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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 (*tk*RNAi). 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 *tk*RNAi 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 *tk*RNAi is suitable for modulation of "classical" MDR in human cancer cells. Overall, the prototype *tk*RNAi system tested here did not yet attain the levels of gene silencing seen with conventional siRNAs nor virally delivered shRNAs; but the *tk*RNAi 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 (*tk*RNAi).[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 *tk*RNAi 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 β1-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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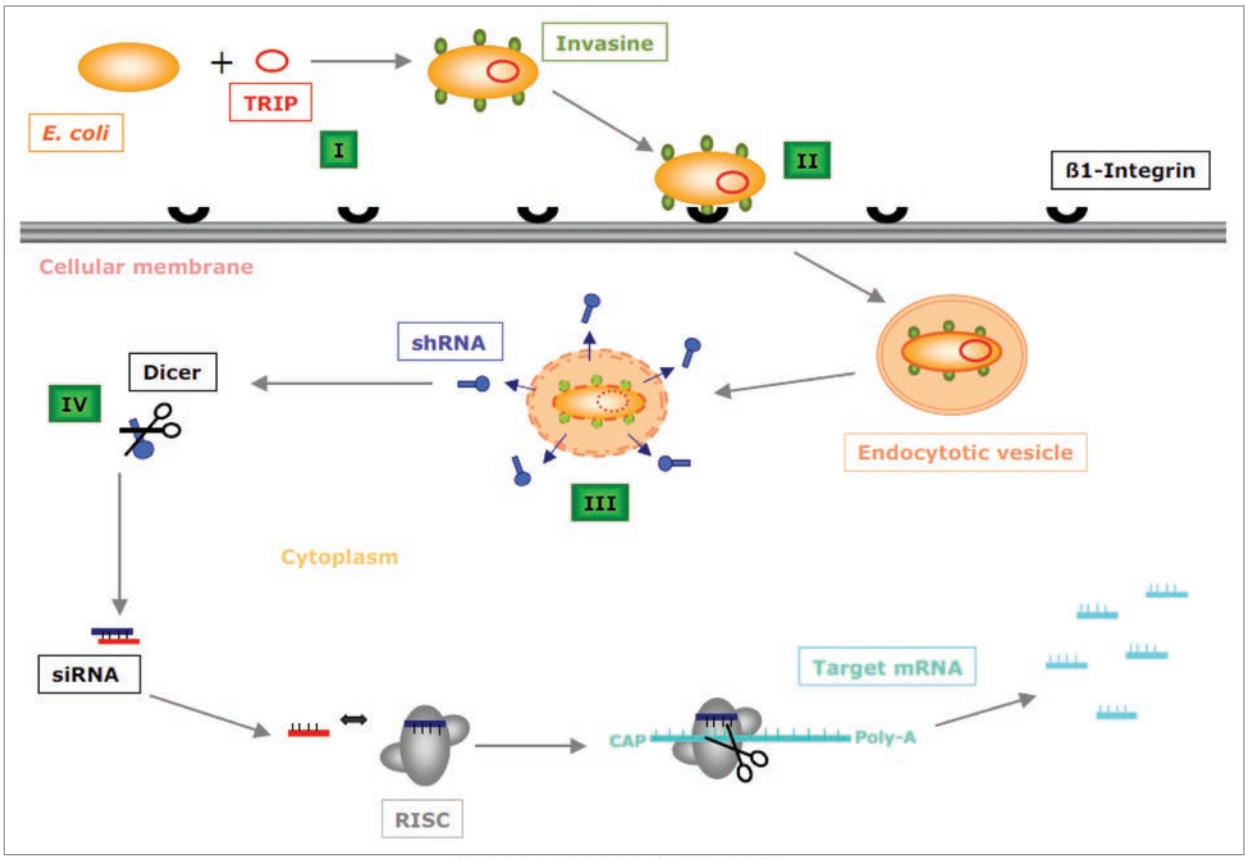

Figure 1. Schematic overview about the *tk*RNAi mechanism. (I) Transformation of *E. coli* with the *tk*RNAi vector TRIP encoding invasin, listeriolysin O and the shRNA directed against the target mRNA. (II) *E. coli* cells expressing invasin on their surface interact with cytoplasm membrane-embedded β1-integrin of human cells. Following β1-integrin binding, *E. coli* cells are internalized by formation of endocytotic vesicles. (III) Following lysis of the vesicle membranes by Listeriolysin O, therapeutic shRNAs escapes from the endocytotic vesicles into the cytoplasm of the target cells. (IV) Intracellular Dicer processes therapeutic shRNAs into the corresponding siRNAs that trigger the RNAi pathway for specific degeneration of target mRNAs.

the T7 RNA polymerase necessary for the T7 promoter-driven synthesis of shRNAs. So far, the *tk*RNAi approach has been successfully applied for silencing catenin-β1 in human colon cancer cells in in vitro as well as in in vivo models.[3]

To evaluate its potency and efficacy in a completely different model system, with clinical impact, the *tk*RNAi 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 *tk*RNAi 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 $2^{-\Delta C_T}$ 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 *tk*RNAi-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 *tk*RNAi 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 *tk*RNAi 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.

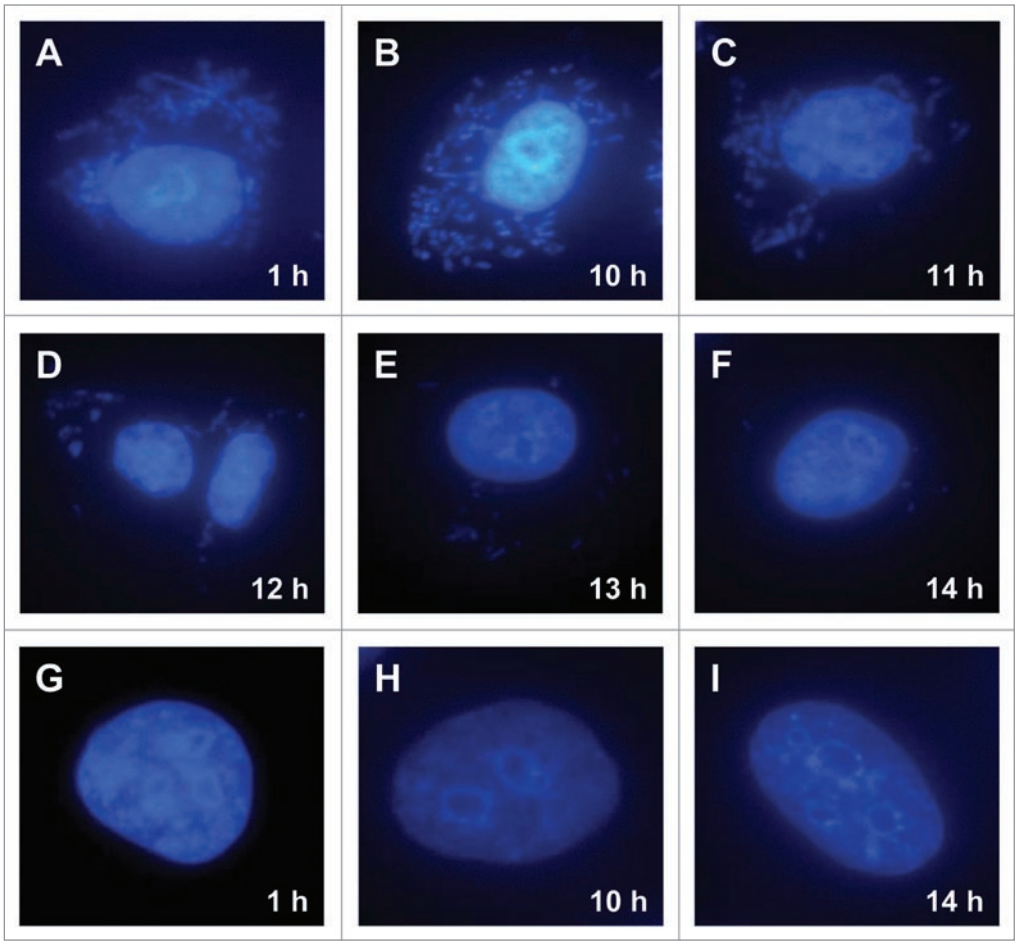

Figure 2. Invasion and lysis of TRIP-containing bacteria detected by DAPI staining. Infection of multidrug-resistant human cancer cells (EPG85-257RDB) was performed using MOI 1:1,000 of anti-ABCB1 shRNA-ecncoding TRIP-containing *E. coli*. Human carcinoma cells are shown (A) 1 h, (B) 10 h, (C) 11 h, (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.

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 *tk*RNAi 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 *tk*RNAi 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

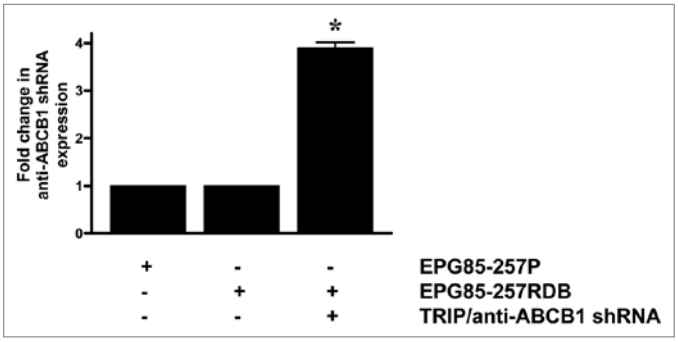

Figure 3. Quantification of anti-ABCB1 shRNA expression level in gastric carcinoma cells 24 h post bacterial exposure using MOI 1:500 determined by quantitative real time PCR. Fold change values were calculated applying the $2^{-\Delta\Delta C_T}$ method.[10] EPG85-257P, non-resistant cancer cells; EPG85-257RDB, 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 (*$p < 0.05$).

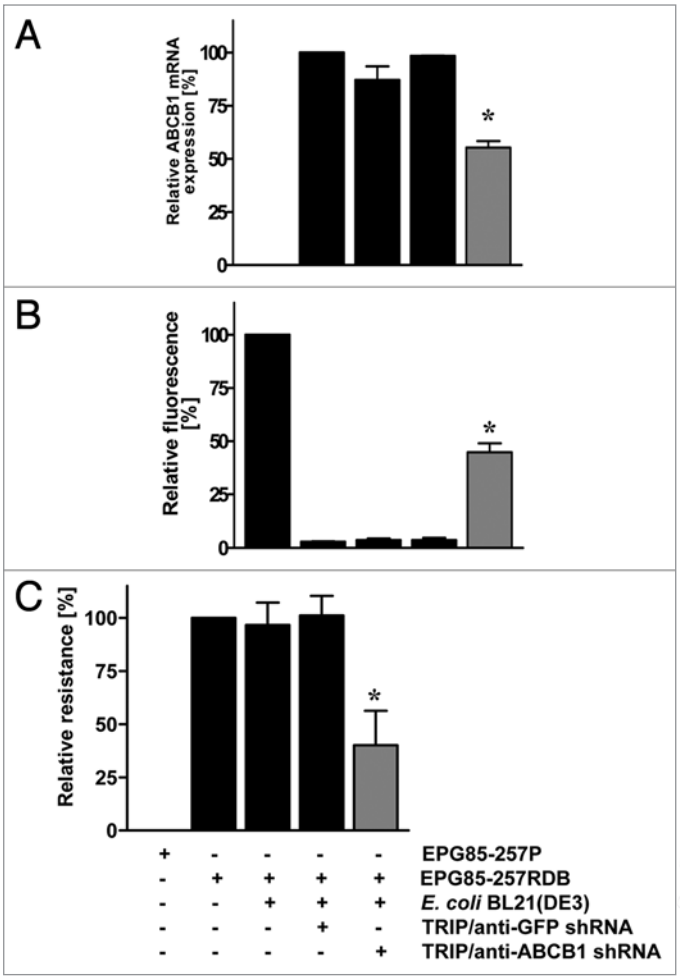

Figure 4. ABCB1 mRNA expression levels and functional analyses in carcinoma cells following bacterial exposure (MOI 1:500). (A) Determination of ABCB1 mRNA levels 72 h post infection using quantitative real time RT-PCR. Normalization was performed using the housekeeping gene aldolase. The ABCB1/aldolase ratio of untreated EPG85-257RDB cells were set to be 100%. (B) Relative anthracycline accumulation of carcinoma cells 72 h after bacterial treatment measured by flow cytometry. Drug accumulation in non-resistant EPG85-257P cells was set to be 100%. (C) Relative drug resistance levels determined by a cytotoxicity assay for cell survival whereby the drug-resistance level of drug-resistant EPG85-257RDB cells was set to be 100%. EPG85-257P, non-resistant cancer cell line; EPG85-257RDB, multidrug-resistant cancer cell variant; *E. coli* BL21(DE3), *E. coli* without *tk*RNAi vector; TRIP/anti-GFP shRNA, anti-GFP shRNA encoding *tk*RNAi vector TRIP/anti-ABCB1 shRNA, anti-ABCB1 shRNA encoding *tk*RNAi vector. p-values were calculated using the student's t-test (*$p < 0.05$).

exhibited a 470-fold higher resistance against daunorubicin than its parental counterpart EPG85-257P. As shown in **Figure 4C**, treatment of EPG85-257RDB cells with the control vector or the cultivation of this cell line in the absence of anticancer agents have negligible influence on the IC_{50}-values. In contrast, treatment of the cell line EPG85-257RDB with the anti-ABCB1 shRNA expression vector altered the drug sensitivity to 60% of the IC_{50}-value of the drug-sensitive cells, i.e., the drug-resistant phenotype was reversed by 40%.

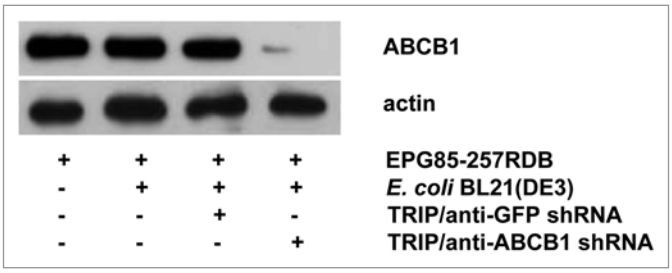

Figure 5. Western Blot analysis for detection of ABCB1 protein expression 96 h following bacteria treatment. As control for equivalent protein loading, western blot membranes were stripped and incubated with an actin-specific mAb. EPG85-257RDB, multidrug-resistant cancer cell variant; *E. coli* BL21(DE3), *E. coli* without *tk*RNAi vector; TRIP/anti-GFP shRNA, anti-GFP shRNA encoding *tk*RNAi vector TRIP/anti-ABCB1 shRNA, anti-ABCB1 shRNA encoding *tk*RNAi vector.

Discussion

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 (*tk*RNAi). Although the proof of concept that the *tk*RNAi 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.[3] Therefore, this study was conducted to assess the biological activity of *tk*RNAi in a well characterized model, i.e., for reversal of the ABCB1-dependent MDR phenotype of the human gastric carcinoma cell line EPG85-257RDB.[7] This cell model has previously been evaluated for different RNAi strategies such as transient in vitro downregulation of ABCB1 by chemically synthesized siRNA molecules,[15] adenovirus-administered shRNAs,[13] as well as stable knock down by plasmid encoded shRNAs.[8] Identical shRNA sequences had also shown efficacy for in vivo targeting of ABCB1 by RNAi.[10]

This study confirmed that the *tk*RNAi 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 *tk*RNAi was less pronounced than levels observed from chemically synthesized siRNA molecules (45% and 87%, respectively).[15] 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.[8,13] The extent of inhibition of drug transport activity and reversal of drug resistance by the *tk*RNAi platform was less pronounced compared to existing RNAi strategies, and the amount of anti-ABCB1 shRNA recovered from the *tk*RNAi-treated gastric carcinoma cells was lower compared to that in cells treated with shRNA-encoding adenoviruses.[13]

Several reasons are thought to be responsible for the less pronounced gene silencing activity of the *tk*RNAi strategy in the investigated cell model; we speculate that each of the components of the *tk*RNAi 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 *tk*RNAi system tested here and might present an opportunity to build improved versions.

The proof-of-concept of in vivo efficacy of the first generation *tk*RNAi vector and bacterial strain was already provided.[3] 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 *tk*RNAi 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 affected. Accordingly, a *tk*RNAi-mediated downregulation of ABCB1 in vivo should be done not before the *tk*RNAi approach was improved in pharmacological efficiency.

Overall, this study demonstrates that the *tk*RNAi approach is promising and useful in targeting ABCB1 and for modulation of MDR. It also highlights that the pharmacological efficiency of this prototype *tk*RNAi 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.[16]

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).[7] 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 *Bam*HI and *Sal*I. The RNAi targeting sequence of ABCB1 was 5'-ATG TTG TCT GGA CAA GCA CT-3'.[8] The design of the DNA template encoding for shRNA against ABCB1 was: *Bam*HI site-sense sequence-loop (5'-TTC AAG AGA-3')-antisense sequence-*Sal*I site. Two oligodeoxynucleotides were synthesized. Each was phophorylated at the 5'-end, annealed and then ligated into linear TRIP after digestion with *Bam*HI and *Sal*I. 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. Transkindom shRNA-encoding expression vectors were transformed into competent *E. coli* BL21 (DE3) (Invitrogen) by heat shock using the $CaCl_2$ procedure. Positive clones were selected on LB-agar plates containing 100 μg/ml ampicillin. *tk*RNAi 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 OD_{600}. 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 (OD_{600} = 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 co-incubation cancer cells were washed twice with PBS and once with serum-containing Leibovitz L-15 medium supplemented with 100 u/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).[9] 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 A-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 (C_T) calculation.[11] C_T 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 $2^{-\Delta C_T}$ method.[11]

Quantification 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 of the housekeeping enzyme aldolase. Specific oligodeoxynucleotide primers used for amplification of each target were as follows: ABCB1-fw 5'-CAG CTA TTC GAA GAG TGG GC-3' and ABCB1-rev 5'-CCT GAC TCA CCA CAC CAA TG-3' (amplification product of 299 bp); Aldolase-fw 5'-ATC GTG GCT GCA CAT GAG TC-3' and Aldolase-rev 5'-GCC CTT GTC TAC CTT GAT GC-3' (amplification product of 249 bp). Cycling conditions for both amplification products were: 10 min at 95°C initial enzyme activation, followed by 40 cycles of 15 s denaturation at 95°C, 5 s annealing at 58°C (ABCB1 mRNA) or 56°C (Aldolase mRNA), 10 s elongation at 72°C. Gene-specific fluorescence was measured at 87°C. Specificity of amplification products was confirmed by melting curve analysis. Quantitative measurement of each mRNA expression level was performed in three independent experiments. Levels of statistical significance were evaluated by calculation of the two-tail p-value by performing the unpaired t-test.

Western blot analysis for detection of ABCB1 protein. Expression of ABCB1 was detected by western blot analysis as described previously.[8,13,14] In brief, 20 µg of membrane proteins of each sample were separated on 4% stacking and 7.5% resolving SDS-PA 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-Cl; 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 equivalent 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 10^2 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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