SILENCE FOR HEALTH

development of RNA interference based cancer therapy

RNA interference (RNAi) has become an invaluable tool for biomedical research. It is already routinely used as an indispensable approach for gene function analysis in basic biological research and for target identification as well as validation in drug discovery programmes, respectively. Remarkably, RNAi advances to an alternative strategy for therapeutic application in medicine. The impact of this powerful technology becomes evident in view of the fact that RNAi presents a naturally occurring mechanism with the chance to virtually target any, even non-druggable, gene products with high efficiency allowing the development of specific drugs in much shorter time compared to classical therapeutics. The clinical translation of this technology from bench to bedside presents an outstanding endeavour in today's medical and pharmaceutical research. The generality of RNAi makes this strategy an applicable intervention for all different kinds of human diseases. The recent approval of RNAi-based therapeutics for phase I/II clinical trials in ocular disease caused big enthusiasm, so that this technology has been heralded to possibly become a new 'magic bullet' also for cancer therapy in the future. This review summarises the current perspectives on application, challenges and potential of RNAi-based therapeutics in oncology.

By Dr Ansgar Santel

RNA interference is an evolutionary conserved cellular mechanism for regulated silencing of gene expression. The 'gene [expression] silencing' phenomenon acts on the post-transcriptional level through sequence specific degradation of mRNAs which in turn interferes on the mRNA-level with the *de novo* synthesis of the corresponding protein. This abrogation of protein synthesis leads to depletion of the respective 'enzymatic' protein activity and its consequences. Scientifically speaking, loss of function can result in impaired cellular function(s).

The major steps of this particular mRNA degradation mechanism have been elucidated biochemically (**Figure 1**, and accompanying explanation). The key player in this mechanism turned out to be

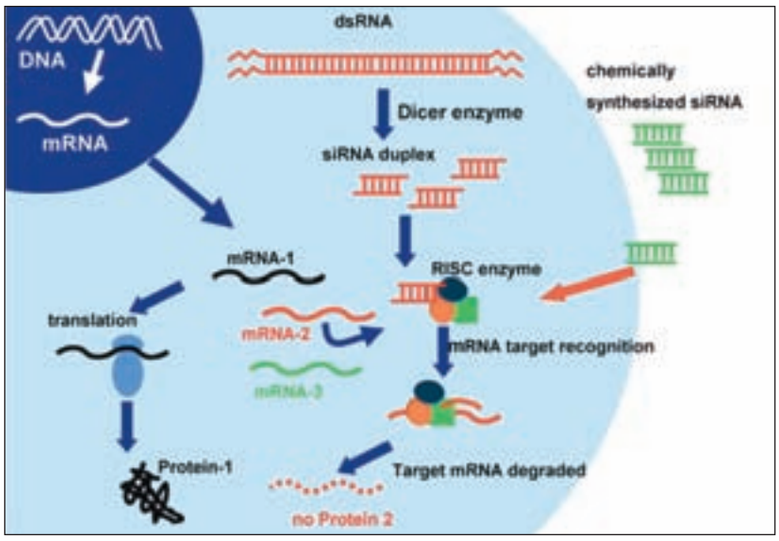

Figure 1
The mechanistic steps of the RNAi pathway. Double-stranded RNA (dsRNA) is generated or taken up by a cell and becomes processed by an enzyme called Dicer, generating a pool of short double-stranded RNAs (siRNA) about 19-23 nucleotides in length. The generation of siRNAs is a prerequisite of RNAi. They become recognised and loaded into the RISC multi-protein enzyme complex, which helps to find the right mRNA homologous to the siRNA and executes the endonucleolytic degradation of the mRNA after unwinding and binding of one of the siRNA strands complementary to the corresponding mRNA sequence. The RNAi pathway can be triggered when chemically synthesised siRNA molecules (green) become introduced into the cell

a novel class of double-stranded RNA molecules, the so-called short interfering RNA, siRNA. The siRNA has to identify the corresponding homologous mRNA destined for degradation by recruiting the degradation machinery as well as sustaining the degradation of the target messenger (the reader is referred to the following reviews to obtain a more detailed overview on the RNAi mechanism and its application[1,2].

The initiation of the RNAi mechanism can be by-passed *in vitro* (for example cultured cells) when exogenous, chemically synthesised siRNA molecules are being introduced into a eukaryotic cell. Since every eukaryotic cell appears to be equipped with the RNAi mediating enzymes, expression of virtually any known gene can be inhibited with siRNAs in all cell and organ types.

The loss-of-protein function due to RNAi-mediated gene silencing impairs, in analogy to a genetic mutation, the cell's physiology which in turn leads to a gene-specific phenotype. The generated loss of function phenotype uncovers the protein's biological importance for the cells/organs physiology (such as effects on metabolism, proliferation, survival or viability) providing the first clue of a novel protein's cellular function. Therefore, RNAi can be used to study the molecular function of a single gene, but can also be scaled up for high-throughput to specifically search for genes of interest in a genome wide approach.

Loss of function analysis by RNAi presents a powerful methodology for functional genomics, gene discovery and target validation (see also an excellent summary on this in the Spring 2005 issue of ***DDW***). Moreover, RNAi gene silencing allows the down-regulation of derailed gene expression which is in many cases causative for the onset of a disease. Therefore, RNAi does not only represent a versatile research tool but has the potential to become a specific and potent 'drug'.

The application of this potential 'drug' is not limited to certain diseases, but can essentially be transferred on to any disorder with altered gene expression (for example viral gene expression after infection). The machinery for RNAi is actually present in every cell of the body including tumour cells, but the sequence specific degradation of an mRNA can only be triggered in the presence of siRNA molecules. These molecules can be generated *in vitro* either by chemical synthesis or recombinant expression from particular DNA templates. Next, RNAi-mediated degradation of the cognate mRNA occurs once the *in vitro* generated siRNAs become introduced into a cell.

Cancer diseases are characterised by the uncontrolled division and invasion of tumour cells often followed by the dissemination to other parts of the body (metastasis). The development of cancer is accompanied by dysregulated gene expression of the tumour cells in contrast to the healthy normal tissue from its origin, making RNAi an attractive therapeutic intervention for treating cancer.

RNA interference, a novel modality for cancer therapy?

Cancer arises from a multi-step process called carcinogenesis. During carcinogenesis originally normal cells turn into defective tumour cells as a result of accumulation of several mutations. This transformation causes autonomous tumour cell growth with unlimited proliferative potential, evasion of programmed cell death, and finally invasion and metastasis. The acquired genetic abnormalities such as point mutations, chromosomal deletions, translocations as well as duplications frequently result in the overexpression of certain genes. Overexpression of these so-called oncogenes gives rise to abnormal uncontrolled growth due to high quantities of the oncogenes product (transcription factor, signalling proteins, cell cycle regulators), which in turn may stimulate the expression of another set of genes. In theory, RNAi might be the appropriate way to abolish in particular the derailed expression of those key genes involved in carcinogenesis. In other words, the rationale underlying the potential therapeutic application is the inhibition of any disease causing or promoting gene expression by destruction of the corresponding mRNA. Moreover, RNAi-mediated gene degradation can be used to selectively eliminate

diseased cells by interfering with the expression of those gene products critical for cell viability as wished for cancerous cells for example. Therefore, owing to the natural occurrence and generality of the RNAi mechanism and proposed application, this approach can be regarded as a novel therapeutic modality. Hence, RNAi-effectors like siRNA molecules emerge as a novel class of 'therapeutic inhibitor' considered for blocking cancer cell proliferation and survival.

Currently, classical anti-cancer therapies used for chemotherapy rely on the inhibitory or cytotoxic action of cytostatic agents (eg 5'-fluoruracil, cisplatin), small molecules (eg imitamib), or monoclonal antibodies (eg trastuzumab, cetuximab) designed to kill tumour cells. These agents interfere with general but important cellular activities such as metabolic pathways or cell cycle progression. Therefore, their inhibitory and cytotoxic effect is often not restricted to tumour cells, but also interferes with healthy cells leading to adverse side-effects in patients. In contrast, targeting the cancer-specific expression of the key molecules offers a novel opportunity for a more selective therapeutic intervention. In addition, many tumours develop over time resistance to chemotherapeutic agents by upregulation of anti-apoptotic gene expression. In this case, RNAi might effectively contribute to classical chemotherapy as an additional approach for restoring chemosensitivity by inhibiting those mechanisms governing chemotherapeutic resistance of tumour cells.

Finally, the big potential of RNAi-based cancer therapeutic and also its advantage over classical cancer drugs becomes apparent in the fact that different tumour types differ in their gene expression profiles as revealed by microarray based genomic surveys. This molecular means is used to obtain a 'genomic overview' for individual tumour types or even patients providing specific data sets on dysregulated gene expression. Accordingly, an RNAi-based intervention would eventually allow a tailor-made therapy due to the flexibility and generality of action by specifically targeting the mRNA of the key molecules.

Targets for RNAi based cancer therapy

As alluded to above, carcinogenesis is accompanied by multiple changes in cellular processes and pathways. These molecular changes are not restricted to the tumour cells but are also present in the surrounding tumour environment. As depicted in **Figure 2**, a solid tumour is composed of diverse cell types (endothelial cells, fibroblast and leucocytes) and structures (stromal tissue, extracellular matrix, necrotic tissue). The uncontrolled growth of cancer cells is only one aspect of tumour development, but the loss of differentiation and the recruitment of new blood vessels (angiogenesis) are other important determinants for tumour malignancy and progression. Consequently, the selection of the right target gene for cancer therapy does not necessarily need to be restricted to oncogenes or other dysregulated tumour target genes. Undoubtedly, gene products involved in oncogenic (receptor tyrosine kinase signalling) and anti-apoptotic (Bcl-2) signalling pathways, or molecules participating in cell-cycle control (RB-, p53-pathways, cyclins), cell invasion (cell adhesion proteins; matrix proteases) and motility merit consideration as first line tumour targets[3]. The potential usage of RNAi for the inhibition of tumour target genes has been addressed in proof of concept studies with tumour xenograft (implants of murine or human cancer cell lines on to immune-compromised mice strains) mouse models[4]. Nonetheless, RNAi mediated inhibition of tumour-host interactions offers another large quantity of attractive target genes. Tumour angiogenesis, the recruitment and formation of new blood vessels for oxygen and nutrition supply, is another hallmark event during cancer pathogenesis, which has been proven to be essential for tumour growth and metastasis. In this context, many angiogenic gene products are being used in this process of stimulating vascular proliferation, growth and remodelling. A prominent player in this process is VEGF (VEGF-A), a mainly tumour-derived factor, crucial for the stimulation of resident vascular endothelial cells to proliferate and migrate. Interestingly, phase-I/II clinical

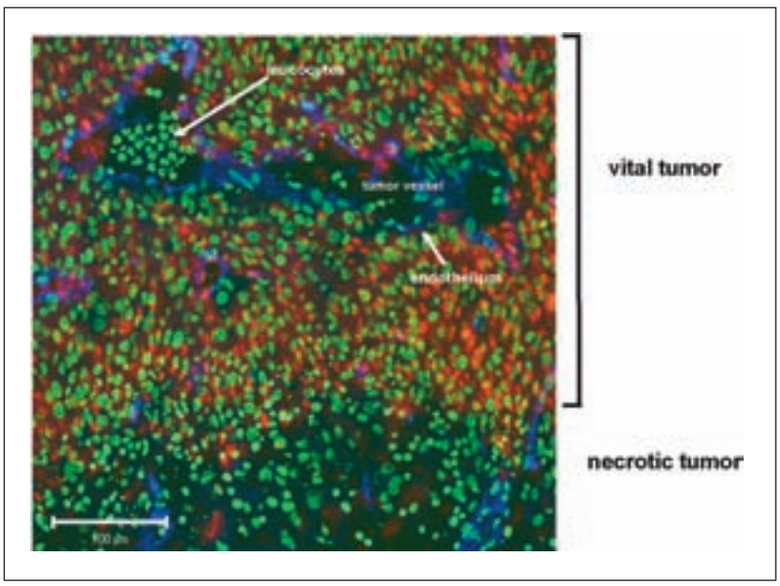

Figure 2
Several different sites of the tumour can theoretically be targeted by RNAi mediated therapy. Any tumour displays a complex tissue containing multiple different cell types and structures. By using RNAi the target gene expression in different cells of the tumour can be addressed. The microscopic picture shows a very small portion of a tumour section derived from an experimentally grown mouse tumour xenograft. The healthy tumour cells are shown in red, the small endothelial cells of the tumour vasculature are shown in blue. Nuclei are shown in green. Note the accumulation of leucocytes in the big tumour vessel and necrotic tumour cells in the lower part of the picture

Table 1: Strategies for siRNA formulation developed for cancer therapy

FORMULATIONS
Diverse cationic liposomes
Nanoparticle (RGD peptide -PEG-polyethyleneimine)
Polyethyleneimine (PEI)
Cyclodextrin-polycation
Protamin-antibody-fusion protein
Histidine lysine

trials have been recently approved to evaluate the treatment of angiogenesis dependent ocular neovascularisation disease AMD (age-related macular degeneration) with siRNA molecules against VEGF. The inhibition of tumour angiogenesis might be an alternative and promising therapeutic strategy. In summary, RNAi offers the opportunity to interfere on many different levels with tumourigenesis making this technology adaptable and flexible according to therapy demands. Above all these considerations regarding the 'right' targets, the nuts and bolts of RNAi-based therapeutics for cancer therapy are the development of applicable and efficacious modes of siRNA delivery and administration.

Delivery of siRNA-based cancer therapeutics

Many efforts are currently being undertaken to find successful strategies for the functional siRNA delivery *in vivo*. In contrast to RNAi applications 'in the test tube', this technology faces many obstacles when applied *in vivo*. The siRNA molecules have to be resistant against RNases of the serum when entering the bloodstream and need to escape from instant clearance by the kidney. Another major hurdle to be overcome for a feasible siRNA-mediated cancer therapy remains the efficient delivery of the RNAi-effector molecules to the tumour tissue destination and subsequent functional cellular uptake, when administered intravenously. For these two reasons, stabilised RNA-molecules carrying backbone or internal nucleotide modifications and diverse delivery vehicles have been developed and suggested to be functional as experimentally demonstrated in several recent publications[5]. Although early studies with cholesterol-modified siRNAs were suggested to be sufficient for triggering RNAi *in vivo* after systemic (intravenous) administration, conjugations to and formulation of the negatively charged siRNAs besides some topical tumour treatment with siRNA collagen deposits, are commonly used for experimental *in vivo* cancer therapy in mouse models. It is, however, currently not clear how the siRNA molecules escape the circulation and cross the endothelial cell barrier of the vasculature to reach tumour or stromal cells. In addition, the molecular mechanism, by which the cell co-ordinates the siRNA uptake, remains obscure and stipulates further experimentation. Remarkably, formulation of siRNAs is thought to improve the stability and pharmacodynamics of these molecules due to the shielding properties of the complexation reagent, but also to facilitate the site-specific delivery of the tumour, cellular uptake and release of the therapeutic siRNAs, especially when administered in the clinically relevant intravenous mode. For this purpose the negatively charged siRNAs are complexed preferentially with cationic reagents such as listed in **Table 1**, which are capable to bind the siRNA and reversibly release them once entered the cell after crossing the cell membrane barrier. For example, specific peptide or antibody ligand fusions aim to ensure cell-type specific tumour compartments targeting for selective induction of RNAi. In this case cell surface receptors are supposed to trap the siRNA-complex. By contrast, cationic liposomes are discussed to have affinity to tumour endothelium cells making this attractive for anti-angiogenesis therapy. Moreover, liposomes appear to have additional advantages for *in vivo* application, such as the bio-degradability (in contrast to PEI) and the lack of immunogenic properties (in contrast to peptides). This approach is also considered for guaranteeing a functional, RNAi-triggering cellular delivery, which includes the proper endocytosis of the liposomal siRNA-complex and the escape of the siRNA molecules from the endocytotic/lysosomal degradation pathway (endosomal release). For other indications (viral lung infections), local administration (pulmonary and intranasal) routes are likely to be adequate to induce siRNA delivery and uptake, whereby the intravenous infusion will be the preferred administration route for future siRNA-based cancer therapy approaches. In conclusion, the formulation of siRNAs 'kills two birds with one stone', overcoming the two major obstacles illustrated above, namely improved serum stability and delivery .

Above and beyond these concerns of siRNA design, stability and functional delivery, the efficacy of the RNAi technology for tumour growth inhibition will be the most important determinant for qualifying RNAi as a valuable anti-cancer approach. Preclinical experiments with tumour xenograft animal models explored the capability of this technology to inhibit tumour growth targeting many different targets considered to be relevant for

tumour growth. These studies proved RNAi to be effective in tumour growth inhibition and therefore places cancer in line with other indications (ocular, inflammatory, infections for example) to be treated by prospective RNAi-based therapies.

Even though promising progress has been made toward the development of RNAi-based cancer therapeutics in different experimental settings, many questions remain to be addressed concerning the delivery, availability and activity of siRNAs at the tumour site upon systemic administration.

Outlooks

The RNAi technology has evolved from a versatile *in vitro* tool for the molecular dissection of cancer pathways to a potential novel cancer therapeutic modality. More than a dozen publications in the past two years report in proof-of-principle studies the *in vivo* applicability and anti-tumoural effects of RNAi approaches. Regardless of the used siRNA-target sequence and formulations as well as the experimental set up of these *in vivo* studies, these data encourage developing the RNAi technology as a novel cancer therapeutic agent. Nevertheless, our understanding of RNAi reflects just the tip of the iceberg and future research will help to uncover the real impact of this technology. Especially with respect to the current efforts to design tailor-made therapeutics for the individual cancer patient, RNAi bears a huge potential as a 'novel class of therapeutics'. It is still noteworthy, that in theory any novel cancer-relevant target (even classical non-druggable targets) can be quickly addressed with the RNAi technology. There is no doubt that this technology needs further developments especially with respect to administrations routes, pharmacologic/pharmacodynamic properties of the diverse RNAi effectors (formulated, modified or none) in order to learn more about appropriate dosing regimens for efficacious treatment. Then, RNAi approaches will have the impact to become an alternative therapeutic modality to complement and augment anti-cancer therapies. There is still a long way ahead of us to fully exploit RNAi for cancer therapy, but there is clearly light at the end of the tunnel. DDW

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