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GMP manufacturing of Vvax001, a therapeutic anti-HPV vaccine based on recombinant viral particles

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ABSTRACT

Therapeutic vaccination is being explored as a treatment strategy for the treatment of patients with primary or metastatic tumours. We developed a vaccine targeted to Human papillomavirus (HPV)-induced tumours based on recombinant Semliki Forest virus (rSFV) encoding a fusion protein of the E6 and E7 proteins of HPV type 16. To enable a phase I clinical trial with this vaccine, Vvax001, a Good Manufacturing Practice (GMP)-compliant manufacturing process was set up and clinical material was produced.

Upstream production of the clinical material resulted in viral titers from 2.4×10^7 to 1.3×10^9 infectious particles/ mL in the harvest. The total volume of 6.0 liter crude virus was purified in 13 consecutive downstream purification runs. The mean titer after purification was 4.0×10^8 infectious particles/ mL and the mean recovery was 19%. Finally, clinical material was filled at a target concentration of 1.25×10^8 infectious particles/mL. Release testing included tests for viral titer and virus identity, biological activity, sterility, bacterial endotoxins, adventitious viruses and absence of replication competent virus. The product complied with all specifications and was released for use as an investigational medicinal product.

This is the first GMP production process developed for a SFV-based therapeutic vaccine. The vaccine, Vvax001 is targeted to HPV and has shown promising results in preclinical studies. The GMP-produced Vvax001 material met the quality criteria and was of sufficient quantity to enable assessment of its immunogenicity, safety and efficacy in a clinical setting.

1. Introduction

Human papillomavirus (HPV) infection is an important cause of premalignant lesions and cancer in genital and oropharyngeal region (e.g. cervical cancer, vulvar cancer, and oropharyngeal cancer), and HPV-induced cancer is the fourth largest cause of cancer deaths in women worldwide (Bray et al., 2018). Current treatment relies on surgery and/or radiotherapy combined with chemotherapy. Surgery is highly discomforting and carries a risk of complications and also radiotherapy and chemotherapy are associated with severe side effects. Above all, the treatments often do not eradicate all HPV infected cells.

Cellular immune responses play an important role in the immunological control of HPV infections and early (pre)malignant lesions.

In immune-competent women, many low-grade (CIN1) HPV-induced lesions regress spontaneously. Also higher grade lesions (CIN2/3) have been reported to regress spontaneously, although regression rates vary and are dependent on HPV type, viral load and HLA type (Trimble et al., 2005; Melnikow et al., 1998). Furthermore, HPV-associated tumours occur more frequently in immune-suppressed individuals (Ferenczy et al., 2003; Frisch et al., 2000; Sillman et al., 1997; Sun et al., 1997), whereas the presence of tumor-infiltrating T cells in patients with cervical carcinoma predicts a better clinical outcome (Gorter et al., 2015; de Vos van Steenwijk et al., 2013; Komdeur et al., 2017). Therefore, enhancement of anti-tumour immune responses by immunotherapy could significantly improve treatment of patients with HPV-induced lesions and cancer (Frazer et al., 2011).

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High-risk HPVs such as types 16, 18, 31, 33 or 45 have the capacity to transform cervical epithelial cells by integrating the open reading frames encoding the viral early proteins E6 and E7 into the host cell genome. This integration may lead to constitutive overexpression of E6 and E7, mediating transformation of the cells to a malignant phenotype. Since the continued production of E6 and E7 is required for the maintenance of the transformed phenotype, these antigens represent potential targets for therapeutic immunization strategies, aiming at the induction or stimulation of cytotoxic T lymphocyte (CTL) activity against HPV-transformed cells.

Therapeutic vaccines based on Semliki Forest virus (SFV) represent an ideal system for the induction of cellular immune responses against the oncoproteins HPV E6 and E7 since SFV RNA does not integrate and SFV infection is cytolytic by apoptosis. Thus, it is very unlikely that the genetic information for E6 and E7 will persist for prolonged periods of time within the organism. Furthermore, SFV-based vaccines induce high-level expression of the encoded proteins and are able to activate both the innate and the adaptive immune system due to the formation of dsRNA intermediates (Riezebos-Brilman et al., 2006). Previously, we have demonstrated that SFV particles encoding an HPV E6 and E7 fusion protein showed an efficient induction of CTL responses and anti-tumour activity in preclinical animal models (Daemen et al., 2002; Daemen et al., 2003; Daemen et al., 2004). Importantly, repeated immunization is not hampered by neutralizing antibodies or cellular immunity directed at the vector (De Mare et al., 2008). When SFV-based immunization was compared to immunization with the widely applied recombinant adenovirus system (recombinant adenovirus type 5), immunization with recombinant SFV particles induced higher CTL responses and more efficient anti-tumour activity, even with 100- to 1000-fold lower doses compared to the adenoviral vector. This difference in efficacy was likely to be due to anti-adenovirus antibody responses, which impaired the effect of booster immunizations, a well-known limitation in the clinical application of the adenovirus system (Riezebos-Brilman et al., 2007).

In this paper we describe the GMP manufacture of an SFV-based therapeutic vaccine against HPV, Vvax001. Vvax001 is a suspension of recombinant SFV particles encoding a fusion protein of the proteins E6 and E7 of HPV type 16. It is aimed at in vivo gene transfer of a therapeutic gene, and therefore considered as an Advanced Therapy Medicinal Product (ATMP). The process development and preclinical evaluation of Vvax001 have been described elsewhere (Singh et al., 2019). Vvax001 will be used in a phase I clinical trial for assessment of HPV-specific immune responses and safety.

2. Materials and methods

2.1. Materials

Plasmid DNA for the three constructs (pSFV3e6,7, pSFV-helper-S2 and pSFV-helper-C-S219A) (Smerdou and Liljeström, 1999) was produced by PlasmidFactory (Bielefeld, Germany). Production was performed without using any animal-derived substance. Each plasmid has been tested for concentration, identity, DNA sequence, purity, bacterial endotoxins, host cell protein and bioburden.

The cell bank used for the manufacture of Vvax001 was a Vero cell line Working Cell Bank (Intravacc, Bilthoven, the Netherlands). The cell line and the derived Master and Working Cell Banks have been tested for adventitious viruses, sterility, mycoplasma, and tumorigenicity according to the European Pharmacopeia and ICH guidelines (European Pharmacopoeia monograph 2.6.16, European Pharmacopoeia monograph 5.2.3, ICH Q5A (R1)).

All other materials were of European Pharmacopoeia quality, or have been tested according to in-house specifications and have been released for use in the production of parenteral preparations.

2.2. Production process

Vvax001 Drug Substance production was performed in a ML-I facility with dedicated upstream- and downstream processing areas (the Unit Biotech & ATMPs, Department of Clinical Pharmacy and Pharmacology, University Medical Center Groningen, the Netherlands). Production was performed under the responsibility of the Department of Clinical Pharmacy and Pharmacology, manufacturing authorization 108964F.

2.2.1. Vvax001 production (upstream processing)

DNA of each individual plasmid was linearized via a digestion reaction using the BcuI restriction enzyme (Fermentas; ER1251), which has one recognition site in each plasmid. Linearized DNA was purified by ethanol precipitation. RNA was synthesized via in vitro transcription using SP6 RNA polymerase, which recognizes the SP6 promoter in each of the linearized DNA constructs. Next the RNA was incubated with TURBO DNase to remove template DNA (mMESSAGE mMACHINE®kit Thermofisher Scientific; AM1340). The purified RNA was recovered by lithium chloride precipitation and centrifugation and the resulting pellet was dissolved in nuclease-free water. The purified RNA was tested for concentration, purity and endotoxins.

Vvax001 replicon particles were produced in Vero cells after electroporation of the three RNA transcripts: the pSFV3e6,7 transcript encoding the replicase proteins nsp1-4 and the E6,7 fusion protein, one transcript encoding the spike proteins and one transcript encoding the capsid proteins. Vero cells were cultured in culture medium (Medium 199 w/o phenol red (Lonza BE12-119F)) with 10% FBS (Sigma 12007C) and 2 mM L-glutamine (Lonza BE17-605E). Vero cells from sub-confluent flasks were detached with Tryple Select (ThermoFisher Scientific; 12563029) and resuspended in Biorad electroporation buffer in a concentration of 20×10^6/ mL. Cells were transferred to Gene pulser cuvettes and electroporated with the RNA transcripts using a Gene pulser Xcell electroporation unit (Bio-Rad). Each electroporation was performed with 16×10^6 cells and these cells were electroporated with 11.3 μg helper C, 15.4 μg helper S and 36.0 μg E6,7 RNA. The transfected Vero cells were cultured for up to two days at 37 °C and 5% CO2 in culture medium containing 5% FBS and 2 mM L-glutamine. Viral particles were harvested after 24 and 48 h by collection of the supernatant and centrifugation to remove Vero cells/cell debris. Viral titer and bioburden were determined for each harvest. Individual harvests were stored at $\leq -60\,°C$ until purification. Supplementary Fig. 1 shows the flow chart of the viral particle production in Vero cells.

2.2.2. Vvax001 purification (downstream processing)

Individual crude Vvax001 harvests were pooled for purification. Purification of viral particles was initiated with thawing of the virus harvests at 37 °C. The thawed harvests were pooled in a sterile glass container. The pooled harvest was treated with benzonase (50 U/mL; Merck, 1.01695 0001)) in 2 mM magnesium chloride for 30 min at 37 °C, in order to degrade all forms of host cell DNA and RNA. The benzonase reaction was stopped by 1:1 dilution with 10 mM EDTA in 50 mM HEPES buffer. The benzonase treated material was prepared for the first purification step by diluting with 50 mM HEPES buffer (ThermoFisher Scientific; 15630) to a conductivity of 4.5–5.0 mS/cm. The diluted pool was loaded on a solid state ceramic anion exchange column (Monolithic column CIM QA-8-cGMP; BIA Separations), which was used in a binding mode. After loading, the column was first washed with a 50 mM HEPES buffer. Subsequently the viral particles were eluted using a 50 mM HEPES buffer containing 0.3 M NaCl. The anion exchange purified fraction was further purified by cation exchange chromatography (Monolithic column CIM SO3-8-cGMP; BIASeparations), again in binding mode. Before loading of the column, the eluate was diluted with a 50 mM HEPES buffer to a conductivity of 7.5–8.2 mS/cm. After loading, the column was first washed with 50 mM HEPES buffer. Impurities were removed by elution using a 50 mM

HEPES buffer containing 0.3 M NaCl. Subsequently the viral particles were eluted with a 50 mM HEPES buffer containing 0.6 M NaCl. The eluted fraction was collected in sub-fractions, which were individually analysed for viral titer before pooling. Finally, 1% Human Serum Albumin (HSA) was added to the purified fractions as a cryoprotectant, and the fractions were stored at ≤ −60 °C for purity and quantity assessment prior to processing to the formulated Drug Substance. Supplementary Fig. 2 shows the flow chart of the viral particle purification.

2.2.3. Formulation and filling

Purified fractions were thawed at 37 °C. The thawed fractions were pooled in a sterile glass container. The pooled bulk Drug Substance was stored at ≤ −60 °C prior to processing to the formulated Drug Substance and samples were analyzed for infectious particles. Subsequently, the bulk Drug Substance was thawed at 37 °C and diluted with a 1% HSA solution to the desired concentration of infectious particles in a formulation buffer consisting of 20 mM Hepes, 1% HSA and 0.24 M NaCl, at pH 7.0. The formulated Drug Substance was immediately processed to the final Drug Product by filtration through a 0.2 μm filter (Millex GP SLGP033RS, Millipore) and dispensing into sterile glass vials (Vial easy fill 22.0 × 40.0 6R, Aluglass). After filling, stoppering and capping, vials were stored at ≤ −60 °C. The flow chart of the formulation and filling process is given in Supplementary Fig. 3.

2.3. Quality control

Critical Quality Attributes (CQA) were defined based on the Target Product Profile (TPP) of Vvax001, considering the route of administration, required titer and stability, and safety aspects (sterility, adventitious agents, impurities) (ICH Q8 (R2)). Subsequently, the quality control strategy was designed to determine Critical Quality Attributes such as viral particle characteristics, purity, microbiological contamination and viral safety. Testing for SFV virus identity and titer assesses viral particle characteristics. Testing for process-related (e.g. host cell DNA and proteins and medium components) and product-related impurities (e.g. Total viral particles) assesses purity. Microbiological quality is assessed by testing for bioburden, bacterial endotoxins and mycoplasma at several points in the production process. In addition, sterility is tested on the filled Drug Product. Performing virus testing on the Vero Master Cell Bank and adventitious virus testing on the crude viral harvests assesses viral safety. In addition, absence of replication competent virus is tested in the formulated drug substance. The quality control parameters and corresponding methods and specifications are listed in the Supplementary Figs. 1-3.

2.3.1. Analytical methods

Tests for Mycoplasma (Culture Method and PCR, Ph. Eur. 2.6.7), bioburden (Ph. Eur. 2.6.12, membrane filtration method), in vitro adventitious virus (Ph. Eur. 5.2.3), pH (Ph. Eur. 2.2.20), Osmolality (Ph. Eur. 2.2.35), and appearance (Ph.Eur. 2.2.1), are compendial methods described in the European Pharmacopoeia. The test for in vivo adventitious virus was performed according to the European Pharmacopeia (Ph. Eur. 5.2.3 and 2.6.16), testing was restricted to adult mice and guinea pigs.

Absence of porcine viruses by using indicator cells was performed according to the method described in 9 CFR 113.47 (Code of Federal regulations, title 9). A quantitative PCR method was used for the detection of Porcine Circovirus, Porcine Rotavirus type A and Hepatitis E virus. The PCRs methods were based on real time PCR technology and Taqman chemistry. First, nucleic acids were extracted from the test sample. Next, a PCR reaction was performed using primers and probes designed to a specific region of the target genome. The real time reaction was performed at 48 °C for 30 min, followed by a Taq DNA polymerase activation step at 95 °C for 10 min. The PCR reaction conditions were as follows: 40 cycles, consisting of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min.

The Picogreen assay was performed to determine the level of host cell DNA in purified fractions. The assay makes use of the Quant-iT™ PicoGreen® dsDNA reagent, which is a fluorescent nucleic acid stain for quantifying double-stranded DNA in solution. Fluorescence was detected using a fluorescence microplate reader with excitation wavelength ~480 nm and emission wavelength ~520 nm. DNA concentration was determined using a DNA standard curve.

The viral titer assay was used to determine the amount of infectious particles per milliliter (titer), as well as the identity of the SFV particles. The assay was performed by titration of viral particles by serial dilution on monolayers of BHK cells. Infection by SFV particles was determined by immunofluorescence using an antibody against SFV-nsp3 (replicase). This antibody was chosen because replicase is present in all cells infected with recombinant SFV. Positive staining therefore confirms the identity of the viral particles. Titers were calculated by counting positive cells and correction for the dilution factor.

The MicroBCA assay was used to determine the level of HSA in Vvax001 formulated Drug Substance. The MicroBCA assay determines the total protein concentration in a solution. However, since the concentration of HSA in the final Drug Product was 10 mg/mL, the contribution of other proteins in the solution (i.e. SFV-derived proteins, host cell proteins) was negligible. The MicroBCA assay makes use of bicinchoninic acid (BCA) as the detection reagent for $Cu+1$, which is formed when $Cu+2$ is reduced by protein in an alkaline environment. A purple-colored reaction product was formed by the chelation of two molecules of BCA with one cuprous ion ($Cu+1$). Absorbance was detected at ~562 nm using a fluorescence microplate reader. Protein concentration was determined using a BSA (bovine serum albumin) standard curve.

In addition to the MicroBCA assay, a test on Vero-specific host cell proteins was performed in order to distinguish between host cell derived proteins and HSA and SFV-derived proteins. The assay is based on an ELISA with Vero-specific antibodies (Cygnus Technologies ELISA kit). The antibodies directed against Vero cell proteins were attached to the solid phase and captured Vero proteins in the test article were detected by interaction with a horseradish peroxidase (HRP)-labeled anti-Vero antibody. Reaction of HRP with a 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate results in a color change that can be measured spectrophotometrically at 450 nm. Host cell protein concentration was determined using a Vero cell HCP standard curve.

The identity of the recombinant E6E7 SFV virus in the formulated product was determined using a Western Blot according to protocol 21885 (Department of Virology). BHK21 cells were infected with the recombinant SFV virus at different MOI (multiplicity of infection). BHK21cells were lysed after 24 h of incubation. The cell lysate was analyzed for E7 with Western Blot gel electrophoresis, using a mouse-anti-HPV16-E7 primary antibody and a goat-anti-mouse-AP (alkaline phosphatase) secondary antibody. Finally, E7 containing bands on the Western Blot were visualized by addition of an AP substrate, resulting in a chemiluminescent reaction.

Replication competent viruses were detected using a cell based assay. Vero cells were seeded in roller bottles to allow detection of a single replication competent virus in the maximum anticipated human dose of 2.5×10^8 infectious particles. Spiking studies demonstrated that replication competent viruses outgrow the replication incompetent virus rapidly causing cytopathic effects and destruction of the Vero cells. After expansion of Vero cells in roller bottles, the product was inoculated into the roller bottles and incubated for 14 days. Within the culture period the cells were passaged once. Cells were evaluated for cytopathic effects at the end of the incubation period, which is indicative for the presence of replication competent viruses.

The residual amount of benzonase was tested in an enzyme immunoassay, using the Benzonase ELISA II kit supplied by Merck (product number 1.01681). The kit uses specific polyclonal antibodies to capture benzonase, where present.

2.4. Stability studies

Stability studies were performed with the non-clinical material stored at actual storage conditions ($\leq -60\,^{\circ}\mathrm{C}$) for a period of 24 months, and at accelerated conditions (2–$8\,^{\circ}\mathrm{C}$) for a period of 6 months. Tests were performed periodically according to the time schedule as advised in ICH Q1A and Q5C (ICH Q1A (R2), ICH Q5C Quality of biotechnological products). Material for this stability study was used at a concentration of 5×10^{8} infectious particles/mL, which is 4-fold higher than the concentration in the clinical material. The formulation buffer, volume and vial/closure system were identical to the clinical formulation. In addition, real time stability studies are ongoing for the clinical material and will be performed for a period of 36 months.

3. Results

3.1. Development and technology transfer

The Vvax001 production process consisted of viral particle production in Vero cells upon electroporation of RNA transcripts (encoding the replicase and E6,7 fusion protein, the capsid protein and the spike proteins), purification by ion exchange chromatography and final formulation and filling. See Fig. 1 for an overview.

The production process was based on the laboratory procedure as described elsewhere (Daemen et al., 2002), and was further developed into a process suitable for technology transfer to the GMP facility. A detailed description of the development of the production process is provided elsewhere (Singh et al., 2019). Upon transfer to the GMP facility, the production process of Vvax001 was scaled up with regard to the viral particle production and purification steps, in order to increase the yield. A benzonase treatment step was added to the purification process to remove residual DNA. The purification by ion exchange chromatography was optimized with regard to column load and loading conditions.

3.2. Production of non-clinical and clinical material

3.2.1. Vvax001 production (upstream processing)

Viral particle production was initiated by electroporation of Vero cells in the presence of the three independent RNAs. In each electroporation round, viral supernatant was harvested at 24 and 48 h after electroporation. For the manufacturing of non-clinical material two campaigns were performed consisting of 7 and 4 electroporation rounds respectively, with an average of 4.8×10^{8} electroporated Vero cells per round. Average yield of an electroporation round was 424 mL of crude harvest, with titers ranging from 1.0×10^{8} to 3.4×10^{8} infectious particles/ mL. Total yield of the non-clinical production runs was 4.6 liter of crude harvest containing approximately 1.0×10^{12} infectious particles.

In the manufacturing of clinical grade material, culture medium without phenol red was used since this was considered an unnecessary additive that could potentially result in an impurity and hamper downstream processing. The performance of the culture medium without phenol red was confirmed with a growth promotion test. No other cell culture process changes were introduced.

For the manufacturing of clinical material, three campaigns were performed consisting of 10, 7 and 5 electroporation rounds respectively, with an average of 4.0×10^{8} electroporated Vero cells per round. Average yield of an electroporation round was 350 mL of crude harvest, with titers ranging from 2.2×10^{7} to 1.3×10^{9} infectious particles/ mL. In particular for the clinical material, viral titers found in the supernatant after 24 h were lower than found after 48 h (mean yield of 9×10^{7} infectious particles / mL at 24 h and 3.8×10^{8} infectious particles/ mL at 48 h). The total volume of the harvested clinical batches was 6.7 liter, equaling 1.6×10^{12} infectious particles. The average titer of the harvests was comparable for the non-clinical (2.2×10^{8} infectious particles/mL) and clinical material (2.4×10^{8} infectious particles/mL), indicating that the removal of phenol red from the culture medium did not impact the yield of the electroporation process.

3.2.2. Vvax001 purification (downstream processing)

At the start of further downstream processing, a subset of individual harvests was pooled and purification was performed by anion and subsequent cation exchange chromatography. For the non-clinical material, a total of 7 purification runs were performed. In the first purification run, individual harvests were pooled to a total volume of 2000 mL corresponding to approximately 5×10^{11} infectious particles.
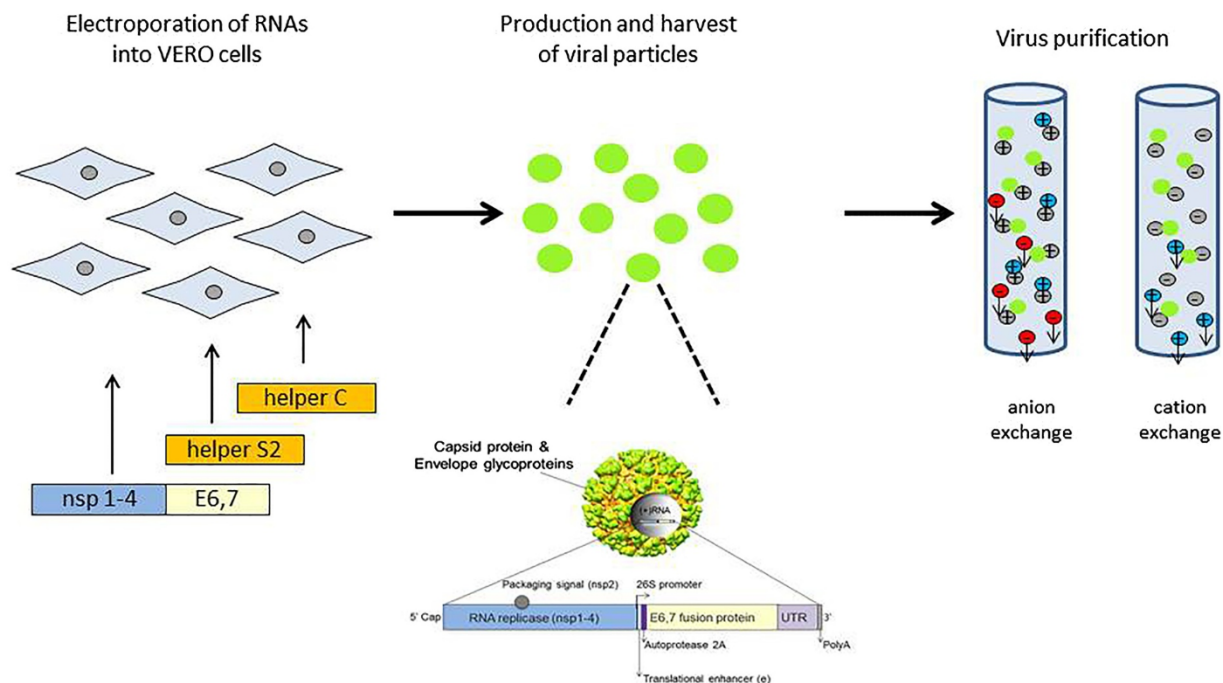

Fig. 1. Overview of the Vvax001 production process.

Table 1

Quality control testing at different stages of nonclinical and clinical Vvax001 purification.

Test	Specification	Nonclinical Material*	Clinical Material**
Crude pooled harvest			
Mycoplasma	No mycoplasma detected	Conform	Conform
Bioburden	< 10 CFU/mL	0 CFU/ mL	0 CFU/ mL
In vitro adventitious virus	No adventitious viruses detected	Conform	Conform
Porcine viruses (indicator cells)	No porcine viruses detected	Not tested	Conform
Porcine circovirus	No viruses detected	Not tested	Conform
In vivo adventitious virus	No adventitious viruses detected	Not tested	Conform
Total proteins (BCA test)	For information	40 μg/mL (15–125 μg/mL)	21 μg/mL (8–29 μg/mL)
Vero host cell protein	For information	Not tested	0.84 μg/mL
Purified bulk			
Host cell DNA	<10 ng/ dose	3.4 ng/mL (0.8–11.0 ng/mL)	4 ng/mL (2–7 ng/mL)
Viral titer (infectious particles/mL)	For information	8.1* 108 (3.4×10^8–16.0×10^8)	4.0 * 108 (0.8×10^8–6.4×10^8)

* Average results and range are shown for 7 nonclinical purification runs.
** Average results and range are shown for 13 clinical purification runs.

However, recovery of this purification run was low (9%), and additional experiments indicated that this was caused by the relatively high conductivity of the pooled harvest (8.9 mS/cm). All other purification runs were performed using 350–400 mL of pooled harvest containing 0.5 to 1×10^{11} infectious particles and diluted to a conductivity of around 5 mS/cm. Mean recovery of these runs after the two column chromatography steps was 21%, with a mean titer of the purified material of 6.8×10^8 infectious particles/ mL. Results of release testing of the non-clinical material are shown in Table 1, in process control results are shown in supplementary Fig. 1.

Also for the clinical material, loading of the column was based on conductivity of the pooled harvest . For the clinical purification runs the individual harvests were combined into a total of 13 pooled harvests, each containing 350–400 mL and 0.5 to 1×10^{11} infectious particles and diluted to a conductivity of 4.5–5.0 mS/cm, and. The mean titer after purification was 4.0×10^8 infectious particles/ mL and a mean recovery of 20%. Losses mainly occurred during purification over the second column (cation exchange) since average recovery of this column was 39% compared to 78% of the first column (anion exchange). In addition, loss was seen during dilution of the purified fraction of the first column and loading onto the second column (average recovery 70%). Release testing consisted of viral and microbiological tests on the pooled harvest before purification, and a test on residual host cell DNA after purification. Batches were demonstrated to be free of adventitious agents, mycoplasma and microbiological contamination. Residual host cell DNA was below the limit of 10 ng/dose, as defined by EU and US guidelines for vaccines (see Table 1). Results of in process testing during the different purification steps are provided in supplementary Table 2. Preclinical and clinical batches showed comparable results, demonstrating that the changes introduced between the nonclinical and the clinical manufacture did not affect product quality of Vvax001.

3.2.3. Formulation and filling

Following purification, the collected fractions were pooled and used for further formulation and filling. Non-clinical material was used for stability studies, non-clinical safety studies, and as reference standard in analytical testing. The material used for stability studies (lot 14F17-013) was filled at a target concentration of 5×10^8 infectious particles/ mL in the formulation buffer consisting of HEPES 12.5 mM, NaCl 150 mM and HSA 1% in WFI. In addition, lots 14F06-013 and 14F17-013 have been filled in different concentrations (5×10^6 and 5×10^7 infectious particles/mL respectively) to allow for lower dose groups in the toxicology study. The volume (1 mL per vial) and vial/closure system was identical to the clinical formulation. Small differences in the concentration of the components of the formulation buffer were introduced between the non-clinical and clinical lots. These differences were adequately covered by the stability data of both non-clinical and clinical lots, demonstrating that there was no effect on product quality.

The clinical material was filled at a target concentration of 1.25×10^8 infectious units/mL in formulation buffer consisting of HEPES 19 mM, NaCl 227 mM, and HSA 1% in WFI at pH 7.0. HEPES functions as buffer component, NaCl to adjust tonicity and HSA as cryoprotectant . The composition of the final formulation buffer was based on development work and the required dilution for an acceptable osmolarity. Two batches of filled Drug Product were generated, of 267 and 263 vials respectively, each vial containing 1.1 mL. Release testing was performed on formulated Drug Substance and filled Drug Product. Formulated Drug Substance complied with all specifications including those for viral titer and SFV virus identity, biological activity and absence of replication competent virus (Table 2). The batches of filled Drug Product also complied with all specifications including those for viral titer and identity, bacterial endotoxins and sterility (Table 3). Importantly, the ratio of 0.27 of total: infectious particles demonstrated proficient biological activity of the purified Vvax001. Given the

Table 2

Quality control of nonclinical and clinical Vvax001 formulated Drug Substance.

Test	Specification	Nonclinical Material	Clinical Material
Virus identity	Confirmed ID	Confirmed ID	Confirmed ID
Viral titer	$1.25 \times 10^8 \pm 0.3$log	5.6×10^8	1.9×10^8
Human serum albumin	10 mg/mL ± 20%	not tested	11 mg/mL
Bioburden	≤ 1 CFU/mL	0 CFU/mL	0 CFU/mL
Biological activity/ identity E6E7	Protein expressed	Expressed	Expressed
pH	7.0 ± 0.3	7.0	7.0 / 6.9 *
Osmolality	390 ± 40 mOsmol/kg	268 mOsmol/kg	395 /347 mOsmol/kg *
Appearance	Clear or slightly opalescent solution	conform	Conform
Absence of replication competent viruses	Not present in one human dose	conform	conform
Benzonase	Not detected LOD = 0.2 ng/mL	not tested	not detected

* Data for both clinical batches are shown.

Table 3
Quality control of nonclinical and clinical Vvax001 filled Drug Product.

Test	Specification	Nonclinical Material	Clinical Material
Sterility (Ph. Eur.)	no growth	no growth	no growth
Endotoxin concentration (Ph. Eur.)	<5 EU/mL	<5 EU/mL	<5 EU/mL
Visible particles (Ph. Eur.)	Essentially free of visible particles	Conforms	Conforms
Extractable volume (Ph. Eur.)	$\geq$ 1 mL/vial	not tested	$\geq$ 1 mL/vial
Viral titer (infectious particles/mL)	$1.25 \times 10^8 \pm 0.3\log$	5.8×10^8	$1.7 \times 10^8 / 1.6 \times 10^8$ *
Virus identity	Confirmed ID	Confirmed ID	Confirmed ID
Appearance (visual inspection)	Clear or slightly opalescent solution	Conforms	Conforms

 * Data for two clinical batches are shown.

variation of both assays a ratio of 0.27 reflects that all physical particles are infectious.

3.3. Stability studies

Stability testing was performed on the nonclinical material, both at real time storage conditions and at 2–8 °C. To date, data are available for up to 24 months at ≤ -60 °C and 6 months at 2–8 °C. For the material stored at ≤ -60 °C, all data are within specifications for all methods and no trends have been observed. For the material stored at 2–8 °C, a clear downward trend in the viral titer has been observed after 6 months of storage (data not shown).

Real time stability studies are ongoing for the two batches of clinical material and to date, data are available for up to 18 months (stored at ≤ -60 °C), demonstrating no non-conformances or trends and confirming the long-term stability data. See Table 4 for results.

In addition to long term and accelerated stability testing of the final drug product, stability of process intermediates was evaluated by verifying the viral titer before and after storage at different points in the production process, based on guidance in ICH Q5C (ICH Q5A (R1)). Information on the stability of the crude harvest was collected by comparing the viral titer of the material directly after harvest, and after storage at ≤ -60 °C just before the start of purification. Data from this evaluation indicate that the crude harvest is stable for at least 6 months (data not shown). Stability of purified fractions has been demonstrated by comparing the average titer of the fractions before storage, and after storage just before formulation. Results demonstrate that the purified viral particles are stable for up to 80 days (data not shown). No stability studies were performed on the Formulated Drug Substance, since this is directly processed into Vvax001 Drug Product.

For clinical administration, Vvax001 is administered by intramuscular injection using a standard syringe and needle. If necessary, based on the clinical protocol, Vvax001 is diluted in formulation buffer (19 mM HEPES buffer, 227 mM NaCl, 1% HSA, at pH 7.0) to the required dose. To assess in-use stability (i.e. compatibility and potential absorption of Vvax001 to the syringe), an in-use compatibility study was performed. The study included the lowest and highest anticipated clinical doses, and the preparation of the doses was identical to the clinical study (i.e. mixing procedure, type of materials, holding temperature). The data from this study demonstrated that there was no significant sorption or change in infectivity when in contact with/stored in the syringe for up to 8 h at 2–8 °C. See table 5 for results. It was concluded that Vvax001 is compatible with the proposed administration device and stable during in-use conditions.

4. Discussion

In this study we describe for the first time the translation and GMP production of an SFV-based therapeutic vaccine. The vaccine, Vvax001, is targeted to HPV and has shown promising results with regard to antitumor immunogenicity in preclinical studies (Daemen et al., 2002; Daemen et al., 2003; Daemen et al., 2004). The availability of GMP-produced material now will enable assessment of its immunogenicity,

safety and efficacy in a clinical setting.

In order to establish a production process suitable for clinical application, several changes were introduced compared to the process as it was originally developed for preparing research batches of recombinant SFV vectors, including changes to the producer cell line, the SFV expression system and the purification process. These changes in the production process were implemented because of safety concerns or because of technical issues. 1) Vero cells were used as a producer cell line instead of baby hamster kidney (BHK) cells because Vero cells are routinely used for vaccine manufacturing and were available in cGMP-grade. 2) In the SFV expression system applied for GMP production, the capsid and spike proteins were encoded on two separate plasmids instead of on one plasmid. Thus, the production of SFV replicon particles is based on co-transfection of packaging cells with three independent RNAs instead of two independent RNAs. This minimizes the risk of generation of replication competent SFV during production (Smerdou and Liljeström, 1999). 3) The purification process was changed to ion exchange chromatography instead of using a discontinuous sucrose density gradient. Using chromatography as a purification method enables easier scale-up and circumvents problems related to ultracentrifugation in a clean room facility. In addition, phenol red was removed from the culture medium, because this was found to be disadvantageous for the purification process.

In the transfer of the production process to the GMP facility, the biggest challenge was to scale-up the different process steps while maintaining a sufficient viral titer in order to reach the highest anticipated clinical dose. For the viral particle production step, this was merely a scale-out, i.e. increasing the number of electroporations performed. Since electroporation is the most time-consuming step in the production procedure, this step would require adjustments in order to generate sufficient material for Phase III studies and the market. Therefore it is our intention to scale up this process using an inline electroporation system (i.e. flow electroporation allowing transfection of more cells and larger cell volumes). In addition, optimization of the viral particle production process would also include change to a serum free, chemically defined medium as has recently been demonstrated by others (Rourou et al., 2019). For the purification process, scale-up consisted of implementation of chromatography columns, which required optimization of several process parameters (e.g. loading capacity and conductivity). The yield of the purification process averaged around 20%, and would require further improvement to enable large-scale production. Since the lowest yield was observed after purification over the cation exchange column, this could potentially be caused by a deteriorating effect of the high salt concentration since in this step the viral particles were eluted with 0.6 M NaCl.%. This will be investigated in further development studies. The production process as described in the present study enabled us to produce enough material for use in early clinical studies. In addition, the obtained viral titers are expected to be sufficient to administer doses that induce a clinical immunological response in humans, based on preclinical results obtained thus far. If these first clinical studies prove to be successful, the recombinant SFV-based vaccination strategy could be easily extended to other targets and pathological conditions. For larger scale clinical application we foresee

Table 4
Real time stability results for Vvax001 clinical material stored at $\leq -60\,^{\circ}\mathrm{C}$.

Test (and method)	Release specification	End of shelf life specification	Time (months)					
			0	3	6	12	18	24
Appearance(visual inspection)	Clear or slightly opalescent solution	Clear or slightly opalescent solution	Conforms	Conforms	Conforms	Conforms	Conforms	pending
pH(Ph. Eur. 2.2.20)	7.0–7.5	7.0 –7.5	7.0	n.a.	7.0	7.0	7.0	pending
Osmolality(Ph. Eur. 2.2.35)	320–370 mOsmol/kg	within 10% of release value	342 mOsmol/kg	n.a.	335 mOsmol/kg	341 mOsmol/kg	342 mOsmol/kg	pending
Viral titer (infectious particles/mL)(viral titer assay)	$1.25 \times 10^8 \pm 0.3\log$	$\geq 1.25 \times 10^8 - 0.3\log\ (\geq 0.7 \times 10^8)$	1.58×10^8	1.67×10^8	1.79×10^8	2.0×10^8	1.83×10^8	pending
Biological activity/ identity E6E7(Western Blot)	Expression of protein confirmed	Expression of protein confirmed no extra bands	Confirms	n.a.	Confirms	Confirms	Pending	pending
Sterility (Ph. Eur)	No growth	No growth	n.a.	n.a.	n.a.	n.a.	n.a.	pending

Table 5
Results in-use compatibility study.

Nominal concentration (infectious particles/mL)	Recovery after preparation and storage in the syringe at 2–8 °C		
	$T = 0$[1]	$T = 4$ hrs [2]	$T = 8$ hrs [2]
2.5×10^5	100%	117% and 98%	100% and 115%
1.25×10^8	100%	95% and 126%	93% and 112%

[1] Titer at $T = 0$ after preparation for use was set at 100%.
[2] duplicate determinations.

that the production process will need to be further optimized in order to allow efficient up scaling.

5. Conclusion

This study demonstrated successful development of a cGMP compliant process and production of a SFV-based viral vaccine for first-in-man use.

Conflict of interest

Toos Daemen and Hans Nijman are stock holders/founders of ViciniVax.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2019.105096.

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ICH Q8 (R2)Pharmaceutical development.

ICH Q1A (R2)Stability testing of new drug substances and products.

ICH Q5C Quality of biotechnological products:Stability testing of biotechnological/biological products.

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