Pre-Clinical Proof of Concept and Characterization of AEZS-120, a Therapeutic Oral Prostate Cancer Vaccine Candidate Based on Live Recombinant Attenuated Salmonella

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Introduction

AEZS-120 (St MoPC) is a novel therapeutic recombinant live oral prostate cancer vaccine. It is based on the attenuated carrier strain *Salmonella typhi* Ty21a, which is already approved as an oral vaccine against typhoid fever. *S. typhi* Ty21a has an excellent safety profile with more than 250 million doses already applied. AEZS-120 is genetically modified to express a PSA-CtxB fusion protein in order to induce a PSA-specific cellular immune response specifically directed towards prostate cancer cells expressing PSA and to stimulate innate components of the immune system.

To evaluate the pre-clinical safety and efficacy of AEZS-120, a program has been designed in agreement with the regulatory authorities. Due to the lack of a relevant animal model for *S. typhi*, a key element of the non-clinical program is the comparison of the recombinant vaccine strain with the carrier strain. Depending on the experimental setting, *S. typhi* Ty21a, or the mouse homologue *S. typhimurium* aroA is employed. The ultimate goal of the program is to evaluate whether there is a difference between the recombinant strain or corresponding carrier strain, which would be indicative of a potential safety difference with respect to the carrier.

Mode of Action

The molecular principle of AEZS-120 is the secretion of a fusion protein of prostate specific antigen (PSA) and cholera-toxin B (CtxB) by the recombinant carrier strain (fig. 1).

Features of AEZS-120 key for the mode of action are summarized in table 1.

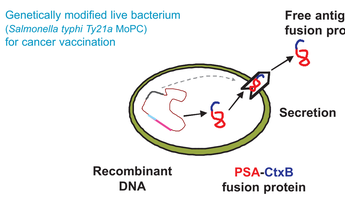

Figure 1: Molecular principle of AEZS-120

Feature	Function
Carrier *S. typhi* Ty21a	Enables oral application; targeting of M-cells / immune system in the gut; induction of innate immunity
Recombinantly produced PSA	Induction of PSA specific CD8 immune response
Secretion of fusion protein	Optimized CD8 response
CtxB fusion	Targeting of MHC class I pathway, optimized CD8 response

Table 1: Molecular features of AEZS-120 and their function with respect to the MoA

Bacterial strains

AEZS-120 is composed of the live carrier *S. typhi* Ty21a containing the recombinant plasmid pMohly Kan PSA-CtxB (MoPC). The plasmid encodes the *E. coli* hemolysin secretion machinery and a fusion protein of PSA with CtxB. In addition, the plasmid encompasses a kanamycin resistance gene as selection marker.

As *S. typhi* is neither pathogenic nor immunogenic in mice, non-clinical studies were also performed employing the attenuated *S. typhimurium* aroA strain as carrier for the plasmid. The recombinant strains applied in this study are summarized in table 2.

Bacterial strains were propagated in a shaking culture process similar to the GMP process employed for AEZS-120 for clinical use and released according to specification.

Bacterial strains (ctd.)

Name	Carrier	Resistance	Plasmid	Remarks
AEZS-120, St MoPC	*S. typhi* Ty21a	kanamycin	pMohly Kan PSA-CtxB	Human vaccine strain
St MoAPC	*S. typhi* Ty21a	ampicillin	pMohly Amp PSA-CtxB	Human vaccine strain with ampicillin resist.
Sl MoPC	*S. typhimurium* aroA	kanamycin	pMohly Kan PSA-CtxB	Mouse vaccine strain
Sl pTolC	*S. typhimurium* aroA	kanamycin	pMohly Kan TolC	Mouse control strain

Table 2: Recombinant strains used for non-clinical evaluation of AEZS-120.

Antitumor efficacy

AEZS-120 is not immunogenic in mice, therefore *S. typhimurium* aroA is used as carrier strain for assessing the immunogenicity and protective efficacy of AEZS-120.

In a first proof-of-concept study, Sl MoPC was shown to induce a PSA specific CD8 T-cell response (Fensterle et al., 2008). In addition, immunization protected against a challenge with tumor cells expressing PSA. In this study it was also shown that the recombinant construct employed in AEZS-120 is superior to plasmids encoding PSA or CtxB alone or separately.

Another study was conducted in order to compare different immunization schedules with one or two immunization cycles. As depicted in figure 2, immunization with one and two cycles resulted in a profound and significant block of tumor growth with the PSA expressing recombinant mastocytoma tumor cell line P815 implying that immunization with Sl MoPC is both effective and not sensitive to epitope masking after repeated applications. Protective efficacy was similar to the efficacy obtained with intramuscular naked DNA vaccination, which is known to induce robust CD8 T-cell responses.

Of note, also immunization with the carrier alone induced a small, albeit significant delay in tumor growth. This delay is indicative of the induction of the innate arm of the immune system, which is considered as important for successful tumor immunotherapies.

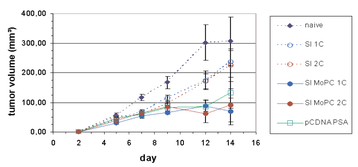

Figure 2: Protective activity of vaccination with *S. typhimurium* aroA MoPC (Sl MoPC) in one (1C) and two cycle (2C) immunization schedules. pCDNA PSA: positive control naked DNA immunization. Values represent median tumor volumes +/- SD.

Biosafety

Shedding and horizontal gene transfer in the gut are potential biosafety risk factors for a recombinant, orally applied Salmonella based vaccine.

Horizontal gene transfer has been assessed employing an enforced filter-mating method (fig. 3). In this experiment, the strain St MoAPC was used which carries an ampicillin-resistance cassette. Plasmid transfer was not detectable and therefore maximal acceptor transfer rates can be considered to be lower than 2.5x10-^{10}, representing the assay specific limit of detection.

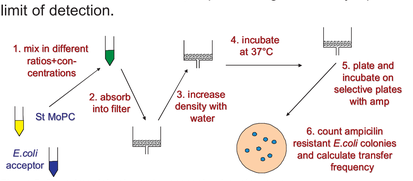

Figure 3: Experimental scheme for assessing transfer rates.

Biosafety (ctd.)

Shedding has been assessed in mice using *S. typhi* Ty21a and *S. typhimurium* aroA (worst case scenario) as carriers. In the case of *S. typhimurium* aroA, shedding was compared between recombinant strains carrying the plasmid MoPC or a irrelevant plasmid with kanamycin resistance (pTolC).

For AEZS-120 at single oral doses of 1x10^{10} CFU, shedding was not detectable in faeces, urine and saliva (data not shown). Sl MoPC, as well as the control strain Sl pTolC, showed the expected substantial shedding in faeces on day 1 after application at an oral dose of 1x10^{10} CFU (fig. 4). At day 6, shedding in faeces is marginal and at day 29, shedding is no more detectable. In urine, kanamycin resistant colony counts are close to the detection limit and most likely due to contamination by faeces. In saliva, no shedding of *S. typhimurium* is observed. Importantly, there is no difference between the shedding of Sl MoPC and the control strain Sl pTolC.

Taken together, the data suggests that shedding of the recombinant vaccine AEZS-120 is similar to the approved carrier strain *S. typhi* Ty21a. For this strain, shedding in humans only occurs at doses above 1x10^{10} CFU, which is 5 fold higher to the recommended dose, and is limited to one day after application.

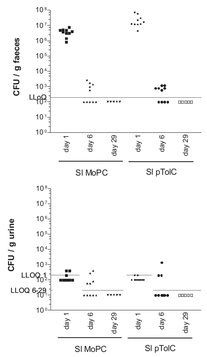

Figure 4: Counts of kanamycin resistant colonies in faeces and urine of mice treated orally with 1x10^{10} CFU Sl MoPC or Sl pTolC. LLOQ: limit of quantification.

Biodistribution

Biodistribution was assessed in mice using the carrier strain *S. typhimurium* aroA again comparing the recombinant strain with the carrier strain. Based on historical data, this mouse adapted strain is known to infect spleen and liver after oral application.
As expected, bacterial and/or plasmid DNA could be detected in lung and spleens in some mice infected orally with 5x10^{9} live bacteria 6 days after infection (table 3). Positive signals were close to the detection limit of the method. Neither bacterial DNA nor plasmid DNA was detected in liver, blood, kidney, brain or testis 6 days after infection. 30 days after infection, neither bacterial nor plasmid DNA was detectable in any organ tested.

Group	Day	Spleen	Heart	Brain	Liver	Testis	Lung	Kidney	Colon
Sl MoPC	6	0/2/5	0/0/5	0/0/5	0/0/5	0/0/5	1/2/4	0/0/5	0/0/5
	30	0/0/3	0/0/3	0/0/5	0/0/4	0/0/5	0/0/5	0/0/5	0/0/3
Sl	6	0/0/5	0/0/4	0/0/5	0/0/4	0/0/5	0/1/5	0/0/5	0/0/5
	30	0/0/3	0/0/5	0/0/4	0/0/4	0/0/5	0/0/5	0/0/5	0/0/0
Detection limit		3.0x10^{3}	1.8x10^{3}	4.8x10^{3}	8.6x10^{3}	1.5x10^{3}	2.1x10^{3}	2.3x10^{3}	n.a.

Table 3: Detection of plasmid DNA / bacterial DNA 6 or 30 days after infection. Number of PCR positive organ samples for plasmid DNA / *Salmonella* DNA / spiked *E. coli* DNA are tabulated. Samples positive for spiked *E. coli* DNA are considered as evaluable. Groups with organs positive for plasmid or Salmonella DNA are highlighted in orange. Detection limit in copies of DNA per organ.

Toxicology

Safety and toxicology studies were performed in mice comparing the recombinant strain carrying the plasmid MoPC with the corresponding non-recombinant carrier strain to assess potential safety risks attributable to the plasmid.

After single oral application up to the highest applied dose of 1x10^{10} live bacteria per animal, neither relevant toxicological findings nor differences between animals dosed with AEZS-120 or the carrier *S. typhi* Ty21a were observed. AEZS-120 was not detectable in the blood 8 hours and 24 hours after infection.

In a **repeat dose toxicological** study, the carrier strain *S. typhimurium* aroA was used and also immunotoxicological parameters were assessed. Neither the recombinant nor the carrier strain in doses up to 1x10^{10} live bacteria per animal resulted in relevant toxicological or immunotoxicological findings. Recombinant *S. typhimurium* was not detectable in the blood 8 hours and 24 hours after infection. There was no difference observed between the strains.

In mice **after intraperitoneal (IP) infection**, the clearance of *S. typhi* Ty21a in spleen and liver is much faster (1000x) compared to the virulent parental strain Ty2, which is indicative of its reduced virulence and used as a model to estimate the degree of attenuation (Germanier 1983). After IP challenge, there was no difference in clearance between AEZS-120 and the carrier *S. typhi* Ty21a in this system suggesting that the attenuation pattern of the approved carrier strain *S. typhi* Ty21a is retained in AEZS-120 (fig. 5).

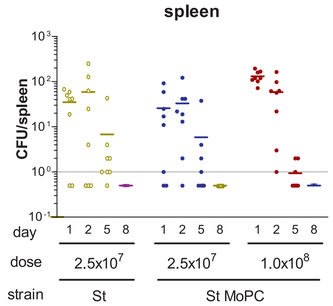

Figure 5: Elimination kinetics in spleen and liver after IP challenge of mice with St or St MoPC. Each value represents CFU counts of spleen samples of individual animals.

Summary & Conclusions

- The production, release, pharmacology, safety and toxicology program has been conducted in agreement with the regulatory authorities and succesfully finalized:
  - The **proof of concept** has been shown in a tumor challenge model and the anticipated clinical application schedule could be confirmed.
  - **Biosafety and biodistribution** studies did not reveal a different safety profile compared to the carrier strain
  - **Pharmacological and toxicological** studies did not reveal differences to the approved carrier strain
- In total, the non-clinical studies suggest that the safety and toxicological profile of AEZS-120 is similar to the approved carrier strain *S. typhi* Ty21a, which has been already safely applied in >250 million doses
- GMP material for clinical use has been produced and released
- CTA filing for phase I clinical study is planned in 4Q2012

Methods

Preparation and application of bacteria in mice. For use, bacteria were thawed and diluted in PBS to the appropriate concentration. Oral application was performed as intragastric application in a volume of 200 µl. 5-10 minutes prior to oral application, 50 µl 7% $NaHCO_3$ was applied PO to reduce gastric pH. Intraperitoneal application was performed in PBS. DBA/2 mice or C57BL/6 mice were kept under specific pathogen free conditions. Animal experiments were performed according to local regulations and laws.

Antitumor efficacy. DBA/2 mice were immunized orally with 5x10^{9} CFU St or St MoPC at days 1 and 8 (1 cycle) or at days 1, 8, 57 and 64 (2 cycles). As a positive control, animals were immunized by intramuscular injection with 50 µg pCDNA PSA for three times in a three weeks interval (DNA immunization). pCDNA PSA is a eukaryotic expression vector encoding PSA. Eight days (2 cycles groups) or 14 days after the last immunization, animals were challenged with the antilogous, recombinant PSA expressing mastocytoma cell line P815-PSA (PPSA). Tumor growth was followed for 21 days. After day 14, parts of the animals had to be sacrificed for animal welfare reasons in the control groups. Therefore, only the time period from day 1-14 is depicted.

Plasmid transfer. Plasmid transfer studies were performed as described by Sasaki (1988) using the method of enforced filter mating as a worst case scenario for plasmid transfer in the gut. In brief, bacterial suspensions of donor strains and the acceptor strain *E. coli* 1243 (streptomycin resistant) were mixed and adsorbed on filter membranes and incubated overnight at 37°C. After incubation, bacteria were suspended and plated on streptomycin / ampicillin plates and colony growth was counted. As a positive control, the *E. coli* strain W945 R1drd16 containing a F-like transmissible plasmid was used. Transfer rates for the positive control were close to 100% in each experiment.

Shedding. Shedding was assessed under GLP by plating serial dilutions of saliva, urine and stool samples of C57BL/6 mice infected orally with a single dose of 1x10^{10} CFU St MoPC, Sl MoPC or Sl pTolC as a control. Urine samples were collected non-sterile by abdominal pressure, stool samples were collected over 24 hours, resuspended in PBS and processed further.

Biodistribution was evaluated after single oral infection of C57BL/6 mice infected orally with one dose of 1x10^{10} CFU Sl MoPC or Sl. The animal part was performed under GLP. Biodistribution in different organs was assessed using a previously validated PCR method. In brief, organ DNA was extracted using a Qiagen tisue kit and than analyzed by PCR. The sensitivity of the method in spleens was determined as approx. 30-50 copies per µg DNA. The sensitivity per organ is calculated according to the fraction of organ processed for DNA extraction. Before DNA extraction, each sample was spiked with 1x10^{4} *E. coli*, which is close to the detection limit. Only samples tested positive for *E. coli* DNA are considered as evaluable.

Acute toxicology was assessed after single oral infection of C57BL/6 mice with 1x10^{9}, 3x10^{9} or 1x10^{10} CFU St or St MoPC under GLP. Animals were monitored for clinical signs over the complete observation period of 16 days. At day 7 and 16, necropsy and hematology was performed. Assessment included standard toxicology assessments, histopathology, full hematology and detection of bacteria in the blood.

Repeated dose toxicology was assessed in C57BL/6 mice under GLP using *S. typhimurium* aroA as a carrier. Animals were infected orally at day 1, 8, 57 and 63 with 1x10^{9}, 3x10^{9} or 1x10^{10} CFU Sl or Sl MoPC. Necropsy was performed on day 71 (main necropsy) or at the end of the recovery period on day 106 for pathology and histopathology. Animals were monitored for clinical signs over the complete observation period. Blood monitoring included standard toxicological assessments, haematological parameters (necropsy dates), live bacterial counts and immunotoxicological parameters (8 and 24 hours after each infection).

IP challenge. Animals were infected IP with 2.5x10^{7} CFU St or St MoPC and 1x10^{8} CFU St MoPC. 1, 2, 5 and 8 days after infection, spleens and livers were removed asseptically and CFU in liver and spleens was assessed by plating 100 µl suspension of organs mashed in 2 ml PBS on agar plates with and without kanamycin selection. In animals infected with St MoPC, values with or without selection were similar suggesting a full plasmid stability. CFU values represent direct counts in 100 µl.

References

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