Evaluating the immunogenicity of an intranasal vaccine against nicotine in mice using the Adjuvant Finlay Proteoliposome (AFPL1)

Nya L. Fraleigha, Justin Boudreaub, Nitin Bhardwaja, Nelson F. Enga, Yanal Murada, Robert Lafreniec,a,b, Reinaldo Acevedod, Reynaldo Olivad, Francisco Diaz-Mitomab,c, Hoang-Thanh Lea,c,*

a Health Sciences North Research Institute, 41 Ramsey Lake Road, Sudbury, ON P3E 5J1, Canada
b Laurentian University, 935 Ramsey Lake Road, Sudbury, ON P3E 2C6, Canada
c Northern Ontario School of Medicine, 935 Ramsey Lake Road, Sudbury, ON P3E 2C6, Canada
d Finlay Vaccine Institute, Ave. 27 No. 19805, La Habana, AP 16017, Cuba

* Corresponding author.
E-mail address: hle@hsnri.ca (H.-T. Le).

Abstract

Tobacco smoking is recognized as a global pandemic resulting in 6 million deaths per year. Despite a variety of anti-smoking products available to aid with tobacco cessation, the majority of people who attempt to quit smoking relapse within 6 months due to the addictive nature of nicotine. An immunotherapy approach could offer a promising treatment option by inducing a potent selective antibody response against nicotine in order to block its distribution to the brain and its addictive effects in the central nervous system. Our nicotine vaccine candidate was administered intranasally using the Neisseria meningitidis serogroup B Adjuvant Finlay Proteoliposome 1 (AFPL1) as a part of the delivery system. This system was designed to generate a robust immune response by stimulating IL-1β production through Toll-like receptor 4 (TLR4), a potent mechanism for mucosal immunity.
The vaccine induced high antibody titers in mice sera in addition to inducing mucosal antibodies. The efficacy of our vaccine was demonstrated using in vivo challenge experiments with radioactive [3H]-nicotine, followed by an analysis of nicotine distribution in the lung, liver, blood and brain. Our results were encouraging as the nicotine concentration in the brain tissue of mice vaccinated with our candidate vaccine was four times lower than in non-vaccinated controls; suggesting that the anti-nicotine antibodies were able to block nicotine from crossing the blood brain barrier. In summary, we have developed a novel nicotine vaccine for the treatment of tobacco addiction by intranasal administration and also demonstrated that the AFPL1 can be used as a potential adjuvant for this vaccine design.

Keyword: Immunology

1. Introduction

Tobacco smoking continues to be a worldwide epidemic with over 1 billion people who currently smoke and an estimated 6 million deaths per year [1]. Each year in Canada alone, more than 45,000 people die because of tobacco related diseases [2], with an additional annual economic burden of over $17 billion [3]. Smoking cessation remains a challenge due to nicotine, the addictive substance present in tobacco. Nicotine replacement products currently available to assist with quitting smoking are only partially successful, and pharmacotherapeutics pose the risk of serious side effects [4]. Attempts to develop a therapeutic vaccine that can generate antibodies capable of sequestering nicotine prior to crossing the blood-brain barrier and reaching the brain have been promising, with three reaching various stages of clinical trials [4, 5]. Despite promising results in preclinical and clinical trials, no nicotine vaccine has been commercially licensed. It is believed that a vaccine capable of generating antibodies with higher affinity and titers against nicotine could help achieve higher abstinence rates and facilitate smoking cessation [4, 6, 7, 8, 9, 10].

In order to induce systemic anti-nicotine antibodies to sequester nicotine from circulation, current vaccine formulations place emphasis on hapten molecule design, adjuvant(s)/carrier systems and the number of haptns per carrier molecule [7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21]. A drawback of parenteral nicotine vaccines could be that they are targeting nicotine once in the circulatory system, which might not be sufficient or fast enough to neutralize nicotine and prevent it from reaching the brain; this occurs within 7–10 seconds of cigarette smoke inhalation [22]. We believe that a nicotine vaccine will be most effective when antibodies are available to block nicotine at two levels: A) in mucus secretions of the respiratory tract to block nicotine absorption into the blood, and
B) in the blood, so that if nicotine is absorbed, it will be neutralized and sequestered in the blood before it reaches the brain.

In this study, we developed a stable adjuvant delivery system using particle assembly strategies for use as an intranasal nicotine vaccine. Intranasal vaccines have become an attractive alternative to needle-based vaccines due to their ability to stimulate both mucosal and systemic immune responses [23], while also preventing the spread of disease and having greater patient compliance [24]. The adjuvant particle was composed of AFPL1, a natural proteoliposome, which was detergent-extracted from the outer membrane vesicle of live *N. meningitidis* serogroup B (Finlay Vaccine Institute, Cuba) chemically linked to peptides, and nicotine. Previously, AFPL1 has been shown to be efficient at upregulating co-stimulatory molecules [25, 26, 27], proinflammatory cytokines and other cytokines involved in adaptive immune responses [27]. AFPL1 activation of TLR4 on antigen presenting cells (APCs) is similar to lipopolysaccharide (LPS), leading to a predominantly Th1 response [27, 28]. AFPL1 has been extensively studied and while it is a Th1 adjuvant, it can induce various different IgG isotypes including IgG2a and IgG1 which are associated with Th1 and Th2 responses respectively [29, 30]. In addition, the compound has already been instilled intranasally and intramuscularly [31], resulting in an adjuvant that is able to induce both mucosal and systemic immune responses.

We examined the levels and isotypes of anti-nicotine antibodies produced by our nicotine vaccine *in vivo* and determined the efficacy of anti-nicotine antibodies to neutralize nicotine through [*H]-nicotine challenge experiments. We also evaluated the immunomodulatory potential of our system by examining its effect on IL-1β production *in vitro*. IL-1β is a proinflammatory cytokine that is produced by APCs among others. The adjuvanticity of IL-1β can be explained by its ability to increase the expression of a variety of different cytokines [32]; enhance the antigen presenting capabilities of APCs [33], influence T cells [34, 35, 36] and humoral immune responses [35, 36].

2. **Materials and methods**

2.1. **Vaccine formulation**

The vaccine was prepared by the conjugation of 3′-aminomethylnicotine with the AFPL1 component in the presence of peptides [37]. The peptide structure and the proportions of nicotine hapten, short peptide and adjuvant in addition to the coupling technique are undisclosed as they are being submitted for patent review. AFPL1 from *N. meningitidis* serogroup B (meningococcal strain Cu385 (B4: P1.19,15)) was manufactured under Good Manufacturing Practice conditions and provided by the manufacturing plant of Finlay Vaccine Institute, Cuba. Nicotine was also quantified by using UV adsorption of derived nicotine as a standard at
265 nm on UV-3600 (Shimadzu) and by TLC on Silica gel 60 F254, EM Science with Dragendorff reagent (Sigma, Mississauga, ON) for detection. Particle size as a parameter for stability analysis was analyzed on Malvern Zetasizer ZS. The nicotine vaccine was stored at room temperature for one month and then stored at 4 °C before in vitro testing. We performed in vivo tests using only the vaccine stored at 4 °C.

2.2. Mice, immunizations and sample collection

Female BALB/c mice were purchased from Charles River (QC, Canada) at 6–8 weeks of age. Mice were housed at the Laurentian University Animal Care Facility and supplied food and water ad libitum. All experimental protocols were approved by the Biosafety committee of HSNRI and the Animal Care Committee at Laurentian University. Briefly, mice were anaesthetized using a ketamine/xylazine (Wyeth Canada, Guelph, ON/LLOYD Inc, Shenandoah, IA) cocktail and the vaccine was administered drop-wise intranasally at a dose of 10 μg/20 μL, with the concentration based on the amount of nicotine present, or 20 μL of PBS once every three weeks for a total of four vaccinations. Blood was collected retroorbitally in microtainer serum separator tubes (BD, Franklin Lakes, NJ) two weeks after each vaccination. The blood was centrifuged at 10,000 rpm for 5 min and sera was stored at −20 °C.

2.3. Fecal collection and processing

Fecal samples collected approximately 4 weeks after the final vaccination were processed as described previously [38]. The supernatants were stored at −80 °C and later analyzed by ELISA for nicotine-specific IgA.

2.4. Cell line and in vitro cell culture treatments

JAWS II cells are a bone marrow derived dendritic cell line from p53 −/- C57BL/6 mice. Cells were grown in RPMI 1640 (HyClone, Logan, UT) supplemented with 8% FBS (Gibco, Grand Island NY), 1% penicillin/streptomycin (HyClone, Logan UT) and 5 ng/mL GM-CSF (Invitrogen, Fredrick, MD) in the presence of 5% CO2 at 37 °C. Cells were grown to confluency, harvested and then seeded on new 12 well culture substrates at a concentration of 10^6 cells/mL for 24 or 48 hours and left untreated or treated with either, LPS from E. coli 0111:B4 (Sigma, St. Louis, MO), vaccine components (AFPL1, peptide and stabilizing compound) or the vaccine.

2.5. Splenocyte isolation

Spleens were isolated from naïve mice, pooled, and placed in cold Hanks’ balanced salt solution (HBSS) (Sigma, St Louis, MO). Whole spleens were minced between the ends of two frosted slides until a cell suspension was made. The cell suspension
was filtered through a 0.7 μm filter and the residual cells were washed with cold HBSS and re-filtered through a 0.7 μm filter. The cells were centrifuged at 4 °C for 10 min at 1500 rpm. The supernatant was removed and the remaining pellet was resuspended in ACK lysis buffer (Lonza, Walkersville, MD) to lyse the erythrocytes. Cold HBSS was added to the ACK lysis buffer suspension after 1 min and the cells were centrifuged again. The pellet was resuspended in RPMI 1640 supplemented with 8% FBS and 1% penicillin/streptomycin. Cells were seeded at a density of 10^6 cells per 200 μL of media in a 96 well microplate and were then left untreated or treated for 24 hours with LPS, a vaccine component or the complete vaccine. Supernatants were collected and the levels of IL-1β were analyzed by ELISA.

2.6. TLR4 inhibition

JAWS II cells were seeded in 12 well tissue-culture plates, in triplicate, at a density of 10^6 cells/mL and pretreated with 2 μg/mL CLI-095 (InvivoGen, San Diego, CA) for 6 hours. The cells were then left untreated or treated with LPS, a vaccine component or the vaccine for another 24 hours. Supernatants were collected and stored at −20 °C for future analysis.

2.7. IL-1β ELISA

Supernatants from treated splenocytes and JAWS II cultures were analyzed for levels of IL-1β using IL-1β ELISAs (eBioscience, San Diego, CA). The assay was performed as per the manufacturer’s instructions and the limit of detection was 7.8 pg/mL.

2.8. Anti-nicotine ELISAs

Sera collected from mice were analyzed using a homemade nicotine-specific ELISA. Costar® 96-well microtiter plates were coated with a poly-lysine nicotine conjugate in carbonate buffer [0.015 M Na₂CO₃, NaHCO₃ 0.035 M, pH 9.6] overnight at 4 °C. The sera samples were diluted in Tris buffered saline, pH 7.4 (TBS)-0.05% Tween 20 (Sigma, St Louis, MO) and probed with biotinylated goat anti-mouse IgG (Invitrogen, Frederick MD), IgG1 (Invitrogen, Eugene OR), IgG2a or IgA (Invitrogen, Frederick, MD) antibodies according to manufacturers’ specifications. Following incubation with streptavidin alkaline phosphatase, samples were washed and incubated with para-nitrophenylphosphate (pNPP) (Sigma, St. Louis, MO) in substrate buffer [diethanolamine (Sigma, St. Louis, MO), 500 mM MgCl₂, pH 9.8] and left to incubate in the dark for 30–60 mins. The reaction was stopped with the addition of 0.3 M EDTA (Fisher, Frederick, MD) and the optical density was measured at 405 nm with a correction at 490 nm using the Synergy H4 Multi-mode Hybrid Microplate Reader (Biotek). Unless otherwise
stated all incubations were performed for one hour at room temperature and
washing was performed with the ELx405 Select Deep Well Microplate Washer
(Biotek). End point titrations were performed with sera derived from the
vaccinated mice, using the PBS immunized mouse sera as a baseline control to
measure fold induction.

2.9. $[^3]$H-nicotine challenge

Specifics of the procedure have been slightly modified from a previous reference
[16]. Unvaccinated and vaccinated mice were anaesthetized with a ketamine/
xylazine cocktail and challenged 6 weeks after the last vaccination with 3 μCi $[^3]$H-
nicotine/mouse (925 kBq, Perkin Elmer) in 100 μL via a cardiac injection. Two
minutes after challenge the mice were sacrificed and blood, brain, lung and liver
were collected and disaggregated using scissors and a motorized pestle
homogenizer and equal volumes of scintillation fluid were added to each organ.
Levels of $[^3]$H were measured with a scintillation counter (Beckman LS6000IC).

2.10. Statistical analysis

Statistical analyses were performed using Graph Pad Prism 5.

3. Results and discussion

3.1. AFPL1 and the vaccine are able to induce IL-1β production

In order to determine whether the adjuvant delivery system was an effective
stimulant of IL-1β secretion, cultures of JAWS II dendritic cells were treated with
suspended media, LPS, the different adjuvant delivery components or the complete
vaccine. It was evident that the stabilizing compound and the linking peptide were
unable to induce levels of IL-1β above basal levels. Alternatively, the AFPL1 from
N. menigitiditis was able to induce a significant amount of IL-1β after 48 hours.
The nicotine vaccine was able to stimulate levels of IL-1β after both 24 and 48
hours (Fig. 1).

Splenocytes were isolated from naïve mice and treated with components of the
nicotine vaccine or with the complete vaccine, and the culture supernatants were
analyzed by ELISA after 24 hours. Treatment with LPS was able to stimulate
significant levels of IL-1β secretion from the naïve splenocytes (Fig. 2). Both the
AFPL1 and the nicotine vaccine were able to increase levels of IL-1β, while the
vaccine induced a significantly higher level of IL-1β compared to AFPL1 (Fig. 2).

AFPL1 has been previously tested as an adjuvant [39] and has been shown to
induce dendritic cell activation, antigen presentation, TNF-α and IL-12(p70)
production [27, 28]. LPS is a component of the bacterial outer membrane, a potent
danger signal and inducer of innate immune responses. We chose to use LPS as a
Fig. 1. Levels of IL-1β produced by JAWS II after 24 (A) and 48 (B) hours. JAWS II, immortalized bone-marrow derived dendritic cells, were seeded at a concentration of 10^6 cells/mL/well in a 12 well plate and left untreated or treated with either 1 μg/mL LPS from *E. coli* 0111:B4, vaccine components (AFPL1: 1 μg/mL, peptide and stabilizing compound: 10 μg/mL) or the vaccine (1 μg/mL based on AFPL1). Supernatants were collected from the cells after 24 or 48 hours of treatment. N = 3, ±SEM, but representative of multiple experiments and statistical significance was determined by an ANOVA with a Tukey HSD. %%% p < 0.001, %% p < 0.01, % p < 0.05 as compared to the no treatment group; ### p < 0.001 and # p < 0.05 as compared to the stabilizing compound; &&& p < 0.001, && p < 0.01 and & p < 0.05 as compared to peptide 1; *** p < 0.001, ** p < 0.01 and * p < 0.05 as compared to AFPL1 and aaa, $$$ p < 0.001 as compared to the vaccine.

Fig. 2. Levels of IL-1β from naïve splenocyte cultures after 24 hours. Spleens were isolated from 3 naïve mice, pooled and seeded at a density of 10^6 cells per well, and stimulated for 24 hours either alone or with 1 μg/mL LPS from *E. coli* 0111:B4, a vaccine component (AFPL1: 1 μg/mL, peptide and stabilizing compound: 10 μg/mL) or the complete vaccine (1 μg/mL based on AFPL1). After 24 hours the supernatants were analyzed by ELISA. Splenocytes were plated in triplicate and error bars are represented as ±SEM and ###, $$$, %%%,*** and aaa p < 0.001 as determined by ANOVA with a Tukey HSD compared to the no treatment, stabilizing compound, peptide 1 and AFPL1 and vaccine respectively.
control for our in vitro experiments as LPS is known to induce proinflammatory cytokines and AFPL1 are essentially bacterial membranes. It is important to note that the doses used for the AFPL1 and LPS are not comparable as the AFPL1 is a heterogeneous mixture of components including small amounts of LPS [28]. We were interested in using IL-1β to test the immunogenicity of the vaccine as it is a potent adjuvant for mucosal immune responses. AFPL1 alone was able to stimulate a moderate IL-1β response from the JAWS II cells; however, it was the conjugate nicotine vaccine that produced the highest levels of IL-1β from the stimulated JAWS II after 24 and 48 hours (Fig. 1). This trend between the AFPL1 and the vaccine was also observed in the ex vivo splenocyte cultures as the vaccine was able to induce significantly higher levels of IL-1β compared to the AFPL1 alone (Fig. 2). Since the vaccine contained nicotine, we also measured the level of IL-1β when JAWS II cells were treated with nicotine, which remained at basal levels (data not shown).

3.2. IL-1β from JAWS II relies on TLR4

We investigated whether the immune response generated by treatment with AFPL1 or the vaccine was dependent on TLR4. JAWS II cells were pretreated for 6 hours with CLI-095, a TLR4 inhibitor [40] that blocks downstream signaling. After 6 hours the JAWS II cells were treated for an additional 24 hours and the supernatants were analyzed for levels of IL-1β. LPS is a well characterized agonist for the TLR4 receptor and was used as the control to ensure that the receptor was inhibited. The levels of IL-1β were significantly decreased by pre-treatment with CLI-095, although not to basal levels. The levels of IL-1β produced after treatment with AFPL1 or the complete vaccine were significantly impaired in the presence of the inhibitor (Fig. 3).

The levels of IL-1β appear to be dependent on TLR4 activation since in the presence of the inhibitor there was significant inhibition (Fig. 3). CLI-095, in the presence of AFPL1 or the vaccine, was able to almost completely abrogate IL-1β measured in the supernatant. LPS-induced IL-1β was not completely inhibited which may be due to the fact that we used a higher level of LPS than suggested by InvivoGen (10 ng/mL) (Fig. 3) resulting in saturation of the receptors and causing breakthrough.

3.3. Antibody responses after vaccination protocol

Mice were vaccinated four times over a period of 12 weeks. Two weeks after each vaccination, blood was collected and sera were analyzed for anti-nicotine IgG by ELISA. There was a gradual increase over the vaccination period with a significant increase in anti-nicotine IgG that peaked by day 56 (Fig. 4A). Total anti-nicotine IgG titers further indicate increasing levels of IgG, which peaked at day 56.
We also investigated levels of anti-nicotine IgG1 and IgG2a from the sera of vaccinated mice. Levels of anti-nicotine IgG1 and IgG2a increased, but were not significantly different (Fig. 4C). Since the intranasal vaccination protocol would induce both systemic and mucosal antibody responses, we evaluated levels of anti-nicotine IgA collected from fresh fecal samples. The level of nicotine-specific IgA present in the fecal supernatant of the vaccinated mice was significantly higher than the PBS control group (Fig. 4D).

Despite appearing reliant on TLR4 in vitro, the vaccine was able to induce both IgG1 and IgG2a in vivo, suggesting a potentially balanced Th1/Th2 response (Fig. 4C). In general, adjuvants comprising TLR4 ligands yield Th1 polarized responses [41]. This balance is desired for protective immunity against bacterial infections; however, for nicotine vaccine development it is critical to have neutralizing antibodies—including IgA and IgG. Previous investigations have demonstrated that the AFPL1 is able to induce a predominantly Th1 response [26, 27, 28, 39]. The ability of the intranasal nicotine vaccine to potentially induce both Th1 and Th2 responses as seen through IgG2a and IgG1 is similar to previous responses induced by AFPL1 when used as a mucosal adjuvant [29, 30]. The vaccine was administered intranasally in order to induce both a mucosal and systemic immune response in vivo. We measured significant anti-nicotine IgA from fecal samples and significant anti-nicotine IgG from the sera of mice vaccinated 4 times at 3 week intervals. Fecal IgA levels are generally higher with

![Graph](http://dx.doi.org/10.1016/j.heliyon.2016.e00147)

**Fig. 3.** Effect of TLR4 inhibition on the ability of JAWS II to produce IL-1β after 24 hours. JAWS II, immortalized bone-marrow derived dendritic cells, were seeded in triplicate at a concentration of 10⁶ cells/mL/well in a 12 well plate and treated with or without CLI-095 (TLR4 inhibitor) for 6 hours. After 6 hours, the cells were left untreated or treated with either 1 μg/mL LPS from *E. coli* 0111:B4 vaccine components (AFPL1: 1 μg/mL, peptide and stabilizing compound: 10 μg/mL) or the vaccine (1 μg/mL based on AFPL1) 24 hours at 37 °C, 5% CO₂. Data are represented as ±SEM, and are representative of several experiments. An unpaired t test was performed and ** p = 0.0014. Unpaired t tests were performed with a Welch’s correction and ## p = 0.0061, $$ p = 0.0025.
oral vaccines as the mucosal response from the mucosae of the stomach would be shed in the feces. However, the entire mucosal immune system is connected [42] and responses generated in the lung will yield distant antigen-specific IgA [43], including fecal IgA. Together the data suggest that the small molecule complex was able to enhance the immunogenicity of nicotine, which is otherwise inert as an antigen, resulting in the production of both anti-nicotine IgG and IgA (Fig. 4).

3.4. Neutralizing of nicotine during challenge

In order to determine whether the antibodies generated by the intranasal nicotine vaccine were effective at blocking nicotine from crossing the blood-brain barrier we challenged the mice with [3H]-nicotine. Prior to the [3H]-nicotine challenge, a competitive ELISA was performed with cotinine to determine that there was no competition between nicotine and a predominant metabolite like cotinine (data not shown). Mice that did not receive a nicotine vaccine and were challenged with nicotine had the highest recovery of nicotine from the brain with similar amounts recovered from the blood, heart, lungs and liver. Mice that were vaccinated prior to nicotine challenge showed a significant decrease in brain nicotine recovery compared to the control group. The data suggest that the antibodies generated by the vaccine are capable of neutralizing nicotine in the brain, thereby preventing its absorption into the bloodstream.
with the nicotine vaccine showed much lower levels of nicotine in the brain and nicotine remained predominantly in the lungs and the blood (Fig. 5).

Although the production of antibodies is a necessary response after vaccination, it is crucial that antibodies be functional and effective. We used $[^3]$H-nicotine to challenge the mice and determine whether anti-nicotine antibodies were capable of sequestering nicotine prior to crossing the blood brain barrier. Vaccinated mice challenged by injection with $[^3]$H-nicotine had significantly less $[^3]$H-nicotine in the brain compared to the non-vaccinated group (Fig. 5). There were also higher levels of $[^3]$H-nicotine remaining in the lung. While the difference was not significant, it is never the less a vital piece of evidence that demonstrates the functionality of the vaccine in vivo. In order for a nicotine vaccine to be a suitable smoking cessation therapeutic it must be able to block the addictive substance from releasing dopamine and adrenaline, which drives the addiction [4].

4. Conclusion

In this work we have demonstrated a novel approach to nicotine addiction therapeutics by developing an intranasal nicotine vaccine that contains AFPL1. The vaccine complex is able to induce significant levels of IL-1\(\beta\) in vitro that was dependent on TLR4, which may make it an attractive mucosal adjuvant. The vaccine was able to induce neutralizing anti-nicotine antibodies that are able to limit nicotine from crossing the blood brain barrier. Furthermore, we have for the first time reported the potential of AFPL1 to improve the efficacy of an intranasal nicotine vaccine. Future studies will focus on testing the toxicity and efficacy of this vaccine in vivo. The efficacy of the vaccine will be tested in an animal model for nicotine self-administration and seeking [44].

![Fig. 5. $[^3]$H-nicotine challenge. Female BALB/c mice were given intranasal instillations of PBS or the nicotine vaccine once every three weeks. Three $\mu$ Curie of $[^3]$H-nicotine was instilled via cardiac injection, after 2 min the mice were sacrificed and levels of beta radioisotopes were measured in each organ. Data are represented by ±SEM with groups of n = 3–5. * p = 0.0357 as determined by a Mann-Whitney U Test.](image)
Declarations

Author contribution statement

Nya L. Fraleigh, Justin Boudreau: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nitin Bhardwaj, Nelson F. Eng, Yanal Murad: Performed the experiments.

Robert Lafrenie: Performed the experiments; Analyzed and interpreted the data.

Reinaldo Acevedo: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Reynaldo Oliva: Contributed reagents, materials, analysis tools or data.

Francisco Diaz-Mitoma: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Hoang-Thanh Le: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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