REVIEW

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Actively personalized cancer vaccines – the step into clinical application

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Received June 30, 2015, accepted August 19, 2015

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Pharmazie 71: 43–47 (2016) *doi: 10.1691/ph.2016.5739*

Cancer vaccine development enters a new phase of innovation based on the development of modern sequencing technologies and novel RNA-based synthetic drug formats which enable the analysis and therapeutic targeting of every patient's tumor genome. By applying and combining these innovations, we have brought the concept of "actively personalized cancer vaccines" to clinical testing. Synthetic RNA is used as the drug format, allowing affordable, individual "on demand" manufacturing of tumor-optimized vaccines.

1. Introduction: RNA-based vaccines hold promise for innovation

The approach to utilize the human immune system to battle tumors through vaccination can be traced back to William B. Coley in the late 19th century. The American physician published the injection of attenuated bacteria as an immunostimulant for cancer therapy in 1893 (Coley 1991). Following his approach, various concepts using molecularly defined vaccines were developed to expand T cells which recognize tumor-specific antigens and thereby facilitate tumor cell lysis. T cells can be "primed" through various antigen formats (peptides, proteins, DNA, RNA) against various antigen classes (differentiation antigens, cancer germline antigens, mutated antigens). Investigation and application of synthetic *in-vitro* transcribed RNA as an antigen format in cancer immunotherapy has been initiated more than two decades ago. As early as 1995, Conry et al. could prove that synthetic messenger (m)RNA induces antibody responses after injection into muscle tissue. A year later, Boczkowksy et al. (1996) observed the induction and anti-tumor activity of specific T cells after vaccinating mice with mRNA-loaded dendritic cells (DCs). Based on this work, RNA vaccination was further developed in the academic and industrial setting into a clinically applicable treatment modality. The use of synthetic mRNA in cancer therapy is appealing in many ways. The antigen format has been clinically proven to be safe. Moreover, since RNA can be produced by *in-vitro* transcription in a faster, more cost-effective and better scalable way than any other immunotherapeutic agent, this drug format is currently under development for many innovative vaccine concepts.

2. Molecular characteristics and the concept of RNA-based cancer vaccines

A therapeutic cancer vaccine enables the targeted activation of the immune system against cancer cells by stimulating and expanding antigen-specific immune cells through the vaccine administered to the patient. The goal is to mount an adaptive immune response that effectively and specifically eliminates

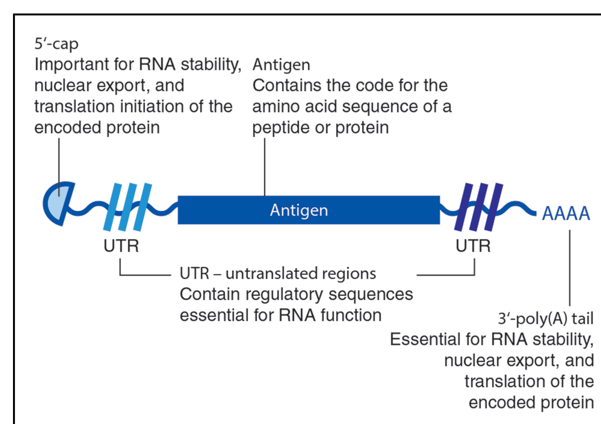

Fig. 1: Structure of a synthetic mRNA. The genetic information necessary to induce an immune response is encoded in the mRNA's open reading frame (antigen) flanked by regulatory elements as indicated.

cancer cells. For this purpose, tumor antigen-encoding synthetic RNA vaccines can be applied. The molecular structure of the synthetic RNA used for this purpose is very similar to that of a native mRNA. It consists of a single RNA strand, carries a 5'-cap structure, untranslated regulatory sequence elements (UTRs) upstream and downstream of the open reading frame (ORF) for the respective encoded protein, as well as a poly(A) tail at the 3'-end (Fig. 1).

To induce a T-cell response the immune system requires the presentation of antigenic peptides on MHC (major histocompatibility complex) molecules (see Fig. 2). To this end, the synthetic mRNA has to be taken up by antigen-presenting cells, preferentially dendritic cells, which synthesize the protein and process it into smaller peptides. The mRNA uptake can also occur *ex vivo*. DCs belong to the so-called "professional" antigen-presenting cells of the immune system, which can induce a very efficient primary immune response through the presentation of peptides. Moreover, they are the only cell type that is able to activate naive T cells. The protein translated from the synthetic mRNA is the true antigen and thus represents the bioactive substance

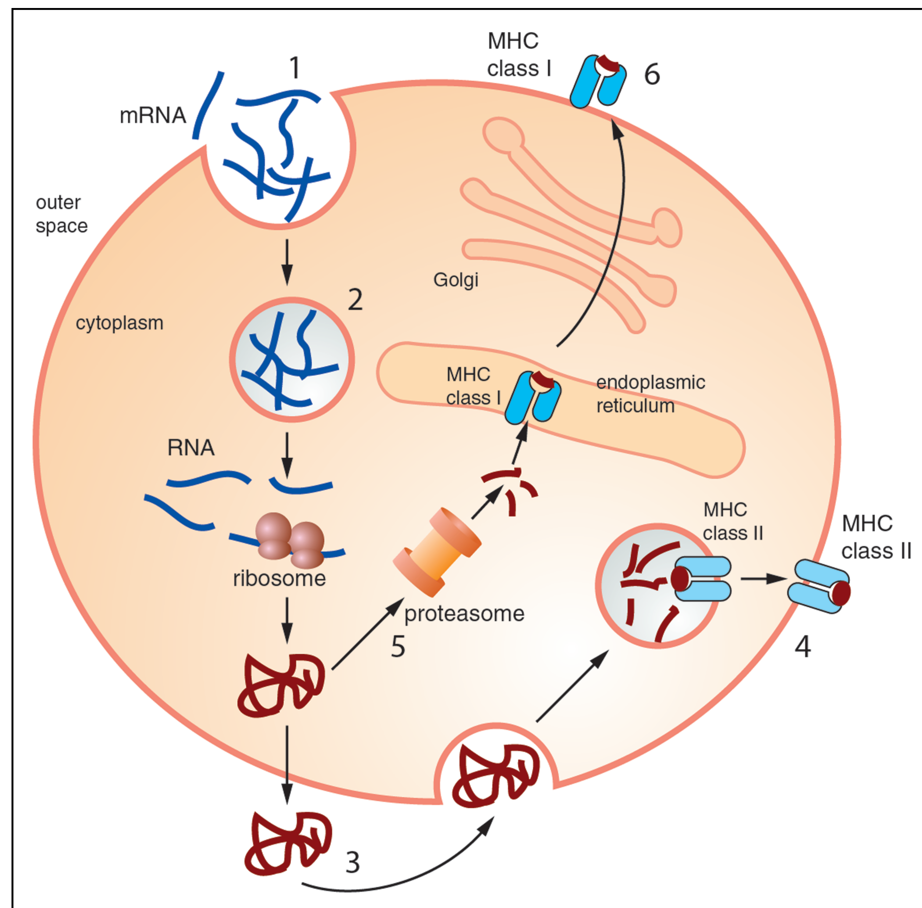

Fig. 2: Principle of the pharmacodynamic activity of synthetic mRNA: (1) absorption of mRNA through endocytosis; (2) cytoplasmic translocation and translation of the RNA into protein; (3) protein secretion; (4) epitope processing and presentation of protein-derived epitopes by MHC class II molecules; (5) intracellular protein processing and (6) subsequent presentation via MHC class I molecules.

of the vaccine. Analogous to proteins translated from endogenous mRNA, the protein encoded by the synthetic mRNA is processed and antigenic peptide fragments (epitopes) are generated and presented to the immune system's effector cells on MHC molecules residing in the cell membrane. Cytoplasmic proteins are primarily processed by the proteasome and the derived peptides are transported to the endoplasmic reticulum where loading onto MHC class I molecules takes place. Such peptide:MHC I complexes are then transported to the cell membrane to stimulate $CD8^+$ cytotoxic T cells. $CD4^+$ T helper cells are activated and expanded through recognition of peptide:MHC class II complexes that are mainly expressed by professional antigen-presenting cells such as DCs.

For a potent vaccination, $CD8^+$ as well as $CD4^+$ T cell responses have to be induced (Schoenberger et al. 1998). DCs are able to simultaneously present epitopes on MHC class I and II molecules and are therefore the primary targets of an RNA-based vaccination therapy (Fig. 2). Optimal MHC class I and II presentation of antigens by DCs can be further enhanced by linking the antigen to intracellular targeting signal sequences (Kreiter et al. 2008). The principle of immune system activation through RNA-based vaccination is presented in Fig. 2.

Unlike endogenous mRNA which is generated through transcription in the nucleus and then transported into the cytoplasm, synthetic mRNA must enter the cell from the extracellular space. This can in principle be done, as is common practice, by loading the patient's cells *ex vivo* using electroporation (Kyte and Gaudernack 2006). Such approaches require a GMP facility for cell therapeutics, which is logistically and financially demanding. A promising alternative is the direct *in vivo* application of the synthetic mRNA. For pharmacologic activity, it must be ensured that a sufficient RNA dose reaches the cytosol of the target cells. The hurdles to be overcome are the cell membrane as a diffusion barrier, as well as abundant RNases in extracellular environments which cause rapid degradation of the RNA. We have recently established a potent vaccination method that is based on direct injection of synthetic RNA directly into lymph nodes (Sahin et al. 2014; Kreiter et al. 2010, 2011). This so-called intranodal immunization was extensively tested in pre-clinical studies and successfully moved to clinical testing (NCT01684241). The high immunogenicity and vaccine potency of this approach is based on the fact that the injected mRNA is primarily taken up by immature DCs. This process is efficient because immature DCs constitutively take up RNA by macropinocytosis, a specialized form of endocytosis (Diken et al. 2011). After translation, the resulting proteins are processed and vaccine epitopes are presented. Simultaneously, the dendritic cell is activated by interaction of the synthetic RNA with Toll-like receptors (autochthonous adjuvant function; 10). These features are ideally suited for the stimulation of antigen-specific T cells.

3. Manufacturing of synthetic mRNA for vaccine development

Application in humans requires manufacturing of synthetic mRNA in accordance with GMP guidelines. The manufacturing process includes synthesis of the DNA template, production

of the synthetic mRNA through *in-vitro* transcription using a bacteriophage RNA polymerase, purification of the product, as well as accompanying analytics. A major challenge for the manufacturing process is to establish the various quality controls for RNA product identity, integrity, sterility, filling quantity, as well as exclusion of other RNA/protein traces or endotoxin contamination. For RNA approval in clinical trials, its functionality must be guaranteed („potency assay"). Importantly, purified RNA – in the absence of contaminating RNases which affect the *in-vivo* stability of RNA as described above – is a chemically stable molecule. Depending on the temperature, it can be stored for several years without a significant loss of quality.
As soon as the manufacturing process for a synthetic mRNA has been established and a corresponding manufacturing license according to GMP rules has been obtained, any other mRNA can be produced, tested and released within a few weeks by slightly adapting the process. This flexible platform setup combined with cost-effective and fast producibility provides excellent conditions for the application of synthetic mRNA in personalized medicine.

4. Induction of antigen-specific immune responses through somatic tumor mutations

The above mentioned characteristics and the advantageous safety profile make synthetic mRNA an ideal antigen format for the clinical development of actively personalized vaccines (Pascolo 2008). Depending on the tumor entity, human tumors carry between approximately ten to several hundred mutations that lead to changes in the amino acid sequence of tumor-relevant proteins (Alexandrov et al. 2013). Independent of the type of tumor, each tumor has a characteristic individual "mutation signature" or "mutanome" (Wood et al. 2007). Thus, somatic non-synonymous mutations, which change the amino acid sequence of proteins and thus give rise to novel epitopes, represent an attractive antigen source for actively personalized cancer vaccines. Such cancer mutations enable a selective targeting of tumor cells by T cells specific to the novel epitopes. Conceptually, such epitopes are attractive target structures for a cancer vaccine therapy: since they are not subject to central tolerance induction in the thymus (such as the individual's native epitopes), they should be able to trigger the expansion of highly reactive T cells. Furthermore, as expression of the mutated antigen is restricted to the tumor, no off-target side effects are to be expected. Indeed, various studies have shown that epitopes derived from somatic non-synonymous mutations are frequently targeted by T cells (Sjoblom et al. 2006; Pleasance et al. 2010; Lee et al. 2010; Shah et al. 2009; Robbins et al. 2013; Lennerz et al. 2005).
To be used in actively personalized cancer vaccine therapies, the mutations have to be identified by sequencing of the individual cancer genome. A selection of immunogenic mutations then provides the basis for the tailored vaccine whose rapid production favors the prospect of therapeutic success.
Next-generation sequencing (NGS) is the method of choice for the identification of mutations. The rapid development of this technology has enabled us to sequence complete genomes within a few days, with continuously decreasing costs (Koboldt et al. 2013). After sequencing, enormous data sets must be processed in order to identify specific mutations that have to be differentiated from technical artefacts and single nucleotide polymorphisms. In the next step, the identified mutations are prioritized to lay the foundation for the selection of mutations that define the lead structure of the RNA vaccine. We and others have been able to achieve relevant advances in epitope predictions, mutation evaluation and prioritization of potentially immunogenic mutations, which now allows potent designs of mutanome vaccines (Scholtalbers et al. 2013; Lower et al. 2012; Lundegaard et al. 2008; Cibulskis et al. 2013).
In pre-clinical experiments, we were able to prove for the first time that the identification, validation and prioritization of somatic non-synonymous mutations is feasible and can provide the basis for the development of an antitumorally effective vaccine (Castle et al. 2012). Toward this goal, DNA of murine melanoma B16F10 cancer cells was sequenced using NGS and the somatic non-synonymous mutations were validated *via* Sanger sequencing. Mutated protein-coding sequences were then analyzed in a systematic manner for the immunogenicity and antitumoral potency of the respective epitopes. Utilizing RNA vaccines, which could be manufactured within a few days after identification and verification of the mutations, it became evident that about 20% of the identified non-synonymous mutations are immunogenic and can induce T cell specific immune responses against the corresponding neo-antigens (Kreiter et al. 2015). Although the B16F10 tumor model only very weakly expresses histocompatibility molecules that can be recognized by T cells (Boegel et al. 2013), this therapeutic strategy resulted in potent tumor growth inhibition.

5. Clinical translation of mutanome vaccines

The preclinical work described above allowed us to translate the "mutanome engineered RNA immuno-therapy" (MERIT) concept into clinical trials. A fast supply of RNA-based vaccines that turn the patient's immune system against her/his individual tumor provides the basis for the personalized treatment. The clinical translation of such mutanome vaccines represents a paradigm shift from a product-centered to a patient-centered drug development.
For the manufacturing of mutanome vaccines, tumor material and healthy tissue (blood) obtained from the patient after diagnosis are subjected to a quality control in the biosampling unit. DNA is extracted from both, tumor and blood cells, while RNA is extracted from tumor cells alone. For the DNA, the protein-coding sequences (exons) are enriched. RNA and DNA libraries are then established and analyzed using NGS. Applying specialized algorithms, the identified non-synonymous mutations specifically expressed in the tumor are further analyzed for their prioritization with regard to their potential immunogenicity (potency to elicit an immune response against the respective epitope). Based on this generated information, a tailored vaccine is designed for each individual patient. For the vaccine, the mutated coding sequences (each encoding 25-35 amino acids connected by linkers) are sequentially arranged in the open reading frame. The sequences, codon-optimized for the use in humans, are then manufactured in line with GMP regulations. After release, packaging and shipping, the tailor-made mutanome vaccines are available for the treatment of patients (Fig. 3).
Various groups all over the world are currently pursuing the clinical development of personalized vaccines, using RNA or peptides as antigen formats. To our knowledge, we are the first group worldwide that succeeded in bringing RNA-based mutanome vaccines into clinical trials. Since late 2013, several patients with malignant melanoma have been treated in a first-in-man study (NCT02035956). Initial results on the safety and induction of targeted immune responses in patients are expected soon.

6. Perspective

The clinical development of mutanome vaccines is the first implementation of the "actively personalized therapy" princi-

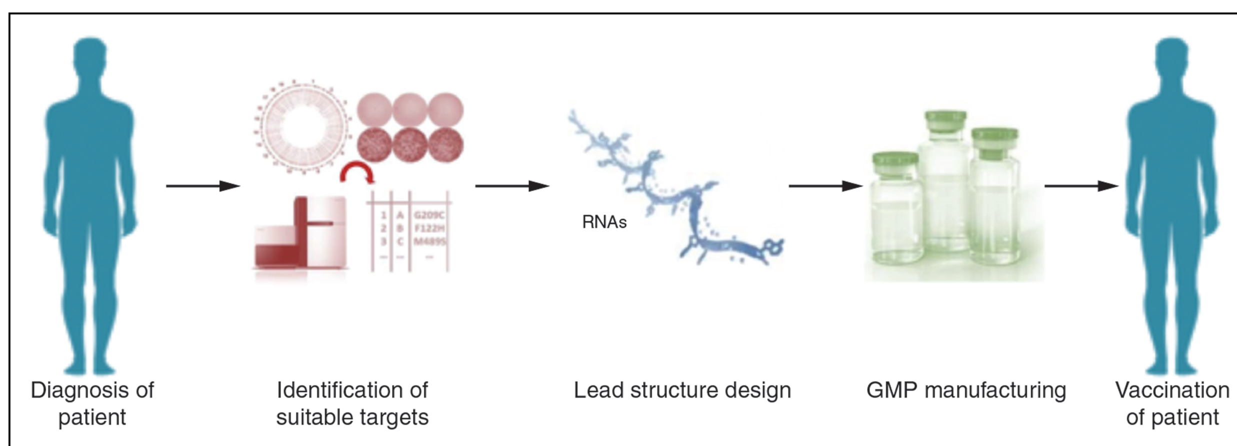

Fig. 3: After diagnosis, patient-specific tumor mutations are mapped by NGS and bioinformatic analyses in order to identify potentially immunogenic mutations. Sequences encoding such mutated peptides are then combined in an mRNA (RNA lead structure) and applied as a tailor-made mutanome vaccine.

ple that can be universally applied to all common as well as rare cancer diseases. Mutanome vaccines represent a highly innovative therapeutic concept that goes along with a paradigm shift in industrial drug development. The principle of targeting the micro-heterogeneity of individual patient tumors opens up the perspective to prevent the development of resistance to therapy and overcomes the limited availability of "druggable" target structures in oncology. The ongoing first-in-man clinical study substantiates that this concept can be translated into the clinic. The next steps towards the clinical implementation of mutanome vaccines are the clinical proof of therapeutic efficacy, followed by efforts aiming at marketing approval and eligibility for reimbursement by health insurance organizations.

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