Evaluation of the *in vitro* and *in vivo* anti plasmodial effect of water treated with PMM (Photonic Multiphase Modulators) designed with Advanced Physics System Engineering (APSE™ and BioPhoton-X™)

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ABSTRACT

Background

In vitro and *in vivo* testing of new technology was performed to evaluate the antiplasmodial activity of PMM (Photonic Multiphase Modulators) in cultures and in mice previously infected with *Plasmodium falciparum* and *Plasmodium berghei* parasites

Methods

Cultures of *P. falciparum* infected erythrocytes were exposed overnight to two generations of different APSE™ and BioPhoton-X™ (Photonic Multiphase Modulators) (C#1, R#1, R#2, D8, D9 and AGN10). Growth of the parasites was determined through flow cytometry or microscopy. Mouse of the strain C57BL/6 were infected and treated with water exposed to a second generation of APSE™ and BioPhoton-X™ PMM's and the parasitemia and weight loss was monitored throughout the infection until deceased or point of euthanasia was reached. After death, necropsy was performed in all animals and their survival computed.

Results

In vitro and *in vivo* testing using different APSE™ and BioPhoton-X™ and designed PMM (Photonic Multiphase Modulators) revealed an effect of D8 on lowering the growth of the parasite *in vitro* while the best effect in mice was observed with D9 PMM's, with a reduced weight loss and an increase in survival.

Conclusions

APSE™ and BioPhoton-X™, biophotonic technology can affect the growth of parasites and shows protective effects on mice drinking from water treated with its PMM's.

INTRODUCTION

Currently, the first-line treatment used to solve health problems of populations suffering from malaria is a therapy based on a combination of medicines that include artemisinin-based drugs (WHO, 2015). However, resistance to artemisinin and its associated derivative medicines threaten the management and control of malaria**.** The perversity and resilience of this parasite and its prevalence in the developing countries require innovative approaches to produce affordable treatments and safeguard those available. With this in mind, we explored a new tool with the aim of finding one such solution.

The use of light to tackle health issues can be best exemplified by photodynamic therapy (PDT) which is a form of non-invasive intervention currently used in pathologies that range from infectious diseases to various types of cancer (Huang, 2005).

PDT has been used successfully in dermatology, oncology, gynecology, urology studies (Kwiatkowski et al., 2018), and in cutaneous leishmaniasis (Aureliano et al., 2015), as well as in the photo inactivation of *S. aureus*, *E. coli*, *C. albicans* and *T. rubrum* (Bornstein et al., 2009).

Some materials are inherently sensitive to light and they might not need a photosensitive material to be affected by photonics (Digre et al, 2012). The Advanced Physics System Engineering APSE™ and BioPhoton-X™ (technology uses portable PMM's that embed photonic arrangements which, when activated by visible light, emit electromagnetic signals configured to deliver the targeted results and/or desired use.

Treatment of water using APSE™ and BioPhoton-X™, technology is proposed to cause a change in the surface tension of the body of water and a hydration of microspheres that surround molecules in water, as described by Bhalerao and Pollack in 2010, through an effect in the Brownian displacement of the molecules (Bhalerao and Pollack, 2010). The energy given off can inhibit reproductive cycles of several microorganisms (Victor De Franco Levi, unpublished communication) and regulate the pH levels and oxidation / reduction of water surface tension and other water physicochemical parameters. Thus, the APSE™ and BioPhoton-X™ PMM's, with its emission of low intensity (below infra-red) levels of electromagnetism, can influence the functioning of biological systems.

Malaria is a disease that affects 40% of the population living in endemic regions where 228 million new cases and 435,000 related deaths were reported in 2017 (World Malaria Report, 2018). Drugs commonly used to treat the disease are toxic and parasites have shown constant resistance to their effect. Studies carried out by Vanderesse et al. (2016), propose two strategies to inactivate blood with malaria in the blood, which are photodynamic therapy and inhibition of hemozoin formation, both in the mosquito.

We decided to test the APSE™ and BioPhoton-X™ technology in our laboratory as for its possible antimalarial action, starting with *in vitro* tests which, in lieu of the results obtained, moved us to continue to the *in vivo* phase. Here we report the results of those tests.

The objective of this study was to evaluate the effect of the PMM's directly on *Plasmodium falciparum* cultures or on water that had been treated with the APSE™ and BioPhoton-X™ PMM's and then given to *C57BL/6* mice infected with *Plasmodium berghei.*

MATERIALS AND METHODS

All reagents were from Sigma-Aldrich (St. Louis,MO, USA), unless otherwise specified.
APSE™ and BioPhoton-X™ Photonic Multiphase Modulators PMM's are designed by Engeenuitly S.A Financial Tower, 35th floor, Calle 50, Panama City, Panama.

with an electromagnetic base of biological origin that has incorporated components such as nanoparticles, optical fiber, chips, circuits and a photovoltaic substrate (proprietary information, Engeenuity S.A. First generation PMM's for antiplasmodial tests were coded C#1, R#1, R#2 and AGN10. C#1 measured 1 cm (diameter); R#1 and R#2, 1cm^2. AGN10 was a semi-rigid film measuring 4.5" x 3". Second generation PMM's were coded D8 and D9 and they measured 2 cm in diameter each (Fig. 1).

Figure 1. Photonic Multiphase Modulator PMM's. A) First generation PMM's with the APSE™ and BioPhoton-X™ technology, with different characteristics. From left to right: C#1, R#1, R#2 and AGN10. B) Second generation PMM's: left, D8 and right, D9. The sizes shown are relative to each other except for AGN10. For this last one, an inset of R#2 is shown in the lower left corner for size comparison purposes.

Parasite culture

The chloroquine sensitive *P. falciparum* HB3 strain was cultured following the conventional method of Trager & Jensen (Trager, W. and Jensen, J.B. 1997) with modifications described in Almanza et al. (Almanza et al. 2011), that include the use of modified RPMI 1640 medium, 25 mM HEPES, 15 μM hipoxanthine, 50 mg/ml gentamicine sulfate, and 200 mM L-Glutamine, supplemented with 10% human serum, 0.2% sodium bicarbonate and a mix of gases (90% N_2, 5% O_2 and 5% CO_2). Synchronization was performed in a temperature-cycling incubator (TCI) (Sanyo, Model MIR-154) and by the addition of 0.3 M alanine. Uninfected red blood cells (uRBCs) were cultured with the same hematocrit (2%) and media conditions.

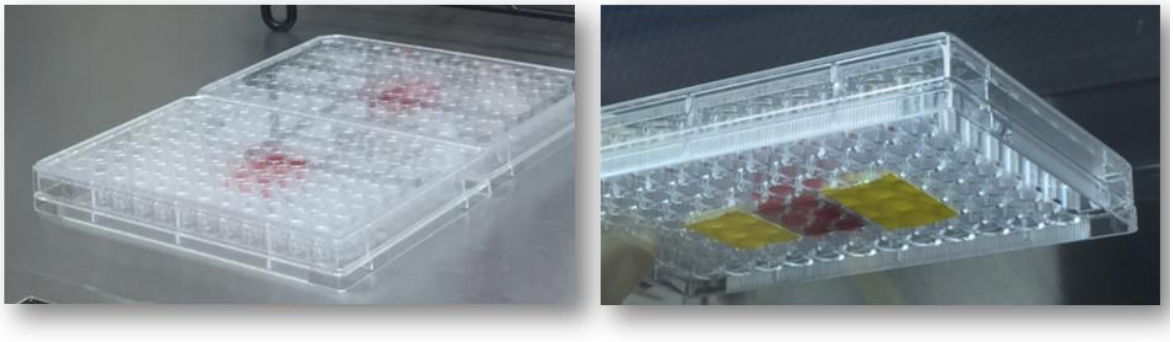

Figure 2. Lay out of the *in vitro* treatment of cultures with PMM. Localization of the wells with samples of *P. falciparum* infected erythrocytes in a 96-well plate. The adherent PMM's were placed underneath the plates, coinciding with the position of the wells in use.

***In vitro* exposure**

200 ul of cultures infected or not with 1% *P. falciparum* parasites in schizont stages were used. APSE™ and BioPhoton-X™, PMM's were adhered to the bottom of the plates as shown in Figure 2. Plates were left in a 37 ^{0}C incubator overnight and the parasitemia was read next day either by microscopy after staining a smear with Giemsa, or by flow cytometry after staining with Hoechst 33342 (Invitrogen, Carlsbad, CA, USA) and reading in a CyFlow Space cytometer (Sysmex Partec, Görlitz, Germany).

***In vivo* exposure**

Mice and parasite strain

Male C57BL/6 animals were kept in rooms with light/darkness daily cycles of 12 hours. The room were kept at a constant temperature of 21 °C and a relative humidity of 65%. Sanitary barriers that included change of clothes by the handling staff, sterilization of input and consumption materials, such as boxes, chips and food were always followed . Water and food were supplied for consumption *at libido*.

Activation of the parasites of the *Plasmodium berghei* ANKA strain, stored in liquid N_2, was done through consecutive mouse-to-mouse passages in animals from 6 to 8 weeks old.

After passages, animals were anesthetized with a ketamine / xylacin anesthetic mixture at a concentration of 105/5 mg / kg of weight. Once the state of surgical anesthesia was verified, the thoracic cavity was opened, and blood was extracted via intracardiac puncture with a 3 mL syringe coupled to a short 21G needle containing 0.02 mL of anticoagulant (citrate phosphate dextrose adenine solution). The blood of all mice used to reproduce the parasites was pooled and 0.1 ml of this were used to inoculate the different groups of mice for the experiments.

Experimental procedure

The protocols used *in vivo* were approved by the IACUC of INDICASAT AIP (CICUA 18-003, 18-006, 19-003 and 19-003R) The experimental design was as follows: Animals were distributed and grouped at random with no more than six (6) animals per cage and were tattooed in order to monitor the variation in weight throughout the extent of the trial. Thirty (30) animals were used for the first

experiment that included the three different PMM's, distributed as follows: healthy mice with the effect of the D8 PMM (n = 4); mice parasitized, treated with D8 PMM (n = 5); healthy mice with D9 PMM (n = 5); mice parasitized, treated with D9 (n = 5); healthy mice, treated with D10 (n = 4); mice parasitized, treated with D10 (n = 5); untreated parasitized mice (n = 4). Water treated with the different PMM's was supplied eight (8) days (experiments 1-3) and 16 days (experiment 4) prior to the infection of the animals. The supply of treated water was continued after the infestation of the animals and until the end of the test, as determined by the death of the last animal of the parasitized group without treatment.

Photonic PMM's were adhered to the bottles containing the water supply to the cages as seen in Figure 3. They were exposed to the ambient light of the animal's room during the light cycles.

Figure 3. Lay out for *in vivo* exposure. PMM's were adhered to the feeding bottle (pointed with the red arrow) that supplied water to the mice in the cages. The photo is taken from above the cage.

Parasitemia was measured every 3 days after infection, placing three drops of blood from the tail of the mice in a 125 µL tube containing 25 µL of anticoagulant (citrate phosphate dextrose adenine). A flow cytometer and/or a microscope were used to calculate the parasitemia, as described elsewhere. Parasitemia was followed up to eight days after infection.

Mice were weighed weekly on the same day of the week and around the same time of the day on a portable electronic balance of 0.1 g sensitivity (Scout II, OHAUS Corp, Darmstadt, Germany).

Mouse behavior was monitored and focused particularly in parameters like displacement patterns around the cage, considering as normal a movement around it. Still positions, placement in a corner

or piloerection were considered as expressions of severe health problems and indicated the need to apply the Endpoint Protocol.
The survival of the animals was calculated as the percentage of living animals in each group. This number considered death equally, regardless of whether it was spontaneous or applied through the endpoint protocol.
When the surviving animals required euthanasia (endpoint protocol), they were sacrificed using excess anesthesia. Necropsies were performed to evaluate macroscopically the state of major organs.

RESULTS

To test the antiplasmodial activity of the PMM's, the first generation of PMM were first used to expose cultures of *P. falciparum*-infected human erythrocytes. Figure 4 shows the results obtained with C#1, R#1 and R#2. Two of them slightly helped the parasites grow more and one of them slightly inhibited their growth, but no experiment showed significant differences (Fig. 4). Second generation PMM, improved in their design, were also tested *in vitro.* D8 and D9 produced opposite results, with D8 helping the growth and D9 showing a tendency to inhibit it. Upon this result, another assay was run superimposing two of the PMM, D8 or D9, under the culture plate. This lay out showed a significant reduction of growth for D8 and a tendency to help the growth for D9 (Fig. 5).

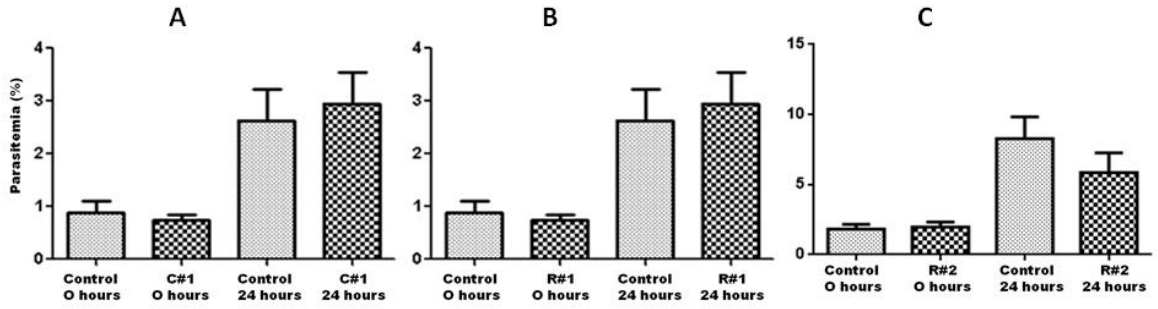

Figure 4. Antiplasmodial activity of first generation PMM. The PMM's were used to expose *P. falciparum* infected cultures in 96-well plates. They were exposed for 24 h and compared to untreated controls. Depicted are representative experiments, run in duplicates, of A) Film C#1, n=5; B) Film R#1, n=4 and C) Film R#2, n=4

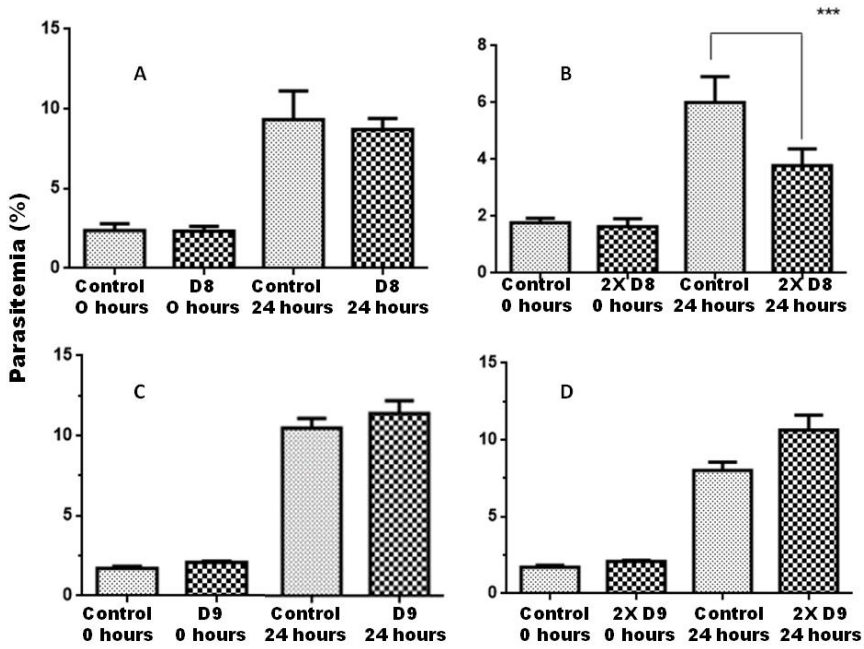

Figure 5. Antiplasmodial activity of second generation PMM's. Improved PMM's were used to expose *P. falciparum* infected cultures in 96-well plates. They were placed under 96-well plates in one or two layers. Cultures were exposed for 24 h and compared to untreated controls. Depicted are representative results of three experiments, run in duplicates, of A) PMM D8; B) Two layers of PMM's D8, C) PMM D9 and D) Two layers of PMM's D9.

With the *in vitro* results, and aware of the leap between culture and animal experimentations, both second PMM generation and one first generation, AGN10, were tested *in vivo.* In the first experiment with mice, all three PMM's were tested in parallel. Figure 6 shows that treatment with D8 and AGN10 increased the level of parasitemia in comparison with the untreated control, while D9 decreased them. D8 treatment showed an initial slowdown of the growth but at the end it surpassed that of the untreated controls.

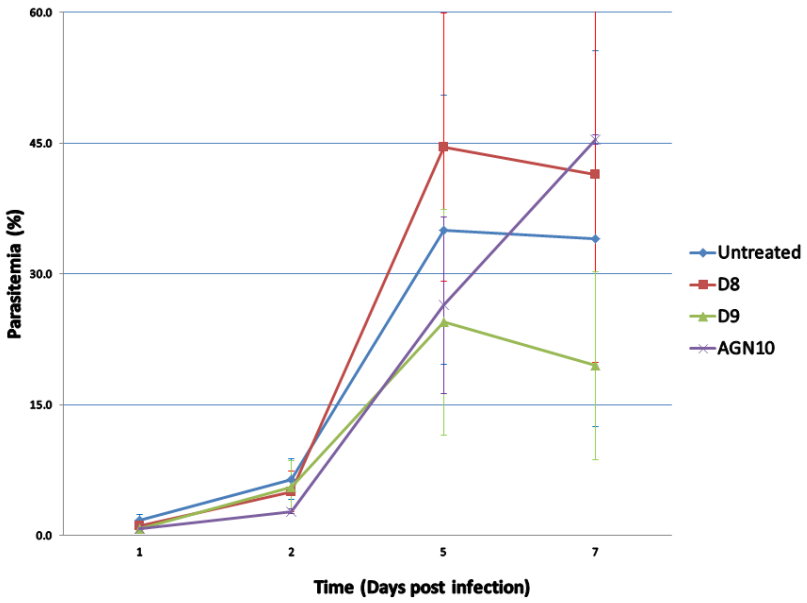

Figure 6. **Development of infection after treatment with PMM's.** Parasitemia was monitored at days 1, 2, 5 and 7 post infection of mice with malaria parasites. The parasitemia of the animals in each group was averaged for each one of the points and the results shown in the graph. The growth of the parasites shown here corresponds to those days when all mice were still alive in all groups.
The mean survival of the groups treated with different PMM's was also analyzed. D9 showed to present the best effect in terms of extending the life of mice as is shown in Figure 7.

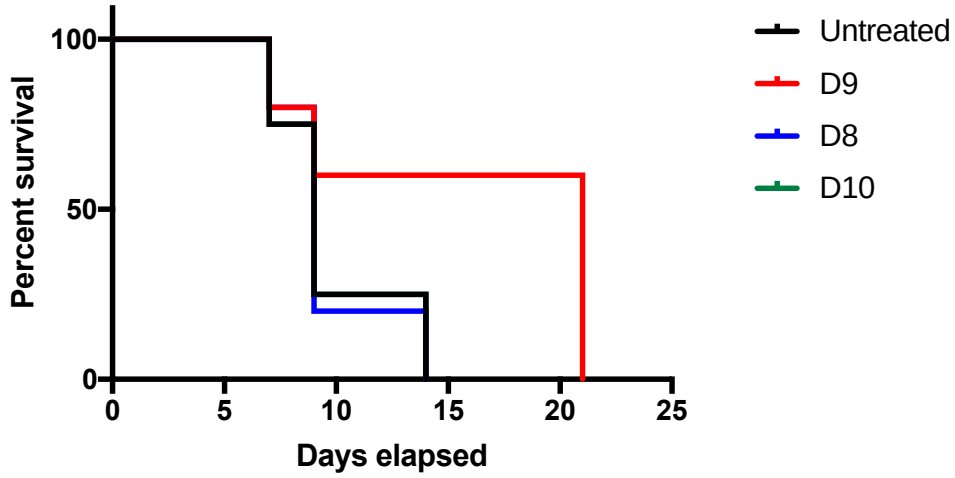

Figure 7. Survival of mice treated with different PMM's. Mice infected with *P. berghei* were treated with water exposed to different APSE™ and BioPhoton-X™ PMM's. The percentage of mice alive in each group was recorded.

After analyzing the results of all PMM's tested in mice, all subsequent experiments were continued with D9. Four experiments were carried out in total and the influence of this PMM in the parasitemia of mice on each of four experiments can be observed in Figure 8. The growth seen in experiment differs between treated and untreated during the first days, but as the days pass the treated parasites achieve the levels of the untreated ones.

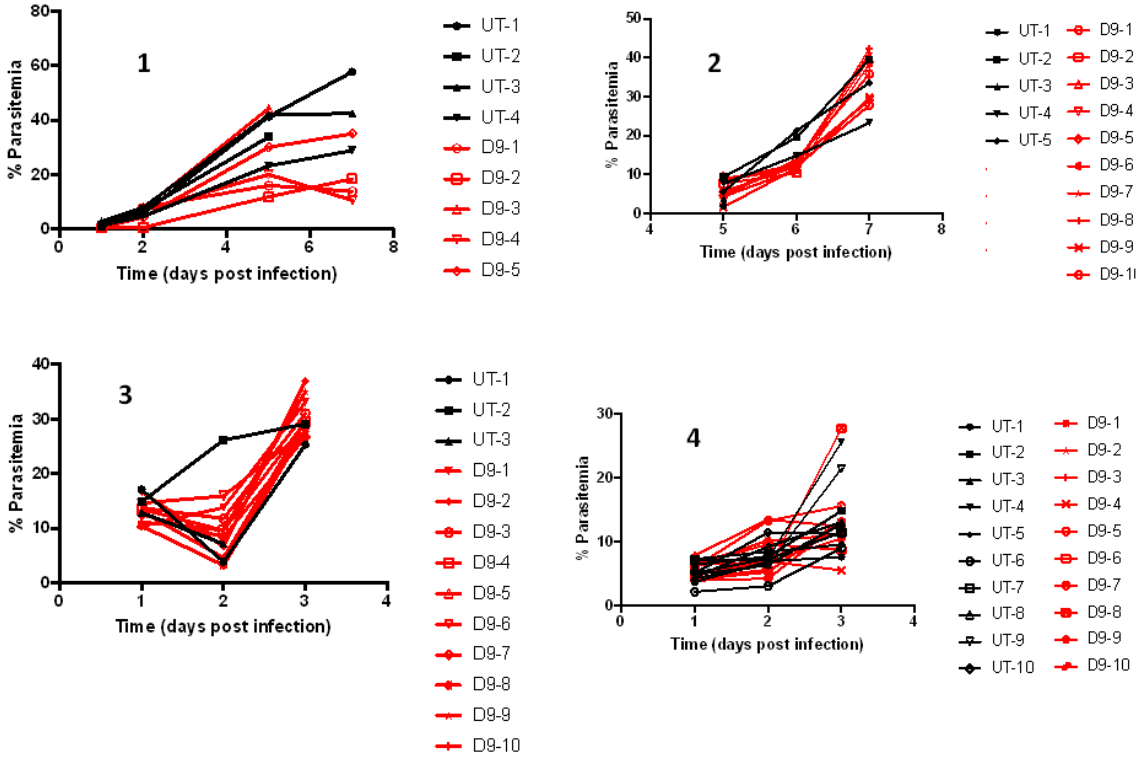

Figure 8. Effect of treatment on growing curve. Four different experiments were carried out to follow the growth of the parasites upon treatment with D9. Untreated infected mice were used as control. The following number of mice were used in each group: 1. Untreated, n=4; treated, n=10; 2. Untreated, n=5; treated, n=10; 3. Untreated, n=3; treated, n=9; 4. Untreated, n=10; treated, n=10.

When using growth rate to offset probable differences in initial parasitemia, the numbers look very similar for all groups. However, the percentage of dead mice by the day the endpoint protocol was applied, varied between the groups, with increased mortalities in the untreated groups.

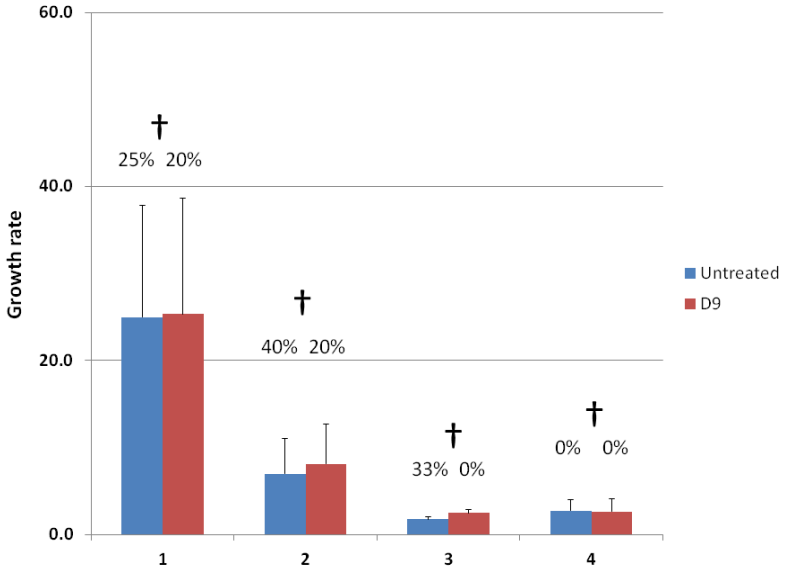

Figure 9. Growth of parasites in mice treated with PMM D9. Four experiments were carried out infecting mice with *P. berghei*. A comparison between the parasitemias achieved on the last day of the experiments between those surviving animals treated with D9 vs those left untreated is shown. The following number of mice were used in each group: 1. Untreated, n=4; treated, n=10; 2. Untreated, n=5; treated, n=10; 3. Untreated, n=3; treated, n=9; 4. Untreated, n=10; treated, n=10. † indicate the percentage of dead animals on the day that was used as cut off to measure growth rate.

In relationship to body weight, in all the experiments the body mass was measured from the day that the treated water started being supplied to the animals, to the last day that all mice were still alive in both untreated and D9 treated parasitized groups. The difference between those two days is presented in Fig 9. The media of the weight of all mice in each group was used in the calculations. Only in experiment 3 the measurement was taken when one mouse from the untreated group had already died, due to the lack of data from another date closer to the onset of the severity of the disease.

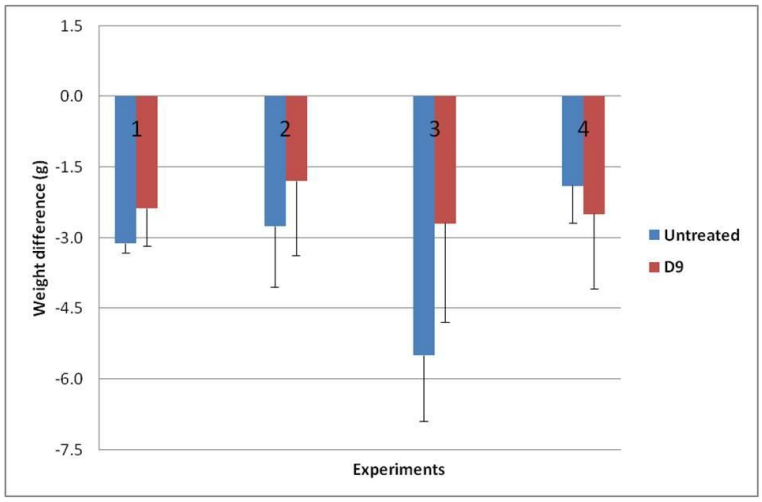

Figure 9. **Change in weight in parasitized mice**. The loss of weight due to the malaria infection was calculated subtracting the average weight of all mice in each group on the day they were infected from that of the last day all mice in each group were still alive. For the different experiments the span of days was the following: **1**: 8 days; **2**: 5 days; **3**: 7 days; **4**: 15 days.

Regarding the general wellness of the animals as evaluated through their behavior, parasitized animals decreased their movement around the cages compared to the healthy, uninfected group, as expected. However, it was the untreated animals which first lost their normal behavior among all the parasitized ones. In the first experiment, when three PMMs were used, those that were drinking water with the effects of AGN10 and D8 PMM's presented piloerection, indicating health problems, and the endpoint protocol had to be applied to them on day 9 after infection. In the rest of the experiments, D9 treatment consistently proved to favor the survival of the animals, despite no individual experiment showing significance on its own, according to a Kaplan Meier analysis of each graph data (Fig. 10). D9 treated animals (consuming water under the effect of the PMMs) which showed no clinical signs of deterioration were allowed to live until endpoint protocol was necessary.

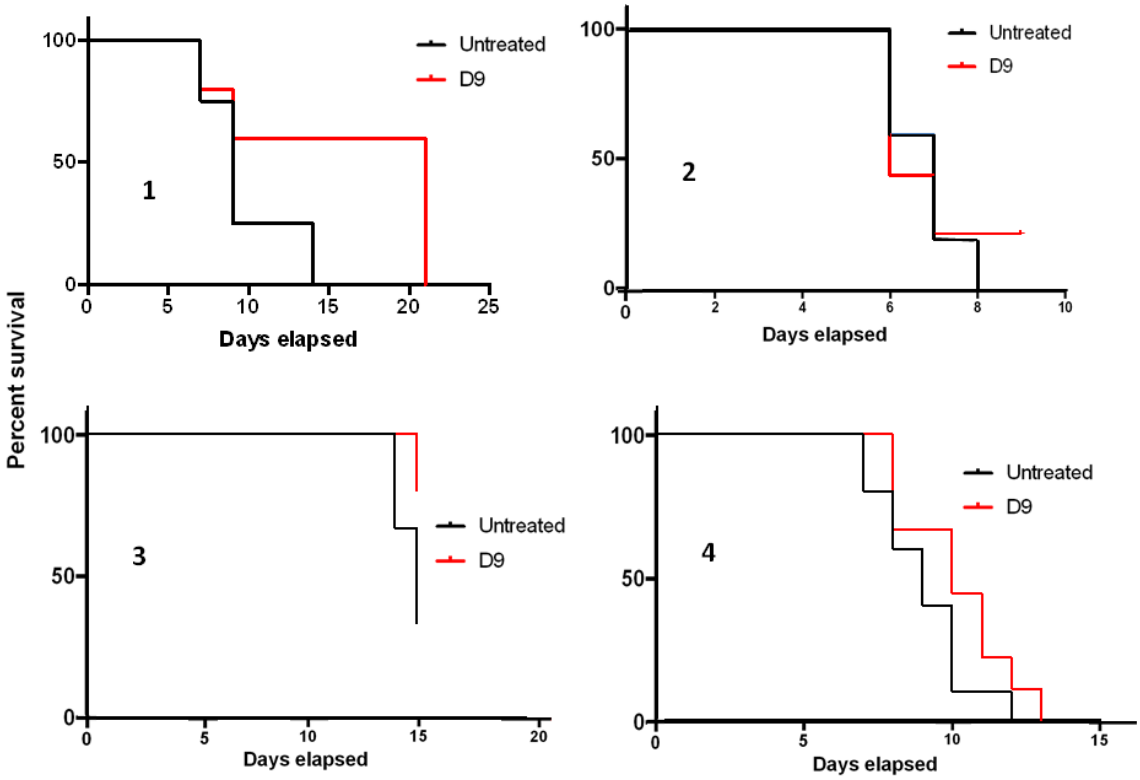

Figure 10. Survival of animals with treatment of water with D9 PMM's. Percentage of mice surviving malaria infection with *P. berghei* parasites comparing treatment with drinking water treated with D9 PMM's versus regular water. Four experiments were carried out. Kaplan Meier Analysis of each individual curve: non-significant.

Necropsies were performed on all animals. Through macroscopic observation of the organs, hypertrophy of the spleen and discoloration of the liver was found in all infected mice, in comparison with those that were not parasitized, but no change between those treated and s

DISCUSSION

Quantum biology refers to applications of quantum mechanics and theoretical physical chemistry to biological systems and problems (Univ. of Illinois, Theoret. and Comput. Biophys. Group, 2010) . Each living cell communicates with others with precision and accuracy to maintain synchrony, unity of

purpose and health. These are fundamental factors for the maintenance of homeostasis in living cells, tissues and organs. Cell-to-cell signaling occurs by chemical reactions, electromagnetic waves, by quantum transfer or by all of them. Electromagnetic signals for biological systems are very fast in their actions and have coherent behavior. The speed of communications may be that of light for biophotons or even instantaneous for quantum transfer (Cifra et al., 2011) (Scholkman et al., 2013) (Sanders et al., 2014) (Xuan et al., 2013) (Zeilinger, 2010). Photons are units of light energy packets and a light beam represents a stream of photons which can exert their effects from the atomic to macroscopic levels. Biophotons perform communications between cells based on speed of light transmission of signals and in coherent fashion to regulate many life processes (Popp, 1988a) (Popp, 1988b). They are ultra-low light photons which are capable of affecting DNA processes such as mitosis and apoptosis, as well as intercellular and inter-organ interactions. Thus, the application of quantum biology to the solution of pathological processes is a new and promising therapeutic approach in modern medicine. The APSE™/BioPhoton-X™ technology utilized in our experiments as previously described, is based on the generation and quantum transfer of photonic and electromagnetic energy to living systems in order to manipulate and correct dysfunctional states with precise aim and simple but effective and non-invasive procedures. These technologies have designed and developed PMM's capable of inducing changes in the physical-chemical properties of water and aqueous solutions of microorganisms and living cells (Victor De Franco, unpublished results). It is therefore plausible that the results observed in the experimental models tested, may be due in part to positive modulating effects exerted by the energy delivered by the PPMs to experimental cells and animals, resulting in the improvement of their physiological homeostasis.

Our results *in vitro* pointed to PMM R#2 as the one that seemed to inhibit parasitemia the best. With this knowledge, a second generation of PMMs were built and again they were first tested *in vitro*, to find that D8 tended to inhibit the parasitemia and placing two PMM's of D8 together under the plates where cultures were incubated produced a better inhibition of parasites. On the other hand, though, D9 seemed to help parasites grow.

As the final goal was to test the PMM's *in vivo*, and aware of the gap between culture testing and animal experimentation, we first performed a head-to-head experiment with both of the second generation PMMs and one the first generation, not tested before, to better assess their effects after the changes introduced between generations. For the animal testing, the strategy was to apply the adherent PMM's to the bottles of water where the mice obtained their liquid. As the *in vitro* experiments suggested that the effect of the PMMs was stronger when placing more than one film during the treatment, to each water reservoir three PMM's were adhered around the bottle.

PMM AGN10 was ineffective in slowing the parasite growth or increasing the survival of the animals. As for the other two biophotonic PMM's, the *in vivo* results differed from the *in vitro* ones. D8 did not help inhibit the parasitemia and did not help the animals survive longer, either. On the other hand, D9 inhibited moderately the parasitemia in the comparative experiment and also extended the life of the animals. For these two reasons, the rest of the *in vivo* experiments were continued with the APSE™ and BioPhoton-X biophotonic D9 PMM.

Three other experiments were added to the comparative one. The inhibition of parasitemia by D9 could not be replicated in the rest of the tests: growth of the parasites varied considerably between the animals in each experiment and no clear conclusion could be drawn from this observation alone. Nonetheless, when comparing the weight loss upon being overtaken by disease, it is seen that treatment with PMM D9 helped the animals keep their weight from crashing as fast. In experiment 3, where this difference is more evident, one of the untreated animals had died when taking the average weight of the group. This fact, however, does not change the outcome for if this mouse was so sick as to have succumbed to malaria, his weight would have probably skewed the average of the group to even lower levels. This observation seems to suggest that D9 treated water was somehow beneficial to the animals.

The analysis of survival of the animals adds to the above suggestion. When analyzed individually, because of the difference in the number of animals in each group, the results point to a non-significant difference between them. However, in every one of the four experiments the tendency is clear: those animals that were drinking water treated with D9 fared the disease better, regardless of how high their parasitemia was. In one experiment, even 60% of the animals in the treated group lived up to 21 days after inoculation of the parasite load. The normal survival period of C57BL/6 after Infection with *Plasmodium berghei* ANKA (PbA) goes from 5 to 10 days, when they usually succumb to cerebral malaria (de Oca et al., 2013).

Upon seeing this result and the increased parasitemia this treatment caused *in vitro*, perhaps the homeostasis of the cells in general is favored by this water, resulting in a better function of the metabolic processes. Interestingly, in the treated groups, those animals that started lowering their consumption of water with the onset of symptoms seemed to accelerate their death from that moment, as if the PMM D9 treated liquid was in fact retarding the lethal effects of the pathogen.

The evidence shown in this work, however inconclusive with regards to malaria infection, clearly points to an effect *in vivo* being exerted from these water treated with the PMM's This work adds to the constantly growing evidence that living beings can be altered through its interaction with light

and most probably other sources of energy. The observations of this study will help continue adapting and optimizing the APSE™ and BioPhoton-X™ technology to achieve the effects desired from the application of the PMM's as alternative treatments that circumvent resistance, as is the case of malaria. However, the technology can be used in many other applications. The use of light to treat diseases is not new. Photodynamic therapy (PDT) is one such alternative used in the treatment of cancer in varied parts of the human body (Kwiatkowski et al., 2018). Biophotons have many properties that still need to be harnessed for the benefit of humans (Popp, 2003).

CONCLUSIONS

In this work, different biophotonic PMMs R#1, C#1 and C#2 were evaluated *in vitro* and D8, D9 and AGN10, *in vivo,* examining their effect on malaria parasites. Their effects in parasites in culture and in infected mice point to a protective effect of PMM D9 on the cells and individuals. Animals that consumed water treated with the biophotonic film PMM D9 withstood better the attack of the pathogen, evidenced by the not so pronounced weight loss as compared to the untreated controls and a repeated increase in survival. These results give hope that with the right manipulation and optimization of the PMMs, photonics can be used to help combat diseases.

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AUTHOR CONTRIBUTIONS

LP was in charge of *P. falciparum* cultures. RI carried out the *in vitro* work. LC helped supervise the *in vitro* work and participated in the analysis of the data. VD provided the PMM PMM's and, together with JL, participated in the supervision of the work. CS was in charge of supervising the *in vitro* work and worked in the analysis and writing of the manuscript. RD carried out all the animal work and wrote the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

Victor De Franco Levy is the CEO of Engeenuity S.A. and Engeenuity Life Sciences S.A., proprietary of the APSE™ and BioPhoton-X™ technology. Julio Lavergne works for Engeenuity S.A.

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