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Pre-clinical development of a hydrogen peroxide-inactivated West Nile virus vaccine

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Abstract

West Nile virus (WNV) is a mosquito-transmitted pathogen with a wide geographical range that can lead to long-term disability and death in some cases. Despite the public health risk posed by WNV, including an estimated 3 million infections in the United States alone, no vaccine is available for use in humans. Here, we present a scaled manufacturing approach for production of a hydrogen peroxide-inactivated whole virion WNV vaccine, termed HydroVax-001 WNV.

Vaccination resulted in robust virus-specific neutralizing antibody responses and protection against WNV-associated mortality in mice or viremia in rhesus macaques (RM). A GLP-compliant

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toxicology study performed in rats demonstrated an excellent safety profile with clinical findings limited to minor and transient irritation at the injection site. An *in vitro* relative potency (IVRP) assay was developed and shown to correlate with *in vivo* responses following forced degradation studies. Long-term *in vivo* potency comparisons between the intended storage condition (2–8°C) and a thermally stressed condition (40±2°C) demonstrated no loss in vaccine efficacy or protective immunity over a 6-month span of time. Together, the positive pre-clinical findings regarding immunogenicity, safety, and stability indicate that HydroVax-001 WNV is a promising vaccine candidate.

Keywords

West Nile virus; hydrogen peroxide; vaccine; vaccination; rhesus macaque; antibody

1. Introduction

West Nile virus (WNV) is a member of the genus *Flavivirus* (family *Flaviviridae*), a group that includes many clinically relevant arthropod-borne viruses such as yellow fever (YFV), dengue (DENV), Japanese encephalitis (JEV), tick-borne encephalitis, and Zika viruses[1]. All members are single-stranded positive-sense RNA viruses with genomes of approximately 11 kilobases that form enveloped mature infectious particles roughly 50 nm in diameter. The genome is translated into a large polyprotein that is subsequently processed by both cellular and viral proteases into 3 structural proteins (capsid, premembrane, and envelope [Env]) and 7 nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). WNV is now endemic throughout the continental United States, with periodic peaks and troughs in disease incidence[2]. Although ~75% of infections are believed to be asymptomatic[3], symptomatic infection can be severe with a mortality rate among reported WNV cases of approximately 4%[4], increasing to as high as 23% among elderly patients 70 years of age[5].

Currently, no vaccine is available to prevent WNV disease in humans. Although a correlate of WNV immunity has not been established formally, it is recognized that neutralizing antibodies have an important role in protective immunity[6–12]. We previously have shown that neutralizing antibodies are sufficient for protection against WNV infection in mice[6, 13, 14] and have reported on the development of a hydrogen peroxide (H₂O₂) inactivated WNV vaccine candidate that demonstrated robust neutralizing antibody responses in mice, with protection against stringent intracranial challenge in both young and aged animals[15]. Herein, we describe the pre-clinical development and testing of this vaccine candidate and provide supportive evidence and proof-of-principle for the use of H₂O₂-based virus inactivation as an effective approach to develop a new WNV vaccine for humans.

2. Materials and Methods

2.1. Vero cell stocks and banks

WHO Vero Seed Lot 10-87 was selected as the parental cell line for vaccine production on the basis of the extensive historical testing and characterization associated with this line[16].

Serum-free cell bank development was carried out under contract by SAFC Pharma (Carlsbad, CA) using optimized procedures established by NTI, with both a master cell bank (MCB) and working cell bank (WCB) manufactured in compliance with current good manufacturing practices (cGMP). The MCB was tested and confirmed to be negative for adventitious agents including porcine circovirus types 1 and 2.

2.2. Virus stocks and banks

The chemically resistant model virus, porcine parvovirus (PPV, strain NADL-2) was obtained from the American Tissue Culture Collection (Manassas, VA) and titered on swine testicle cells. WNV strains Texas TX02, Egypt101, New York 1999 (NY99), Madagascar AnMg798 and South Africa H442 SA58, have been described previously[14, 17, 18]. A low-passage, attenuated, lineage 1 Kunjin virus isolate (WNV-KV, strain CH16532[15]) was selected for vaccine development. The WNV-KV master seed was sequentially triple-plaque-purified on serum-free adapted Vero cells and used to develop a pre-master virus bank (pre-MVB). The WNV-KV pre-MVB, propagated on the Vero WCB, was used to prepare the WNV-KV master virus bank (MVB), which was in turn used to manufacture a working virus bank (WVB). SAFC Pharma performed all manufacturing steps under cGMP compliance. Both virus banks were sequenced according to good laboratory practices (GLP, Genewiz, Inc.) and confirmed to be free of potential adventitious agents.

2.3. Inactivation kinetics and tests for residual live virus

Inactivation kinetics of WNV-KV by H₂O₂ were studied under small-scale conditions using our previously described hydrogen peroxide-based approach with catalase treatment to remove residual H₂O₂ prior to performing live virus quantitation [15]. To confirm complete inactivation for manufacturing purposes, 5% of the total bulk drug substance (BDS) was tested using a validated residual live virus assay developed in conjunction with WuXi AppTec, similar to the approach recently described for an inactivated YFV vaccine candidate[19].

2.4. Vaccine

HydroVax-001 WNV is a whole virion vaccine inactivated with H₂O₂ and adsorbed to aluminum hydroxide adjuvant. Two sequential manufacturing lots (Lot 159-13001 and Lot 159-13005) were produced by SAFC Pharma. Lot 159-13001 was manufactured in cGMP facilities using draft batch records to confirm the manufacturing process at scale, with Lot 159-13005 produced under cGMP compliance for subsequent clinical use. For each production, aliquots of the WCB were thawed, expanded in serum-free medium, and passaged into a GE WAVE bioreactor using microcarrier beads. Cells were infected with the WNV-KV WVB, with virus harvests collected at the anticipated peak of viral replication using a batch-refeed method. Harvests were clarified, pooled, and treated with Benzonase to minimize host-cell DNA contamination. The Benzonase-treated, clarified pooled harvest was then concentrated and buffer-exchanged using tangential flow filtration. Just prior to initiating virus inactivation, material was filtered and H₂O₂ was added at a final concentration of 3.0%. Inactivation proceeded with gentle agitation at a controlled temperature (25±2°C) for 7 hours. Halfway through inactivation, an additional filtration was performed to limit potential aggregates. Directly following inactivation, viral material was

purified using ion-exchange chromatography with a subsequent tangential flow filtration diafiltration step for formulation of BDS into a proprietary stabilization buffer, followed by final sterile filtration. BDS was then formulated with 0.1% aluminum hydroxide (Alhydrogel®, Brenntag Biosector, Denmark) to produce the final drug product (DP) and filled into stoppered glass vials. The BDS and DP conformed to specifications on appearance, osmolality, pH, protein concentration, residual Benzonase, residual host cell DNA, residual protein, residual growth medium components, potency, residual live virus, identity (SDS-PAGE and confirmatory Western blot using the WNV-specific MAb, 7g11 [15]), endotoxin, general safety and sterility.

To assess vaccine potency a WNV-specific *in vitro* relative potency (IVRP) assay was developed incorporating both the measurement of total protein and WNV-specific antigen via ELISA, similar to other non-replicating vaccines [19, 20], with direct testing of the BDS or desorbed DP. The WNV Env-specific, neutralizing mouse monoclonal antibody (MAb) 7g11 [15] was used as both the capture and detection reagent. Log-log transformed ELISA optical density (OD) and protein concentration were plotted in the linear portion of the curve. The titer was defined as the protein concentration needed to reach an OD of 0.50 and normalized to a reference standard established using an engineering lot of BDS (similar to [20]). Samples from clinical-grade vaccine lots were tested in triplicate by WuXi AppTec using GLP-compliant practices.

2.5. ELISA and neutralization assays

Epitope mapping of WNV-KV after different modes of inactivation was performed with previously described WNV Env-specific monoclonal antibodies (MAbs) [13, 21], following a 1:250 dilution of the target antigen to reduce residual inactivation components. Immunity following vaccination was assessed by ELISA using WNV-KV lysate similar to previous studies [14]. Serum neutralization titers (NT₅₀) for WNV-NY99 were determined using a plaque reduction assay as described previously [14], whereas neutralization titers for all other WNV strains were performed using a focus-forming assay (FFA) [15]. WNV-KV neutralization titers were initially tested and found comparable using either the plaque assay or FFA (Supplemental Figure 1), with all subsequent testing performed using FFA.

2.6. Human subjects

Human subjects who had experienced symptoms of WNV infection were recruited approximately 1 year after a WNV outbreak in Colorado [14]. Naïve control subjects who had not lived in WNV-endemic areas had no detectable neutralizing antibody response (NT₅₀ < 10) [14]. WNV-exposed subjects ($n = 11$) who demonstrated seropositivity to WNV (NT₅₀ > 10) and had no history of prior YFV or JEV vaccination were included in the study (dengue exposure history is unknown). Each subject provided written informed consent before participating in the study, and the Oregon Health & Science University (OHSU) Institutional Review Board approved all human studies.

2.7. Animals

Female BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME). For challenge studies, mice were inoculated via the i.p. route with 200 plaque forming units

(PFU; 20 LD₅₀) of WNV-NY99 as described[14]. For passive immunity studies, monoclonal or polyclonal antibody was purified by Protein A chromatography according to the manufacturer's instructions (Bio-Rad) and administered to mice by the i.p. route one day prior to challenge. WNV-infected mice that became moribund or experienced greater than 25% weight loss were euthanized according to protocol. BALB/c mice become progressively more susceptible to lethal WNV-NY99 infection with age (Supplemental Figure 2), and we therefore typically challenged vaccinated (active or passive) mice at 16 weeks of age. Non-human primate studies utilized adult (6–13 years of age) male and female rhesus macaques (RM, *Macaca mulatta*) bred and housed at the Oregon National Primate Research Center (Beaverton, OR). For RM challenge studies, animals were inoculated subcutaneously (s.c.) with a total of 2×10^5 PFU of WNV-NY99, administered across four sites on the back. All mouse and RM studies were overseen and approved by the OHSU Institutional Animal Care and Use Committee (IACUC) in accordance with the National Institutes of Health guide for the care and use of laboratory animals. The safety/toxicology study performed in male and female Sprague-Dawley rats was conducted under GLP compliance by Charles River Laboratories following IACUC approval (Spencerville, OH).

2.8. Safety/Toxicology study

The finalized toxicology study was performed by Charles River Laboratories following Good Laboratory Practices for Nonclinical Laboratory Studies, as specified by the Code of Federal Regulations, Title 21, Part 58. HydroVax-001 WNV was administered on study days 1, 8, 15 and 22 to 10 female and 10 male Sprague-Dawley rats (10 – 11 weeks old) as two 0.25 mL i.m injections (left and right thigh muscles) for a total dose of 6 µg of purified viral protein at each vaccination time point. An equal number of control animals received 0.9% saline using the same volume and route. The parameters and end points evaluated included clinical signs, dermal scoring, body temperatures, body weights, body weight changes, food consumption, ophthalmology, clinical pathology parameters (hematology, coagulation, and clinical chemistry), α₂-macroglobulin (performed by BioReliance, Rockville, MD), WNV-specific antibody analysis (NT₅₀, performed by NTI), gross necropsy findings, organ weights, and histopathologic examinations.

3. Results

3.1 WNV characterization

WNV-KV was triple plaque purified and single step (multiplicity of infection [MOI] = 1.0 PFU/cell) or multi-step (MOI = 0.10 PFU/cell) growth curves were determined (Figure 1A). WNV titers peaked between 1–2 days post-infection, and averaged approximately 5×10^8 PFU/ml at the peak. Similar to the parental WNV-KV strain, the TPP WNV-KV clone was attenuated *in vivo*, showing no mortality in adult mice at doses as high as 10^6 PFU (Figure 1B). By comparison, the virulent WNV-NY99 strain demonstrated 80% mortality at a dose of 200 PFU.

The MVB was sequenced (GenBank JX276662.1) and demonstrated 98.5% and 99.2% nucleotide and amino acid identity across the entire genome in comparison to a full-length prototypical WNV-KV clone (pAKUN, GenBank AY274505.1), and 99.8% amino acid

identity across the Env protein. There was a single amino acid difference in the Env (F156S) that appears to be a common polymorphism among Kunjin strains of WNV [22]. In comparison to WNV-NY99 (GenBank AF202541.1), nucleotide and amino acid identities were 88.0% and 97.8%, respectively, similar to previous reports[23]. An aliquot of the MVB was expanded for 5 more passages under GLP-compliant conditions (WuXi AppTec) to develop an end-of-production (EOP) virus bank. The EOP was sequenced (10,948 nt, 99.3% total genome coverage) with no nucleotide differences observed between the MVB and the EOP samples, indicating genetic stability of the virus even when carried multiple passages beyond the intended production level.

3.2 Virus inactivation with H₂O₂

To assess the robustness of the H₂O₂-based manufacturing process, we performed inactivation studies testing a range of conditions that might influence inactivation. We first examined reproducibility across two pilot lots of high-titer WNV-KV, and observed consistent results with an average inactivation half-life ($T_{1/2}$) of 2.6 minutes (Figure 2A). To determine if H₂O₂-based inactivation was protein concentration-dependent, WNV-KV was spiked with increasing levels of the model protein, bovine serum albumin (BSA; 0.01, 0.10, 1.0 and 10 mg/mL). No concentration of additional protein showed a measurable impact on virus inactivation kinetics (Figure 2B). Similarly, variations in temperature (23–27°C) or pH (range = 7.0–8.0) did not impact inactivation rates (Figures 2C and D). A standard 3% H₂O₂ concentration led to complete inactivation of WNV-KV within 7 hours (Figure 2E), and additional studies indicated that H₂O₂ concentrations were stable during virus inactivation (data not shown). To evaluate whether H₂O₂ retained virus inactivation activity over time, a 1/10th dose of infectious WNV-KV (1×10^8 PFU) was added at either 1 or 4 hours after the initiation of H₂O₂ inactivation of a representative vaccine lot. These spiked samples also showed complete virus inactivation at the final 7-hour time point. In addition, we examined the ability of H₂O₂ to inactivate the chemically-resistant model virus, PPV[19], and demonstrated complete inactivation of the PPV-spiked samples within a 7 hour H₂O₂ inactivation period.

3.3 WNV antigenicity and stability

We investigated the effects of different inactivation approaches on specific WNV Env epitopes using MAbs specific for the major Env domains (Figure 3A). As expected, some inactivation approaches such as heat (56°C, 2 hours) or UV irradiation (5 joules) damaged neutralizing epitopes. Formaldehyde and β -propiolactone (BPL) are among the most common inactivation approaches used for vaccine development but they were typically more damaging than H₂O₂ in terms of maintaining the integrity of WNV Env epitopes. H₂O₂-induced changes were examined in more detail using an expanded panel of MAbs (Figure 3B). Domain I/II (DI/II) epitopes appeared more sensitive to H₂O₂-based disruption, especially if the inactivation period was extended to 12 or 20 hours (Figure 3B). However, this pattern varied according to finer epitope specificity. For example, some DII fusion-loop (DII-fl) MAbs such as E18 were resistant to damage, whereas the epitope bound by MAb E53 demonstrated a significant loss of signal after 4 hours of treatment. These results suggest E53 contacts oxidizable amino acid residues, and indeed it has been shown to make close contact with Met77 of the Env[24]. By comparison, E16 (located in domain III, DIII)

was largely unaffected by oxidative inactivation, which agrees with prior structural studies demonstrating residue contacts that generally are resistant to oxidation[25]. Indeed, the majority of domain III (DIII)-specific MAbs were resistant to H₂O₂-induced changes, suggesting this region of the WNV Env remained largely intact even after extended exposure to H₂O₂.

To quantitate viral antigen in our vaccine lots, and to assess stability over time, we developed an IVRP assay using the MAb 7g11[15] that efficiently neutralizes both WNV-KV and WNV-NY99 virus strains (Supplemental Figure 3). Alum-formulated vaccine drug product (DP) was stressed by exposure to increased temperatures (56°C or 80°C) for 1 hour and this demonstrated a progressive loss in potency as judged by the IVRP assay (Figure 3C). In parallel, control and heat-stressed vaccine preparations were used to vaccinate mice to assess *in vivo* immunogenicity. Thermally stressed vaccine showed decreases in IVRP signal, which correlated with decreased *in vivo* neutralizing responses. The IVRP assay subsequently was used to assess the stability of the BDS for up to 18-months and the final DP for up to 26-months post-manufacturing and the results remained within provisional stability limits (Figure 3D and E).

3.4 GLP safety/toxicology study

All vaccinated animals survived to scheduled euthanasia on Day 24 (main termination; 2 days after the last injection) or 36 (recovery termination; 14 days after the last injection). Clinical findings were limited to local irritation at the injection site, which was minimal and transient in both the control and treated groups. There were no vaccine-related changes in mean body temperatures, mean body weights, mean body weight gains, or food consumption as compared to controls. There were no vaccine-related findings noted during ophthalmic examinations. Vaccine-related changes in hematology data included minimal increases in fibrinogen (up to 1.3×) and eosinophils (up to 2.5×) in treated males and females as compared to the control group on Days 24 and 36. However, all mean values were within the historical control ranges and therefore these findings were considered minimal and non-adverse. There were no apparent vaccine-related changes in clinical chemistry parameters during the study. There were no vaccine-related gross pathology changes. Vaccine-related splenic weight increases were noted at the end of the 14-day recovery period in males (Day 36). However, there were no correlating histopathology findings in recovery males and no splenic weight changes in recovery females. Microscopically, at test article injection sites, minimal to moderate histiocytic infiltrates or histiocytic infiltrates admixed with varying amounts of lymphocytes and plasma cells in muscle bundles or along fascial plains were seen at the end of the main phase. Similar findings were noted at the end of the recovery period; however, the severities were reduced. Results from the α₂-macroglobulin analysis indicated that there were few samples with results above the limit of quantitation (6.25 ng/mL). The few results obtained were observed with similar magnitudes in both the vaccinated animals and controls on Day 24 (36.5 µg/mL vs. 29.82 µg/mL, respectively) and on Day 36 (27.93 µg/mL vs. 20.35 µg/mL, respectively). No animals exhibited α₂-macroglobulin levels above the reported range of 30 to 40 µg /mL for normal Sprague Dawley rats[26]. For WNV-specific neutralizing antibody titers, all controls (vaccinated with 0.9% saline) were seronegative (NT₅₀ 20) whereas all of Hydrovax-001 WNV vaccinated

animals seroconverted with geometric mean neutralizing titers of 2,389 (95% CI: 1,164–4,900) and 5,881 (95% CI: 2,829–12,225) on Days 24 and 36, respectively (Supplemental Figure 4).

3.5 Protection against WNV infection

To determine if *in vivo* potency was maintained over extended periods of storage, mice were immunized with vaccine material stored for 6 months at either 2–8°C (intended condition) or 40±2°C (stressed condition) (Figure 4A). Peak WNV-specific ELISA titers were higher in mice that received the 2–8°C vaccine material, but NT₅₀ titers were similar between groups by the 90-day point, with no significant differences in protection against lethal WNV challenge ($P = 0.32$, Mantel-Cox log rank test). Together, these data show that HydroVax-001 WNV is heat-stable, immunogenic, and capable of conferring protective immunity against lethal WNV-NY99 challenge even after 6 months of storage at elevated temperatures (40°C/104°F), equivalent to an extended break in cold-chain.

Dose-escalation studies were performed using 1 µg, 2 µg or 4 µg of HydroVax-001 WNV. WNV-specific ELISA responses were similar between the 2 µg and 4 µg dose groups, whereas animals in the 1 µg dose group demonstrated lower titers at 60 and 90 days post-vaccination (Figure 4B). NT₅₀ titers revealed similar trends, though all groups were comparable at the 90-day point. All groups demonstrated protection against lethal WNV-NY99 infection (80–100% survival compared to 0% in naïve animals) with no significant differences observed between vaccinated groups: 4 µg vs. 2 µg, $P = 0.32$; 4 µg vs. 1 µg, $P = 0.32$; 1 vs. 2 µg, $P = 1.0$, Mantel-Cox log rank test). A similar study was performed with vaccine material stored for 26 months at 2–8°C, and although titers at the 1 µg and 2 µg dose were somewhat reduced compared to T=0, the 4 µg dose elicited comparable neutralizing antibody responses and provided full protection against lethal challenge (Supplemental Figure 5), further supporting the long-term *in vitro* stability results (Figure 3D).

Vaccine dose-response studies were also performed in adult RM, with animals demonstrating robust WNV-specific ELISA and neutralizing antibody responses at either the 1 µg or 4 µg dose (Figure 4C). At 2-months post-secondary immunization, 4 vaccinated animals and 4 age-matched naïve controls, were challenged with WNV-NY99. Serum samples were collected on days 0, 1, 2, 3, 4, 6, 8, 10 and 14 post-challenge and assessed for viremia either through a plaque assay or co-culture. Animals were considered viremic if they demonstrated any live virus during this time frame (Figure 4C). All naïve monkeys (4/4) were viremic, with 2 animals that were positive by plaque assay (peak viremia range: 18–45 PFU/mL, similar to previous reports[27]), and all 4 animals were positive by WNV co-culture. In the 1 µg vaccine group, none of the RM were positive by plaque assay, but 50% (2/4) were viremic by co-culture. None of the 4 µg vaccine group (0/4) demonstrated viremia by either detection method at any time point, and were significantly protected in comparison to naïve animals ($P = 0.029$, two-tailed Fisher's exact test). To examine the breadth of antibody neutralization, a panel of WNV strains encompassing the two major WNV lineages was tested using NHP serum collected at 2-months post-secondary immunization (Figure 4D). Neutralizing titers for Lineage 1 WNV strains showed no substantial differences across virus strains, with neutralizing GMTs ranging from 190–453.

As expected, neutralizing responses against Lineage 2 WNV strains trended lower (GMTs ranging from 57–113). Nevertheless, all vaccinated animals mounted a measurable neutralizing response against all tested WNV strains, indicating that the HydroVax-001 WNV vaccine induced broadly neutralizing and WNV-specific antibody responses.

In the 4 µg dose RM group, neutralizing responses also were measured against the virulent WNV-NY99 strain and compared to a group of human subjects who had been infected with WNV during a 2003 outbreak in Colorado[14] (Figure 4D). Geometric mean titers in vaccinated RM were lower than that observed in convalescent humans (248 [95% CI: 63–985] vs. 773 [95% CI: 463–1293]) but the differences were not statistically significant ($P=0.081$). To compare protective capacity *in vivo*, serum IgG was purified and pooled from *a*) four naïve RM, *b*) four vaccinated RM (4 µg dose), or *c*) four WNV-immune human subjects with similar neutralizing antibody titers and tested in a passive transfer mouse protection model. As expected, naïve RM IgG from unvaccinated animals had no measurable impact on WNV disease progression (Figure 4D). Purified convalescent human IgG at 0.5–1 mg per mouse resulted in 75% survival, whereas 3 mg was required to achieve full protection. Purified IgG from HydroVax-001 WNV vaccinated RM protected 50% of mice at the 0.5 mg IgG level, and 100% of mice when administered at 1 mg per mouse. In total, these results indicate that HydroVax-001 WNV elicited neutralizing antibody titers that were functionally comparable to those observed in naturally infected WNV-immune human subjects.

4. Discussion

WNV is endemic to the US, with an estimated 3 million infections and >1,900 reported deaths since its introduction in 1999[28, 29]. Despite this burden, there are no licensed therapeutics or vaccines available to combat WNV in humans. In these studies, we report the pre-clinical development, clinical manufacturing, and testing of HydroVax-001 WNV, and demonstrate immunogenicity as well as protective efficacy, long-term stability, and safety in a pivotal repeat-administration *in vivo* toxicology study. These immunogenicity, stability and safety results indicate that HydroVax-001 WNV is capable of inducing a protective WNV-specific immune response, and support the recent initiation of a Phase I clinical trial ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02337868) Identifier: NCT02337868).

The development of a novel vaccine entails numerous considerations, with safety a chief concern. For our inactivated WNV vaccine candidate, we chose the naturally attenuated WNV-KV strain as our seed virus[30], which is attenuated in birds[31, 32] and immunocompetent mice[33]. Unlike virtually all other WNV strains, it is designated as a BSL-2 agent[34], which improves feasibility of manufacturing. We used Vero cells for WNV production since flaviviruses grow to high titers on this cell line[19, 35, 36], and it has a long history in clinical vaccine production[16, 37]. We have previously reported on the H₂O₂-based inactivation approach for vaccine development and demonstrated rapid inactivation of multiple viruses[14, 15]. H₂O₂ is a potent oxidizer utilized by neutrophils and other mammalian cells to inactivate intracellular/phagocytosed pathogens [38, 39] and has been shown *in vitro* to inactivate a broad range of microbes [40–42]. Oxidation by H₂O₂ is believed to inactivate microbes by several mechanisms, including disruption of lipid membrane bilayers, oxidation of protein backbones and amino acid side chains, and

oxidation of nucleosides [43, 44]. Damage to nucleic acids, including the induction of single-stranded and double-stranded breaks in genomic RNA or DNA is the most probable mechanism of irreversible inactivation [45, 46]. This oxidation-based inactivation approach appears distinct from more traditional approaches such as formaldehyde, wherein complete virus inactivation often requires several weeks of treatment[35]. BPL, another common inactivation agent, degrades in solution to undetectable levels within 10–12 hours, and in some cases is unable to fully inactivate virus spiked into vaccine lots at late points in the inactivation process[19]. By contrast, H₂O₂ retains its inactivation potential at later time points during the manufacturing process, providing a robust approach to virus inactivation and vaccine development while still maintaining antigenic epitopes. In a recent example, an independent group compared γ -irradiation to H₂O₂ treatment for the development of a vaccine against Ebola [47]. Interestingly, γ -irradiation resulted in a damaged vaccine that provided no protection against viremia and all of the vaccinated NHP died or required euthanasia after lethal Ebola challenge. In contrast, a similar vaccine that was treated with H₂O₂ provided complete protection against viremia and the H₂O₂-Ebola immunized NHP survived a normally lethal dose of Ebola, suggesting that key antigenic epitopes were maintained following H₂O₂ exposure.

The clinical potential of inactivated vaccines to combat flavivirus infections is well-established, with inactivated vaccines against TBEV and JEV having been in use for >30 years [48]. Evidence for the feasibility of an inactivated WNV vaccine comes from the success of a crude, formalin-fixed veterinary vaccine, Ft. Dodge Innovator®, which is commercially available for the protection of horses from WNV [49]. An important consideration for any vaccine is the number of doses needed to achieve protective, durable immunity. In this preclinical study, we demonstrated that two doses of vaccine were sufficient to achieve protection in two animal species, with neutralizing antibody responses remaining stable for 2–3 months post-vaccination, but more studies are needed to determine the longevity of vaccine induced serum antibody responses. In comparison, the currently licensed inactivated JEV vaccine (IXIARO®) recommends a two-dose primary series, with >97% seroconversion (NT₅₀ 10) observed at 7 days following completion of this series[50]. However, immunity wanes rapidly, dropping to only 58% seroconversion within one year[51]. Nevertheless, an additional booster dose at 15 months following the primary series quickly recovered immunity (100% seroconversion) and 96% of subjects remained seropositive for up to 6 years after booster vaccination [52]. Similarly maintenance of long-term immunity was observed with TBEV booster vaccination following a primary vaccination series [53], and together indicate that long-term immunity is achievable for inactivated flavivirus vaccines.

The development of a safe and effective WNV vaccine for use in humans remains an important public health goal, with several candidates in various stages of clinical development [4]. As discussed previously [4], a targeted vaccine program could be cost-effective, and interest has been renewed since the surge in US infections in 2012, which represented the largest outbreak since 2003 with the most WNV-associated deaths on record [4]. However, due to the intermittent nature of WNV infections in the continental US, multiple hurdles remain in the pursuit of an effective, licensed vaccine. For example, a WNV vaccine would represent a niche market compared to other large market vaccine products,

and government support may be necessary to incentivize commercialization. Another challenge is developing a practical approach for conducting pre-licensure efficacy trials, since WNV outbreaks are sporadic in nature and not easily predicted [54, 55]. One alternate approach may be to apply the FDAs “Animal Rule”, which was established to allow licensure of vaccines or therapeutics using animal systems in cases where human efficacy studies are not ethical or feasible [56].

While most WNV infections are clinically inapparent, ~25% of cases will present as West Nile fever[5], with ~0.5 to 1% of infections leading to severe neuroinvasive disease[5, 28]. The incidence and severity of neuroinvasive disease increases steadily with age, putting elderly populations at particularly high risk [5]. For example, of the 112 cases of neuroinvasive disease identified in patients <20 years of age, there were no fatalities (0% mortality). By comparison, for the 818 cases identified in those ≥70 years of age, 190 died (23% mortality)[5]. Given the serious threat posed by WNV in the elderly, protection of this population should be a key consideration for any new vaccine[4]. Prior studies have investigated the efficacy of HydroVax-001 WNV in aged mice, and demonstrated protection against a stringent intracranial infection model (10,000 LD₅₀ doses)[15]. Furthermore, the vaccine was able to induce polyfunctional antigen-specific CD8⁺ T cell responses in human HLA-A2 transgenic mice, and primed a cellular immune response against the human HLA-restricted immunodominant WNV epitope SVG9, suggesting it has the potential to stimulate a similar CD8⁺ T cell response in humans[15]. In total, these results support the continued development of this vaccine candidate, which could provide an important tool for protecting elderly and other vulnerable populations against this persistent public health threat.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BDS	bulk drug substance
BPL	β-propiolactone
cGMP	current good manufacturing practices
DENV	dengue virus
DP	drug product
Env	envelope protein

FFA	focus forming assay
GLP	good laboratory practices
H₂O₂	hydrogen peroxide
IVRP	<i>in vitro</i> relative potency
JEV	Japanese encephalitis virus
LOD	limit of detection
MAb	monoclonal antibody
MCB	master cell bank
MOI	multiplicity of infection
MVB	master virus bank
NT₅₀	50% neutralizing titer
PFU	plaque forming unit
PPV	porcine parvovirus
pre-MVB	pre-master virus bank
s.c.	subcutaneous
T_{1/2}	half-life
TPP	triple plaque purification
WCB	working cell bank
WNV	West Nile virus
WNV-KV	WNV Kunjin strain
WVB	working virus bank
YFV	yellow fever virus

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Figure 1. *In vitro* growth kinetics and attenuated phenotype of WNV-KV

(A) Growth kinetics for the WNV-KV parental strain (CH16352) and the triple-plaque purified WNV-KV clone (TPP WNV-KV) were performed in Vero cells under serum-free conditions at two MOI levels. (B) BALB/c mice (6–7 weeks of age, $n = 5$ mice per group) were injected intraperitoneally with 200 PFU of virulent WNV-NY99, 10^6 PFU of the parental WNV-KV strain, or 10^6 PFU of the TPP WNV-KV vaccine strain. After infection, mice were monitored for 28 days for morbidity and mortality. The data are representative of two independent challenge experiments, both demonstrating maintenance of an attenuated *in vivo* phenotype by the plaque-purified WNV-KV clone selected for preparation of the HydroVax-001 WNV MVB. Significance testing was made for each WNV-KV group in comparison to WNV-NY99 using the Mantel-Cox log rank test, with significant differences indicated by an asterisk (* $P < 0.05$).

Figure 2. H₂O₂ treatment results in robust virus inactivation under varying experimental conditions

(A) H₂O₂ was added at a final concentration of 3% (w/v) to two pilot lots of WNV-KV under standard buffer conditions (Tris-HCl, pH = 8.0) at room temperature. The $T_{1/2}$ was calculated from the average of the two runs. The impact of different variables on the rate of virus inactivation was tested including (B) increasing concentrations of exogenous protein (bovine serum albumin, BSA), (C) small temperature variations within the range used during cGMP manufacturing ($25 \pm 2^\circ\text{C}$), and (D) buffer and pH composition. The average

results from Panel A are included in each subsequent panel for comparison. The limit of detection (LOD) for each experiment is indicated by the dashed line, with samples that were negative for infectious virus shown as open symbols. **(E)** WNV-KV was inactivated with 3% H₂O₂ at room temperature for 7 hours. WNV-KV (10⁸ PFU) was spiked into the initial bulk material (1.1×10⁹ PFU/mL) at 1 or 4 hours post-H₂O₂ treatment. Alternatively, porcine parvovirus (PPV, 10⁷ PFU/mL) was spiked into WNV-KV (1.1×10⁹ PFU) at 0 hour. WNV-KV titers were measured from untreated samples at 0 or 7 hours after H₂O₂ addition. For H₂O₂-treated samples, catalase was used to neutralize the H₂O₂ prior to testing for infectious virus by plaque assay. The LOD of <6 PFU/mL is indicated by the dashed line. Shown are the average results (± standard deviation) of two independent biological experiments. N/A; Not Applicable.

Figure 3. H₂O₂-inactivated WNV-KV retains Env-specific neutralizing epitopes and maintains long-term stability

(A) WNV-KV (in PBS) was treated with heat (56°C, 2 h), UV irradiation (5 joules), BPL (0.10%, 16 h at RT), formaldehyde (0.01%, 20 days at 37°C) or H₂O₂ (3%, 6 hrs at RT). ELISA results are expressed as a percentage of live, untreated virus. (B) WNV-KV was treated with 3% H₂O₂ for 4, 12 or 20 h and compared to live WNV-KV using a panel of domain-mapped WNV Env-specific MAbs[13, 21]. The maximum ELISA titer was established for each MAb based on ELISA plates coated with untreated live virus and the

percent of this live virus signal was calculated for each inactivation condition. **(C)** HydroVax-001 WNV (Lot 159-13001) was maintained under intended storage conditions (2–8°C) or thermally stressed for one hour at elevated temperatures (56°C or 80°C) and tested by the *in vitro* relative potency (IVRP) assay. In parallel, vaccine material was used to immunize BALB/c mice (3 µg per mouse, $n = 4$ per group) and WNV-specific serum neutralizing antibody titers were assessed 4.5 months following this single immunization. IVRP values were compared with serum neutralization responses following vaccination and modeled through linear regression of the log-transformed values. Neutralizing titers are presented as group GMTs $\pm$ 95% confidence intervals. **(D)** HydroVax-001 WNV (Lot 159-13005) was manufactured as a bulk drug substance (BDS) and formulated with aluminum hydroxide to produce the final vaccine drug product (DP). Both the BDS and DP were placed on a long-term stability study at the indicated storage conditions with samples removed at pre-specified time points for assessment by the IVRP assay. The solid line represents the time zero value for this vaccine lot, with provisional upper- and lower-bound stability limits indicated by the dashed lines. DI-lr, Domain I lateral ridge; DII-ci, Domain II central interface; DII-di, Domain II dimer interface; DII-hi, Domain II hinge interface; DII-fl, Domain II fusion loop.

Figure 4. HydroVax-001 WNV vaccination induces protective neutralizing antibody responses
 (A) HydroVax-001 WNV DP (Lot 159-13001) was maintained at either the intended storage condition (2–8°C) or a stressed temperature (40±2°C) for 6-months, and subsequently used to immunize groups of female BALB/c mice ($n = 10$ per group) at 3 µg per mouse by the i.p. route at day 0 and day 28 (black arrows). (B) Similarly, HydroVax-001 WNV (Lot 159-13005) was used to immunize groups of female BALB/c mice at the indicated doses ($n = 5$ –10 per group) by the i.p. route. For both groups, serum samples were collected and tested by WNV-specific ELISA or neutralizing activity using live WNV-KV. At 3-months

post-primary vaccination, all animals were challenged i.p. with 200 PFU (20 LD₅₀) of WNV-NY99 and monitored for 28 days. **(C)** RM ($n=4$ per group) were immunized i.m. with 1 or 4 μ g of HydroVax-001 WNV (Lot 159-13005). Serum samples were tested by WNV-specific ELISA or plaque neutralization using WNV-KV. At 3 months post-primary immunization, vaccinated RM and seronegative controls ($n=4$) were challenged s.c. with 2×10^5 PFU of WNV-NY99. **(D)** RM serum samples collected at 2 months after secondary vaccination were tested for neutralizing activity against the indicated WNV strains. Neutralizing titers against WNV-NY99 from the 4 μ g HydroVax-001 WNV-immunized RM group were compared to human subjects at one year following natural WNV infection (Student's *t*-test, two-tailed). Protein A-purified IgG was pooled from naïve RM controls or from the indicated RM or human samples ($n = 4$ per group, half-filled circles) for use in subsequent mouse WNV-NY99 challenge studies. BALB/c mice (16–27 weeks of age, $n = 3$ –15 per group) received purified RM or human IgG at the indicated doses, 7g11 MAb (50 μ g, positive control) or were untreated (no IgG, negative control). Animals were challenged intraperitoneally with 200 PFU (20 LD₅₀) of WNV-NY99 one day following treatment and monitored for 28 days. For serological assays, data is presented as group GMTs ($\pm$ 95% confidence intervals). Naïve serum samples (day 0 time points) from unvaccinated mice score <100 by ELISA and <200 for RM, and both species score <10 by NT₅₀. Assay-specific LOD thresholds were established based on these naïve testing results and are shown as dashed lines, with arrows indicating vaccination time points. For each mouse challenge study (panels **A**, **B** and **D**) comparisons were made to naïve animals using the Mantel-Cox log rank test, with significant differences indicated by asterisks (* $P < 0.05$; ** $P < 0.01$). Challenge studies in panel **A** and **B** were carried out concurrently and utilized the same naïve control group.