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Vaxfectin: a versatile adjuvant for plasmid DNA- and protein-based vaccines

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Importance of the field: Many vaccines require the use of an adjuvant to achieve immunity. So far, few adjuvants have advanced successfully through clinical trials to become part of licensed vaccines. Vaxfectin® (Vical, CA, USA) represents a next-generation adjuvant with promise as a platform technology, showing utility with both plasmid DNA (pDNA) and protein-based vaccines.
Areas covered in this review: This review describes the chemical, physical, preclinical and clinical development of Vaxfectin for pDNA-based vaccines. Also included is the preclinical development of Vaxfectin-adjuvanted protein- and peptide-based vaccines.
What the reader will gain: The reader will gain knowledge of vaccine adjuvant development from bench to bedside.
Take home message: Vaxfectin has effectively boosted the immune response against a range of pDNA-expressed pathogenic antigens in preclinical models extending from rodents to non-human primates. In the clinic, Vaxfectin-adjuvanted pDNA-based H5N1 influenza vaccines have been shown to be well tolerated and to result in durable immune responses within the predicted protective range reported for protein-based vaccines.

Keywords: adjuvant, cationic lipid, plasmid DNA, vaccine

Expert Opin. Drug Deliv. (2010) 7(12):1433-1446

1. Introduction

Vaccine development historically utilized live-attenuated as well as inactivated viruses and bacteria, but with the advent of molecular biology techniques, it has evolved to include recombinant proteins and nucleic acid-based vaccines. One of the main challenges for recombinant protein-based vaccines is the preservation of the native conformation of the antigen derived from a pathogen. This is especially challenging for integral membrane proteins, such as viral envelope proteins, that are single or multiple subunit proteins. Plasmid DNA (pDNA)-based vaccines circumvent these challenges by directly expressing in injected subjects the protein antigen in its native configuration. Furthermore, because all pDNAs have the same chemical composition and structure regardless of the encoded transgene, it is very easy to combine multiple plasmids expressing different protein antigens. Alternatively, one may express more than one antigen from a single pDNA.

It has been shown that direct intramuscular (i.m.) injection of pDNA results in gene expression at a level sufficient to induce an immune response to the expressed protein [1,2]. However, as is true for inactivated and subunit protein-based vaccines, adjuvants may be required to potentiate the immune response to pDNA-based vaccines, though not all adjuvants used for protein-based vaccines are suitable for use with pDNA. Vaxfectin® (Vical, CA, USA) is a second-generation cationic lipid-based suspension that has been shown to adjuvant effectively both pDNA and protein-based vaccines. This review describes the clinical development of

10.1517/17425247.2010.538047 © 2010 Informa UK, Ltd. ISSN 1742-5247

Article highlights.

- In this review, the development of Vaxfectin as an adjuvant for DNA as well as protein- and peptide-based vaccines is discussed.
- Preclinical results for DNA vaccines show that formulation with Vaxfectin increases humoral and cell-mediated immune responses to the expressed antigen(s). The value of Vaxfectin-enhanced immune activation is shown through disease protection after pathogen challenge in animal models ranging from mice to non-human primates.
- Results from two Phase I clinical trials for Vaxfectin-formulated plasmid for immunization against avian influenza (H5N1) are discussed. Vaxfectin was well tolerated when administered intramuscularly using either a conventional syringe or a needle-free device; responses observed included up to 67% of patients with neutralizing antibodies and 75 – 100% with antigen-specific T cells.
- The mechanism of action by which Vaxfectin enhances immune system activation is discussed. The results so far suggest that Vaxfectin's adjuvant activity is not produced through a Toll-like receptor (TLR) pathway.
- Vaxfectin is also an effective adjuvant for protein- and peptide-based vaccines. Results are presented using a conventional seasonal influenza vaccine showing not only significantly increased antibody titers but also antigen dose-sparing. Results from a cancer peptide mouse model are presented demonstrating that optimal T-cell activation can be achieved by adjusting Vaxfectin/antigen ratios.

This box summarizes key points contained in the article.

Vaxfectin for boosting the immune response to expressed protein antigens and also describes Vaxfectin's adjuvant activity for protein- and peptide-based vaccines.

1.1 Chemical composition

Vaxfectin is a cationic lipid-based formulation composed of GAP-DMORIE (CAS name: (±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-bis(9-*cis*-tetradecenyloxy)-1-propanaminium bromide), a synthetic cationic amphiphile, and DPyPE (CAS name: 3,7,11,15-tetramethylhexadecanoic-*sn*-glycero-3-phosphoethanolamine), a neutral phospholipid, at a 1:1 molar ratio. The chemical structures of GAP-DMORIE and DPyPE are shown in **Figure 1**. The salient features of GAP-DMORIE are: i) a hydrophobic domain composed of two C_{14} hydrocarbon chains with a single double bond attached to a glycerol backbone by means of ether linkages; and ii) a hydrophilic head group consisting of a quaternary amine and a primary amine separated by a C_3 hydrocarbon chain, yielding a net positive charge. DPyPE contains a hydrophobic domain consisting of two tetramethylhexanoic acyl chains and a hydrophilic domain consisting of a glycerophosphatidylethanolamine.

Vaxfectin lipid suspensions are formed by first dissolving both lipids in a compatible organic solvent, such as chloroform. After the two lipids are mixed together, the organic solvent is removed by evaporation, or other comparable methods for drying organic lipid mixtures, producing a lipid film. The lipid film is stable for at least 2 years when stored frozen (< -20°C). Hydration of the lipid film with saline or isotonic sucrose under controlled mixing yields multilamellar vesicles (MLVs). The liposome suspension can be buffered in several pharmaceutically acceptable buffers, such as HEPES, Tris or citrate buffer. Liposome size distribution is dependent on lipid concentration, hydration medium composition and the amount of energy input with agitation. For example, vortexing an aqueous 2 mg/ml suspension of Vaxfectin for 5 min can yield MLVs with an average diameter of 2 µm ranging from 0.5 to 4 µm. The size can be reduced to < 200 nm diameter liposomes by applying higher energy, such as sonication. Zeta potential measurements showed that the equimolar ratios of the two lipids in aqueous media yield liposomes with a net positive surface charge.

Lipids in a bilayer configuration can exist in fluid or gel phases. Lipids freely diffuse translationally within the plane of the bilayers in a liquid phase, whereas translational diffusion is greatly reduced in a gel phase. GAP-DMORIE, having a single double bond, should freely diffuse within bilayer planes. However, the bulky methyl groups in the tetramethylhexanoic acyl chains of DPyPE result in tight packing of the acyl chains, thus restricting the diffusion of GAP-DMORIE and resulting in fixed positive surface charge rather than a transient charge distribution. This lack of surface charge diffusion influences the ionic interaction with negatively charged molecules, such as nucleic acids.

Through an extensive screen of many cationic lipid/neutral lipid formulations with pDNA for immune response in mice, Vaxfectin was found to produce the greatest adjuvant effect with several different expressed antigens [3]. Plasmid DNA, a negatively charged biopolymer, spontaneously binds to Vaxfectin cationic liposomes, resulting in the formation of discrete lipid/pDNA complexes, primarily through ionic interactions. Administration of these transfection complexes by intradermal (i.d.), subcutaneous (s.c.) or i.m. injections results in gene transfer (from the plasmid expression cassette encoding the antigen of interest) to cells at the site of injection. The cell types available as potential transfection targets include stromal cells such as myocytes, fibroblasts and endothelial cells, as well as resident and infiltrating immune cells.

The adjuvant activity of Vaxfectin is not limited to antigens expressed from pDNA, it also occurs with protein- and peptide-based vaccines [4]. Simple formulation options exist depending on the physical parameters, such as hydrophobicity, hydrophilicity, net charge at neutral pH and secondary or tertiary structure of the protein or peptide. Hydrophobic proteins and peptides can be cosolubilized with the Vaxfectin lipids before forming a lipid film and liposome formation with aqueous media results in incorporation of the hydrophobic protein domains and peptides into the liposome bilayers. Hydration of Vaxfectin lipid films with either hydrophilic

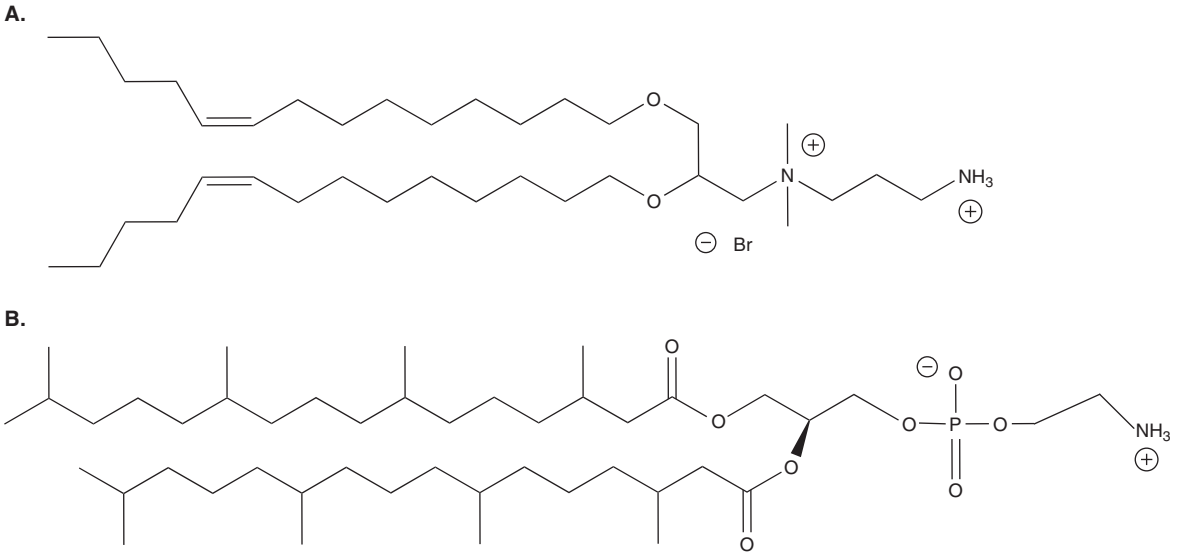

Figure 1. Chemical structures of (A) GAP-DMORIE: (±)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-bis(9-*cis*-tetradecenyloxy)-1-propanaminium bromide and (B) DPyPE: 1,2-diphytanoyl-*sn*-glycero-3-phosphoethanolamine.

proteins or peptides aqueous solutions followed by agitation results in entrapment of the proteins and peptides within the liposomal aqueous space. In addition, if the proteins or peptides are negatively charged, they can electrostatically bind to the liposome surface [5,6].

2. Non-clinical immunological studies of plasmid DNA formulated with Vaxfectin

The identification of an equimolar combination of GAP-DMORIE and DPyPE (i.e., Vaxfectin) as a pDNA vaccine adjuvant was the result of *in vivo* screening of > 100 cationic lipid formulations in mice [3]. A mouse intramuscular immunization protocol was used to screen candidate formulations for their ability to enhance humoral responses directed against influenza A nucleoprotein (NP). The physical parameters optimized for maximal immune responses were: pDNA concentration, molar ratio of cationic lipid to neutral co-lipid, and molar ratio of cationic lipid to pDNA nucleotide. For Vaxfectin, the optimal parameters for a 1 mg/ml pDNA concentration were a 1:1 mole ratio of GAP-DMORIE to DPyPE and a 4:1 mole ratio of pDNA nucleotide to GAP-DMORIE. These formulation parameters produced a 7- to 50-fold increase in antibody titers to influenza NP compared with pDNA alone in both mice and rabbits (**Figure 2**). A durable immune response was observed after 2 injections delivered 3 weeks apart, with titers remaining stable for at least 14 weeks after the initial injection. Formulation of Vaxfectin with pDNAs encoding human factor IX, beta-galactosidase or hen egg lysozyme resulted in a 3- to 10-fold increase in antibody titers against these foreign proteins, thus showing that Vaxfectin had the potential to be a broad adjuvant for pDNA-expressed antigens [7].

Subsequent non-clinical evaluations of pDNA vaccines formulated with Vaxfectin have been conducted in a variety of animal models, including mice, rats, rabbits, ferrets, sheep and non-human primates (**Table 1**). Multiple disease indications, plasmid constructs, routes of administration (i.m., i.d. and intranasal) and dosing schedules were investigated. These studies demonstrated that, relative to non-adjuvanted pDNA, Vaxfectin can significantly increase antibody and T-cell responses to pDNA-encoded antigens, provide a dose-sparing effect, and enhance survival or protect from disease in challenge models (**Table 1**). For example, Vaxfectin has been shown to increase antibody titers up to 200-fold, increase IFN-γ ELISPOT responses up to 10-fold, provide up to 25-fold dose-sparing effect, and enhance protection in a sporozoite mouse challenge model when formulated with pDNA encoding malaria (*Plasmodium yoelii*) circumsporozoite protein [8]. More recently, Vaxfectin has been shown to enhance both antibody and cellular immune responses in mice to each component of a multi-antigen vaccine mixture consisting of five different pDNAs encoding malaria (*Plasmodium falciparum*) antigens [9]. Furthermore, a significant reduction in viral load in peripheral blood mononuclear cells (PBMCs) was observed in a challenge model in baboons vaccinated with HIV-2 pDNA vaccine consisting of HIV-2 *env*, *tat*, *nef* and *gag/pol* genes [10]. Studies performed in rabbits [3] and non-human primates (Vical, unpublished results) (**Figure 3**) have also demonstrated that Vaxfectin formulations are compatible with needle-free jet injectors.

Vaxfectin has also been tested for boosting the immune response to measles antigens expressed from pDNA in a macaque measles virus challenge model, which uses the same strain of measles virus that infects humans producing a similar

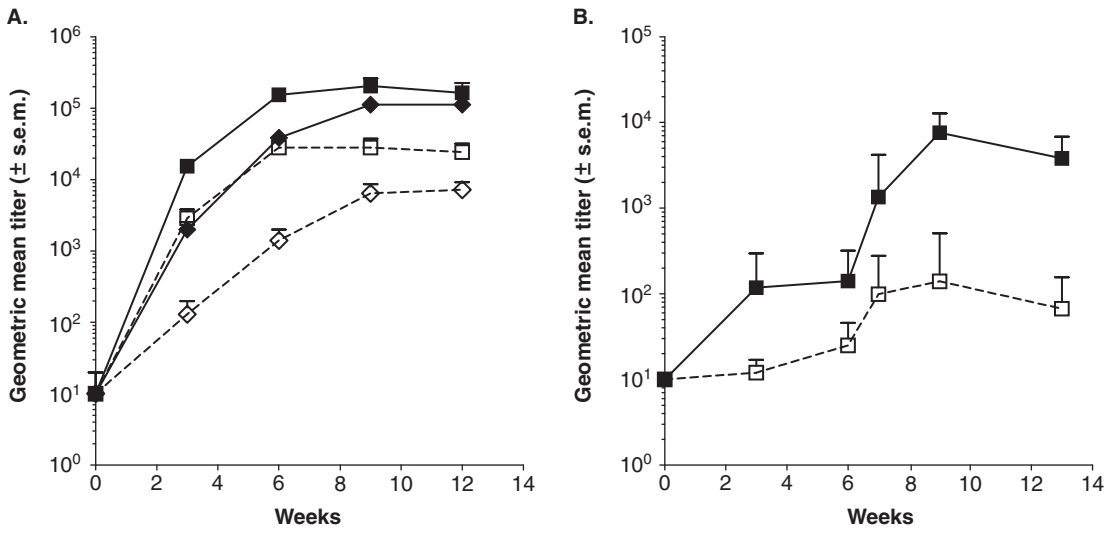

Figure 2. Adjuvant effect of Vaxfectin in mice and rabbits. A. Mice were immunized using an influenza NP (nucleoprotein) DNA vaccine, delivered either as pDNA alone (plasmid resuspended in saline, dashed line/open symbols) or adjuvanted with Vaxfectin (unbroken line/filled symbols). Plasmids were administered four times as a prime/boost schedule 3 weeks apart, at doses of either 1 or 25 μg (◇ or ■, respectively). Anti-NP-specific antibody titers are shown as the geometric mean titer ± s.e.m. Note how the use of Vaxfectin provides both an adjuvant effect (increasing overall titers) and a dose-sparing effect (higher titers achieved with lower doses). **B.** Rabbits were immunized and data are presented in the same manner as shown in **A**, except that a dose of 150 μg was used and rabbits were injected on Days 0 and 42. (□) pDNA alone; (■) Vaxfectin-adjuvanted pDNA. These results have been reported previously [3].

disease etiology. One advantage of a pDNA-based vaccine compared with the current live measles vaccine is the potential to bypass interference from maternal neutralizing antibodies when vaccinating infants, hence the reason for vaccinating juvenile and infant macaques in the following studies. Formulation of measles H and F pDNAs with Vaxfectin produced a robust immune response with regard to neutralizing antibody, shown in **Figure 3A** [11]. Anti-measles neutralizing antibody titers were measured using a standard plaque reduction neutralizing antibody titer assay [11]. All vaccinated juvenile and infant macaques developed protective levels of neutralizing antibodies within 1 month after the first of two vaccinations, and developed antigen-specific IFN-γ and IL-4 T-cell responses. After the second injection at 4 weeks, the peak antibody titer was equivalent to that obtained with the current live measles vaccine in both infant and juvenile macaques, and neutralizing antibody responses were maintained above the protective level of 120 mIU/ml for at least 1 year. When vaccinated juvenile and infant macaques were challenged intratracheally 12 – 15 months after vaccination, all vaccinated animals had undetectable viremia and no rash [11]. The results are shown in **Figure 3B**. In a subsequent pDNA dose ranging study in macaques where pDNA doses were reduced 10- to 25-fold, formulation of pDNA with Vaxfectin increased neutralizing antibody titers compared with plasmid DNA alone (unpublished results/pers. commun.), thus showing that Vaxfectin can boost immune response to expressed antigens in a non-human primate model.

For vaccine candidates that have advanced to clinical testing, preclinical studies were conducted to assess the ability of Vaxfectin to enhance the immunogenicity and protective efficacy of pDNA vaccines encoding influenza antigens. Compared with pDNA administered in PBS, pDNA formulated with Vaxfectin demonstrated dose-sparing properties and provided a statistically significant enhancement in the protective efficacy of an H3 hemagglutinin (HA)-encoded pDNA vaccine. Less than a microgram of HA pDNA vaccine formulated with Vaxfectin provided 100% protection after a single i.m. injection in a mouse model [12]. In subsequent studies, the ability of Vaxfectin-formulated pDNA vaccines to provide protection in mice and ferrets was evaluated using a highly pathogenic A/Vietnam/1203/04 (H5N1) virus challenge model. Complete protection from death and disease was achieved in mice with two doses of a Vaxfectin-formulated vaccine containing H5 HA, NP and M2 plasmids, and in ferrets with only a single pDNA dose. Furthermore, a single dose of vaccine significantly reduced viral titers in nasal wash samples from ferrets within 5 days after infection [13]. More recently, the ability of Vaxfectin-formulated vaccines to generate robust antibody responses was demonstrated using pDNA encoding the HA of the swine-origin influenza A (H1N1) virus. Three weeks after the first vaccination, 88% of mice

Table 1. Non-clinical immunological studies of pDNA vaccines formulated with Vaxfectin.

Species	Pathogen source	End point	Ref.
Mouse	Influenza	Ab, T cell, dose-sparing	[3]
Mouse	Influenza	Ab, T cell	[7]
Mouse	Tuberculosis	Ab, T cell, protection	[35]
Mouse	JEV	Ab, dose-sparing	[36]
Mouse	HIV-2	Ab	[10]
Mouse	Rabies	Ab	[37]
Mouse	Tuberculosis	T cell	[38]
Mouse	Malaria	Ab, T cell, dose-sparing, protection	[8]
Mouse	Influenza	T cell, dose-sparing, protection	[12]
Mouse	Influenza	Protection	[15]
Mouse	Influenza	Ab, protection	[13]
Mouse	Measles	Ab, T cell	[11]
Mouse	Influenza	Ab	[14]
Mouse	Malaria	Ab, T cell, dose-sparing	[9]
Rat	Influenza	Ab	[39]
Rabbit	Influenza	Ab	[3]
Rabbit	Anthrax	Ab, protection	[40]
Rabbit	Influenza	Ab	[14]
Ferret	Influenza	Ab, protection	[13]
Sheep	Anthrax	Ab	[41]
Baboon	HIV-2	Protection	[10]
Macaque	Measles	Ab, protection	[11]

Ab: Increased antibody titer and/or achieved protective level of neutralizing antibody; Dose-sparing: Demonstrated dose-sparing effect; JEV: Japanese encephalitis virus; Protection: Reduced viral load, improved and/or complete protection in a challenge model; T cell: Enhanced T-cell response and/or maintained strong cytotoxic T lymphocyte response.

and 75% of rabbits reached a hemagglutination inhibition (HI) titer of ≥ 40 (the putative threshold for a protective immune response), and 2 weeks after the second vaccination, all mice and rabbits reached an HI titer of ≥ 40. This study also demonstrated that a pDNA vaccine against an emerging pandemic influenza virus can be manufactured and tested in animals within weeks of the availability of the HA DNA sequence from the virus [14] without the need for the pathogen itself. Vaxfectin not only enhances immunogenicity of antigens expressed from a covalently closed double-stranded circular plasmid DNA, but it has also been shown to enhance protection in a lethal mouse challenge model when formulated with a polymerase chain reaction (PCR)-generated linear expression cassette encoding influenza H3 HA [15].

2.1 Toxicology, biodistribution and integration studies

Several good laboratory practice-compliant toxicology and biodistribution studies have been performed using Vaxfectin-adjuvanted pDNA vaccines as a prerequisite to conducting human trials. In all these studies, favorable profiles have been observed. In repeat-dose toxicity studies (conducted in rabbits), both an influenza vaccine [16] and a cytomegalovirus (CMV) vaccine (unpublished data) were well tolerated at the top doses administered (1 mg). The primary observations made were mild inflammatory responses at injection sites accompanied by a limited set of associated clinical pathology changes (e.g., increases in circulating leukocyte and plasma creatine kinase levels). These observations, indicative of a local reactogenicity, were of limited scope and duration, resolving in a few days to weeks, and are consistent with the responses expected after i.m. vaccination. Reactogenicity did not increase with repeated injections, indicating a lack of any hypersensitization response. Acute dose toxicology studies in which Vaxfectin alone (without added pDNA) was delivered intramuscularly to rabbits at doses up to 2 mg produced similar responses. This indicates not only that Vaxfectin displays a well-tolerated safety profile even when delivered in the form of cationic liposomes (rather than the positively charged complexes formed on mixing with pDNA), but also that the adjuvant itself contributes directly to the nascent inflammatory/immune response induced following vaccine injection.

Biodistribution and integration studies, again conducted in rabbits, followed the fate of Vaxfectin-adjuvanted vaccines using quantitative PCR [16]. A trivalent H5N1 influenza vaccine adjuvanted with Vaxfectin was injected i.m. in both forelimbs, after which plasmid levels were followed over a 60-day period in the injection site skin and muscle as well as a panel of tissues and organs (blood, heart, lung, liver, kidneys, spleen, lymph nodes, bone marrow, brain and gonads). Plasmids remained primarily at injection sites, where levels declined steadily over the 2-month period; distribution to blood or distal organs was minimal and short-lived. PCR analyses also confirmed a negligible risk of genomic integration, as separating total DNA extracted from injected tissues into low- and high-molecular-mass fractions clearly segregated plasmid from genomic DNA signals. Gene-based vaccines present the hypothetical risk of transgene integration into the host genome, which were it to occur could theoretically lead to chromosomal instability or even oncogenesis. Based on the data generated so far, a consensus has developed that the probability of this event occurring with pDNA is extremely low [17-21]; it nevertheless remains a question to be addressed, and therefore the demonstration that Vaxfectin does not induce any extra integration risk is of note.

3. Clinical development of Vaxfectin

Vaxfectin-formulated pDNA vaccines encoding several influenza antigens have completed testing in two double-blind, placebo-controlled Phase I clinical trials [22]. Safety and immunogenicity, respectively, were the primary and secondary end points of each trial. One trial tested i.m. injection by needle whereas the second trial tested i.m. injection by a needle-free device, Biojector® 2000 (Bioject Medical Technologies, Inc. (Tualatin, OR)). The clinical utility of this device was explored based on non-clinical studies showing enhanced antibody titers in rabbits compared with needle delivery [3]. Both trials tested two different pDNA vaccine

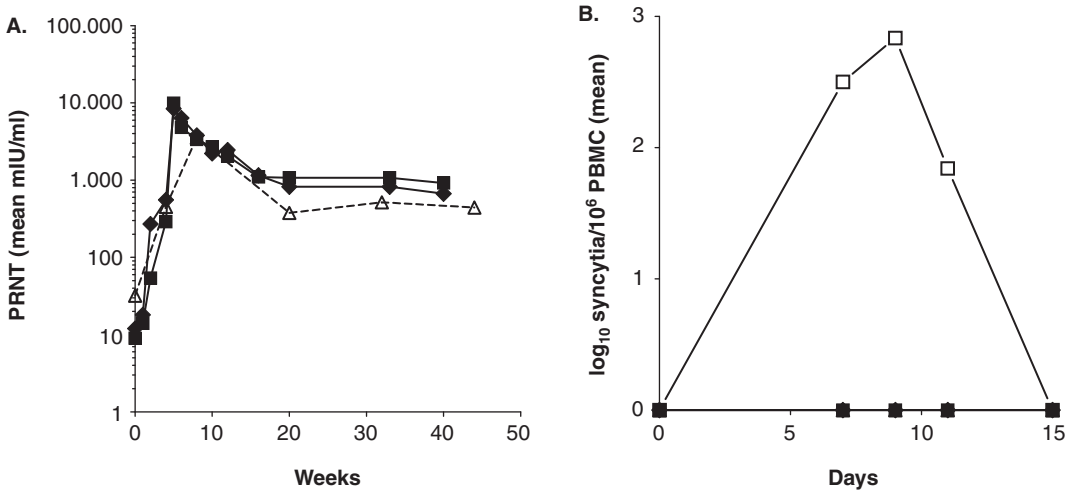

Figure 3. Induction of viral neutralizing antibodies in macaques. A. Groups of 5 juvenile and 4 infant rhesus macaques were immunized with 1 mg i.m. (■) or 500 μg i.d. (◆) of Vaxfectin-formulated pDNA encoding measles H and F antigens. Infant immune response (500 μg i.d.) is (△) with the dashed line. Both juvenile and infant macaques received 2 vaccinations 4 weeks apart. One infant died 10 weeks after vaccination of unrelated causes. **B.** Measles virus-specific neutralizing antibodies were measured by plaque reduction (PRNTs). Data are presented as the geometric means in milli-international units per milliliter. **B.** 9 vaccinated juvenile (■) and infant (△) rhesus macaques and 2 unvaccinated control macaques (□) were challenged 12 – 15 months after vaccination. Viremia was measured by coculture of serially diluted (10^5 – 10^1) PBMCs with B95-8 cells. The mean number of syncytium-forming cells per million PBMCs is shown. These results have been reported previously [11].

PBMC: Peripheral blood mononuclear cell; PRNT: Plaque reduction neutralizing titer.

products administered on a 0- and 21-day schedule. A monovalent product contained one pDNA encoding a codon-optimized version of the full-length H5 HA gene from the clade 1 H5N1 influenza virus strain, A/Vietnam/1203/04. A trivalent product contained equal mass mixtures of three plasmids, each encoding a codon-optimized version of the following: the H5 HA mentioned above, a consensus NP sequence and a consensus M2 sequence [12].

As these were first-in-human trials for Vaxfectin-formulated pDNA vaccines, doses were escalated from 0.1 to 1 mg of total pDNA/injection for the needle study and from 0.5 to 1 mg for the Biojector 2000 study. All pDNA doses were formulated with Vaxfectin at a 4:1 pDNA:cationic lipid molar ratio based on non-clinical studies [3]. A total of 78 normal healthy subjects aged 18-45 years received Vaxfectin-formulated pDNA vaccines in this dosing range. No vaccine-related clinical or laboratory serious adverse events were reported, no subject discontinued owing to receipt of investigational product, and, overall, both monovalent and trivalent products were well tolerated [22]. The most commonly reported local and systemic events occurring within 7 days of each dose were pain at the injection site, headache, malaise and myalgia. All events were of brief duration and resolved within a few days. Overall, the safety findings of these studies supported the subsequent allowance of two extra investigational new drug applications (INDs) to test up to 1 mg doses of two other infectious disease pDNA vaccines formulated with Vaxfectin.

Figure 4 shows the antibody and T-cell responses for each of the three monovalent cohorts measured at seven time points up to 6 months. An important functional serological assay used to measure the potency of influenza vaccines is the HI assay. An accepted surrogate of protection for influenza vaccines has been defined as HI titers of ≥ 40 after vaccination [23]. Inactivated H5N1 protein-based vaccines have been characterized as inherently poor immunogens [24]. Despite this, the percent of subjects achieving HI titers of ≥ 40 after vaccination (up to 67%) as well as the geometric mean antibody titers, among the three monovalent cohorts, were in the range of those reported for inactivated protein-based H5N1 vaccines [24]. The use of the Biojector 2000 did not appear to enhance significantly antibody titers compared with needle delivery. The number of IFN-γ-producing T cells ranged from 75 to 100% among the three monovalent cohorts, with peak frequencies achieved by day 42 (**Figure 4B**). The HI antibody response rate was lower in the trivalent cohorts (≤ 20% for any cohort); however, the total antiviral antibody and/or T-cell responses reached 72% among all four trivalent cohorts [22]. Based on these immunogenicity findings following two doses of H5 HA pDNA vaccine, the potential exists for even stronger responses against more immunogenic antigens, particularly if optimized further by administering a third dose, increasing the DNA dose and/or testing different ratios of pDNA:Vaxfectin adjuvant.

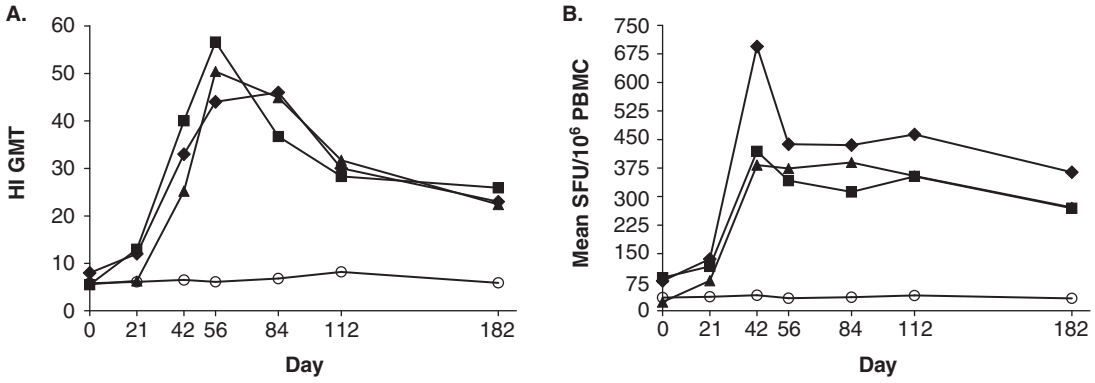

Figure 4. Clinical responses to a Vaxfectin-adjuvanted H5N1 influenza vaccine. Subjects received either placebo or Vaxfectin-adjuvanted influenza H5 HA pDNA vaccine on days 0 and 21, delivered by either needle injection or a needle-free device. Immune responses were followed out to day 182. **A.** Longitudinal GMTs of HI antibodies. **B.** Mean SFU of IFN-γ-producing T cells per million PBMCs. (■) 1 mg by needle; (♦) 1 mg needle-free; (▲) 0.5 mg needle-free; (○) placebo pDNA. These results have been reported previously [22].

GMT: Geometric mean titer; HI: Hemagglutination inhibition; PBMC: Peripheral blood mononuclear cell; SFU: Spot forming unit.

4. Non-clinical immunological studies of protein-based vaccines formulated with Vaxfectin

There is a growing need for new adjuvants that enhance the potency of poorly immunogenic protein-based vaccines and/or to increase dose-sparing capabilities to increase vaccine supply. A wide variety of adjuvants have been evaluated with seasonal and pandemic influenza vaccines with the primary goal to increase antibody titers against hemagglutinin. Hence, a licensed seasonal trivalent inactivated influenza vaccine (TIV, Fluzone®, sanofi pasteur) was used as a model antigen for testing the adjuvant activity of Vaxfectin. TIV was combined with Vaxfectin by simply mixing the antigen with the adjuvant. Hemagglutination inhibition and anti-TIV ELISA titers were increased by up to 200-fold in mice [4], with increasing amounts of Vaxfectin resulting in stronger enhancement of antibody responses (**Figure 5A**) and at least a 10-fold dose-sparing effect. Vaxfectin preferentially increased IgG2 titers compared with IgG1 titers, resulting in a more balanced IgG isotype distribution compared with TIV alone. Lower amounts of Vaxfectin did not significantly enhance antibody responses, but did increase the number of IFN-γ-secreting T cells up to 18-fold (**Figure 5B**). These data demonstrate that Vaxfectin enhances Th1 responses with protein-based seasonal influenza vaccine, and suggest that cellular or humoral immune responses may be preferentially improved by modifying the Vaxfectin:antigen ratio in the vaccine formulation [4].

The ability of Vaxfectin to enhance T-cell responses was evaluated further using a peptide derived from tyrosinase-related protein 2 (TRP-2) as a model for a peptide-based cancer vaccine. In a mouse study, formulating an MHC class I-restricted peptide TRP-$2_{180-188}$ with Vaxfectin resulted in up to 7-fold higher responses than obtained with Freund's adjuvant (**Table 2**). In another study, a dose response was observed with increasing vaccine doses generating up to a 68-fold increase in the number of TRP-2-specific IFN-γ-producing T cells compared with peptide injected in PBS (**Table 2**).

In addition to the TRP-2 peptide, two peptides from ovalbumin, $OVA_{323-339}$ and $OVA_{257-264}$, and a multimerized five tandem repeat MUC-1 peptide [25] were formulated with Vaxfectin. The $OVA_{257-264}$ and the tandem repeat MUC-1 peptides were water soluble, whereas the $OVA_{323-339}$ peptide was not. Mouse ELISPOT assay results for the OVA peptides were equivalent to those obtained with the same peptides formulated with complete Freund's adjuvant (Vical, unpublished results), and the tandem repeat MUC-1 peptide formulated with Vaxfectin produced anti-MUC-1 peptide-specific IgG response. The non-clinical proof-of-concept studies demonstrate that Vaxfectin has great potential for being a powerful adjuvant for protein- or peptide-based antigens that may benefit from dose-sparing or enhanced antibody or T-cell responses.

5. Mechanism of action

Non-adjuvanted pDNA vaccines typically induce strong cytotoxic T-cell as well as humoral immune responses, both characterized in mice by Th1 phenotypes [26]. The Th1/Th2 balance induced by Vaxfectin-formulated pDNA vaccines is antigen dependent, but in general i.m. administration of Vaxfectin-adjuvanted pDNA vaccines induces antigen-specific T cells that produce IFN-γ (rather than IL-4), whereas IgG2a (rather than IgG1) dominates the antibody response [7]. Serum levels of IL-6 are also enhanced, and Vaxfectin's adjuvant effect was observed to be reduced ~ 70% in IL-$6^{-/-}$ mice [7], arguing for a functional role by this cytokine.

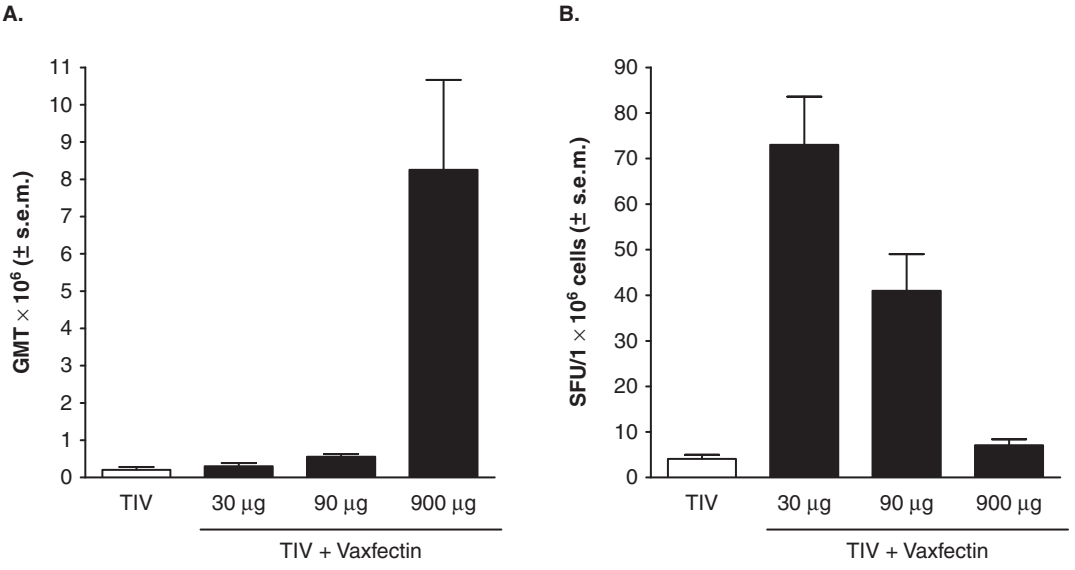

Figure 5. Adjuvant effect of Vaxfectin with a protein-based vaccine. Mice were immunized using either TIV alone (open bars) or Vaxfectin-adjuvanted TIV (filled bars) in a prime/boost schedule. For the adjuvanted groups, a fixed dose of TIV (0.1 μg) and varying co-doses of Vaxfectin (30 – 900 μg) were administered. Both humoral and cellular responses were then assessed. **A.** Anti-TIV antibody titers (GMT ± s.e.m.). **B.** TIV-specific T cells were detected by an ELISPOT assay to measure IFN-γ production following antigen stimulation of splenocytes. Data shown as spot forming units per 1 × 10^6 cells (s.e.m.). These results have been reported previously [4].

GMT: Geometric mean titer; TIV: Trivalent inactivated influenza vaccine.

One indirect means to enhance pDNA vaccine immunogenicity is to improve transfection efficiency, by increasing either the number of transfected cells or their production of transgene-encoded protein, and as a result, enhance overall antigen production. Vaxfectin, however, does not utilize this mechanism, as it does not enhance transgene expression at the site of administration [3,27].

Adjuvants may enhance immune responses by mechanisms applicable to all vaccine types, not just those based on pDNA: increasing antigen presentation (by increasing MHC expression), modulating the local immune environment (by altering the pattern of cytokine secretion), or increasing immune cell recruitment (by upregulation of chemokines and/or adhesion molecules). A gene and cytokine profiling study [27] suggested that the immune response to Vaxfectin-adjuvanted vaccines utilizes all these mechanisms. By comparing mice treated with non-adjuvanted versus Vaxfectin-adjuvanted pDNA for the upregulation of gene transcripts in injected muscles and cytokine levels in sera, Vaxfectin was observed to upregulate four functional groups (**Table 3**). Upregulated immune response genes included both MHC class I and class II genes, antigen transporters (TAP1 and TAP2), and a regulator of T-cell activation (CD274). Cytokines present at increased levels included not only the previously mentioned IFN-γ and IL-6, but also chemokines such as CCL2 (MCP-1) and several members of the CXC family. Several members of the apoptotic cascade were upregulated, of interest as apoptosis under the correct conditions can enhance immune responses [28]. Finally, upregulation of Toll-like receptor (*TLR*) genes as well as the TLR adaptor gene *MyD88* may reflect a remodeling of the innate immune response at the cellular or receptor signaling level.

A second profiling study performed after i.m. injection of a protein-based vaccine (TIV) with or without Vaxfectin also demonstrated upregulation of gene transcripts across a range of functional groups [29], many in common with those observed in the pDNA vaccine study (**Table 3**). Further studies using TIV protein to examine the influence of particle size on immunogenicity showed that the higher ratio of Vaxfectin to TIV produced large particles with diameters > 1 μm whereas the lower ratio of Vaxfectin to TIV produced smaller particles with an average diameter between 400 and 500 nm. These differences in particle size may affect how the vaccine interacts with the immune system. For example, smaller particles (< 200 nm) may be more readily endocytosed, resulting in more efficient antigen processing and therefore potentially more robust immune responses.

The role of the TLR pathway in the adjuvant activity of Vaxfectin was directly addressed by comparing immune responses with a Vaxfectin-adjuvanted protein vaccine (TIV) in C57BL/6 wild-type versus $MyD88^{-/-}$ mice, the knockout mouse lacking an adaptor protein required for the functioning of most TLRs [30]. Gene profiling revealed few differences in gene transcript upregulation between the two mouse strains

Table 2. Vaxfectin enhances T-cell responses with a cancer-associated peptide.

Study	Antigen	Adjuvant	SFU/10^6 splenocytes	Fold increase
1	TRP-2 peptide (30 μg)	None	3 ± 1	–
	TRP-2 peptide (30 μg)	Freund's	18 ± 16	6×
	TRP-2 peptide (30 μg)	Vaxfectin	129 ± 37	45×
2	TRP-2 peptide (30 μg)	None	4 ± 1	–
	TRP-2 peptide (30 μg)	Vaxfectin	101 ± 29	28×
	TRP-2 peptide (60 μg)	Vaxfectin	194 ± 44	53×
	TRP-2 peptide (90 μg)	Vaxfectin	249 ± 50	68×

Mice received s.c. injections of TRP-$2_{180-188}$ peptide unadjuvanted or adjuvanted with either Freund's adjuvant or Vaxfectin (10:1 Vaxfectin:TRP-2 mass ratio). The number of TRP-2-specific IFN-γ-producing cells, designated as spot forming units (SFU) per million splenocytes, was determined using an ELISPOT assay 1 week after boost injections. Data are presented as means ± s.e.m.; in study 1, the Vaxfectin-adjuvanted group differs from all others by $p < 0.05$ (one-way analysis of variance and Newman-Keuls multiple comparison test).

Table 3. Gene and cytokine profiling: Vaxfectin-adjuvanted pDNA and protein.

Stimulus	Gene/protein functional group			
	Immune response	Cytokine/chemokine	Apoptosis	TLR
Vaxfectin-adjuvanted pDNA	MHC class I	IFN-γ	Caspase 7	TLR2
	β2-Microglobulin	IL-6	Caspase 12	TLR3
	MHC class II	CCL2 (MCP-1)	CD95L (Fas)	MyD88
	TAP1	CXCL9	CD262 (TNFSF10B)	
	TAP2	CXCL10	CFLAR (c-FLIP)	
	CD274 (PD-L1)	CXCL11		
	SAMHD1			
Vaxfectin-adjuvanted protein	CD274 (PD-L1)	IFN-γ	(Not analyzed)	TLR2
		IL-4		TLR3
		IL-5		TLR4
		IL-6		TLR9
		TNF-α		MyD88
		CCL2		TRIF
		CXCL9		

Gene microarray, cytokine multiplex and polymerase chain reaction analyses were used to profile the gene transcripts and cytokines upregulated in response to Vaxfectin-adjuvanted pDNA (encoding hCMV gB protein) or protein (influenza TIV). Adjuvanted pDNA or protein was injected i.m. in either BALB/c or C57BL/6 mice, respectively, and the prominent transcripts and cytokines upregulated (compared with mice injected with non-adjuvanted pDNA or protein) within the next 48 h period are presented according to their overall functional group.
TLR: Toll-like receptor.

(**Table 4**). As for vaccine immunogenicity, whereas the wild-type mice mounted the greater overall humoral response, Vaxfectin successfully acted as an adjuvant in both strains (**Table 5**). These data are in agreement with a previous report that concluded that TLR signaling is contributory to but not required for the activity of a diverse range of adjuvants [31]. Also in agreement with this previous report, the authors observed when subtyping the antigen-specific antibodies in the knockout mice that the IgG2c isotype was relatively underrepresented as compared with IgG1, IgG2b and IgG3.

5.1 Competing technologies for enhancing plasmid DNA-based vaccines

Vaxfectin is now being developed as an adjuvant for plasmid DNA-based vaccines. Although it is also a potent adjuvant for protein- and peptide-based vaccines, the competing technologies are examined only for pDNA-based vaccines. Plasmid DNA-based vaccines are commercially available in the animal health field, including a vaccine to protect farm-raised salmon against infectious hematopoietic necrosis virus (Apex® IHN, Novartis Animal Health) and a canine therapeutic cancer vaccine to treat oral melanoma (Oncept®, Merial Limited, Duluth, Georgia). Both of these vaccines are non-adjuvanted plasmid DNA; no adjuvanted plasmid DNA-based vaccines are commercially available at present. In addition to Vaxfectin-containing vaccines in clinical trials, other plasmid DNA vaccines have advanced into man using agents designed to increase vaccine potency. These modifying agents can be divided into two categories: molecular adjuvants and devices.

Molecular adjuvants in development include cytokines, such as IL-12 and IL-15, as well as chemokines, such as granulocyte/macrophage-colony stimulating factor

Table 4. Gene and cytokine profiling: wild-type versus MyD88$^{-/-}$ mice.

Functional group	Gene/cytokine	Fold increase	
		C57BL/6	MyD88$^{-/-}$
Cytokine/chemokine	IL-5	0	0
	IL-6	178	31
	IFN-γ	12	1
	TNF-α	15	19
	CCL2	104	138
	CXCL9	10	7
TLR	MyD88	13	NA
	TRIF	3	2
	TLR2	12	13
	TLR9	20	68
Immune response	CD274	3	14
	NOD1	3	2
	NOD2	8	5

Vaxfectin-adjuvanted TIV protein was delivered by i.m. injection to C57BL/6 or MyD88$^{-/-}$ mice, and 24 h later gene transcripts in injected muscles were quantified by polymerase chain reaction. Data presented as fold increase over animals injected with non-adjuvanted TIV.
NA: Not applicable; TLR: Toll-like receptor.

(GM-CSF). Interleukin-12 is a heterodimeric cytokine that supports the development of cell-mediated immunity [32]. Interleukin-15 differs from IL-12 in that it maintains memory T cells in the absence of antigen [33,34]. These cytokines and chemokines are coadministered as either recombinant proteins or extra expression plasmids along with the antigen expression plasmid. One example of a device technology designed to enhance vaccine immune response is electroporation (EP), the application of an electric charge through needle electrodes to the site of injection following intramuscular administration of plasmid DNA. The electric pulse is designed to facilitate plasmid transfer into the muscle cells, resulting in increased expression. This results in some tissue damage at the EP site that may also result in activation of the immune system. Examples of clinical trials adjuvanting plasmid DNA-based vaccines are listed in **Table 6**. Examples of clinical trials combining electroporation with plasmid DNA-based vaccines are listed in **Table 7**.

Expression of the cytokines and chemokines increases vaccine potency by activating T cells and macrophage (in the case of IL-12 and IL-15) or antigen-presenting cells and neutrophils (in the case of GM-CSF). However, expression of these proteins is unregulated and although they have positive impacts on the immune system, they may induce adverse reactions, thus creating safety concerns. Hence, most studies are conducted in Phase I clinical trials for life-threatening diseases such as cancer and HIV. Electroporation has been shown to increase DNA expression in animal models by one to two orders of magnitude. The adjuvant effect when applied to vaccines has been attributed at least in part to tissue damage at the administration site. Hence, electroporation, although having positive benefits, also presents safety and tolerability challenges for clinical use.

6. Conclusion

Vaccine development for animal and human health to prevent or treat a variety of diseases, in particular infectious diseases and cancer, can benefit from the discovery of new synthetic adjuvants such as Vaxfectin. The simplicity of Vaxfectin synthesis and its characterization as a well-defined lipid-based system, its ease of formulation with a range of biomolecules, and its cost-effectiveness make it a very attractive option for new vaccines in need of improved immune responses or dose-sparing. This review has exemplified the broad applications of Vaxfectin as an adjuvant, not only for pDNA-based vaccines but also for protein- and peptide-based vaccines. The flexibility of Vaxfectin to favor different arms of the immune response, either humoral or cellular, based on the adjuvant-to-antigen ratio, makes it an attractive candidate for the development of next-generation vaccines. A growing safety and toxicology database is being gathered for Vaxfectin alone and for Vaxfectin-formulated vaccines. Vaxfectin's initial intended use to formulate plasmid-based vaccines has now been tested in Phase I clinical trials and has shown a good safety profile and demonstrated immunogenicity on repeated injections in healthy subjects. Vaxfectin presents many of the desired features of a new adjuvant: simplicity, ease of formulation and stability, low manufacturing costs, ability to improve and favor specific immune responses, provide a dose-sparing effect, and promising safety profile. All of these attributes could qualify it as a universal vaccine for pDNA-, protein- and peptide-based vaccines.

7. Expert opinion

The advent of modern vaccines derived from genomics and proteomics has increased the need for safe and effective new adjuvants. Since the discovery in the 1920s by a French veterinarian, Gaston Ramon, at the Pasteur Institute, that substances as diverse as lecithin, saponin, tapioca or even breadcrumbs could augment immune responses, and a few years later that aluminum salts and Quil A (a saponin extracted from the bark of the South American tree Quillaja saponaria) could offer similar benefits, a handful of adjuvants have been used in commercial vaccines. Until the late 1990s when Novartis's (then Chiron's) MF59 oil-in-water emulsion seasonal influenza vaccine (Fluad®) was approved in Italy, no adjuvant other than aluminum salt (or Alum) had reached licensure in Europe as part of a vaccine. Since then, several vaccines using new adjuvants have been marketed in Europe. Fluad has obtained marketing authorization in more than a dozen European countries; Pandemrix™ (GlaxoSmithKline), a mock-up prepandemic H5N1 influenza vaccine adjuvanted with ASO3 (an oil-in-water emulsion containing α-tocopherol, squalene and Tween 80), was

Table 5. TIV-induced humoral responses: wild-type versus MyD88$^{-/-}$ mice.

Mouse strain	Vaccine	Anti-TIV titer	
		GMT	Fold increase
C57BL/6	TIV	9.1×10^3	–
	Vaxfectin-TIV	2.0×10^7	2195 × (p = 0.004)
MyD88$^{-/-}$	TIV	1.1×10^3	–
	Vaxfectin-TIV	1.2×10^6	1024 × (p = 0.045)

Non-adjuvanted or Vaxfectin-adjuvanted TIV protein (1 μg) was delivered by i.m. injection to C57BL/6 or MyD88$^{-/-}$ mice on days 1 and 23, and on day 43 TIV-specific antibody titers were determined by ELISA. Data presented as geometric mean titer (GMT) and the fold increase over non-adjuvanted TIV titer, with significance between treatment groups determined by the Wilcoxon rank sum test.

Table 6. Clinical trials for molecular adjuvants with plasmid DNA-based vaccines.

Type of modifier	Clinical trial	Disease	Start date	Trial status	No. of patients	Sponsor
IL-15 pDNA coadministered with antigen-expressing pDNA	Phase I	HIV preventive vaccine	26 June 2005	Completed 23 September 2009	120	NIAID NCT00115960
IL-15 and IL-12 pDNAs coadministered with antigen expressing pDNA	Phase I	HIV therapeutic vaccine	5 August 2006	Study terminated 3 December 2007	91	Wyeth NCT00195312
IL-12 pDNA coadministered with antigen-expressing pDNA	Phase I	HIV preventive vaccine	23 May 2005	Completed 11 May 2010	144	NIAID NCT00111605
Recombinant human GM-CSF	Phase II	Therapeutic prostate cancer vaccine	19 February 2009	Now recruiting	34	University of Wisconsin and NCI NCT00849121

Information was obtained from www.ClinicalTrials.gov.

Table 7. Clinical trials for electroporation with plasmid DNA-based vaccines.

Type of modifier	Clinical trial	Disease	Start date	Trial status	No. of patients	Sponsor
EP	Phase I	Therapeutic melanoma vaccine	8 May 2007	Study continuing enrollment completed	25	Ichor Medical Systems, Inc. NCT00471133
EP	Phase II	Therapeutic prostate cancer vaccine	10 March 2010	Now recruiting	18	Uppsala University NCT00859729
EP	Phase I	Prophylactic malaria vaccine	22 July 2010	Now recruiting	39	NIAID
EP	Phase I	Prophylactic avian influenza vaccine	27 May 2010	Recruiting as of 16 June 2010	30	Inovio Pharmaceuticals NCT01142362
EP	Phase I	Therapeutic cervical cancer vaccine	22 May 2008	Study continuing enrollment completed	18	Inovio Pharmaceuticals

Information was obtained from www.ClinicalTrials.gov.
EP: Electroporation.

approved in Europe, and two other vaccines from GlaxoSmithKline adjuvanted with ASO4 (alum and monophosphoryl lipid A) – the hepatitis B vaccine Fendrix® and the human papilloma virus vaccine Cervarix® – obtained market approval in Europe and other territories. In the US no new adjuvant-based vaccine was licensed until the late 2009 approval by the Food and Drug Administration of the cervical cancer prophylactic vaccine Cervarix, adjuvanted with ASO4. At present, an adjuvant can be approved only as part of a registered vaccine, not as a standalone product, limiting the development of adjuvants for multiple vaccine applications.

Over the past decade, advances in immunology and a better understanding of the mechanism of action of

traditional adjuvants have led to a more rational design and testing of new adjuvants that can be tailored more specifically to obtained a desired immune response. In particular, the elucidation of certain immunological pathways involving pathogen-associated molecular patterns and pathogen-recognition receptors such as TLRs helped with the design of such new adjuvants. Those adjuvants, whether by providing a direct stimulation of the innate immunity or an enhanced and more selective antigen delivery, are designed to boost the immunogenicity and duration of the desired immune response to the vaccine with a reasonable safety profile. Ideally, adjuvants should have several attributes, including defined physicochemical properties to ensure batch-to-batch vaccine consistency, stability on formulation of a vaccine, scalability, cost-effectiveness, enhanced and sustained immune responses, possible reduction of antigen dose, as well as good safety profile and body clearance. Among a variety of new adjuvants in development, Vaxfectin is well positioned as it meets these requirements for an adjuvant with broad-based applications: it is a synthetic adjuvant with a straightforward scalable synthesis of the two constituting lipids; it formulates reproducibly with different types of vaccine by simple mixing and analytical assays have been developed to ensure lot-to-lot reproducibility. The simplicity of lipid synthesis, ease of production of Vaxfectin and straightforward formulation as part of a vaccine renders this new adjuvant cost-effective. Vaxfectin has shown enhanced specific immune responses, dose-sparing effect and protection against challenge with pDNA-, protein- and peptide-based vaccines in several preclinical models, together with a good safety profile and no overt reactogenicity concerns. In addition, early testing in healthy subjects has shown promising safety and immunogenicity of Vaxfectin-adjuvanted pDNA vaccines. Although some preliminary data have been published on the potential mechanism of action of Vaxfectin-formulated pDNA and protein-based vaccines, showing the effect of the produced particle sizes, the IL-6 dependency and the effect of stimulation of various cytokines and chemokines on immunogenicity in animal models, it does not appear that Vaxfectin mediates its effect through a direct TLR pathway. Further studies will be required to define precisely the mechanism of action of Vaxfectin when formulated with different types of vaccine and at various ratios.

Acknowledgments

The authors thank D Griffin from The Johns Hopkins Bloomberg School of Public Health for her continued collaboration with the measles vaccine. The authors thank E Raz from School of Medicine at the University of California, San Diego, for providing the MyD88$^{-/-}$ mice and B Beutler from The Scripps Research Institute for his advice on the mechanism of action studies.

Declaration of interest

All authors are employees of Vical, Inc. They declare no other conflict of interest.

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