Aus dem Institut für Pathologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Delivery of RNA interfering effectors against HPV-16 E6 and E7
proteins in OSCC cells

zur Erlangung des akademischen Grades
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"Hope is a good thing, maybe the best of things, and no good thing ever dies."

— *Stephen King*

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Abstract (deutsch)

Einleitung: Sicherer und effektiver Transfer von Short-interfering-RNA-/Short-hairpin-RNA-Molekülen (siRNA/shRNA) zum Zweck der RNA-Interferenz war eine der größten Herausforderungen der vergangenen 20 Jahre. Als Alternative zu den herkömmlichen Transferprotokollen wurde eine Transkingdom-RNA-Interferenz (tkRNAi) vorgeschlagen. Die hier vorgestellte Arbeit hat die Effizienz und Wirksamkeit eines verbesserten Bakterien-Übertragungssystems zur Hemmung der humanpapillomvirus-16-(HPV16)-E7-spezifischen mRNA oraler Plattenepithelkarzinom-Zellen (oral squamous cell carcinoma , OSCC) untersucht.

Methodik und Ergebnisse: Zunächst wurden Plasmide konstruiert, welche neben einer therapeutischen shRNA zwei unterschiedliche Proteine kodieren. Das erste „Invasin" verleiht nichtinvasiven, nichtpathogenen Bakterien einen invasiven Phänotyp. Das zweite Protein „Listeriolysin O" zeigt sich dafür verantwortlich, dass die therapeutischen shRNA-Moleküle nach bakterieller Invasion in der Zielzelle freigesetzt werden. Nach bakterieller Transfektion der Tumorzellen, wurde die Fähigkeit der modifizierten Bakterien, in Tumorzellen einzudringen und sich dort zu sammeln mittels Fluoreszenzmikrokospie nachgewiesen. Die Effizienz des Transfers und der Expression der shRNA wurde durch die Messung des Expressionsniveaus von kleiner siRNA und der Zielgen-mRNA mit qRT-PCR bestimmt; bestätigt per Western-Blot. Ein signifikanter Abfall des IC_{50} von anti-HPV16-E7 CEQ221-E7 in oraler Plattenepithelkarzinom-Zellen im Vergleich zu CEQ221-GFP; und eine höhere Gesamtapoptose sowie eine aktivierte Caspase-3-Aktivierung bestätigten den funktionellen Effekt der durch die bakterielle Abgabe vermittelten Hemmung des HPV16-E7.

Schlussfolgerungen: Transkingdom-RNA-Interferenz ist ein wirksames Instrument zur Hemmung spezifischer Zielgene in Säugetierzellen. Es wurde zum ersten Mal nachgewiesen, dass tkRNAi das HPV16-E7-Gen beim oralen Plattenepithelkarzinom hemmen, Apoptose auslösen und die Proliferationsaktivität senken kann, was schließlich zum Tod von Zielzellen führt.

Abstract (englisch)

Introduction: Safe and effective delivery of short interfering RNA/short hairpin RNA (siRNA/shRNA) molecules for the purpose of RNA interference was one of the biggest challenges in the past 20 years. Transkingdom RNA interference (tkRNAi) was suggested as an alternative to the traditional delivery protocols. This work aimed to evaluate the efficiency and the potency of an improved bacterial delivery system in inhibiting human papilloma virus 16 (HPV16)-E7-specific mRNA in oral squamous cell carcinoma (OSCC).

Method and Results: Firstly, shRNA plasmids were constructed and transformed in the CEQ221 bacteria to construct the delivery vehicles. After bacterial transfection of the tumor cells, the ability of the modified bacteria to penetrate and collect in the tumor cells was confirmed by fluorescent microscopy. Efficiency of shRNA delivery and expression was confirmed by measuring the expression level of small siRNA and target gene mRNA via qRT-PCR; followed by a confirmation via western blot. Significant drop in the IC_{50} of the CEQ221-E7 targeting HPV16-E7 in oral squamous carcinoma cells as compared to their control counterparts CEQ221-GFP; and higher total apoptosis and activated caspase-3 activation confirmed the functional effect of the bacterial delivery-mediated inhibition of the HPV16-E7.

Conclusion: Transkingdom RNA interference is a potent tool to inhibit specific target genes in mammalian cells. It was proved for the first time that tkRNAi is capable of inhibiting HPV16-E7 gene in oral squamous cell carcinoma triggering apoptosis and lowering proliferation activity and eventually leading to target cell death.

1. Introduction

RNA interference (RNAi) is regarded as one of the most reliable tools to specifically inhibit selected genes in mammalian cells. The mechanism was firstly described by Fire et al., 1998 in the model organism *Caenorhabditis elegans* as a naturally occurring mechanism, depends on introduction of 21 nucleotides (nt) long double-stranded RNAs (dsRNA) into target cells to inhibit a certain gene by post-transcriptionally destroying its messenger RNA (mRNA). Briefly described, the introduced dsRNA is cleaved by Dicer, one of the RNAse III family enzymes, into 21-23 nt long double-stranded small interfering (siRNA). RNA-induced silencing complex (RISC, a protein complex already exists in mammalian cells cytoplasm) incorporates the siRNA and unwinds the double strand. The antisense siRNA combined with RISC targets and cleaves the homologous mRNA by base pairing interaction. The Argonaute 2 (AGO2) component of RISC contains an endonuclease activity that cleaves the target mRNA and the resulting mRNA fragments are destroyed by cellular exonucleases (Rand et al., 2005; figure 1). The imperfect complementarity as in case of microRNA cause translational repression and mRNA decay by deadenylation (Bagga et al., 2005). Eventually, the target mRNA and hence the target gene expression becomes inhibited. For longer gene silencing effects expression vectors encoding short hairpin RNA (shRNA) were developed, where plasmids containing shRNA expression cassettes under the control of strong promotors are delivered to target cells. The shRNA typically consists of a sense strand, a complementary antisense strand and a hairpin loop of 11 nt long (and hence the name). Beside siRNA and shRNA, RNAi can also endogenously be triggered by microRNA, which are produced and processed in the nucleus to be transported to the cytoplasm to bind the RISC protein and initiate the gene silencing pathway. Gene silencing by RNAi strategy has many advantages over the other antisense-strategies since the RNAi effectors are highly specific and easy to be designed along with their long-lasting gene silencing effect as in case of using shRNA (Castanotto and Rossi, 2009). Another advantage is that siRNAs/shRNAs can be recycled after the cleavage of the target mRNA (Burnett and Rossi, 2012). This strategy could be optimized to be used in treatment of many diseases such as cancer since RNAi has the power to target undruggable targets in tumor cells (Stege et al., 2010).
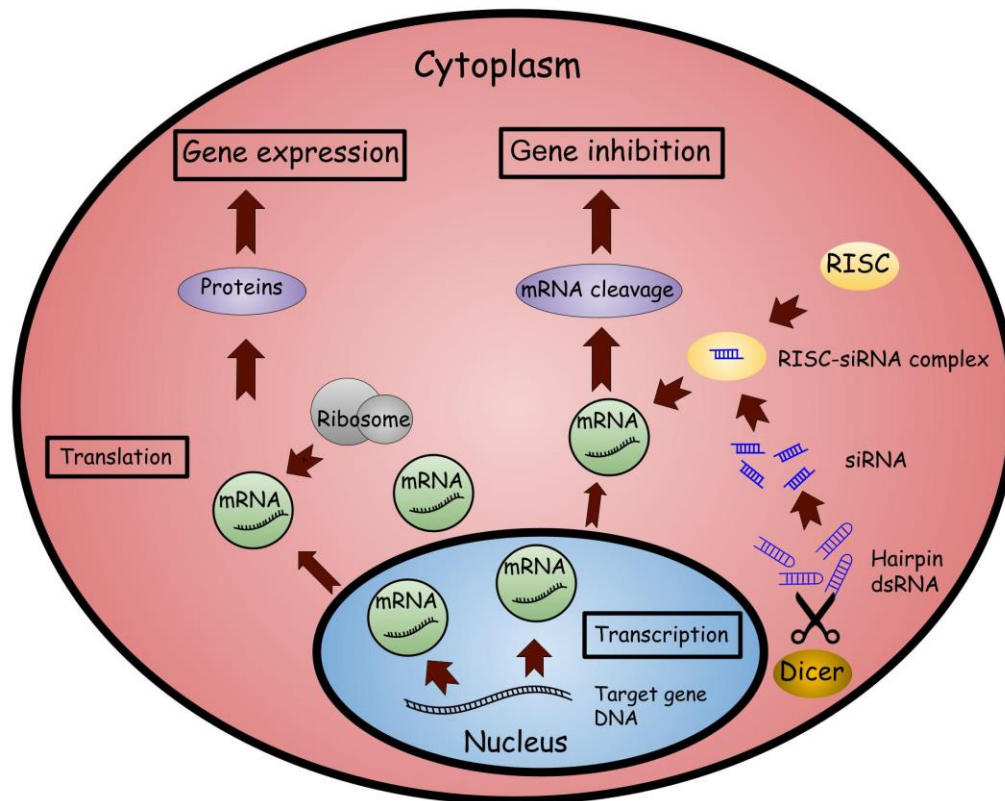
Figure 1: Diagrammatic representation of the RNA interference pathway in mammalian cells using double stranded hairpin RNA. The RISC-siRNA complex interacts with the target gene mRNA according to sequence complementarity and eventually lead to inactivation and hence inhibition of target gene expression (original picture not included in previous publications).

The main challenge for the therapeutic application of RNAi lies in safely and effectively delivering the RNAi effectors to the target cells (Krühn et al., 2009). Despite proven efficiency and specificity of siRNA in gene silencing, some issues needed to be addressed before clinical application. The siRNA has to be delivered to the target cell cytoplasm in order to engage with RISC proteins and proceed with RNAi cascade (Chen et al., 2018). The naked siRNAs are chemically unstable and would be fastly degraded by endonucleases upon systemic administration (Hickerson et al., 2008; Kanasty et al., 2012). Besides, their negative charges and heavy molecular weight make it hard to penetrate the negatively-charged cell membranes. Although the half-life of siRNA can be prolonged by chemical modification, the modified siRNA showed lower potency and specificity (Behlke et al., 2008, Nguyen and Szoka, 2012).

Moreover, the applied RNAi effectors could be distributed to unwanted sites producing unwanted immunogenic, inflammatory or toxic responses (Kong et al., 2007).

After internalization, siRNA will be entrapped in the pinocytotic vesicle. The next challenge will be to escape the internalization endocytotic vesicle to engage with the cell machinery in the cytoplasm to proceed with the RNA interference pathway (Du Rietz et al., 2020). It was suggested that a delivery carrier would help overcoming both cell membrane and endocytotic vesicle penetration barriers. Moreover, the used delivery carrier has to be nontoxic, able to deliver the RNAi effectors to the target cell cytoplasm in decent concentrations, able to penetrate tumor mass, able to penetrate the target cell membrane and the endocytotic vesicle and whenever possible, do not carry the siRNA/shRNA to unwanted sites. Currently used siRNA/shRNA delivery systems can be generally classified into viral and nonviral delivery systems (Chen et al., 2018).

Non-pathogenic viruses have many characters qualifying them to be carriers for RNAi effectors. After the required genetic modification, viruses can carry shRNA expression cassettes under the control of an expression promotor and terminator, able to produce stable and prolonged expression of the required siRNA and subsequently a stable and prolonged inhibition of the target gene in the target cells (Dong et al., 2019). Although viruses represent a very robust siRNA delivery vehicles, some disadvantages appeared. Viruses do not penetrate neither cell membranes nor internalization vesicle and therefore require a delivery vehicle. Moreover, viruses integrate their genome in the target cell genome raising safety issues. Viruses can also result in toxic, immunogenic, mutagenic and off target effects (Gherardini et al., 2014; Schagen et al., 2004).

Several molecules were suggested to be conjugated with siRNA or shRNA to transport them to the cytoplasm of the target cells. For instance, exosomes, nanoparticles and high-density lipoproteins. Exosomes are phospholipid bilayer vesicles and were successfully used to deliver soluble drugs due to their ability to overcome natural barriers. Nanoparticles can be engineered to have lower toxicity and immunogenicity and to overcome the physical barriers facing the successful delivery of siRNAs which makes them good candidates as delivery vehicles. Indeed, systemic delivery of siRNA using nanoparticles as delivery vectors showed successful gene silencing in therapeutic levels (Miele et al., 2012). Lipid-based nanoparticles delivery systems

such as liposomes, solid lipid nanoparticles and nanostructured lipid carriers showed remarkable success especially when modified to be biocompatible and biodegradable. Polymer-based nanoparticle delivery is another category of nanoparticles used in the delivery of RNAi, where it is easy to incorporate the negatively charged nucleic acids on the surface of those nanoparticles due to surface positive charge. In addition, Lipid-polymer hybrid nanoparticles were developed to overcome the disadvantages of lipid- and polymer-based nanoparticles as a delivery system for RNAi. Nanoparticles showed variable chemical and physical properties, good cellular uptake and produced stable and efficient inhibition of the target genes. Otherwise, further research is required to ensure the safety of nanoparticles after systematic administration (Chen et al., 2018; Miele et al., 2012; Reynolds et al., 2017). To minimize the off-target effects of RNAi and the toxicity and immunogenicity of the delivery agents, more research has focused on targeted delivery. Conjugating the siRNA/shRNA-vector to an aptamer, peptide and antibody or to folate will result in tumor-targeted delivery of the siRNA/shRNA to the desired sites (Guo et al., 2017; Powell et al., 2017).

Microbial therapy, includes oncolytic viral therapy and bacterial anticancer therapy, gained a lot of attention in the past 20 years (Forbes et al., 2018). Several modes of action of the bacteria on the target cancer cells after delivery were suggested. Direct killing of the target cells after competing on nutrients, production of toxins, induction of apoptosis, bursting of the target cell by uncontrolled bacteria replication or activation of immune response against the tumor areas were among the mechanisms by which the bacteria kill the target tumor cells (Forbes et al., 2018). Historically ancient Egyptians have connected tumor regression with bacterial infection (Wong and Slavcev, 2015). Willian B. Coley observed as early as 1890 an association between bacterial infection and tumor regression. Afterwards, he tried to experimentally reproduce those findings by injecting patients with live *Streptococcus pyogenes* and later by injecting killed *Streptococcus pyogenes* or *Serratia marcescens* with some success. However, his results were not widely accepted by the scientific community because he was unable to explain the mechanism of tumor regression induced by the bacteria (Felgner et al., 2017). Recently, bacteria have emerged as a potential anticancer therapeutic due to a better understanding of tumors and their microenvironment and the advances in molecular biology techniques allowed the manipulation of bacterial genes and the ability to produces specifically engineered bacteria to better serve the purpose. Several candidates were suggested as bacteria delivery agents. For

instance, *Salmonella spp.* proved successful as a delivery transporter in vitro but provoked relatively strong side effects upon systemic administration which required attenuation before in vivo application. On the other hand, attenuation may drastically affect therapeutic efficiency (reviewed in details in Felgner et al., 2017). Bacillus Calmette-Guérin vaccine (BCG) is the most successful bacterial treatment of superficial bladder cancer (Herr and morales, 2008). Several bacterial candidates provided many advantages as delivery vehicles for anticancer therapeutic such as *Listeria monocytogenes*, *Clostridium novyi, Salmonella spp and E. coli.*

The biggest advantage of the bacteria as delivery vehicles for anticancer therapeutics is that they are easy to be manipulated using standard and cheap molecular biology techniques. Bacteria are engineered either to be attenuated or to enhance their anti-tumor activity. In order to enhance safety and decrease toxicity, certain gene deletions were applied to known pathogens. For instance, deletion of the gene responsible for endotoxin production in *Clostridium novyi*; and the deletion of some genes from *Salmonella spp*. resulting in loss of some certain components of lipopolysaccharides and hence decreasing toxicity (Low et al., 2016). Auxotrophic bacterial mutants were engineered to be dependent on certain nutrients and in the absence of such nutrients, they fail to grow and multiply. Optimally, the needed nutrients would only exist in the tumor microenvironment (Pawelek et al., 1997). Bacteria can also be engineered for the enhancement of tumor targeting ability. *Salmonella* was modified to express integrin-binding RGD on the bacterial surface, which led to more than 1000-fold bacterial accumulation in integrin expressing tumor cells and xenografts as compared to controls (Park et al., 2016).

Bacteria can be used to carry proteins, DNA or RNA exhibiting anti-tumor effects. In most cases, the delivery will be in the form of a plasmid carrying and expression cassette in the control of a strong promotor that uses, upon expression, the target cell machinery to produce siRNA, proteins or directly initiate cell death cascade after bacterial accumulation in the tumor area. Autolysis of the bacteria themselves is granted by the depletion of nutrients (especially in case of auxotrophs) or due to accumulation of byproducts (Forbes, 2010).

Following bacterial accumulation in the tumor area, several strategies were suggested for them to act against tumors. a) Delivery of bacteria expressing cytotoxic agents (Ryan et al., 2009), induce apoptosis (Loeffler et al., 2008) or produce prodrug enzyme (Austin and Huber, 1993). In case of prodrug enzymes, an anticancer agent will be administered systematically as a prodrug in an inactive form, where activation of the prodrug requires and enzyme produced by the therapeutic bacteria locally in the tumor areas only, and hereby decreasing off target effect and toxicity. b) Enhancing immune reaction against tumors by delivery of immune modulators, which lead eventually to immune sensitization against the tumor (Binder et al., 2013). c) Targeting tumor stroma such as destroying the intratumoral blood vessels (Niethammer et al., 2002). d) Targeting specific genes that decrease tumor cell resistance, proliferation or metastasis.

Transkingdom RNA interference technology was developed by Xiang et al., 2006. They succeeded to produce genetically modified bacteria capable of producing shRNA and efficiently capable of penetrating and delivering the RNAi effectors to the target cells. For this purpose, a 'transkingdom RNA interference plasmid' (TRIP) was generated. Basically, this plasmid composed of the shRNA expression cassette controlled by a strong promoter and terminator. Another component of TRIP is the *inv* gene from *Yersinia pseudotuberculosis*, which encodes for the expression of invasin protein on the bacterial cell surface. This protein interacts with β1-integrin receptor on target cell surface and mediates the internalization of the bacterial cell to the target cell (Young et al., 1992). The third component is the *Hly*A gene from *Listeria monocytogenes* which produce listeriolysin O, the pore-forming toxin. When released, listeriolysin O leads to rupture of the endosomal membrane causing the shRNA with other components to reach the cytoplasm (Mathew et al., 2003 and Xiang et al., 2006).

Cancer cells can acquire resistance against chemotherapeutics and subsequently increasing toxicity resulting from modifying the anticancer agents' therapeutic doses. Human papilloma virus-induced carcinogenesis was found to be associated with decrease in levels of TP53 and hypophosphorylated retinoblastoma proteins (pRb). It was found that inhibition of E6/E7 proteins via RNA interference resulted in more than 4-fold increase in sensitivity of HPV-positive cervical cell to cisplatin (Rajasekaran et al., 2017; Xu et al., 2020). Developing new

modulators of p-gp, the efflux pump responsible for the extracellular extrusion of the anticancer agent, is one of the classical approaches to address the persistent problem of chemotherapeutic resistance. Combination of chemotherapeutic agent with one of the p-gp modulators would inhibit the efflux pump and decrease the therapeutic dose of the chemotherapeutic agent and hence decrease the possible toxicity (Xu et al., 2020). Natural products such as Epoxylathyrane derivatives were suggested as p-gb modulators using a mechanism known as collateral sensitivity (Reis et al., 2017, Reis et al., 2020).

2. Aims of the work

This work aimed to design a bacterial transport vehicle carrying shRNA against HPV16-E7 protein in OSCC cells with the intention to answer the following questions:

1- Can the tkRNA bacteria penetrate the OSCC cells?

2- Can the bacteria-carried shRNA inhibit HPV16-E7?

3- Would that inhibition affect the ability of the tumor cells to proliferate?

4- Can Epoxylathyrane derivatives compound modulate the multi-drug resistance phenotype?

3. Materials and Methods

3.1 Cell lines and culture

The HPV16-positive oral squamous cancer cell line (UPCI-SCC-090) was grown on DMEM medium (Biowhittaker, Walkersville, MD) enriched with 10% foetal calf serum (Gibco/BRL, Grand Island, NY) and 1mM L-glutamine. The human gastric carcinoma cells (EPG85-257RDB) and the pancreatic carcinoma cells (EPP85-181P) and their established drug-resistant variants were grown on Leibovitz L-15 medium (Biowhittaker, Walkersville, MD) enriched by 10% foetal calf serum (Gibco/BRL, Grand Island, NY), 1mM L-glutamine, 6.25 mg/L feutin, 80 IE/L insulin, 2.5 mg/L transferring, 0.5 g/L glucose, 1.1 g/L NaHCO3, 20 000 kIE/L trasylol and 1% minimal essential vitamins. All cell lines were grown at 37 °C and 5% CO2 in humified atmosphere.

3.2 Construction of transkingdom shRNA expression vectors.

The construction of the RNAi plasmid was performed according to Ahmed et al., 2015. Briefly, the DNA inserts of two complementary oligodeoxynucleotides (Eurofins MWG, Ebersberg, Germany) containing the shRNA sense strand, a short spacer loop, the shRNA antisense strand, and multiple deoxythymidines as a terminator were designed. The inserts contain as well the complementary sequence of the *Bam*HI at one end and the sequence of *Sal*I at the other end (figure 2). Inserts were prepared by annealing the sense and antisense oligodeoxynucleotides (Eurofins MWG, Germany) targeting HPV-16 E7 (sense: 5'- GATCC TCT CTA CTG TTA TGA GCA ATT ATT CAA GAG TAA TTG CTC ATA ACA GTA GAG ACT TTT TTT TTT G -3' and antisense: 5'- TCG ACA AAA AAA AAA TCT CTA CTG TTA TGA GCA ATT ATC TCT TGA AAA TTG CTC ATA ACA GTA GAG AG-3') or targeting GFP (sense: 5'-GAT CCG ACG GTA TCG ATA AGC TTG ATT TCA AGA GAA TCA AGC TTA TCG ATA CCG TCT TTT TTT TTT G-3' and antisense: 5'-TCG ACA AAA AAA AAA GAC GGT ATC GAT AAG CTT GAT TCT CTT GAA ATC AAG CTT ATC GAT ACC CTG G-3'). After linearizing the short hairpin RNA expression vector pMBV43-H3 (Cequent, Cambridge, MA, USA) using *Bam*HI and SalI, sticky end ligation of the annealed anti- HPV16-E7 and anti-GFP inserts was performed to produce pMBV43-H3-E7 and pMBV43-H3-GFP plasmids respectively. The plasmids were then sent for sequencing (GATC Biotech AG, Konstanz, Germany). After

confirming the sequence, the constructs were transformed into CEQ221 bacteria (Cequent, Cambridge, MA, USA) by heat shock protocol producing CEQ221-E7 and CEQ221-GFP bacteria.

3'- GNNNNNNAAGTTCTCTNNNNNNNAAAAAAAAAAACAGCT-5'

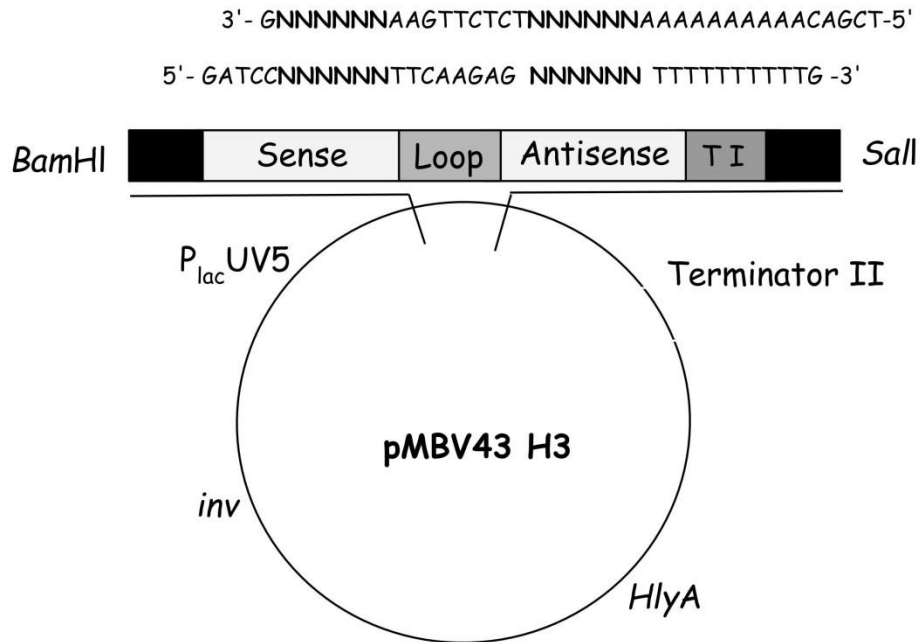5'- GATCCNNNNNNNTTCAAGAG NNNNNN TTTTTTTTTTG -3'

Fig. 2: Diagrammatic representation of the pMBV43 H3 plasmid vector map and the shRNA insert. The insert is flanked by the *Bam*HI and the *Sal*I sequences to facilitate the digestion and the sticky end ligation of a new insert. Typically, the insert comprises a sense strand, antisense strand, the loop and the multiple Thiamin termination site (original picture not included in previous publications).

3.3 Transfection of the oral carcinoma cells with tkRNA bacteria

The CEQ221-E7 and CEQ221-GFP bacterial colonies were inoculated in 7 ml LB medium with 50 mg/ml kanamycin and 50 μg/ml 2,3-Diaminopropionic Acid (DAP; Sigma, St. Louis, MO, USA) incubated overnight at 37 °C with shaking. Meanwhile, the tumor cells were cultured in 10 cm petri dishes, 6-wells plates or LapTek II Chamber slides. From the overnight bacterial culture, 1 ml was inoculated in 100 ml LB medium 50 mg/ml kanamycin, 50 μg/ml 2,3-Diaminopropionic acid and 2mM IPTG (Carl Roth GmbH, Germany) in 1 l Erlenmeyer flask, and incubated on a shaker at 37 °C and 200 rpm until $OD_{600} = 0.5$ is reached. Bacteria were washed twice in PBS, diluted in serum-free DMEM medium and added to human cells at

multiplicity of infection or MOI of 1:500 (which represent the ratio of the infecting bacteria to one cell of tumor), and incubated for 2 hours. Afterwards, washing twice with PBS follows and culture on serum containing DMEM medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin, 150 μg/ml gentamicin, and 100 μg/ml kanamycin.

3.4 Fluorescence microscopy for bacterial penetration detection

In order to confirm the bacterial penetration to the epithelial cells, cells were cultured in LapTek II Chamber slides, treated with the bacteria as described and stained with DAPI after 3, 6, 12 and 15 hours of the beginning of the co-incubation. Cells were washed twice with PBS and once with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride; Sigma, St. Louis, MO, USA) working solution (DAPI 2% in methanol) then covered with DAPI working solution and incubated for 15 minutes at 37°C. DAPI was then discarded and cells were washed with methanol, mounted, examined and photographed using fluorescent microscope (Keyence BZ-8000, Keycence; Ahmed et al., 2015).

3.5 Quantification of shRNA expression

Quantification of the expression level of anti-E7 and anti-GFP shRNAs in treated cells was determined by real-time qRT-PCR as described in details in Ahmed et al., 2015 and Ahmed and Lage, 2019. Forward primers were designed for the detection of the siRNA inserts of HPV16-E7 (5'-TAA TTG CTC ATA ACA GTA GAG A-3') and GFP (5'-ATCAAGCTTATCGATACCGTC-3'), while a universal reverse primer was provided with the QuantiMir RT Kit and was used as a reverse primer. Human U6 snRNA control was used as a reference gene; and its sequence was 5'-CGC AAG GAT GAC ACG CAA ATT C-3'. Fold change values for the anti-HPV16-E7 and anti-GFP siRNA relative to the human U6 snRNA expression as well as to untreated tumor cells for calibration were calculated for each replicate of each sample using LC data analysis software and RelQuant software for relative quantification (Roche Diagnostics, Mannheim, Germany).

3.6 Quantitative Real-Time PCR

As described in details in Ahmed et al., 2015, quantitative analysis of HPV16-E7 gene expression was performed by real-time qRT-PCR with a LightCycler apparatus and SYBR-Green Fluorescent dye (Roche Diagnostics, Mannheim, Germany). The measured expression

levels were normalized for expression of the reference gene enzyme GAPDH1. Specific oligodeoxynucleotide primers used for amplification of each target were as follows: HPV16-E7-fw 5'-AGA AAC CCA GCT GTA ATCAT-3' and HPV16-E7-rev 5'- TTA TGG TTT CTG AGA ACA GA-3'; GAPDH1-fw 5'-TGA ACG GGA AGC TCA CTG G-3' and GAPDH1-rev 5'-TCC ACC ACC CTG TTG CTG TA-3'. Fold change values for the HPV16-E7 gene expression relative to the GAPDH1 gene expression as well as to untreated tumor cells for calibration were calculated for each replicate of each sample using LightCycler data analysis software and RelQuant software for relative quantification (Roche Diagnostics, Mannheim, Germany).

3.7 Western blotting

In Brief, the whole-cell lysates were prepared by RIPA Lysis Buffer (Merck Millipore, Massachusetts, USA) after the previously described culture and bacterial transfection of the tumor cells. Using Pierce BCA Protein Assay Kit (Pierce, Rockford, USA), protein concentration was measured; diluted, denatured and processed in polyacrylamide gel and transferred to an Amersham nitrocellulose membrane (GE Healthcare Life Sciences, UK). Blocking of the membranes followed using Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA). Mouse monoclonal antibody raised against amino acids 1-98 representing full length E7 of HPV16 origin was used to determine the E7 levels (sc-6981, Santa Cruz Biotechnology, Dallas, TX, USA); while anti-GAPDH antibody (ab8245, abcam, Cambridge, UK) was used as an internal control. Immunoblots were visualized and photographed using Odyssey imaging system (LI-COR Biosciences, Lincoln, NE, USA) and analysed with Odyssey software.

3.8 Cell proliferation assay

Sulforhodamine B (SRB) staining-based test was used to evaluate the proliferative activity of the tumor cells. Briefly, 7.5×10^3/mL UPCI-SCC-090 were cultured in 96-well plates in triplicates for 2 days. Bacterial transfection using different dilutions and MOIs of both CEQ221-E7 and CEQ221-GFP bacteria according to the described protocol was performed and plates were incubated for 5 days. 10% cold trichloroacetic acid was used to fix the cells for 1 hour at 4 °C followed by five times washing with tap water. Staining of the cells was done using 0.4% SRB in 1% acetic acid for 10 min at room temperature; cells were then washed by 1% acetic acid and left to dry overnight. Before measurement, cells were suspended in 20 mM Tris-HCl, pH = 10 for 1 hour at room temperature and plates were measured at 562 nm against the reference

wavelength of 690 nm. The IC$_{50}$ represents how much bacterial MOI is needed to inhibit 50% of the tumor cells in vitro. Mean IC$_{50}$ values of at least three independent experiments for both CEQ221-E7 and CEQ221-GFP bacteria were calculated by Graph-Pad Prism5 program.

3.9 Detection of apoptosis using flowcytometry

To measure the intracellular levels of active caspase-3 and total apoptosis, flow cytometry was used. Tumor cells were cultured in six-well plates and incubated for 1 day then transfected with CEQ221-E7 or CEQ221-GFP bacteria according to the above-described protocol. Using FITC Active Caspase-3 Apoptosis Kit and FITC Annexin V apoptosis detection kit (BD PharmingenTM, BD Biosciences), active caspase-3 and total apoptosis was measured respectively. According to the kit manufacturer's instructions, treated cells were trypsinized, washed in PBS and stained. Determination of the levels of active caspase-3 and total apoptosis was done using BD Accuri C6 flow cytometer (BD PharmingenTM, BD Biosciences) by collecting 10000 events. BD Accuri C6 software was used to process the data. Three independent experiments for each cell line were performed and the mean values were calculated.

4. Results

After constructing the transkingdom shRNA plasmids using the standard laboratory protocols, sequencing followed to confirm the correctness of the sequences. Successful clones were transformed in CEQ-221 competent bacteria and hereby, producing the CEQ-221-E7 or CEQ-221-GFP bacterial strains. To test the capability of the bacteria to penetrate and collect in the cytoplasm of the mammalian tumor cells, the engineered bacteria were incubated with the OSCC cells and stained with DAPI stain at different time intervals. Bacteria were seen collecting around the cytoplasm of the transfected cells after 3 hours of the co-incubation (figure 3). Lower numbers of bacteria could be encountered afterwards until no bacteria could be seen after 15 hours of co-incubation. After confirming the arrival of the bacterial transport vehicles, and hence the shRNA plasmids, to the target cells cytoplasm, we needed to evaluate the efficiency of shRNA expression. The generated cDNA produced by small RNA quantification kit (Quantimir) from OSCC cells transfected with CEQ-221-E7 or CEQ-221-GFP plasmids was used in an qRT-PCR test to measure the expression of the anti-HPV16-E7 and the anti-GFP shRNAs. Human U6 snRNA was used as a reference gene to quantify the differential expression of the studied siRNAs. High levels of anti-HPV16-E7 and anti-GFP siRNA were noticed in corresponding transfected cells as compared to untreated cells.

The level of downregulation of the HPV16-E7 gene mRNA by the expressed anti-HPV16-E7 siRNA was evaluated by relative quantification qRT-PCR. The results were normalised against the GADPH1 gene and compared to CEQ221-GFP-treated and untreated tumor cells. After 3 days of co-infection, the E7 mRNA expression in cells received the bacterially delivered shRNA directed against HPV16-E7 was only 61% of E7 mRNA expression in control OSCC cells. Corresponding inhibition of the E7 protein could be confirmed using western blot.

The functional consequences of E7 inhibition on the treated tumor cells was examined by measuring the levels of activated caspase-3, total apoptosis and SRB-dependent cell proliferation assay. Activated caspase-3 in CEQ221-E7-treated tumor cells was 8.8-fold higher than of untreated cells, while CEQ221-GFP-treated showed 2.2-fold increase in caspase-3 expression level as compared to untreated cells. Meanwhile, total apoptosis in CEQ221-E7-treated tumor cells was 6.4-fold higher than of untreated cells, while, CEQ221-GFP-treated cells exhibited 2-fold increase in total apoptosis level as compared to untreated cells. The IC_{50} of the CEQ221-E7

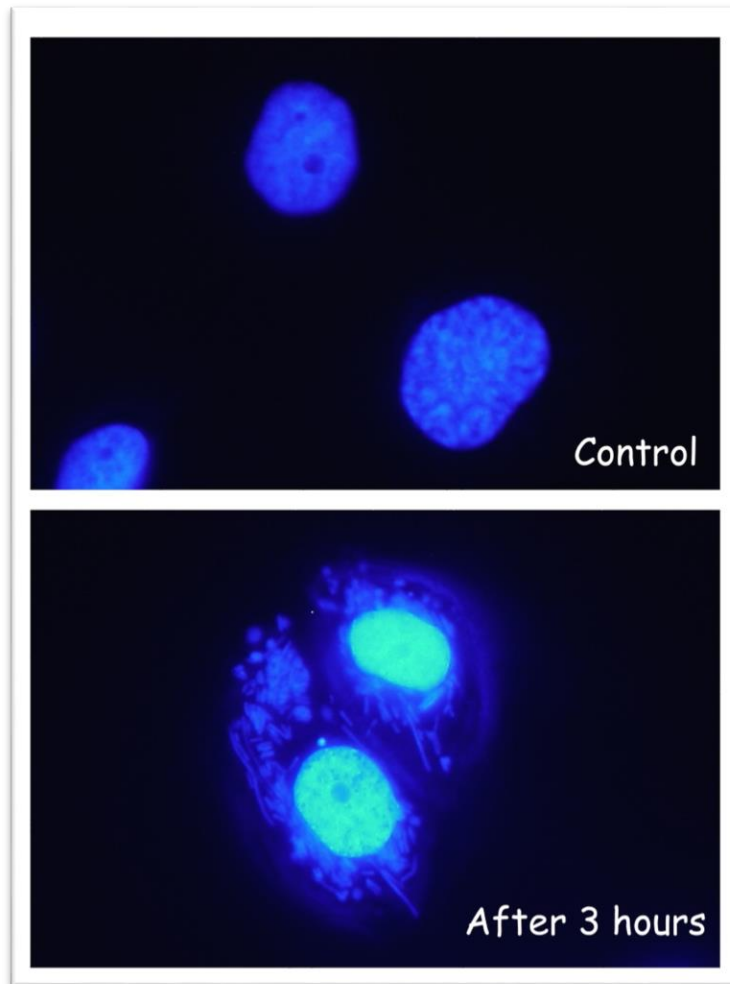bacteria was 1:130 ± 19 MOI fold in comparison to 1:530 ± 93 MOI in the CEQ221-GFP bacteria.

Fig. 3: Representative image of OSCC cells stained with DAPI and examined by fluorescent microscope before and 3 hours after co-incubation with the CEQ221-E7 bacteria to illustrate bacterial penetration (original picture not included in previous publications).

5. Discussion

Several approaches were used to confront the human papilloma virus since it first appeared in 1963. Treatment of HPV-induced carcinogenesis faced some challenges due to lack of inflammatory reaction which delays the immune response against the newly formed tumor cells. Another factor is the high diversity of the tumors and antigen differences that requires subsequently different therapies to different tumors (Chang et al., 2010; Singhania et al., 2012). Therapeutic vaccines against HPV-induced carcinogenesis did not show much success. While prophylactic vaccines proved successful against HPV infection and HPV-induced tumors subsequently, it may require many years of applying good vaccination programs to decrease the infection and the incidence rates (Zhou et al., 2013). Therefore, different approaches were required to provide treatment alternatives. In order to eradicate the tumor cells without affecting the neighboring cells and tissues, researchers focused on molecular targeted therapy, aiming at genes with specific genetic or epigenetic alterations limited to the cancer cells and not present in normal cells. Although molecular therapy proved successful invitro, some problems appeared upon in vivo application such as side effects, resistance, lack of specificity and lack of targetable genetic changes in many tumors (reviewed in Zhou et al., 2018). RNA interference was suggested for the treatment of HPV-induced carcinogenesis and the early work showed induction of apoptosis in the HPV-infected tumor cells invitro (Singhania et al., 2012).

In order to address the persistent issue of delivery of shRNA to target cells, a new bacterial delivery system was designed and optimized for this purpose. Bacterial delivery of shRNA molecules has many advantages over both viral and non-viral delivery system. Beside it is easy and cheap to construct, tkRNA directly overcomes the issue of nuclease degradation since the shRNA will be protected inside the bacteria. Moreover, some bacteria preferentially grow in the hypoxic solid tumors due to focal lack of vascularization (due to fast growth of the cancer is not usually met by fast growing capillary network); which render a systematically administered bacteria more prone to target cancerous tissue rather than normal tissue; and therefore, decreasing off target toxicity (Gardlik and Fruehauf, 2010; guyen and Fruehauf, 2008). Hypoxic tropism has limitations because small tumors lacking necrotic hypoxic will be less invaded by the therapeutic bacteria. This limitation was addressed by using facultative anaerobes such as *Salmonella spp.* and *E. coli* instead of strict anaerobes (Diaz et al., 2005; Pawelek et al., 1997).

In this case, after systemic administration, bacteria will be cleared from the circulation by the immune system after reaching different tissues and organs, but preferentially to the hypoxic tumor masses. The complex and atypical capillary network enhance the process of entrapment of the bacteria within the tumor areas (Forbes et al., 2018). The mode of anticancer effects after localization in the tumor masses is largely dependent on bacterial strain. While many bacterial strains have direct cytotoxic effect, other bacteria such as *Salmonella spp*. do not possess such cytotoxic capabilities due to either their inherent probertites or due to attenuation required before systemic application. In this case, the host immune response decides the potency of the anti-tumor effects of the bacteria (Avogadri et al., 2005).

Because of their size and charge, it is not possible for the siRNA molecules to directly cross the tumor cell membrane. Therefore, Once reaching the tumor mass in a therapeutic concentration, bacteria are designed to cross the tumor cell membrane owing to the invasin protein expressed on the bacterial wall by the *inv* locus of *Yersinia pseudotuberculosis* integrated in the shRNA plasmid. Invasin protein interacts with β1 integrin on the tumor cell membrane; which is upregulated in less differentiated tumor cells providing selective penetration to tumor cells (Buttaro and Fruehauf, 2010; Critchley et al., 2004; Birmingham et al., 2008). In this experiment, the efficiency of delivery at different MOIs and time intervals was showed by DAPI fluorescent staining. The bacteria could be seen in the cytoplasm around the tumor cells nuclei with maximum penetration noticed within the first three hours of the bacteria-tumor cells co-incubation. Bacterial degradation followed, due to lack of nutrients (especially DAP), until almost no bacteria could be seen after 15 hours of co-incubation. For illustration we presented the results of MOI 1:1000 (figure 3). To avoid non-specific tumor cell death due to transfection and based on our previous work (Lage and Krühn, 2010; Ahmed and Lage, 2019), we used the MOI 1:500 for our further experiments.

Following introduction of the shRNA molecules into the target cell, they will be entrapped in a membrane-bound endocytotic vesicle. The RNAi effectors have to escape the pinocytotic vesicle to the cytoplasm in order to interact with the RNAi endogenous machinery and target mRNA; which represent another challenge facing RNAi successful application (Chang et al., 2010). To overcome this issue, our shRNA plasmid is endorsed with *Hly*A gene expressing the pore-forming listeriolysin O which acts on the lysis of the entry vesicle bringing the RNAi effectors in

direct contact with the RNAi machinery. Plentiful expression of the shRNA targeting GFP and E7 after their bacterial delivery was measured by qRT-PCR in oral cancer cells. These results provide evidence of successful endocytotic vesicle escape and shRNA expression.

Our most relevant results showed that the delivered shRNA efficiently knocked out the HPV16-E7 expression in oral cell lines used as confirmed by qRT-PCR and western blot. Other delivery strategies produced comparable levels of E7 inhibition following anti-E7 shRNA delivery (Chang et al., 2010; Zhou et al., 2013). Subsequent lower tumor cell proliferation ability was observed following E7 gene knockdown as anticipated by previous studies (Hong et al., 2009, Xi et al., 2016). High levels of total apoptosis and active caspase-3 were observed on the tumor cells treated with CEQ221-E7 bacteria as compared to those treated with CEQ221-GFP suggesting that apoptosis is the major pathway of cell death after bacterial delivery-mediated HPV16-E7 gene inhibition. Direct correlation was found between E6/E7 inhibition and increased levels of P53 and pRb (Singhania et al., 2012; Xi et al., 2016) explaining the strong apoptotic signal following HPV-E7 inhibition in our experiments. Previous reports suggested that expression of E7 resulted in downregulation of pRB protein leading to G1 checkpoint failure and subsequently leading to uncontrolled cell proliferation (Singhania et al., 2012).

According to Forbes, 2010, the perfect vehicle for anticancer drug delivery should possess the following characters: 1) selectively lethal to the tumor cells thus increasing the therapeutic index; 2) controllable; 3) adjustable to the tumor microenvironment; 4) can be detected from the outside; and 5) self-propelled enabling it to infiltrate the tumor masses. Bacteria can be engineered to perform all the above-mentioned functions. Many bacterial strains tend to accumulate and proliferate in tumor masses due to hypoxia. Besides, bacteria will be entrapped in the complex vasculature of the growing tumor, which normally represent an obstacle against the conventional anticancer agents (Forbes, 2010). Moreover, bacteria can be designed to express chemotactic receptor on their surfaces, which can direct them to certain chemotactic factors in tumors (Kasinskas and Forbes, 2007). As a result, bacteria will be cleared from circulation by the immune system but meanwhile they will thrive in the immune-privileged cancerous masses (Dang et al., 2004; Kasinskas and Forbes, 2007). In addition, the ability of the bacteria to move and to leave the blood vessels to the intercellular space is a key feature of the anticancer bacterial therapy. Upon reaching and colonization in the tumor massed, the inflammatory cells at the

periphery of the tumor masses will inhibit the escape of the bacteria to the systemic circulation. Bacteria can be modified to express prodrugs or prodrugs activating enzymes such as cytotoxic agents (Ryan et al., 2009), cytokine to provoke immune system (Loeffler et al., 2008), and tumor specific antigens and antibodies (Groot et al., 2007). It was also found that bacteria are able to transfer genetic material to mammalian cells such as shRNA for the purpose of RNA interference. While shRNA delivery through expression vector produces strong and more stable effect, it is harder to control. Bacteria were also used to transfer shRNA to target cell for gene silencing and for gene triggering. Another advantage of the bacteria as a delivery vector for shRNA to tumor cells is that bacteria can be identified and monitored from the outside. Bacteria can be labelled by fluorescence, magnetic resonance or positron emission (Min et al., 2008).

Systemic application caused some side effects as well such as off target effect of siRNA, acute liver failure due to toxicity and activation of interferon I (Forbes et al., 2018). Local application of the therapeutic bacteria, as we suggest in our work by choosing an oral tumor as a model, would overcome the three prementioned drawbacks. For instance, oral administration of *Salmonella* showed reduced toxicity and preservation of the anti-tumor activity. However, oral route in human might be different from that of murine models. Topical application of anti-HIV, RNAi-based treatments faced some problems such as low pH of the mucous membranes, degradation by nucleases and difficulties in penetrating the superficial layers especially when hyperkeratotic (Chen et al., 2009).

Although bacteria represent an interesting alternative to the traditional gene delivery and anticancer therapeutics, some drawbacks and challenges appeared. a) bacteria carrying antibiotic resistance genes are generally not considered safe and especially when used in vivo. b) Live bacteria cannot be sterilized; meaning it is very hard to ensure that therapeutic bacteria are not contaminated with other pathogenic or non-pathogenic bacteria. c) Administered dose do not correlate with the therapeutic dose. It depends on many factors sch as the used bacterial strain, the transported anticancer agent, the hypoxic state of the targeted tissue, the inflammatory and immune status of the tumor and other factors, which make it difficult to adjust the therapeutic dose. To overcome this obstacle, case-to-case pre-administration analysis and tumor angiography are required. d) Infection is associated with variable degrees of toxicity. The early use of antibiotic would contain the infection but it will also prematurely abort the therapeutic trial

decreasing its outcome. e) the full mechanism is not totally understood. Besides, specificity and side effects are still unclear. f) despite intensive bacterial anticancer research, few studies successfully reached clinical trials. Clearly what applies in vitro do not essentially applies in vivo. Moreover, murine models are not identical to human models. Besides the heterogenicity of tumor cannot be replicated experimentally. g) Little is understood about bacterial tropism, side effects upon systemic administration, off target effect and the possibility of combining bacteria with other anticancer therapies (reviewed in details in Forbes et al., 2018).

In this work, we aimed to cover another aspect of HPV-related carcinogenesis. Human papilloma virus associated tumors are often associated with multi-drug resistance phenomena, where cancer cells developed resistance against structurally and functionally unrelated compounds (Efferth et al., 2019). Herewith, we tested a group of 16 Epoxylathyrane derivatives to evaluate their possible MDR modulating effect by targeting more than one MDR mechanism. Parental tumor cells were grown alongside with their drug-resistant counterparts and the drug-resistant phenotype was preserved by continuous addition of the corresponding selecting chemotherapeutic agent (either daunorubicin or mitoxantrone). After co-incubating a series of double fold serial dilution of the test substances with the tumor cells, an SRB-based test was performed and the IC$_{50}$ was calculated. The compounds 8, 15 and 16 showed the best results and were tested afterwards for induction of apoptosis using flow cytometry-based total apoptosis and caspase-3 detection kits. Among the three successful candidates was the compound 8 that showed the best overall performance against MDR. The MDR modifying activity was referred to their unique structure especially having hydroxyl function at position C-15 but also due to the aromaticity of the substituent together with the presence of heteroatoms (Reis et al., 2020).

In addition to the previously mentioned results, we used the RNAi technology to tackle the persistent problem of multi-drug resistance of gastric cancer cells to anticancer agents (unpublished data, the manuscript is in preparation). Based on previous publications we selected five microRNA candidates that might be involved in the atypical MDR in cancer cells. The differential expression of the five candidates was performed using qRT-PCR and revealed higher expression of the of mir-548d-3p in atypical MDR BCRP/ABCG2-positive gastric carcinoma cells (EPG85-257RNOV) as compared to their parental drug-sensitive counterpart (EPG85-257P). This was followed by inhibition of the mir-548d-3p and the effects of the inhibition on

the expression of BCRP/ABCG2 was evaluated. Inhibition of mir-548d-3p resulted in downregulation of BCRP/ABCG2 expression level and hence increased the sensitivity of the drug-resistant EPG85-257RNOV cells to mitoxantrone and eventually lead to substantial increase in mitoxantrone-dependent apoptosis.

Bacterial delivery represents a promising alternative for the classic delivery strategies for shRNA. Using the advantage that RNAi effectors molecules are protected inside bacterial cell, beside the comparatively easier way to transfect the bacteria with the required plasmid and its ability to multiply in the bacterial cell and hence multiplying the copies of the interfering RNAi molecules. Bacteria can carry large amounts of heterogenic DNA, which is not the case with other alternatives such as viruses. Taken together with the possibility of integrating some other genes within the infecting plasmid helping the cell to penetrate the target cell and helping the shRNA to escape the pinocytotic vesicles. Although hypoxia and necrosis in the tumor area represent a hurdle against chemo- and radiotherapy, it is considered advantageous for the bacterial therapy since it helps the bacteria to accumulate in the tumor masses and hence enhancing specificity. Bacterial therapy can also be potentiated by chemotherapy or radiation. That would lead to the reduction of the doses and the duration of exposure of chemo- or radiotherapeutic agents required for the patients, and hence decreasing the side effects associated with their use (Dang et al., 2004). Finally, in comparison to viruses, bacteria are safer, well tolerated in in vivo trials and easily controlled using antibiotics. In this work, we highlighted the availability of bacteria as robust, effective and safe delivery vehicles for the interfering shRNA molecules in HPV16-positive oral carcinomas. Inhibition of E7 mRNA resulted in a corresponding triggering to the caspase-dependent apoptosis signal. Transkingdom RNAi represents a good alternative to substitute current delivery strategies of the RNAi effectors especially in topically accessible tumors such as skin and oral cavity tumors.

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7. Anteilserklärung / eidesstattliche Versicherung

Eidesstattliche Versicherung

„Ich, Omar Hashem Bauomy Ahmed, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: Delivery of RNA interfering effectors against HPV-16 E6 and E7 proteins in OSCC cells, Delivery von RNA-interferierenden Effektoren gegen HPV-16 E6- und E7-Proteine in OSCC-Zellen, selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren/innen beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) werden von mir verantwortet.

Ich versichere ferner, dass ich die in Zusammenarbeit mit anderen Personen generierten Daten, Datenauswertungen und Schlussfolgerungen korrekt gekennzeichnet und meinen eigenen Beitrag sowie die Beiträge anderer Personen korrekt kenntlich gemacht habe (siehe Anteilserklärung). Texte oder Textteile, die gemeinsam mit anderen erstellt oder verwendet wurden, habe ich korrekt kenntlich gemacht.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Erstbetreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass ich mich zur Einhaltung der Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis verpflichte.

Weiterhin versichere ich, dass ich diese Dissertation weder in gleicher noch in ähnlicher Form bereits an einer anderen Fakultät eingereicht habe.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§§156, 161 des Strafgesetzbuches) sind mir bekannt und bewusst."

Datum   Unterschrift

08.03.2021

Anteilserklärung an den erfolgten Publikationen

Omar Hashem Bauomy Ahmed hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: Omar Ahmed, Andrea Krühn and Hermann Lage, Delivery of siRNAs to cancer cells via bacteria, Methods In Molecular Biology, 2015

Beitrag im Einzelnen:

The initial Manuscript of this method paper was jointly contributed to by the three authors. Mr. Ahmed wrote the first draft of the Introduction and the manuscript figures and photos were prepared by Mr. Ahmed, revised by Prof. Dr. Hermann Lage and the corrections were addressed by Mr. Ahmed

Publikation 2: Omar Bauomy AHmed, Hermann Lage, Bacteria-mediated delivery of RNAi effector molecules against viral HPV16-E7 eradicates oral squamous carcinoma cells (OSCC) via apoptosis, Cancer gene Therapy, 2019

Beitrag im Einzelnen:

Mr. Ahmed has substantially contributed to this publication. The initial Idea and the overall design of the experiments were proposed by Prof. Dr Hermann Lage and discussed with Mr. Ahmed. The Plasmid construction part including the shRNA design and realizing the experiments and the subsequent plasmid sequencing and the flourcent microscope study for the purpose of confirming bacterial penetration in cancer cells was almost completely independently performed by Mr. Ahmed. The RTqPCR experiments and the western blot study and the subsequent cytotoxicity study and the flow cytometry study for detecting apoptosis was performed completely by Mr Ahmed. The initial copy of the manuscript including the figures and the statistical analysis were prepared independently by Mr. Ahmed, Revised by Prof. Dr

Hermann Lage and the corrections and comments were addressed by Mr Ahmed before it was sent for publication.

Publikation 3: Reis MA, Matos AM, Duarte N, Ahmed OB, Ferreira RJ, Lage H and Ferreira M-JU, Epoxylathyrane Derivatives as MDR-Selective Compounds for Disabling Multidrug Resistance in Cancer, Frontiers In Pharmacology, 2020

Beitrag im Einzelnen :

After the isolation of the test substances and confirming the chemical structures, the test substances were sent to our lab in Berlin. Mr. Ahmed contributed to experimental design but mainly he conducted the toxicity study and the subsequent apoptosis detection using flow cytometry. Culture of the cell line and the subsequent toxicity studies and the flow cytometry-based apoptosis evaluation were almost completely performed by Mr. Ahmed.

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Unterschrift, Datum und Stempel des/der erstbetreuenden Hochschullehrers/in

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Unterschrift des Doktoranden/der Doktorandin

8. Selected publications

Delivery of siRNAs to Cancer Cells via Bacteria

Omar Ahmed [1], Andrea Krühn [1], Hermann Lage [1]

Bacteria-mediated delivery of RNAi effector molecules against viral HPV16-E7 eradicates oral squamous carcinoma cells (OSCC) via apoptosis

Omar Bauomy Ahmed, Hermann Lage

Epoxylathyrane Derivatives as MDR-Selective Compounds for Disabling Multidrug Resistance in Cancer

Mariana Alves Reis, Ana M. Matos, Noélia Duarte, Omar Bauomy Ahmed, Ricardo J. Ferreira, Hermann Lage, and Maria-José U. Ferreira

9. Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elecktronischen Version meiner Arbeit nicht veröffentlicht.

10. List of Publications

- **Ahmed O**, Krühn A, Lage H. (2015) Delivery of siRNAs to cancer cells via bacteria. **Methods mol. biol**. 1218, 117-129. doi: 10.1007/978-1-4939-1538-5_7

- Nafady AM, **Ahmed OB**, Ghafeer HH, (2016) Scanning and transmission electron microscopy of the cells forming the hepatic sinusoidal wall of rat in acetaminophen- and Escherichia coli endotoxin-induced hepatotoxicity. **J Microsc Ultrastruct** (2016), http://dx.doi.org/10.1016/j.jmau.2016.04.003

- Reis MA, **Ahmed OB**, Spengler G, Molnár J, Lage H, Ferreiraa MJU. (2016) Macrocyclic jatrophanes and cancer multidrug resistance – the ABCB1 efflux modulation and selective cell death induction. **Phytomedicine**. 23(9), 968-978.

- **Ahmed OB**, Mahmoud UT, Elganady S, Nafady AM, Afifi SMH. (2016) Immunomodulatory effect of gelatin-coated silver nanoparticles in mice: An ultrastructural evaluation. Nov-Dec 2016;40(6):342-350. doi: 10.1080/01913123.2016.1239666.

- Reis MA, **Ahmed OB**, Spengler G, Molnár J, Lage H, Ferreira MU. (2017) Exploring Jolkinol D Derivatives To Overcome Multidrug Resistance in Cancer. **J Nat Prod**. 2017 May 26;80(5):1411-1420. doi: 10.1021/acs.jnatprod.6b01084. Epub 2017 Apr 19.

- **Ahmed OB**, Lage H. (2019) Bacteria-mediated delivery of RNAi effector molecules against viral HPV16-E7 eradicates oral squamous carcinoma cells (OSCC) via apoptosis. **Cancer Gene Ther**. 2019 May;26(5-6):166-173. doi: 10.1038/s41417-018-0054-x. Epub 2018 Nov 15.

- Reis MA, Matos AM, Duarte N, **Ahmed OB**, Ferreira RJ, Lage H and Ferreira M-JU. (2020) Epoxylathyrane Derivatives as MDR-Selective Compounds for Disabling Multidrug Resistance in Cancer, Frontiers In Pharmacology, 2020, 11:599. doi: 10.3389/fphar.2020.00599.

11. Acknowledgements