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ORIGINAL ARTICLE

Pain reduction in validated rat pain models: radio frequency spectrum targeted at the low and ultra-low ends using the emulate® delivery system

Xavier A. Figueroa, Lucas Lacambra, and B. Michael Butters

Pre-Clinical Development, EMulate Therapeutics, Inc, Bellevue, WA, USA

ABSTRACT

EMulate Therapeutics, Inc. (EMTx) has developed a technology to deliver time-varying magnetic fields as WAV files, emitted in the extremely low through the low spectrum of radio frequencies (DC to 22 kHz), that can be applied to regulate pain sensation. These low power fields (~30-70 milli-Gauss AC RMS) are delivered via a portable, light-weight wearable device (Voyager). A contract third-party animal research organization (ANS Biotech, S.A.) specializing in validated rat pain models, ran the studies independently of the authors. Here we report that a subset of signals demonstrated a statistically significant effect in reducing the sensation of pain in rat models for visceral pain, neuropathic pain and inflammatory pain. Furthermore, removing frequencies above 6 kHz in the original signals improve the pain reducing effects of the unmodified signal.

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KEYWORDS
magnetic; pain; rat; radio; analgesia; frequency

Introduction

For many decades, reports of magnetic fields affecting cell growth (both promoting and inhibiting), altering embryo development (in chicken embryos and nematodes), promoting and inhibiting cancer growth, accelerating wound healing/bone growth and pain reduction are consistently repeated observations in the basic and applied medical research (Mattsson and Simko 2019; Saliev et al. 2019; Strauch et al. 2009; Vallbona and Richards 1999; Zhadin 2001), including veterinary medicine (Gaynor et al. 2018).

Extremely low-frequency, pulsed electromagnetic fields (PEMF) have been studied and tested for their analgesic effects in mice (Shupak et al. 2004) and rats (Ryczko and Persinger 2002). Other PEMF studies using low-frequency PEMF have reported pain reduction and bone preserving effects in complex musculoskeletal conditions, like arthritis (Ganesan et al. 2009).

The use of magnetic fields to delivery pain reducing or inhibiting effects are attractive. The permeability of biological tissue to magnetic fields is the same as that for air (International Commission on Non-Ionizing Radiation P 2010), ensuring deep penetration that is dependent on the antenna geometry and the current applied. The magnetic field has the potential of providing a constant effect to the exposed region, thus overcoming the effects of dilution and clearance that drug compounds experience.

In the field of pain management, the use of opioids has become an essential tool (Vuckovic et al. 2018), but the addictive and health-impacting effects of opioid misuse result in approximately 70,000 deaths per year in the US alone (Scholl et al. 2018). Alternatives exist to opioids, but do not provide substantial pain relief (Yekkirala et al. 2017), (Cuesta et al. 2021; Woolf 2020). New methods or technologies to safely and effectively reduce pain sensation, either as a monotherapy or in combination with lower dosage or safer pain medications are urgently needed in populations suffering from acute and chronic pain.

EMulate Therapeutics (EMTx) has developed a non-sterile, non-invasive, non-thermal, non-ionizing, battery-powered, portable investigational medical device called Voyager, which uses low to ultra-low level (DC-22 kHz) frequencies to deliver specific electromagnetic signals to reduce pain sensation. The system emits a low power field (< 100 mG) that can be applied locally or globally, dependent on the type of therapeutic effect sought. The EMTX signals are stored as WAV files on the Voyager and emitted via a cable to the attached antenna unit.

We report that the unmodified (signals with frequency components from DC-22 kHz) and modified signals (signals with frequency components from DC-6 kHz) – decreased pain sensation after 24 hours of full-body exposure to the magnetic fields, in three of the five validated pain animal models tested. The results from

CONTACT Xavier A. Figueroa xfigueroa@emulatetx.com EMulate Therapeutics, Inc, 13810 SE Eastgate Way, Suite 560, Bellevue, WA 98005, USA
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these pre-clinical animal models suggest that three signals may be effective in reducing pain in a clinical setting.

Materials and methods

Signals

The method for the generation of signals used in the study are described in a previously published article (Butters et al. 2014). Briefly, EMTx's molecular interrogation and data system (M.I.D.S.) was used to record the electromagnetic frequencies (EMF) emitted from pharmacological compounds dissolved in a solvent (water, DMSO or ethanol). These signals were recorded in the time domain and stored in a digital format (WAV) for analysis, selection and testing. The Nyquist rate specifies a sampling rate equal to twice the highest frequency of a given function or signal. With an equal or higher sampling rate, the resulting discrete-time sequence is free of aliasing. The original unmodified signals (WAV 1 – WAV 3, WAV 5 – WAV 6) were recorded at a 44.1 kHz Nyquist sampling rate (DC – 22 kHz). WAV 4 was recorded at a Nyquist rate of 8.0 kHz (DC – 4 kHz).

The complex wave forms recorded by the M.I.D.S. can be filtered or re-sampled post-recording to select or enhance specific features of the signal. The original unmodified signals (WAV 1 – WAV 3, WAV 5 – WAV 6) were re-sampled at 11.25 kHz Nyquist rate. The resampling cuts off frequencies above 6 kHz and generates the modified signals tested in this report (DC – 6 kHz). WAV 4 was resampled at a Nyquist rate of 8 kHz, resulting in a signal with a frequency range of DC – 4 kHz.

Six unmodified signals (designated as WAV 1 – WAV 6) and six modified signals (designated as WAV 1' – WAV 6') were for tested at the ANS Biotech vivarium. A broad banded, white noise control signal (DC – 22 kHz) was generated using a Stanford Research Instruments Arbitrary Waveform Generator (Stanford Research Systems, Sunnyvale CA) and stored as a WAV file. The White Noise signal was used as a general magnetic field control signal.

Signal transmission and coil design

The delivery device is an experimental, third generation system that is described here (Cobbs et al. 2019). Briefly, the signal amplifier/emitters (named "Voyager"; Figure 1a, white devices) are a Class A/B amplifier with the capability of storing signals and emitting the signals with low distortion in the DC-22 kHz range, as well as in the DC-6 kHz range. A single Voyager is plugged in to the antenna system to deliver the digitized signal. Additional features, such as encryption and real-time decryption of the stored signal are built into the Voyager system. Voyagers are assigned in pairs and loaded with the same WAV file. Voyagers can transmit continuously for 15 hours, at which time they need to be replaced with a charged unit, hence the need to supply two Voyagers per every cage coil used (one unit is used, while the other unit charges).

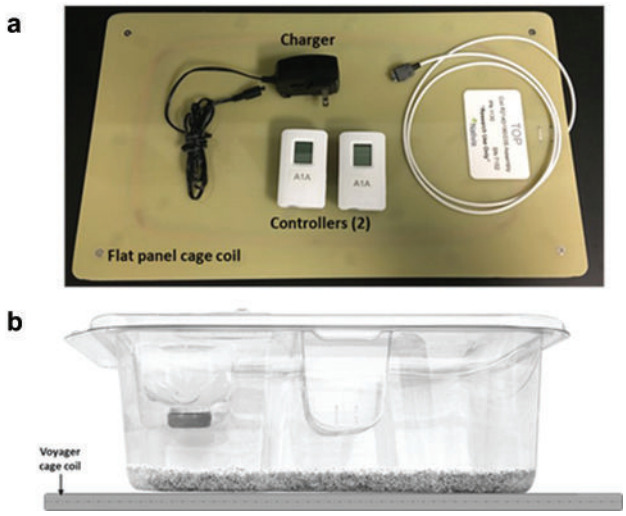

Figure 1. Equipment used to emit signals to rats. a, the equipment used to deliver the signal are composed of two Voyagers (controllers), a wall charger to charge the battery of the Voyagers and the flat panel cage coil. b, an example image of how the rat cages sits on the flat panel cage coil.

The cage coils were designed and built by Sparton (Ohio, USA). The cage coils are flat panels (Figures 1 and 2) that house a wound copper coil antenna that transmits the magnetic field. The wound copper coil is sandwiched between two G10/FR-4 glass epoxy plates (Curbell Plastics, Orchard Park, NY). The rat cages are placed on top and in the center of the cage coil. The magnetic field envelope extends above the surface of the cage coil (see Supplement 1). The magnetic field AC root-mean square (RMS) flux density (at the surface, center cage coil) is in the ~30-70 mG range, depending on the signal being emitted.

A contract pre-clinical laboratory (ANS Biotech SA, RIOM Cedex, FRANCE) was selected to run the validated animal pain models (see **Pain Models** in the Materials and Methods section). An initial high through-put screen (the ALGOGram^{TM}) was used to measure the pain reducing potential of select signals to determine whether an analgesic/anti-inflammatory effect is detected (data not shown). Signals that showed an effect were selected for confirmatory testing powered to detect a statistically significant effect.

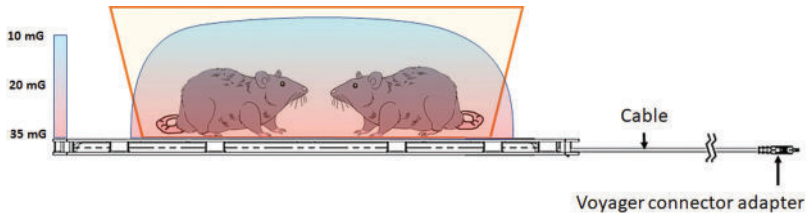

Figure 2. On-edge view of cage coil with a diagram depiction of magnetic field emission. Rat is immersed in the magnetic field of the generated signal for up to 24 hours of continuous exposure. Alternating current root-mean square (AC RMS) of the milli-gauss (mG) field strength averaged ~35 mG for the signals.

Male Sprague-Dawley rats (SPF status, Janvier, France) were used for each exposure to a WAV file emitted as magnetic field (24 hours of constant magnetic field exposure prior to testing) for each test in the ALGOGram^{TM} screen and the second, confirmatory pain assays. Rats were housed in a temperature (20–24°C) and relative humidity (45% – 65%) controlled room and acclimated to an artificial day/night cycle of 12 hours light (6.30 a.m. to 6.30 p.m.)/ 12 hours darkness. Rats had free access to tap water and were fed *ad libitum* with pelleted complete diet (reference A04, S.A.F.E.). Animals were housed 2 or 4 per cage (type E and type III H) during their acclimation period and 1 or 2 per cage type 2150E (18 cm x 30 cm) during the 24 hours of exposure to the magnetic field. Each rat was identified by tail markings. Based on the SPF status of the animal facilities, there is no reason to expect that contaminants were present in the food, water or bedding, at levels capable of interfering with the results of the tests.

The protocols describing the animal model used in these studies were approved by the Animal Ethical Committee (Comité d'Ethique pour l'Expérimentation Animale Auvergne – C2E2A) and accredited by the French Ministry of Education and Research (MESR) under national authorization number #23617. Rats used in this study were treated according to the guidelines of the Committee for Research and Ethical Issue of the I.A.S.P. (1983) and the European guidelines 2010/63/UE. Test facility accreditation number for the use of laboratory animals is D63.300.12.

Confirmatory assays

Twelve signals were tested (WAV 1 – WAV 6; WAV 1' – WAV 6'), along with a White Noise control. For five pain models (acetic acid test, oxaliplatin, carrageenan, TNBS and Bennet paw pressure; see **Pain Models**), ten (N = 10 per signal per assay; N = 440) Male Sprague-Dawley rats were used for each pain model and for each of the signals tested.

(1) Oxaliplatin Paw Withdrawal latency: Total number of rats, N = 110.
(2) Acetic Acid Writhing assay: Total number of rats, N = 90.
(3) Peripheral Mononeuropathy (Bennett Model): Total number of rats, N = 50.
(4) Carrageenan-Induced Mechanical Hyperalgesia: Total number of rats, N = 110.
(5) TNBS-Induced Chronic Colonic Hypersensitivity: Total number of rats, N = 80.

All rats were exposed in pairs (in the same cage) for 24 hours prior to testing (Figure 2). Technicians at ANS Biotech were blinded to the signals the rats experienced. No randomization of rats was done.

Pain Models. The 5 different models are described below:

- Paw pressure test – Static mechanical hyperalgesia is assessed using the Paw Pressure test (Randall & Selitto test). This test relies on the application of an increasing pressure on the hind paw placed between a flat surface and a blunt pointer. This test is usually performed on animals with one hind paw inflamed by an injection or injured by ligation, and one normal hind paw, to evaluate drugs for analgesic action. The apparatus exerts a steadily increasing force and reaction threshold is determined as the pressure (g) required to elicit paw withdrawal and/or vocalization (Randall and Selitto 1957). In the experiment, animals are gently handled by the experimenter and static mechanical hyperalgesia is assessed 2 times for both hind paws.
- Acetic acid test – Abdominal contraction is induced by intraperitoneal injection of 0.6% acetic acid solution in rats (10 mL/kg). The number of contractions are recorded from 5 to 15 minutes after injection of the solution (Koster et al. 1959).
- Bennett model – Peripheral mononeuropathy is induced by loose ligation of the sciatic nerve in anaesthetized rats (Xylazine 10 mg/kg i.p., Ketamine 60 mg/kg i.p.) on D-14. Briefly, the

common sciatic nerve is exposed at the level of the middle of the thigh by blunt dissection through the biceps femoris. Proximal to the sciatic trifurcation, four ligatures are tied loosely around it with about 1-mm spacing. Great care is taken to tie the ligatures such that the diameter of the nerve is seen to be just barely constricted (Bennett and Xie 1988). After surgery, animals are allowed to recover for 4 days.

- Oxaliplatin – (Induction) Acute peripheral neuropathy is induced by a single intraperitoneal injection of oxaliplatin (10 mg/kg, i.p.) 72 hours before testing (Cheng et al. 2017).
  - Paw immersion test – Cold allodynia is measured using the paw immersion test. In this test, the latency of hind paw withdrawal is measured after immersion of the hind paw in the temperature-controlled water-bath (cryothermostat) with a temperature fixed at 10°C (± 0.5°C).
- Carrageenan (Induction): Mechanical hyperalgesia test – Three hours before assessment of the nociceptive threshold using the paw pressure test (Randall and Selitto 1957), 100 µL of a 2% carrageenan suspension is injected into the plantar aspect of the right hind paw. After carrageenan injection, rats were returned to their cages. Rats assigned to a magnetic field exposure, were placed back in to the magnetic field.
- TNBS (Surgery) – Colonic sensitivity is induced by surgical administration of TNBS (2,4,6-trinitrobenzenesulfonic acid solution) seven days before behavioral testing (D_{-7}). Animals are fasted overnight prior to surgery. Animals are anesthetized by injection of xylazine 10 mg/kg/Ketamine 60 mg/kg, then the colon is exposed through a small incision of the abdomen. TNBS (50 mg/kg, 1 ml/kg) is injected into the proximal part of the colon (1 cm from the caecum). After surgery, animals are returned into their home cages in a regulated environment, and are fed *ad libitum* until D-1 (animals were fasted 24 hours before distension) (Diop et al. 2002).
  - Colonic distension – Seven days (D_0) after TNBS injection, colonic sensitivity is assessed on fasted (overnight) animals by measuring the intracolonic pressure required to induce a behavioral response during colonic distension. To perform distension, a 5-cm long balloon is gently inserted into the colon of vigil animals at 10 cm from the anus and the catheter is taped to the base of the tail. After a 30-minute acclimation period with the inserted balloon, colonic pressure is gradually increased by 5 mmHg steps every 30 seconds from 5 to 75 mmHg (cut off) until pain behavior is evidenced. Pain behavior is characterized by an elevation of the hind part of the animal body and a clearly visible abdominal contraction corresponding to severe cramp. Two determinations are performed at 30 minutes and at 50 minutes.

Data presentation and statistical analysis: confirmatory assays

All statistical comparisons were post-hoc and alpha levels for significance were set at $\alpha = 0.05$., apart from the Bennet Model of Peripheral Mononeuropathy ($P = .01$). Each treatment/exposure arm had $N = 10$ rats and all statistical comparisons were made against the saline or MC1% control group arm for the pain model tested, apart from the Bennet Model of Peripheral Mononeuropathy assay. All rats in the pain model groups had a known pain inhibiting positive control arm (either duloxetine, indomethacin, (-)U50, 499 H, or morphine). Descriptions for validating the effects of surgical or chemically induced injury and the statistical tests used are described in each section for each pain model below.

Oxaliplatin paw withdrawal latency

The paw withdrawal latency (mean ± s.d.) in seconds for each group, calculated from individual paw (left and right) withdrawal latency. A single intraperitoneal administration of Oxaliplatin 10 mg/kg induces cold allodynia as evidenced by a marked and significant decrease in the paw withdrawal latency 3 days after injection in the Oxaliplatin/MC-treated group as compared to the pre-induction baseline. $P < .01$ as compared to the pre induction baseline of the corresponding group, Wilcoxon test. Signal exposed groups were compared to the MC1% group, using Dunnett's test after significant one-way ANOVA.

Acetic acid abdominal contraction assay

The number of contractions (mean ± s.d.) for each group, calculated from individual number of contractions observed during the 10 min-observation period. Signal exposed groups were compared to the saline group, using Dunnett's test after significant one-way ANOVA.

Peripheral mononeuropathy (Bennett model)

The paw withdrawal threshold (mean ± s.d.) in grams of contact pressure for each group, calculated from individual paw withdrawal thresholds. Effect of injury induction was assessed by comparing the control paw to the injured paw, using Bonferroni's test after significant two-way ANOVA ($P < .001$). Pair-wise comparison of

pre-treatment versus post-treatment groups (control paw versus control paw; injured paw versus injured paw) using Students T-test (α = 0.01) was done.

Carrageenan-induced mechanical hyperalgesia

The paw withdrawal threshold (mean ± s.d.) in grams of contact pressure for each group, calculated from individual paw withdrawal thresholds. Validity of mechanical hyperalgesia was assessed by comparing the control paw and the injured paw using Mann-Whitney rank sum test. The signal and physical drug groups were compared to the MC1% group and analyzed using Dunnett's test after significant one-way ANOVA.

TNBS-induced chronic colonic hypersensitivity

The colonic distension threshold (mean ± s.d.) for each treatment group (N = 10 groups). Signal and physical drug treated groups were compared to the Saline (Surgery; TNBS Induced Colonic Lesion), using Dunnett's test after significant One-Way ANOVA.

Results

From April 26, 2021, to June 5, 2021, ANS Biotech tested 10 different EMTx signals, White Noise, a drug positive control and sham exposure (no signal) against their ALGOGram™ pain screen.

The initial screen resulted in WAV 1, WAV 2, WAV 3, WAV 4, WAV 5 and WAV 6 being identified as potential candidates for further testing.

Additionally, modified signals (WAV 1' – WAV 6') were tested to determine if the pain inhibiting potential could be increased, relative to the unmodified parent signals (WAV 1 – WAV 6). These studies were done from August 2021 to October 2021.

Oxaliplatin induce cold allodynia

In the oxaliplatin induced cold allodynia assay (Figure 3), prior to oxaliplatin induction, rats in each group were tested to assess their cold response as a baseline measure. A Dunnett's test (Groups = 8, N = 10 per Group, α = 0.05), versus the MC1% group (negative control), demonstrated no significant differences between groups (average time to leg withdrawal: 12.7 second; black columns). Post-oxaliplatin induction (purple columns), the duloxetine drug group (positive control) significantly increased the leg withdrawal time (13.2 ± 1.6 seconds) compared to the MC1% control group (7.9 ± 1.0 seconds). All three modified signals (WAV 1'; WAV 2'; WAV 3') demonstrated significant increase in leg withdrawal delay (11.8 ± 2.3 sec; 11.2 ± 1.9 sec; 11.7 ± 1.8 sec, respectively) versus the MC1% control group (7.9 ± 1.0 seconds; P = .01). Two unmodified signals (WAV 2 and WAV 3) demonstrated significantly increased leg withdrawal delays (10.7 ± 1.7 and 10.7 ± 2.1 seconds, respectively) at P = .05 versus the MC1% control group. The White Noise signal achieved a statistically significant effect (10.6 ± 2.3 seconds; P = .05) versus the MC1% control group. The WAV 1 signal did not reach a statistically significant delay in leg

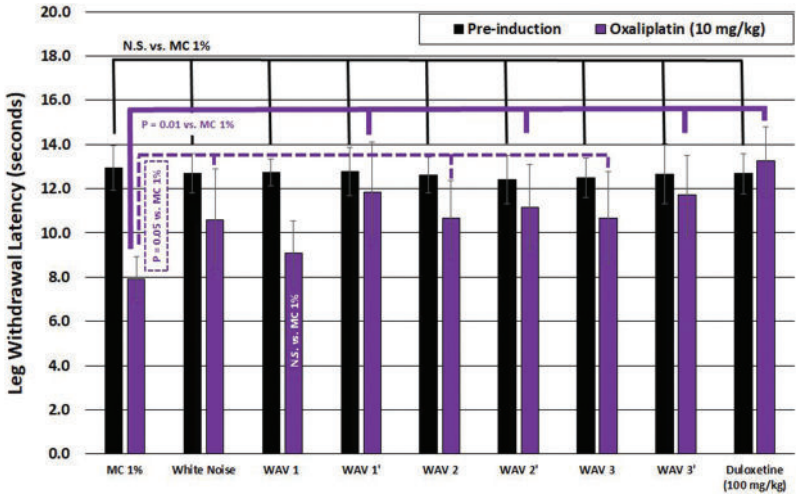

Figure 3. Oxaliplatin induced neuropathic allodynia pain model. Duloxetine 100 mg/kg p.o. was used as a positive control for pain modulation. Black columns are the time-lag for leg withdrawal (left & right paws) from a 10°C water bath at baseline measure before oxaliplatin treatment. Purple columns are the time-lag for leg withdrawal (left & right paws) post-oxaliplatin treatment and post drug injection/signal exposure (2 hours/24 hours). Significance values are a post-hoc analysis using a Dunnett's test for multiple comparisons after a significant ANOVA. All comparisons are made against the MC 1% control (purple column average). Error bars are standard deviations (S.D.). N.S. – not significant.

withdrawal (9.1 ± 1.5 seconds; Not Significant, N.S.) versus the MC1% control group.

Carrageenan induced mechanical hyperalgesia

In the carrageenan induced mechanical hyperalgesia assay (Figure 4) rats in each group were tested to assess their paw pressure threshold response in the un-injected paw (Figure 4, left; Control Paw). A Dunnett's test (Groups = 9, N = 10 per Group, α = 0.05), versus the MC1% group, demonstrated no significant differences between groups, except in the Indomethacin injected group (Indomethacin: 350 ± 14 grams, MC1%: 318 ± 18 grams; P = .01).

Measures in the carrageenan injected paws resulted in WAV 2', WAV 1' and WAV 4' (256 ± 16 grams, 254 ± 16 grams, 252 ± 21 grams, respectively), Indomethacin (306 ± 21 grams) and White Noise (256 ± 18 grams) reaching a statistically significant difference in applied pressure when compared to the MC1% group (218 ± 18 grams; P = .01). The WAV 1 signal (248 ± 19 grams) reached a statistically significant difference against the MC1% group at P = .05 level. The WAV 4 signal did not reach a statistically significant difference in applied pressure (240 ± 24 grams; Not Significant, N.S.) versus the MC1% control group.

TNBS colonic lesion sensitivity assay

In the TNBS colonic lesion sensitivity assay (Figure 5) rats in each group were tested twice (at 30 minutes and 50 minutes after balloon insertion) to assess the pressure threshold response (average of both times). A Dunnett's test (Groups = 8, N = 10 per Group, α = 0.05), versus the TNBS lesion – saline group (25 ± 2 mmHg), demonstrated a significant difference between the WAV 1' (37 ± 5 mmHg), White Noise (39 ± 6 mmHg) signals and the drug compound (-) U50, 488 H kappa-opioid agonist (37 ± 3 mmHg) at a level of P = .01. The sham lesioned control rats (saline, no colonic lesion; 42 ± 3 mmHg) that served as a baseline group, reached a significance level of P = .01.

The WAV 1, WAV 4 and WAV 4' signals (32 ± 4 mmHg, 31 ± 6 mmHg, 29 ± 6 mmHg, respectively) did not reach a statistically significant difference in applied pressure versus the TNBS lesion – saline control group (25 ± 2 mmHg).

Surgically Induced Mono-Neuropathy (Bennet Paw Pressure; Tactile Allodynia)

In the surgically induced mono-neuropathy assay, three signals were tested for activity (WAV 2, WAV 2' and White Noise). Both the control paws (Figure 6, left) and injured paws (Figure 6, right) were tested at two different times: before exposure/treatment (Pre-Tx) and after exposure/treatment (Post-Tx). A one-way ANOVA within the Pre-Tx control paw group and the Pre-Tx injured paw group did not reach statistical significance (data not shown).

The analysis (Student's T-test, two-tailed; α = 0.01) compared the pressure required to lift a leg or induce a vocalization between the control paw group (Pre-Tx versus Post-Tx) and the injured paw (Pre-Tx versus Post-Tx).

Tests on the control paw group (Pre-Tx vs. Post-Tx) resulted in the WAV 2' (292.8 ± 36 grams vs. 244 ± 34

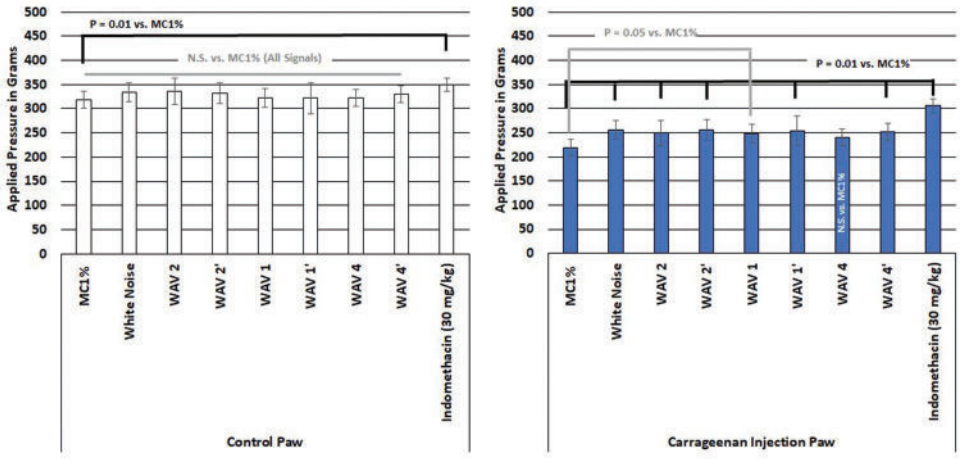

Figure 4. Carrageenan induced mechanical hyperalgesia pain model. Indomethacin (p.o.) was used as a positive control for pain modulation. Left graph, with White columns are the amount of pressure (in grams) applied before paw was lifted (un-injected left paw). Blue columns are the amount of pressure (in grams) applied before paw was lifted (injected right paw). Significance values are a post-hoc analysis using a Dunnett's test for multiple comparisons after a significant ANOVA. All comparisons are made against the MC 1% control. Error bars are standard deviations (S.D.). N.S. – not significant.

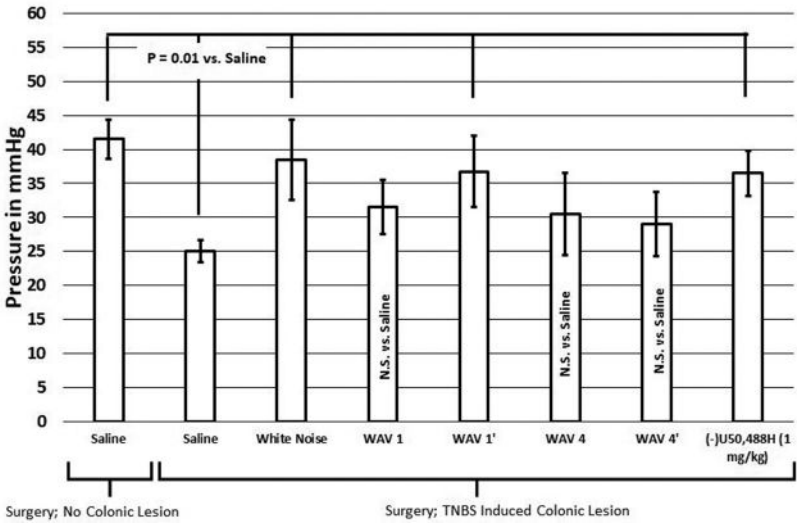

Figure 5. TNBS colonic sensitivity model of visceral pain. The kappa-opioid agonist ((-)U50,488 H) was used as a positive control for pain modulation. Columns are the amount of pressure (in millimeters of Mercury; mmHg) applied to a balloon that induced a stereotyped pain response during colonic distension. Significance values are a post-hoc analysis using a Dunnett's test for multiple comparisons after a significant ANOVA. All comparisons are made against the TNBS induced colon lesion saline group (negative control). Error bars are standard deviations (S.D.). N.S. – not significant.

grams, P = .004) and the morphine (300 ± 30 grams vs. 578 ± 73 grams, P < .00001) positive control group reaching a statistically significant difference in applied pressure (Figure 6, left). None of the other control paw groups (MC1%, White Noise and WAV 2) reached a statistically significant difference.

In the injured paw group, only the morphine treated injured paw group, reached statistical significance Pre-Tx vs. Post-Tx (180 ± 27 grams vs. 438 ± 51 grams). None of the other control paw groups (MC1%, White Noise, WAV 2 and WAV 2') reached a statistically significant difference.

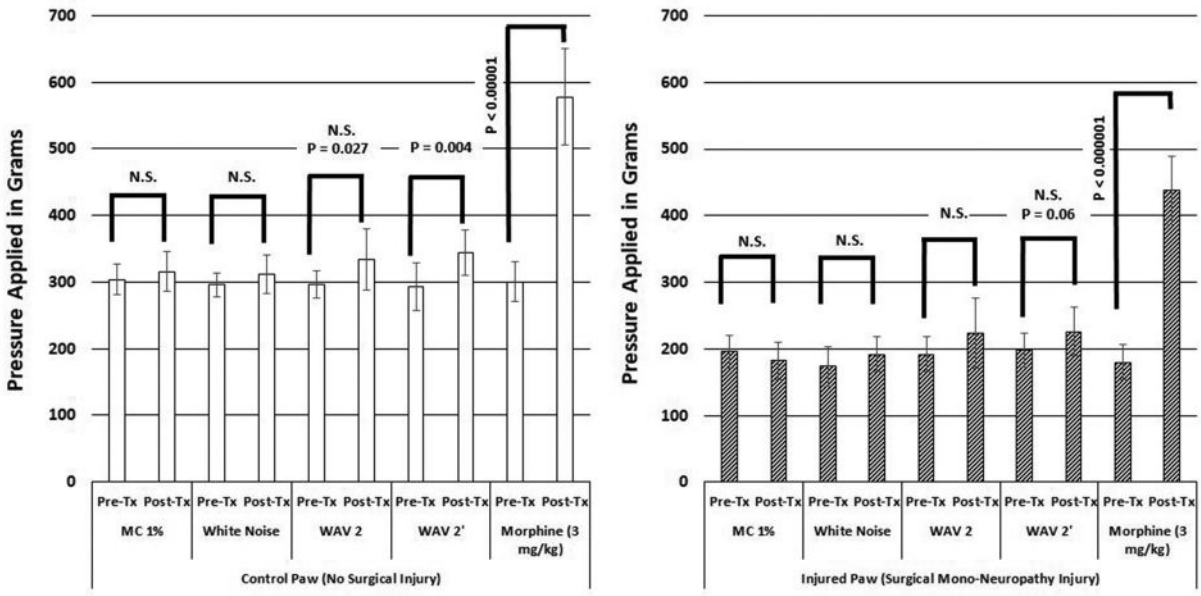

Figure 6. Surgically induced tactile allodynia assessed using the electronic von Frey. Morphine was used as a positive control for pain modulation. All columns are the average grams (g) of applied force that induce paw-withdrawal. Left, grams of pressure applied to the control paw of each treatment before and after applied treatment . Right, grams of applied force to the injured paw that induce paw-withdrawal before and after 30 minutes (morphine)/24 hours after signal exposure. A Students T-test (pair-wise comparison) was done after a one-way ANOVA. Error bars are standard deviations (s.d.). Alpha was set at 0.01. N.S. – not significant.

Acetic acid abdominal contraction assay

In the abdominal contraction assay seven signals (White Noise, WAV 4, WAV 5, WAV 6, WAV 4', WAV 5' and WAV 6') and the (-) U50, 488 kappa-opioid receptor agonist group (positive control) were tested and compared to the saline treated control group (negative control). The (-) U50, 488 kappa-opioid receptor agonist group (Figure 7, left) was the only treatment that reached a statistically significant effect (Groups = 9, N = 10 per Group, $\alpha = 0.05$; P = .01).

The tabulated outcomes for all the assays are displayed in Figure 8.

Discussion

Drug screening in animal models for analgesic and anti-inflammatory effects are an established method in drug development (Middleton et al. 2021) and serve as a critical milestone towards establishing safety and potential efficacy. In the case of the animal models used at ANS Biotech, all the animal models are validated,

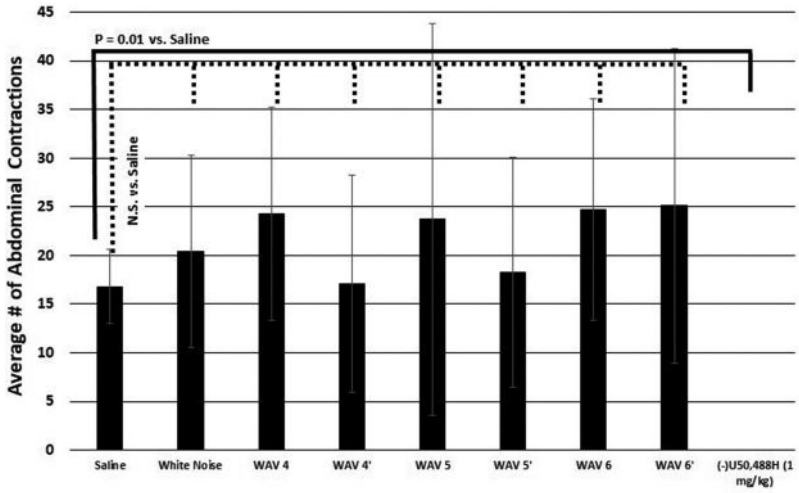

Figure 7. Acetic acid induced abdominal contraction model. The kappa-opioid agonist ((-) U50,488 H) was used as a positive control for pain modulation. Columns are the average number of contractions counted during a 15-minute period. Significance values are a post-hoc analysis using a Dunnett's test for multiple comparisons after a significant ANOVA. All comparisons are made against the 0.9% NaCl control. Error bars are standard deviations (s.d.). N.S. – not significant.

Results of Pain Models

	Pain Model Tested	Oxaliplatin	Acetic Acid	Bennett (Control Paw / Injured Paw)	Carrageenan	TNBS
Signal/Method Delivered	White Noise	P = 0.05	N.S.	N.S.	P = 0.01	P = 0.01
	WAV 1	N.S.	Not tested	Not tested	P = 0.05	N.S.
	WAV 1'	P = 0.01	Not tested	Not tested	P = 0.01	P = 0.01
	WAV 4	Not tested	N.S.	Not tested	N.S.	N.S.
	WAV 4'	Not tested	N.S.	Not tested	P = 0.01	N.S.
	WAV 2	P = 0.05	Not tested	N.S. / N.S.	P = 0.01	Not tested
	WAV 2'	P = 0.01	Not tested	P = 0.004 / N.S.	P = 0.01	Not tested
	WAV 5	Not tested	N.S.	Not tested	Not tested	Not tested
	WAV 5'	Not tested	N.S.	Not tested	Not tested	Not tested
	WAV 3	P = 0.05	Not tested	Not tested	Not tested	Not tested
	WAV 3'	P = 0.01	Not tested	Not tested	Not tested	Not tested
	WAV 6	Not tested	N.S.	Not tested	Not tested	Not tested
	WAV 6'	Not tested	N.S.	Not tested	Not tested	Not tested
ANOVA		Yes	Yes	Yes	Yes	Yes
Statistical Test		Dunnett's	Dunnett's	Students T-Test	Dunnett's	Dunnett's
Comparisson Type		Multiple	Multiple	Pair-Wise	Multiple	Multiple

Figure 8. Outcomes of Pain Models and post-hoc statistical analysis of results after ANOVA.

with a long-standing record of use and well characterized pathways (Gregory et al. 2013). In the case of the EMTx technology, radio frequency signals (applied via magnetic field exposure), produced measurable and statistically significant reductions in pain sensation in the oxaliplatin-induced neuropathy, visceral pain models (TNBS colonic distension) and the inflammatory pain model of carrageenan injections. These signals are complex waveforms, containing frequencies between DC – 22 kHz. In the case of the modified signals (WAV 1' – WAV 6'), frequencies above 6 kHz are removed, measurably increasing the ability to reduce pain sensation and inflammation.

A pattern of activity was noted in four out of the five pain models used. The modified signals (DC – 6 kHz) showed a consistent ability to reach a statistically significant value of activity, when compared to the full-frequency (DC – 22 kHz), unmodified signal. In the Oxaliplatin (Figure 3), Carrageenan (Figure 4) and TNBS (Figure 5) assays and the surgical neuropathy (Figure 6) assays, the modified signals achieved statistical significance at lower P values and had higher average values than the full-length, unmodified signals. The exception to this trend occurred in the TNBS model, in which both WAV 4 and WAV 4' did not reach a statistically significant cut-off value (P = .05). As reported in the Materials and Methods, WAV 4 was recorded with parameters that only captured the frequency range from DC – 4 kHz.

The surgical neuropathy model (Figure 6) achieved statistical significance in the post-treatment control paw (P = .004) for WAV 2' and trended towards significance in the injured paw group (P = .06). The modest increase in applied pressure after WAV 2' exposure in the control paw group indicates that the signal had an analgesic effect in a non-injured limb but could not significantly reduce the pain associated with tactile allodynia in the injured paw. The White Noise signal did not demonstrate any analgesic effect in either paw.

Not all pain models showed a significant effect, such as the acetic acid abdominal contraction model (Figure 7). None of the Signals (WAV 4 – WAV 6 and WAV 4' – WAV 6') including the White Noise signal, demonstrated a statistically significant inhibition in the abdominal contraction assay.

The White Noise signal, which was used as a negative control to account for the generalized effect of a fluctuating magnetic field of similar magnitude, demonstrated a measurable and significant decrease in three of the five pain models tested. This was a somewhat surprising finding, as the White Noise signal had not previously demonstrated a significant effect in the initial screen run at ANS Biotech and in other internal studies completed (data not shown). Furthermore, the White Noise showed no statistically significant effects on the other two pain models tested (Figures 6 and 7).

A possibility to account for the results of the White Noise exposure is the known effect of stochastic resonance (Adair 2003) in biology. The stochastic resonance effect is a known signal enhancement technique (Krawiecki et al. 2000) in tele-communications and signal analysis, in which White Noise is introduced to a sub-threshold signal to elevate components of the signal above the noise threshold. This has the effect of enhancing coherent signal components above the noise-floor and producing a signal that can be recognized as an actual signal with information.

Exposure of the rats to the signals occurred in resin-type, tiered racks. Rat cages and cage coils were stacked on shelves. The cage set ups for the pain assays had a mixture of different signals on the same rack. The cage arrangement at ANS varied, based on the test that was being run on the date of exposure and testing. Due to the proximity of the cages, there was a potential for one of the signals (WAV 1', as an example) to be near enough to the White Noise cage coil for the stochastic resonance effect to occur. This is a potential confounding factor that could have led to one of the signals interacting and producing the pain inhibiting effects seen with White Noise.

We tested the potential of the signals to extend beyond 6 inches from the cage coil (Supplement 1). Using a commercial magnetometer and a data logger, we tested the ability of the magnetometer to pick up signals at 6, 12 and 18 inches from the source coil. A cross-correlation program was used to identify the signals emitted from a coil (WAV 1' – WAV 4') to determine if a match with a reference recording (WAV 1' – WAV 4') could be detected at the 6, 12 and 18 inches from source coil. The analysis revealed that a better than 90% match could be achieved at 12 inches and at 18 inches a better than 70% match could be achieved (Supplement Tables 1–4). These measures indicate that coherent and detectable signals potentially could reach the White Noise emitting coils during the exposure period.

Alternatively, the White Noise may have actual pain reducing effects. This seems unlikely, given the lack of specificity and the nature of the broad-band energy applied to all the frequency components in the White Noise. In future assays, the White Noise signal may need to be isolated from other signals being emitted or another type of control signal, such as the recording of water or an inactive compound (like a physiologic saline signal) could be used.

Given that modified signals consistently produced statistically significant effects in 4 out of 5 pain models (with the exception of White Noise and WAV 4), these results suggest that some signals with frequencies of DC – 6 kHz can provide an efficacious reduction in pain sensation in these short-term pain studies. WAV 4, with a frequency range of DC – 4 kHz and WAV 4' (DC-2.0 kHz) suggest that there is a lower bound for frequency efficacy. Future testing with combinations of signals may result in improved or broader effects in reducing pain sensation and inflammation.

Future plans to test the potential analgesic effects of the White Noise signal includes running a control study with only the White Noise signal tested in these models or with the White Noise isolated in a separate room.

Additional work to determine the potential toxicological and safety profile and time to onset/offset of these signals is required. Continuous or discontinuous (i.e. – one hour on/one hour off) exposure to the signals needs to be addressed for potential long-term use, as well as motor and cognitive effects that may be induced in either exposure scenario.

The intensity of the applied magnetic field (AC RMS; milli-Gauss) of the Voyager system is several orders of magnitude lower than the reported effects using magnetic fields in polyneuropathy clinical trials (2000–3000 Gauss) (Geiger et al. 2015) or analgesia induction in healthy volunteers (10 Gauss) (Kortekaas et al. 2013). This suggests that induced electrical currents are not the driving mechanism that produce the results we observed in the rats using our technology. Other mechanisms may be essential to produce the reported effects.

Conclusion

The results of these assays indicate that these signals have pain reducing effects in specific pain models in rats without any apparent safety concerns. A next step is to confirm the safety of these signals with a toxicology and histology series of prolonged exposure. We fully expect that the results will provide a safety profile that will allow for clinical testing.

Furthermore, modification of the original signals (DC – 22 kHz) by reducing the frequency range (DC – 6 kHz) produced a consistent pain inhibitory effect and highlights the potential for further signal enhancement after recording. The ability to induce analgesia and reduce nociception via the application of a magnetic field, without (potential) systemic side-effects, would be a significant improvement in pain management, diversion control and stock maintenance of pharmaceutical compounds, without the limitations of systemic dilution, delivery and metabolites.

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Data availability

Results from work done at ANS Biotech can be made available upon request to the corresponding author.

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