1 protein expression levels were reduced to a point at which merely a weak band could be detected. Drug accumulation was enhanced 11-fold, to an extent that it reached 45% of the levels in non-resistant cells and resistance to daunorubicin was decreased by 40%. The data provide the proof-of-concept that tkRNAi is suitable for modulation of “classical” MDR in human cancer cells. Overall, the prototype tkRNAi system tested here did not yet attain the levels of gene silencing seen with conventional siRNAs nor virally delivered shRNAs; but the tkRNAi system for gene-silencing of ABCB1 is still being optimized, and may become a powerful tool for delivery of RNAi effectors for the reversal of cancer MDR in future.

Introduction

Since the initial demonstration that gene-silencing RNA interference (RNAi) pathways can be triggered in mammalian cells through treatment with double-stranded short interfering RNA (siRNA),1 RNAi technology was introduced in many bio-medical research laboratories. In addition to the application of the RNAi platform for high-throughput analyzes and functional investigations, the RNAi approach has also been considered for the development of the next new class of targeted drugs. In principle, RNAi effectors can be used to knock down expression of one or several genes involved in a pathophysiological process. For treatment of malignant diseases, genes promoting cancer cell growth or survival, chemo- and radiotherapy resistance, or metastasis all represent ideal targets for RNAi-based therapeutics. Small RNAs may also be useful to mimic or antagonize micro RNAs (miRNAs) involved in regulation of oncogenic or tumor suppressor pathways. While developments in the clinical application of RNAi have progressed rapidly in recent years, the major obstacle of this technology has not been resolved. The issue lies in the difficulty in delivering the RNAi effectors to target cells and tissues thereby resulting in a bottleneck for successful pharmacological usage.2 This problem must to be solved before RNAi technology can become a realistic therapeutic option in clinical practice.

Although many published studies reported that in vivo delivery of RNAi effectors is possible, effective delivery to tissues in vivo remains a challenge. Thus numerous efforts are underway to develop both more efficient methods and new delivery concepts to target therapeutic RNAi effectors to cells of interest-such as cancer cells. One of these promising new delivery strategies is transkingdom RNAi (tkRNAi).3 This technology uses non-pathogenic bacteria to produce and deliver therapeutic short hairpin RNA (shRNA)-encoding plasmid DNA into target cells to hijack the cellular RNAi machinery (Fig. 1). In this first generation tkRNAi vector, TRIP, the expression of the shRNA of interest is controlled by the bacteriophage T7 promoter. Furthermore, the TRIP vector contains the Inv locus from Yersinia pseudotuberculosis that encodes invasin, which permits the noninvasive bacteria to enter ß1-integrin-positive mammalian cells and the HlyA gene from Listeria monocytogenes, which produces listeriolysin O thereby permitting the shRNA to escape from entry vesicles. TRIP constructs are introduced into a competent non-pathogenic Escherichia coli strain BL21(DE3), which contains

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the T7 RNA polymerase necessary for the T7 promoter-driven synthesis of shRNAs. So far, the tkRNAi approach has been successfully applied for silencing catenin-β1 in human colon cancer cells in vitro as well as in vivo models.1

To evaluate its potency and efficacy in a completely different model system, with clinical impact, the tkRNAi strategy was applied for targeting the multidrug resistance (MDR)-mediating drug extrusion pump ABCB1 (MDR1/P-gp) in multidrug-resistant cancer cells. Notwithstanding the introduction of new targeted anti-cancer drugs, MDR still constitutes one of the main obstacles to the successful chemotherapeutic treatment of cancer.4 One of the best characterized mechanisms involved in MDR is the enhanced activity of the membrane-embedded ATP binding cassette (ABC)-transporter ABCB1. Reversal of MDR by RNAi-mediated inhibition of ABCB1 has already been characterized in detail in various cell models.5 Thus, ABCB1 represents an ideal target molecule for independent evaluation of the tkRNAi approach.

Results

Internalization and lysis of E. coli in cancer cells. Internalization and lysis of anti-ABCB1 shRNA-expressing E. coli in multidrug-resistant EPG85-257RDB cells was detected by fluorescent microscopy following DAPI staining. The time course of E. coli lysis was performed after bacterial exposure at MOI 1:1,000. As shown in Figure 2A, E. coli were internalized by multidrug-resistant gastric carcinoma cells. After stepwise lysis no bacteria could be detected 14 h post bacterial infection as shown in Figure 2B–F.

Expression of anti-ABCB1 shRNAs in E. coli-treated cancer cells. For quantification of the anti-ABCB1 shRNA expression levels in E. coli-treated multidrug-resistant EPG85-257RDB cells, a real time RT-PCR-based protocol was applied. The PCR was designed for specific detection of the antisense anti-ABCB1 siRNA strand that was produced by intracellular Dicer activity from the anti-ABCB1 shRNA molecule. As shown in Figure 3, 24 hours post infection using MOI 1:500 of E. coli resulted in a 3.8-fold increase of the expression of the antisense anti-ABCB1 siRNA strand as determined by the ΔΔCT method.11 Specific detection of the corresponding anti-ABCB1 siRNA sense strand showed identical results (data not shown).

Downregulation of ABCB1 mRNA expression by E. coli-mediated shRNA delivery. The efficiency of the tkRNAi-mediated downregulation of the expression level of the ABCB1 encoding mRNA was analyzed by quantitative real time
RT-PCR. For calculation of relative expression levels, ABCB1 mRNA expression values were normalized against mRNA expression of the housekeeping enzyme aldolase. As shown in Figure 4A, the classical multidrug-resistant gastric carcinoma cell line EPG85-257RDB exhibited a 346-fold overexpression of the ABCB1-encoding mRNA compared to the drug-sensitive, parental variant EPG85-257P. Treatment with an anti-ABCB1 shRNA-encoding tkRNAi vector TRIP decreased the ABCB1 mRNA level to 55% of the original expression value, i.e., 45% gene-silencing activity on mRNA level in this cancer cell line.

Downregulation of ABCB1 protein expression by E. coli-mediated shRNA delivery. For analyzing the biological effects of the anti-ABCB1 shRNA-encoding tkRNAi vector TRIP on the cellular protein content, western blot analysis was performed. As shown in Figure 5, western blot experiments demonstrated that the ABCB1 protein expression was reduced to a point at which merely a weak band could be detected.

Anthracycline accumulation in E. coli-treated carcinoma cells. The relative cellular accumulation of the anthracycline daunorubicin in nonresistant, parental cells, in “classical” multidrug-resistant cells, and in anti-ABCB1 shRNA treated cells was examined by flow cytometry. As shown in Figure 4B, the drug-sensitive gastric carcinoma cell line EPG85-257P exhibited a considerable accumulation of the anthracycline, that is, 30-fold higher in comparison to the ABCB1-overexpressing MDR variant EPG85-257RDB. The MDR cell line EPG85-257RDB merely shows a negligible drug accumulation. By treating EPG85-257RDB cells with the anti-ABCB1 shRNA-encoding tkRNAi vector TRIP, the drug accumulation could be enhanced to 45% of the drug accumulation of drug-sensitive cells.

Modulation of the drug-resistant phenotype in E. coli-treated carcinoma cells. The anti-ABCB1 shRNA-encoding tkRNAi vector TRIP-mediated reversal of the multidrug-resistant phenotype in gastric carcinoma cells was assessed by comparison of the anthracycline-specific IC_{50} values determined by a cell proliferation assay. By comparing the IC_{50} values, the “classical” multidrug-resistant gastric carcinoma cell line EPG85-257RDB

Figure 2. Invasion and lysis of TRIP-containing bacteria detected by DAPI staining. Infection of multidrug-resistant human cancer cells (EPG85-257RDB) was performed using MOI 1:1,000 of anti-ABCB1 shRNA-encoding TRIP-containing E. coli. Human carcinoma cells are shown (A) 1 h, (B) 10 h, (C) 11 h, (D) 12 h, (E) 13 h and (F) 14 h after bacterial exposure. Non-exposed control cells (EPG85-257RDB) are shown after (G) 1 h, (H) 10 and (I) 14 h. Magnification, 40x.

Figure 3. Quantification of anti-ABCB1 shRNA expression level in gastric carcinoma cells 24 h post bacterial exposure using MOI 1:500 determined by quantitative real time PCR. Fold change values were calculated applying the \(2^{- \Delta \Delta C T}\) method.\(^{10}\) EPG85-257P, non-resistant cancer cells; EPG85-257RDB, multidrug-resistant variant; TRIP/anti-ABCB1 shRNA, anti-ABCB1 shRNA encoding vector administrated by E. coli. P-values were calculated using the student’s t-test (*p < 0.05).
Delivery of therapeutic RNAi effectors to cells of interest remains the main obstacle in the development of new targeted RNAi-based therapeutics. Thus, efforts are ongoing to develop improved as well as new delivery concepts. One of these novel promising RNAi therapeutic delivery technologies is transkingdom RNA interference (tkRNAi). Although the proof of concept that the tkRNAi approach shows gene-silencing activity in vitro and in vivo was produced independent evaluation of the pharmacological efficiency in a completely different model has been missing. Therefore, this study was conducted to assess the biological activity of tkRNAi in a well characterized model, i.e., for reversal of the ABCB1-dependent MDR phenotype of the human gastric carcinoma cell line EPG85-257RDB. This cell model has previously been evaluated for different RNAi strategies such as transient in vitro downregulation of ABCB1 by chemically synthesized siRNA molecules, adenovirus-administered shRNAs, as well as stable knock down by plasmid encoded shRNAs. Identical shRNA sequences had also shown efficacy for in vivo targeting of ABCB1 by RNAi.

This study confirmed that the tkRNAi approach induces targeted gene-silencing resulting in downregulation of the ABCB1-encoding mRNA and the corresponding ABC-transporter molecule. Furthermore, the drug extrusion activity of ABCB1 was inhibited and resulted in a reversion of the drug-resistant phenotype. Overall, the extent of downregulation of the ABCB1-specific mRNA by tkRNAi was less pronounced than levels observed from chemically synthesized siRNA molecules, adenovirus-administered shRNAs, as well as stable knock down by plasmid encoded shRNAs. Identical shRNA sequences had also shown efficacy for in vivo targeting of ABCB1 by RNAi. When a gene therapy-like approach had been used in conjunction with plasmid- or adenovirus-encoded shRNAs, an even more complete knock down of the ABCB1 mRNA expression was noted. The extent of inhibition of drug transport activity and reversal of drug resistance by the tkRNAi platform was less pronounced compared to existing RNAi strategies, and the amount of anti-ABCB1 shRNA recovered from the tkRNAi-treated gastric carcinoma cells was lower compared to that in cells treated with shRNA-encoding adenoviruses.
Several reasons are thought to be responsible for the less pronounced gene silencing activity of the tkRNAi strategy in the investigated cell model; we speculate that each of the components of the tkRNAi system leave room for optimization, be it the bacterial biosynthesis of therapeutic shRNA molecules from the T7 promoter or the lysis of the shRNA containing entry vesicles and the possible damage to the shRNA through chemical modification in the entry vesicles. Variable lengths of the shRNA could lead to inefficient Dicer processing and influence the amount of resulting active siRNAs. Each of these factors may have contributed to a lower efficacy in the prototype tkRNAi system tested here and might present an opportunity to build improved versions.

The proof-of-concept of in vivo efficacy of the first generation tkRNAi vector and bacterial strain was already provided. In that study, human colon cancer cells were implanted subcutaneously in BALB/c nude mice and grew as xenografts. Mice were injected through the tail vein with E. coli containing TRIP vectors, the first generation tkRNAi vectors, encoding shRNAs against catenin-β1. The experiment demonstrated that catenin-β1 mRNA and protein were downregulated in the xenografts, but the tumor growth was not altered. Accordingly, a tkRNAi-mediated downregulation of ABCB1 in vivo should be done not before the tkRNAi approach was improved in pharmacological efficiency.

Overall, this study demonstrates that the tkRNAi approach is promising and useful in targeting ABCB1 and for modulation of MDR. It also highlights that the pharmacological efficiency of this prototype tkRNAi system can still be improved. Accordingly, efforts are ongoing to improve both the plasmid transkingdom shRNA expression vector as well as the used bacterial carrier strains.3,6

**Materials and Methods**

**Cancer cell lines and cell culture.** Establishment and cell culture of the human gastric carcinoma cell line EPG85-257P was previously described by Dietel et al. (1990).6 The “classical” multidrug-resistant ABCB1-positive subline EPG85-257RDB was established by in vitro exposure to stepwise increased concentrations of daunorubicin (Daunoblastin®; Pfizer Pharma GmbH, Berlin, Germany). In order to ensure maintenance of the MDR phenotype, cell culture medium of EPG85-257RDB cells was supplemented with 2.5 μg/ml (4.31 μM) daunorubicin. Medium was replaced routinely twice a week.

**Construction of transkingdom shRNA expression vectors.** The short hairpin RNA expression vector TRIP contains an expression cassette driven by T7 promoter.3 The cloning site retains two restriction enzyme sequences of BamHI and SalI. The RNAI targeting sequence of ABCB1 was 5'-ATG TTG TCT GGA CAA GCA CT-3'.3 The design of the DNA template encoding for shRNA against ABCB1 was: BamHI site-sense sequence-loop (5’-TTC AAG AGA-3')-antisense sequence-SalI site. Two oligodeoxynucleotides were synthesized. Each was phosphorylated at the 5'-end, annealed and then ligated into linear TRIP after digestion with BamHI and SalI. For plasmid amplification, the reconstructed anti-ABCB1 encoding TRIP was transformed into E. coli TOP 10 (Invitrogen, Carlsbad, CA, USA) and further confirmed by direct sequencing.

**Administration of transkingdom shRNA expression vectors to cancer cells.** Transkingdom shRNA-encoding expression vectors were transformed into competent E. coli BL21 (DE3) (Invitrogen) by heat shock using the CaCl₂ procedure. Positive clones were selected on LB-agar plates containing 100 μg/ml ampicillin. tkRNAi vector-containing E. coli clones were cultured at 37°C in LB medium containing 100 mg/ml ampicillin (Carl Roth GmbH, Karlsruhe, Germany) whereby bacteria growth was measured at OD₆₀₀. For cancer cell infection, overnight cultures of E. coli were inoculated into fresh LB-medium for another 2 h growth. Bacteria of the early log phase (OD₆₀₀ = 0.5) were washed twice in PBS, diluted in serum-free Leibovitz L-15 medium and added to human cells at MOI 1:500. After 2 h of coinoculation cancer cells were washed twice with PBS and once with serum-containing Leibovitz L-15 medium supplemented with 100 μg/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 2.5 μg/ml amphotericin (Biochrom AG, Berlin, Germany), 150 μg/ml gentamycin (Biochrom AG), and 100 μg/ml ampicillin (Carl Roth GmbH).

**Detection of intracellular bacteria.** For detection of intracellular bacteria, cancer cells were grown and bacterially exposed on Lap-Tek II Chamber Slides (Nalgene Inc., New York, USA). DAPI (4',6-Diamidino-2-phenylindol x 2 HCl) staining was performed as described by Jagielski et al. (1976). Cells were washed once with DAPI solution (Sigma, St. Louis, MO, USA) (2% DAPI stock solution v/v in methanol) and incubated with DAPI solution for 30 min at 37°C. Afterwards, cells were washed once with PBS followed by visualization of intracellular bacteria and their lysis using fluorescent microscopy 640 nm emission.

**Quantification of shRNA expression.** Quantification of the expression level of anti-ABCB1 shRNAs in E. coli-treated multidrug-resistant cancer cells was determined by real-time RT-PCR as described by Stein et al. (2008).10 Briefly, RNA highly enriched with miRNAs was extracted from cancer cells using the “mirVana miRNA Isolation Kit” (Ambion, Austin, TX, USA), followed by a reverse transcription reaction on 500 ng of adaptor-tagged miRNA using a “QuantimiR RT Kit” (SBI, Mountain View, CA, USA). Quantitative real-time PCR was carried out using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) with LC FastStart DNA Master Mix (Roche Diagnostics), containing 500 nM of antisense ABCB1 (5'-AGT GCT TGT CCA GAC AAC-3') and 500 nM miR-16 (5'-TAG CAG CAC GTA AAT ATT GGC G-3') as forward primers, respectively, and 500 nM universal reverse adaptor primer (SBI, Mountain View, CA) directed against the tagged miRNA. The PCR program started with 10 min 95°C, followed by 40 cycles 15 s 95°C, 10 s 52°C and 30 s 72°C followed by threshold crossing point (Cₚ) calculation.12 Cₚ values were generated from three replicates for both, the target (ABCB1) and the endogenous control (miR-16) amplification curves for each sample using the second derivate maximum mode of analysis. Fold change values for the anti-ABCB1 siRNA relative to the mir-16 expression as well as to untreated tumor cells for calibration were calculated for each replicate of each sample applying the 2ΔΔCₚ method.11
Quantitative analysis of ABCB1 mRNA expression levels. Quantitative analysis of ABCB1 mRNA expression was performed by real-time RT-PCR with a LightCycler instrument and SYBR-Green Fluorescent dye (Roche) as described previously.12,13 The measured expression levels were normalized for expression data using real-time quantitative PCR and the 2(-Delta Delta Ct) method. Methods 2001; 16:178-86.

Expression of ABCB1 was detected by western blot analysis as described previously.12,13 In brief, 20 μg of membrane proteins of each sample were separated on 4% stacking and 7.5% resolving SDS-PAGE gel and transferred to a 0.2-μm cellulose nitrate membrane (Schleicher and Schuell, Dassel, Germany). To avoid unspecific binding, filters were incubated in 5% skim milk, 0.05% Tween-20 in 1x TBS overnight. Subsequently, filters were incubated with mouse mAbs C219 (Alexis, San Diego, CA, USA) directed against human ABCB1 diluted in 1% skim milk in 1X TBST (20 mM Tris-CI; 137 mM NaCl; 0.05% Tween-20; pH 7.5) (1:100) for 2 h and, afterwards, with peroxidase-conjugated mouse anti-rabbit IgG (1:10,000) (Sigma, St. Louis, MO, USA; #A-1949). As a control for equal protein loading, the filters were simultaneously incubated with a mouse mAb directed against actin (Chemicon, Temecula, CA; #MAB 1501R) diluted 1:5,000. The protein-antibody complexes were visualized by chemiluminescence (ECL system, Amersham).

Anthracycline accumulation assay. Measurement of cellular anthracycline accumulation was performed by flow cytometry as described previously.8,13,14 In brief, 1.5 x 10^6 cells were seeded in 10 cm dishes and infected with E. coli at MOIs of 1:500 on the following day. The cells were exposed to 10 μM (5.8 μg/ml) of daunorubicin for 3 h, 72 h postinfection. Cells were trypsinized, then washed twice with ice-cold phosphate-buffered saline (PBS) and analysed by flow cytometry (Calibur 750; Becton-Dickinson, San Jose, CA, USA). The cells were excited at 480 nm and emission was collected at 550 nm. A minimum of 10^4 cells was analyzed for each sample. Data of at least three independent experiments in duplicate were used to calculate a geometric mean.

Cytotoxicity assay for cell survival. Cytotoxicity of daunorubicin was tested using a proliferation assay as described previously.8,13,14 Briefly, 1.5 x 10^6 cells were treated at MOI of 1:500 with E. coli in 12-well plates in triplicate. 24 h after bacterial exposure cells were trypsinized and 7.5 x 10^4 cells were seeded into 96-well plates per well. 24 h after seeding daunorubicin was added in dilution series 48 h post infection. After 3 days, incubation was terminated by replacing the medium with 10% trichloroacetic acid, followed by incubation at 4°C for 2 h. Finally, the plates were washed, stained, and absorbance was measured at 562 nm against a background at 690 nm. To determine the IC_{50}-values, the absorbance difference of control cells without drug was set to be 1 and IC_{50} values were calculated from multiple, at least three independent experiments.

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