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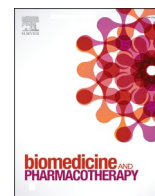
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## Pharmacological antagonism of EP2 receptor does not modify basal cardiovascular and respiratory function, blood cell counts, and bone morphology in animal models

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### ABSTRACT

The EP2 receptor has emerged as a therapeutic target with exacerbating role in disease pathology for a variety of peripheral and central nervous system disorders. We and others have recently demonstrated beneficial effects of EP2 antagonists in preclinical models of neuroinflammation and peripheral inflammation. However, it was earlier reported that mice with global EP2 knockout (KO) display adverse phenotypes on fertility and blood pressure. Other studies indicated that EP2 activation with an agonist has a beneficial effect of healing fractured bone in animal models. These results impeded the development of EP2 antagonists, and EP2 antagonism as therapeutic strategy. To determine whether treatment with EP2 antagonist mimics the adverse phenotypes of the EP2 global KO mouse, we tested two EP2 antagonists TG11-77, HCl and TG6-10-1 in mice and rats while they are on normal or high-salt diet, and by two different administration protocols (acute and chronic). There were no adverse effects of the antagonists on systolic and diastolic blood pressure, heart rate, respiratory function in mice and rats regardless of rodents being on a regular or high salt diet. Furthermore, chronic exposure to TG11-77, HCl produced no adverse effects on blood cell counts, bone-volume and bone-mineral density in mice. Our findings argue against adverse effects on cardiovascular and respiratory systems, blood counts and bone structure in healthy rodents from the use of small molecule reversible antagonists for EP2, in contrast to the genetic ablation model. This study paves the way for advancing therapeutic applications of EP2 antagonists against diseases involving EP2 dysfunction.

**Abbreviations:** ACN, acetonitrile; AD, Alzheimer's disease; AERD, aspirin-exacerbated respiratory disease; ALS, amyotrophic lateral sclerosis; ANOVA, Analysis of variance; A $\beta$ , amyloid-beta; BL, baseline; BPM, beats per minute; BT, body temperature; BW, body weight; CBC, complete blood count; COX-1, cyclooxygenase 1; COX-2, cyclooxygenase 2; DBP, diastolic blood pressure; DI, deionized; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; PGE<sub>2</sub>, prostaglandin-E<sub>2</sub>; EP1, prostaglandin-E<sub>2</sub> receptor-1; EP2, prostaglandin-E<sub>2</sub> receptor-2; EP3, prostaglandin-E<sub>2</sub> receptor-3; EP4, prostaglandin-E<sub>2</sub> receptor-4; HCT, hematocrit; HGB, hemoglobin; HR, heart rate; HSD, high salt diet; H&E, hematoxylin and eosin; IL-6, interleukin 6; IP, intraperitoneal; JAK, janus kinase; LC-MS/MS, liquid chromatography with tandem mass spectrometry; LLOQ, lower limit of quantification; MAP, mean arterial pressure; MV, minute volume;  $\mu$ CT, microcomputed tomography; NaCl, sodium chloride; PBS, phosphate buffered saline; PD, Parkinson's disease; PDWc, platelet distribution width; PEG, polyethylene glycol; PI, post injection; PT, post treatment; PP, pulse pressure; p38 MAPK, p38 mitogen-activated protein kinase; RBC, red blood cells; RF, respiratory frequency; SBP, systolic blood pressure; SD, Sprague Dawley; STAT3, signal transducer and activator of transcription 3; TG6-10-1, potent and selective EP2 receptor antagonist; TG11-77, HCl, potent & selective EP2 receptor antagonist; TV, tidal volume; WBC, white blood cell; VOI, volume-of-interest; Veh, vehicle.

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## 1. Introduction

Inflammation is the natural response of the immune system challenged with either sudden or chronic insult. Inflammation is associated with neurodegenerative diseases, cancer, diabetes, and cardiovascular diseases [1,2]. Signaling pathways involved in inflammation include p38 MAPK, IL-6/JAK/STAT3, PI3K, Hippo [3], and COX-2 [4]. Activation of these inflammatory pathways is an essential step in restoring normalcy and maintaining homeostasis. However, prolonged activation leads to secondary damage in nearby cells and tissues. To prevent deleterious effects of prolonged inflammation, targeting the pathways with small molecule drugs could be an effective strategy. Prostaglandin-E<sub>2</sub> (PGE<sub>2</sub>), a lipid mediator that is ubiquitously present in tissues, is synthesized from arachidonic acid by cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2) as the rate limiting enzymes [2]. PGE<sub>2</sub> exerts its physiological and pathological effects via four G-protein coupled receptors (EP1, EP2, EP3, and EP4) [2,5]. PGE<sub>2</sub> signaling via EP2 is associated with increased inflammation in animal models of status epilepticus [6-9], Alzheimer's disease [10-12], Parkinson's disease [13], amyotrophic lateral sclerosis (ALS) [14], colitis [15], arthritis [16], skin inflammation [17], endometriosis [18], sepsis [19] cancer and tumor progression [20-25]. Moreover, in cell culture and mouse models lacking EP2, activation of innate immunity is accompanied by reduced cerebral oxidative damage [26], enhanced amyloid- $\beta$  (A $\beta$ ) phagocytosis [27], reduced neurotoxicity by A $\beta$ -peptide [27,28], and reduced A $\beta$ -peptide accumulation [29]. Based on these observations, inhibiting EP2 receptor pharmacologically could be an effective therapeutic strategy for reducing inflammation in a variety of diseases [1].

While studies listed above encourage the development and clinical advancement of EP2 antagonists for therapeutic applications, two studies published in 1999 raised serious questions about the viability of this target. Namely, one report showed that global EP2 KO in mice causes reduced reproduction rate and significant elevation in blood pressure with high salt diet compared to mice on regular diet [30]. Similarly, a study by Kennedy et al., showed that EP2 deletion in mice results in salt-sensitive hypertension [31]. Subsequently, two other studies using a selective EP2 agonist reported that activation of EP2 promotes healing of fractured bone in a canine model [32] and prevents the degeneration of articular cartilage during the early stage of osteoarthritis in rabbits [33], suggesting EP2 activation has beneficial roles in bone repair and healing.

Furthermore, evidence suggests that EP2 receptor function is associated with protective effects against fibroproliferative lung disease, aspirin-exacerbated respiratory disease (AERD), rhinitis and asthma [34-38]. However, it was not clear whether EP2 has any role in normal lung function, and if using an EP2 antagonist could lead to any adverse effects on respiratory function.

To promote EP2 antagonists for preclinical and clinical use for any disease indication, we must determine whether the adverse effects seen in EP2 KO animals are replicated by the antagonists in healthy animals. EP2 KO mice developed high systolic blood pressure only when they were fed high-salt diet, but not normal diet [30,31]. Therefore, the overall goal of this study was to test whether two EP2 selective antagonists, TG6-10-1 and TG11-77. HCl, by acute and chronic administration, have any adverse effects on cardiovascular function, respiratory function, bone morphology and cellular composition of blood in normal, non-disease mouse and rat models with or without high salt diet (HSD). We used two structurally different EP2 antagonists with different pharmacological profile; both mice and rats; two dosing protocols acute vs chronic. Gratifyingly, the results presented in this study reveal no adverse effects, and we conclude that the adverse effects observed in EP2 KO animals are not present with pharmacological EP2 antagonism.

## 2. Materials and methods

### 2.1. Materials

EP2 antagonists TG11-77. HCl and TG6-10-1 (chemical structures in [supplementary Table S1](#)) were synthesized and purified in our laboratory [6,39]. Pilocarpine was purchased from Sigma-Aldrich, prepared in saline and injected at dose of 12 mg/kg to induce changes in heart rate and blood pressure. Regular diet, containing 0.3% NaCl, was provided by Emory University DAR, for high salt diet the Teklad diet containing 3.15% NaCl was used.

### 2.2. Animals

All animal procedures were approved by the Emory University Animal Care and Use Committee and conformed to the guidelines of the National Institutes of Health. Animals, C57Bl/6 mice (8-16 weeks old) and SD rats (14-16 weeks old) used in this study were purchased from Charles River Laboratories. Multiple cohorts of animals were used in this study as shown in [Table 1](#).

### 2.3. EP2 antagonist doses tested

We have shown that EP2 antagonist TG6-10-1 is anti-inflammatory, neuroprotective and prevents cognitive deterioration in rats after status epilepticus at a dose of 10 mg/kg via intraperitoneal injection [8,9], therefore to assess the safety of this EP2 antagonist we selected 50 mg/kg (5-fold more than efficacious dose). Moreover, we have also shown that EP2 antagonist TG11-77. HCl.2H<sub>2</sub>O is anti-inflammatory in a mouse model of status epilepticus at 10 (free base 8.4) mg/kg I.P., and can be administered orally by supplanting in drinking water, therefore we used a projected oral dose of 100 (free base 84) mg/kg (>5-fold more than efficacious dose) in this study. We used pilocarpine (a muscarinic receptor agonist) at 12 mg/kg in the blood pressure and heart rate assays to verify the sensitivity of the instrument.

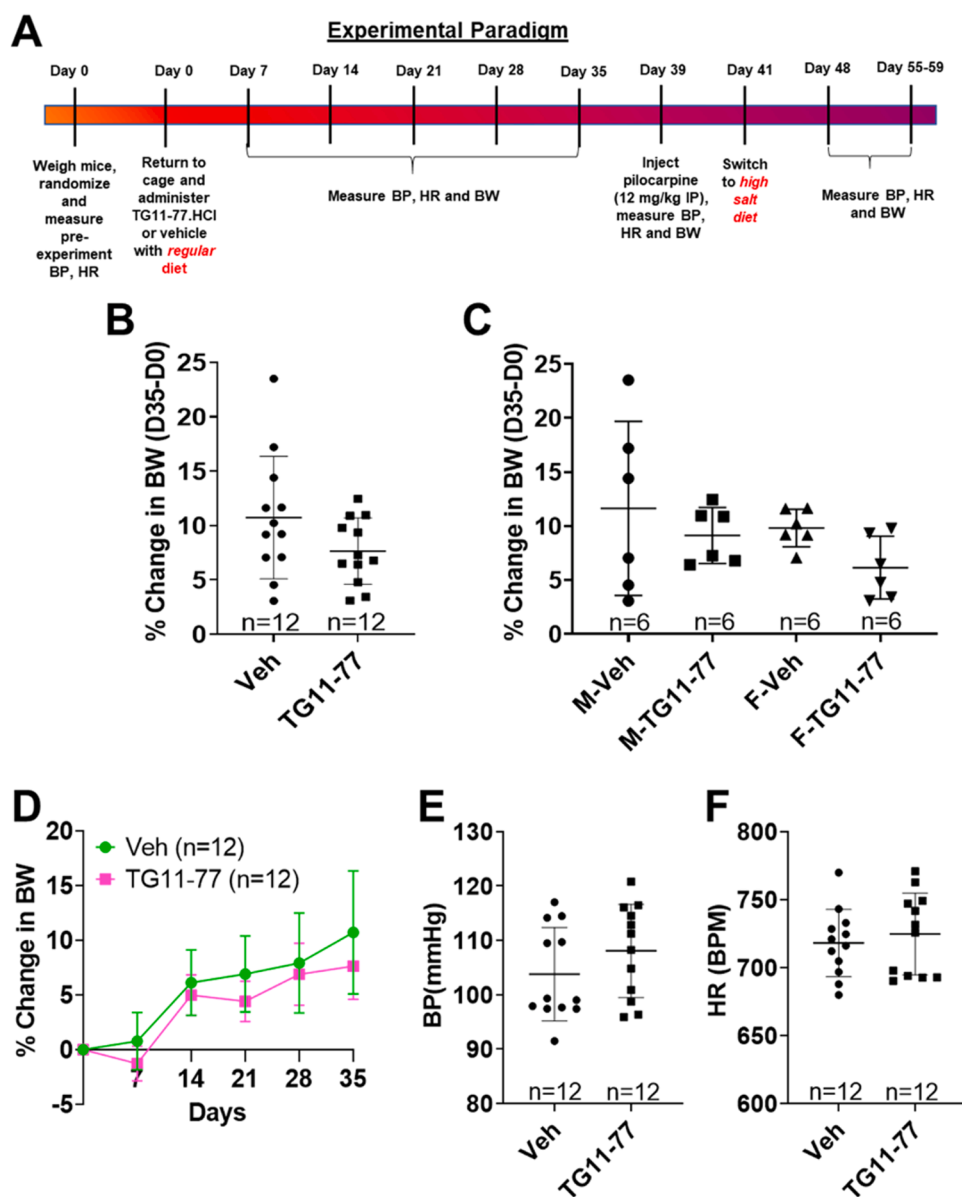
### 2.4. EP2 antagonist administration in mice and rats

TG11-77. HCl was dissolved in water at pH 2.8-3.5 at concentration of either 0.5 or 1.5 mg/ml with an aim to achieve a dosage of either 100 or 300 (free base 84 or 252) mg/kg/day respectively. Either water or water containing TG11-77. HCl was provided to cages containing 3 mice each for drinking. Consumption in ml was measured once a week and the average amount of TG11-77. HCl utilized for each mouse was calculated as mg/kg/day. The actual drug dose delivered was determined taking into account the 92.5% recovery from the drinking water on 7th day at room temperature, measured by HPLC. The resulting doses of drug are shown in [supplementary Table S1](#) and mentioned in the results section for individual experiments. EP2 antagonist TG6-10-1 (a free base) has low aqueous solubility so was dissolved in vehicle and administered via intraperitoneal (IP) injection (see details below).

The antagonist TG6-10-1 (EP2 K<sub>B</sub> = 17.8 nM, > 10-fold selective against other prostanoid receptors) has low water solubility (<25  $\mu$ M) with plasma half-life 1.7 h and brain-to-plasma ratio 1.7 after IP administration [40], whereas TG11-77. HCl (EP2 K<sub>B</sub> = 9.7 nM, > 300-fold selective against other prostanoid receptors) has plasma half-life of 2.4 hrs. brain-to-plasma ratio 0.4 by oral delivery, and high water solubility (2.52 mM), which facilitate chronic dosing in drinking water at the strength of 0.5 mg/ml [39]. These parameters guided us to administer TG6-10-1 acutely by IP-injection and TG11-77. HCl by oral dosing via drinking water. Moreover, the choice of the rodent is based on the technical considerations and test compound requirement for the planned experiments. For example, we preferred mice over rats for chronic dosing of antagonist due to limited amount of compound needed to treat the animals for nearly 2 months ([Fig. 1A](#)).

**Table 1**  
Animals, group numbers and dosing protocols used in experiments reported in this study.

Study (Cohort)	species	Animals size/ group	No. groups	Gender (approx. 1:1)	Dosing protocol	Measurement	Technique used
1	Mice	N = 12	2 (vehicle and one dose antagonist)	males & females	Chronic	BP and HR	Tail-cuff method
2	Rats	N = 12	2 (vehicle and one dose antagonist)	males	acute	BP and HR	Radiotelemetry
3	Rats	N = 8	4 (vehicle, 5, 20 and 50 mg/kg antagonist)	males	acute	Respiratory function	Plethysmography
4	Mice	N = 12	2 (vehicle and one dose antagonist)	males	chronic	Organ and brain toxicity and muscle weakness	Weights of vital organs and Rotarod
5	Mice	N = 28	2 (vehicle and one dose antagonist)	males & females	chronic	Immune cell regulation	CBC
6	Mice	N = 15	2 (vehicle and one dose antagonist)	males & females	chronic	Bone phenotyping	μCT and histology



**Fig. 1.** EP2 antagonist TG11-77.HCl (57 mg/kg/day for 5 weeks), treatment had no significant effect on mice body weight (BW), systolic blood pressure (SBP) and heart rate (HR). (A) Experimental paradigm for treatment of mice with EP2 antagonist, TG11-77.HCl to study its effect on blood pressure (BP), heart rate (HR) and body weight, and test the effect of TG11-77.HCl in comparison to control compound pilocarpine on BP and HR. (B) Change in body weight (BW) (%) in mice treated with either vehicle (water at pH 3.5) or TG11-77.HCl. Compared to vehicle, mice treated with TG11-77.HCl showed lower % increase in body weight over the course of 5 weeks of treatment 10.7 vs 7.64 that was not statistically significant ( $p = 0.11$ , unpaired t-test). (C) Effect of vehicle vs TG11-77.HCl on change in body weight (%) in male and female mice; the difference in weight gain observed in female mice (vehicle vs TG11-77.HCl) was compared to male mice. In female mice, the % weight increase in vehicle vs TG11-77.HCl treated mice was 9.83% vs 6.15% ( $p = 0.054$ , paired t-test), whereas in male mice vehicle treated animal showed an increase of 11.6% vs 9.13% in TG 11-77 treated mice ( $p = 0.73$ , paired t-test). (D) Weekly change in body weight (%) in mice treated with vehicle vs TG11-77.HCl over the period of 5 weeks. Mice treated with TG11-77.HCl showed marginal decrease in weight over the first week of treatment, but it recovered in weeks 2 through 5, with weight gain similar to vehicle treated mice. (E) Effect of vehicle vs TG11-77.HCl on the BP of mice over the course of 5 weeks of treatment. TG11-77.HCl treatment did not have any significant effect on BP compared to vehicle treated mice over the course of 5 weeks of treatment (104 mmHg vs 108 mmHg, vehicle vs TG11-77.HCl,  $p = 0.232$ , unpaired t-test). (F) Effect of vehicle vs TG11-77.HCl on the HR of mice over the course of 5 weeks of treatment. TG11-77.HCl treatment had no significant effect on HR compared to vehicle treated mice over the course of 5 weeks of treatment (718 BPM vs 725 BPM, vehicle vs TG11-77.HCl,  $p = 0.564$ , unpaired t-test).  $p$  value of  $< 0.05$  was considered significant. The data are presented as mean  $\pm$  SD.

## 2.5. Experimental paradigm for chronic treatment of mice with EP2 antagonist

A total of 24 mice (cohort 1) were used in this study,  $n = 12$  (6 males and 6 females) in vehicle group and EP2 antagonist TG11-77. HCl group. On day 0 (D0), after measuring body weight (BW), blood pressure (BP) and heart rate (HR) using a tail-cuff method, mice were randomized and separated in cages containing either vehicle (water at pH 3.5) or TG11-77. HCl (0.5 mg/ml) resulting in a measured average consumption of 57 mg/kg/day free base (see [supplementary Table S1](#)). This dose is 7–14-fold higher than the dose that achieves anti-inflammatory efficacy in an acute brain injury model of status epilepticus (Varvel et al., in preparation). BP, HR, and BW were measured weekly for 5 weeks. On D39, mice were injected intraperitoneally with 12 mg/kg of pilocarpine, a muscarinic receptor agonist that has been shown to elevate blood pressure and reduce heart rate [41–43], and BP and HR were measured as a test of the sensitivity of the apparatus. On D41 mice were switched to a high salt diet (HSD) for 14–19 days with their BP, HR and BW measured weekly and at the end of study (Fig. 1A).

## 2.6. Determination of blood pressure and heart rate in mice (cohort 1)

Systolic blood pressure (SBP) and heart rate (HR) of mice were measured using a tail-cuff method [44] with BP-2000 blood pressure analysis system (Visitech Systems), which measures BP and HR by transmission photoplethysmography. A total of  $n = 24$  animals, 12 males and 12 females were used in the study; animals were randomized into vehicle arm or TG11-77. HCl treatment arm (57 mg/kg/day), with both groups consisting of 6 males and 6 females. On the day of measurement, mice were acclimatized to the procedure room for 30 min before experiment. Then, 6 animals at a time were immobilized on the heated platform (37 °C) using adapters specific to mice. The mouse tail was put into tail-cuff and fixed using tape. Once immobilized, mice were given 10 min to get accustomed to being restrained on the platform before measurements were commenced. BP and HR were measured a total of 20 times with each recording episode being 45 s, with 5 trial readings followed by 15 experimental readings. For data to be considered for final analysis, at least 10 out of 15 readings had to be recorded, with a standard deviation of < 10%. If an animal's readings did not meet the criteria, it was rested for a minimum of 30 min and the measurements were redone. In case of pilocarpine injection, if an animal's readings did not meet the criteria it was excluded from final analysis, as it was not possible to reinject them and redo the measurements. Out of 24 animals, 6 were excluded for BP and 5 were excluded for HR. Output of the experiment was given by the software in the form of individual readings and average reading for BP and HR.

**Statistical analysis:** Paired or unpaired t-test were used for analysis depending on the requirement of the data set. GraphPad Prism was used for analysis.

## 2.7. Determination of blood pressure, pulse pressure, heart rate and body temperature in rats (cohort 2)

This study was performed by SRI Biosciences following our instructions. The objective of this study was to measure the effects of IP injection of TG6-10-1 on blood pressure (systolic, diastolic, and mean), pulse pressure, heart rate, and body temperature in conscious Sprague Dawley rats instrumented with a radiotelemetry transmitter. Pilocarpine was again used as a positive control compound. A total of  $n = 24$  animals were randomized and used in this study. Pilocarpine (12 mg/kg) and TG6-10-1 (50 mg/kg) in its vehicle (10% DMSO, 50% PEG400, and 40% DI water) were administered as a single dose by intraperitoneal injection into male Sprague Dawley rats following maintenance at low and high salt diets. Heart rate, arterial blood pressure (systolic, diastolic, pulse, and mean arterial pressure), and body temperature were collected every 2 min for at least 24 h prior to dietary change or treatment on

study days 7, 10, 11, 18, and 21. The same 12 animals from each group were dosed on study days 7 (normal salt diet) and 18 (high salt diet) with TG6-10-1 and on study days 10 and 21 with pilocarpine. The 24-hour analysis period was divided into three different phases, analysis phase 1 (0–2 h after drug injection), analysis phase 2 (2–14 h) and analysis phase 3 (14–24 h). Data are reported from analysis phase 1 as the measured parameters returned to basal levels by 2 h. On all other days, telemetry data were collected for 5 min every 60 min through the dosing period. Clinical observations were collected once daily beginning with administration of the regular diet (study day 0). Physical examination (piloerection, appearance, scabbing, presence of any anomalies on nose or neck, hair loss) were performed once weekly, and individual body weights were collected on each day of dosing (prior to dosing) and once weekly.

**Statistical analysis:** Analysis was performed for a period of 2 h post injection (PI) and was compared to baseline (BL) values for the corresponding time point before injection. For BL, data was averaged for the first 2 h and for PI data from every 30 min was used. 3-way ANOVA (time, diet, and treatment) with Tukey's multiple comparison test was used to analyze the data. GraphPad Prism was used for analysis.

## 2.8. Determination of respiratory function in rats (cohort 3)

This study was performed by SRI biosciences following our instructions. The objective of this study was to evaluate the acute respiratory effects of TG6-10-1 when given via intraperitoneal injection to Sprague Dawley rats. Vehicle (10% DMSO, 50% PEG400, and 40% DI water) or TG6-10-1 in the vehicle was administered as a single dose by intraperitoneal injection to 4 groups (Groups 1 through 4) of 8 male Sprague Dawley rats/group at 0, 5, 20, or 50 mg/kg, respectively ( $n = 32$ ). For measurement of respiratory function parameters, each rat was placed in a single-chamber, head-out, neck-sealed plethysmograph. Respiratory frequency, tidal volume, and calculated minute volume were collected for at least 60 min prior to dosing and for at least 5 h postdosing.

**Statistical analysis:** Analysis was performed for a period of 1 h post injection (PI) and was compared to 0 mg/kg value for the corresponding time point post injection. 2-way ANOVA with Tukey's multiple comparison test was performed for analyzing the data. GraphPad Prism was used for analysis.

## 2.9. Determination of organ toxicity and brain-to-plasma ratio in mice (cohort 4)

C57BL/6 male mice at 8 weeks of age ( $n = 24$ ) were subjected to either vehicle (i.e., drinking water at pH 3.5) or TG11-77. HCl (average of 62 mg/kg/day) treatment through drinking water. After 4 weeks of treatment, mice were perfused with ice cold PBS to harvest different organs. Before perfusion, blood was collected by cardiac puncture and stored in EDTA tubes for further processing to separate plasma. Heart, liver, kidney, and spleen were collected, and wet weights immediately measured. Brain tissues were dissected to separate into neocortex and hippocampus before measuring their weights. All organ weights were normalized to the body weights of the individual mice. For determination of compound in brain and plasma, blood samples were collected in microcentrifuge tubes containing K2EDTA as anticoagulant. Plasma was separated by centrifugation of whole blood and stored below  $-70$  °C until analysis. Whole brain samples were homogenized using phosphate buffered saline (pH 7.4) and the total homogenate volume was thrice the brain weight. All samples were processed for analysis by protein precipitation using acetonitrile (ACN) and analyzed by LC-MS/MS (lower limit of quantification (LLOQ) was determined to be 1.23 ng/ml). Waters Xterra MS C18 column was used with flow rate 0.8 ml/min using the mobile phase 0.1% formic acid in acetonitrile (pump A) and 0.1% formic acid in water (pump B). Internal standard glipizide (ret. time 1.60 min) was used to quantify the peaks and the ratio was used for concentration

analysis in the brain and plasma. The bioanalysis of the samples was done at Sai-life (CRO company), India.

**Statistical test:** Unpaired *t*-test with Bonferroni correction was applied between groups for different organ weights. GraphPad Prism was used for analysis.

#### 2.10. Rotarod test (cohort 4)

Rotarod test trials were performed using the Rotamex rotarod (Columbus Instruments) in an isolated room. The device was set in an accelerating mode with initial speed at 4 rpm and increasing linearly to 40 rpm over 5 min. On the testing days, mice ( $n = 24$ ) were transported to the rotarod room two hours before the trials started. Either TG11–77.HCl (62 mg/kg/day) or vehicle treated mice were placed on the moving rod (1.5-inch diameter) separated by plexiglass sheets and the latency to fall from the rod was recorded for each mouse using automated timers set at the platform. Each day mice were subjected to 4 trials for 5 min each and after each trial, they were housed in the resting cage for 20 min before their next trial. If the mice did not fall at the end of the trial, their latency to fall was measured as 300 s. Mice were tested on day 0, 7, 14, 21 and 28 and their average latency to fall over days was represented as an index of muscle coordination upon drug treatment.

**Statistical analysis:** Two-way repeated measure ANOVA with Sidak's multiple comparisons test was applied for rotarod analysis. GraphPad Prism was used for analysis.

#### 2.11. Determination of complete blood count in mice (cohort 5)

Samples of fresh arterial blood were analyzed for complete blood count (CBC) in 20-week-old males and females ( $n = 56$ ), treated with vehicle (drinking water at pH 3.5) or TG11–77.HCl (58 mg/kg/day) in drinking water for 12 weeks. CBC analysis was performed by the Quality Assurance & Diagnostic Lab, Division of Animal Resources, Emory University. Blood samples were collected into K2EDTA tubes, stored at 4 °C and analyzed within 24 H by a VetScan HM5 v2.3 Hematology Analyzer.

**Statistical analysis:** Multiple unpaired *t*-test with Bonferroni correction for multiple comparisons was applied between groups.  $p = 0.05$  was selected as the threshold for change. GraphPad Prism was used for analysis.

#### 2.12. Bone phenotyping analysis in mice by MicroCT (cohort 6)

C57BL/6 mice of both sexes at 10 weeks of age ( $n = 30$ ) were randomly subjected to either vehicle (drinking water with adjusted pH 2.8) or TG11–77.HCl (134 mg/kg/day) in vehicle. After 4 weeks of treatment, mice were sacrificed by overdose of isoflurane and bones were collected immediately from both hind limbs. Tibia and femur were separated, and the muscles and ligaments were carefully removed using a tweezer. They were then fixed in 4% paraformaldehyde solution in PBS overnight and stored in 70% ethanol at 4°C till further analyzed.

Bone samples were shipped to the Microcomputed Tomography ( $\mu$ CT) Core at Georgia Tech, Atlanta for bone phenotyping analysis. Tibia and femur pairs were immersed vertically in specimen holders and supported by foam pieces to maintain orientation.  $\mu$ CT scans of the long bone mid-diaphysis and the metaphysis near the knee joint were performed using a Scanco CT40 (6  $\mu$ m voxels, 300 ms). For the diaphyseal scans, the length of the long bone was estimated from scout view images and 158 slices (6  $\mu$ m) straddling the approximate mid-point were acquired. For tibial metaphyseal scans, 368 slices starting at the proximal end of the tibia were acquired to encompass the proximal metaphysis. Similarly, for femoral metaphyseal scans, 525 slices including the distal end of the femur were acquired to encompass the distal metaphysis. Each mid-diaphyseal scan was manually contoured at the initial slice around the outer margins of the cortex and then automatically adjusted with the native software for best fit around the cortex. The contouring

was then automatically progressed to the remaining slices. Bone volume and density parameters were estimated from all mid-diaphyseal scans. For both metaphyseal scans, all images were first run through an auto-contouring script to delineate the boundary of the trabecular region within the cortical shell. Subsequently, 100 slices starting at a fixed number of slices distal to the end of the growth plate (and moving towards the mid-diaphysis, away from the knee joint) were analyzed after further manual adjustments of the contours as needed to better define the trabecular regions or margins. Total bone volume, density, and trabecular network parameters were estimated for all samples (Supplementary Fig S2).

**Statistical analysis:** Unpaired *t*-test with Holm-Sidak correction for multiple comparisons was applied between groups.  $p = 0.05$  was selected as the threshold for change. GraphPad Prism was used for analysis.

#### 2.13. Bone histology in C57BL/6 mice treated either with vehicle or TG11–77.HCl (cohort 6)

The bone samples from the bone phenotyping experiment were used for this analysis. Tibia and femur samples were fixed in 10% neutral-buffered formalin and then stored in 70% ethanol until processed. Samples were decalcified then processed for standard histology and stained with hematoxylin and eosin. Histological sections were examined under a light microscope (Olympus BX41) by a board-certified veterinary pathologist. Representative images were prepared using a digital camera (Olympus DP25) and a connected imaging software (Olympus cellSens Standard). Samples were analyzed blindly. Cancellous bone density for each bone section was qualitatively scored on a scale of 0–5 (0 = most depleted cancellous bone density and 5 = normal density of cancellous bone).

**Statistical analysis:** Unpaired *t*-test with Holm-Sidak correction for multiple comparisons was applied between groups.  $p = 0.05$  was selected as the threshold for change. GraphPad Prism was used for analysis.

### 3. Results

#### 3.1. Chronic exposure to high dose EP2 antagonist did not significantly alter body weight of mice

Compared to vehicle, mice treated with TG11–77.HCl (57 mg/kg/day) showed lower % increase in body weight over the 5 weeks of treatment (7.64% increase versus 10.7%, Fig. 1B). However, this observed difference was not statistically significant ( $p = 0.11$ ). In female mice, the weight increase in vehicle versus TG11–77.HCl treated mice was 9.83% versus 6.15% ( $p = 0.054$ ), whereas in male mice vehicle treated animals showed an increase of 11.6% versus 9.13% in TG11–77.HCl treated mice ( $p = 0.73$ ) (Fig. 1C). It is worth noting that mice on vehicle treatment trended an increase of 0.8% after one week, whereas TG11–77.HCl treated mice trended a decrease of 1.32% body weight (Fig. 1D), but these observations are also statistically non-significant ( $P > 0.05$ , two-way ANOVA).

#### 3.2. Chronic TG11–77.HCl treatment did not alter mouse blood pressure (BP) or heart rate (HR)

To determine the effect on cardiovascular system, we used a tail-cuff method on mice to measure the systolic blood pressure (SBP) and HR every week. After five weeks of treatment with TG11–77.HCl (57 mg/kg/day), there was no significant difference in the blood pressure of vehicle treated mice versus TG11–77.HCl treated mice (Fig. 1E). Furthermore, no significant difference was observed in BP of mice treated with either vehicle or TG11–77.HCl, on any given week during the treatment (Supplementary Fig. S1A).

Similarly, mice treated with TG11–77.HCl for 5 weeks showed no

significant difference in HR compared to vehicle treated mice (Fig. 1F). There was a significant increase in heart rate of mice after one week of treatment, but this increase in heart rate was similar in cohort treated with TG11-77. HCl and vehicle, suggesting the increase in heart rate might be a vehicle (pH 3.5 water) effect (Supplementary Fig. S1B).

To test the effectiveness of our experimental procedure in measuring changes in BP and HR we used pilocarpine (12 mg/kg) as a positive control. Mice showed a significant increase in BP (95.2 mm Hg versus 105 mm Hg,  $p < 0.05$ ) (Fig. 2A) within 30 min of pilocarpine injection. Treatment with pilocarpine resulted in significant reduction of HR (724 bpm versus 419 bpm,  $p < 0.0001$ ) (Fig. 2C). However, in mice treated with vehicle versus EP2 antagonist TG11-77. HCl, there was no significant difference in the pilocarpine-induced change in BP (Fig. 2B) and HR (Fig. 2D). These results together indicate that EP2 antagonist TG11-77. HCl does not alter the cardiac response of mice, either under basal conditions or after pilocarpine.

### 3.3. TG11-77.HCl did not alter blood pressure and heart rate of mice on high sodium (salt) diet (HSD)

As shown in Fig. 1A, we used the same cohort of mice, but switched them from regular diet to high-salt diet on day 41 and continued until Day 55–59 on high-salt diet with vehicle or EP2 antagonist treatment. Mice treated with a diet containing 10-fold elevated sodium showed a significant increase in their systolic blood pressure (SBP) after 14–19 days, compared to pre-HSD systolic blood pressure (109 mmHg versus 102 mmHg,  $p < 0.01$ ) (Fig. 2E). When analyzed separately, this difference in systolic blood pressure observed in males was not statistically significant (113 mmHg versus 109 mmHg,  $p = 0.25$ ) (Fig. 2F), but in females, HSD resulted in significant increase in systolic blood pressure (105 mmHg versus 96.1 mmHg,  $p < 0.05$ ) (Fig. 2G). However, TG11-77. HCl (57 mg/kg/day) did not alter the effect of a high salt diet on systolic blood pressure (Fig. 2H).

There was a significant elevation in HR of mice post-HSD versus pre-HSD (735 bpm versus 684 bpm,  $p < 0.0001$ ) (Fig. 2I). This increase in post HSD HR was independent of sex with significant changes observed in both male (741 bpm versus 708 bpm,  $p < 0.05$ ) (Fig. 2J) and female (729 bpm versus 662 bpm,  $p = 0.0001$ ) (Fig. 2K). However, change in HR of mice treated with vehicle versus TG11-77. HCl, pre- versus post-HSD was not significantly different ( $\Delta$ HR post versus pre-HSD 55.9 bpm versus 45.3 bpm,  $p = 0.54$ ) (Fig. 2L). These data indicate that the cardiac effects of the global EP2 KO [30,31] are not replicated by chronic exposure of adult mice to an EP2 antagonist.

### 3.4. TG11-77.HCl had no adverse effect on motor behavior and vital organ weight in mice

A separate cohort of mice (cohort 2,  $n = 24$ ) was used to ask whether chronic administration of this EP2 antagonist adversely affects overall health, and to determine if therapeutic drug levels are found in the plasma and the brain. In this study, treatment with TG11-77. HCl (62 mg/kg/day) for 4 weeks did not have significant effect on body weight (Fig. 3A) or motor performance of mice when compared to vehicle as determined by the rotarod test by measuring latency to fall (Fig. 3B). Similarly, 4 weeks of TG11-77. HCl treatment did not change the weight of internal organs such as heart, spleen, kidney, and liver along with no differences observed in weight of brain tissues cerebral cortex, hippocampus, and striatum (Fig. 3C), indicating no overt toxicity by chronic treatment with the EP2 antagonist. We analyzed the concentration of TG11-77. HCl in plasma and brain of the mice used in this study and found about 3-fold higher total concentration of the TG11-77 in plasma, and 4.5-fold higher in the brain than its Schild potency against EP2 receptor (Schild  $K_B = 9.7$  nM) [39] with brain-to-plasma ratio of 1.4. (Fig. 3D).

### 3.5. Chronic TG11-77.HCl administration does not affect cellular composition and characteristics of blood in mice

To determine whether chronic administration of TG11-77. HCl altered the cellular composition of blood in mice, we investigated a separate cohort of mice (cohort 3,  $n = 56$ ) after treatment with TG11-77. HCl. Interestingly, TG11-77. HCl treatment (58 mg/kg/day) for 12 weeks did not produce any significant changes in complete blood counts, namely, red blood cells (RBCs), lymphocytes, monocytes, neutrophils and platelets (Fig. 4A), although monocyte counts trended lower. Similarly, hemoglobin, hematocrit levels, red cell distribution width (RDWc) and platelet cell distribution width (PWDs) were unaltered (Fig. 4B). These observations suggest that chronic dosing of EP2 antagonist has no adverse effect on cellular composition of blood.

### 3.6. Acute administration of EP2 antagonist, TG6-10-1, did not alter cardiovascular function in rats

The above results indicate that chronic treatment of mice with the EP2 antagonist produced no effects on BP and HR. To generalize this conclusion we investigated a second EP2 antagonist, TG6-10-1, which displayed anti-inflammatory and neuroprotective efficacy in multiple models of brain injuries [6,8,24,45-49]. Here we utilized rats ( $n = 12$ ) as animal species and used acute high dose administration of TG6-10-1 to study the effect on cardiovascular function. We studied the effects of TG6-10-1 on blood pressure (systolic, diastolic, and mean), pulse pressure, heart rate, and body temperature in conscious Sprague Dawley rats instrumented with a radiotelemetry transmitter.

Acute administration by single intraperitoneal injection of TG6-10-1 at 50 mg/kg showed no significant change in cardiovascular function parameters of rats including heart rate (HR) (Fig. 5A), systolic blood pressure (SBP) (Fig. 5B), diastolic blood pressure (DBP) (Fig. 5C), mean arterial pressure (MAP) (Fig. 5D), pulse pressure (PP) (Fig. 5E) as well as body temperature (BT) (Fig. 5F). This dose is 5-fold higher than the efficacious dose [8,9]. Moreover, when animals after 7 days of high sodium diet (HSD) were injected with TG6-10-1, it had no effect on any of the cardiovascular parameters measured (Fig. 5). As expected, the positive control pilocarpine (12 mg/kg, IP injection) significantly decreased HR and BT (Fig. 5A and F), significantly increased SBP (Fig. 5B), DBP (Fig. 5C), MAP (Fig. 5D) and PP (Fig. 5E) in rats irrespective of their diet.

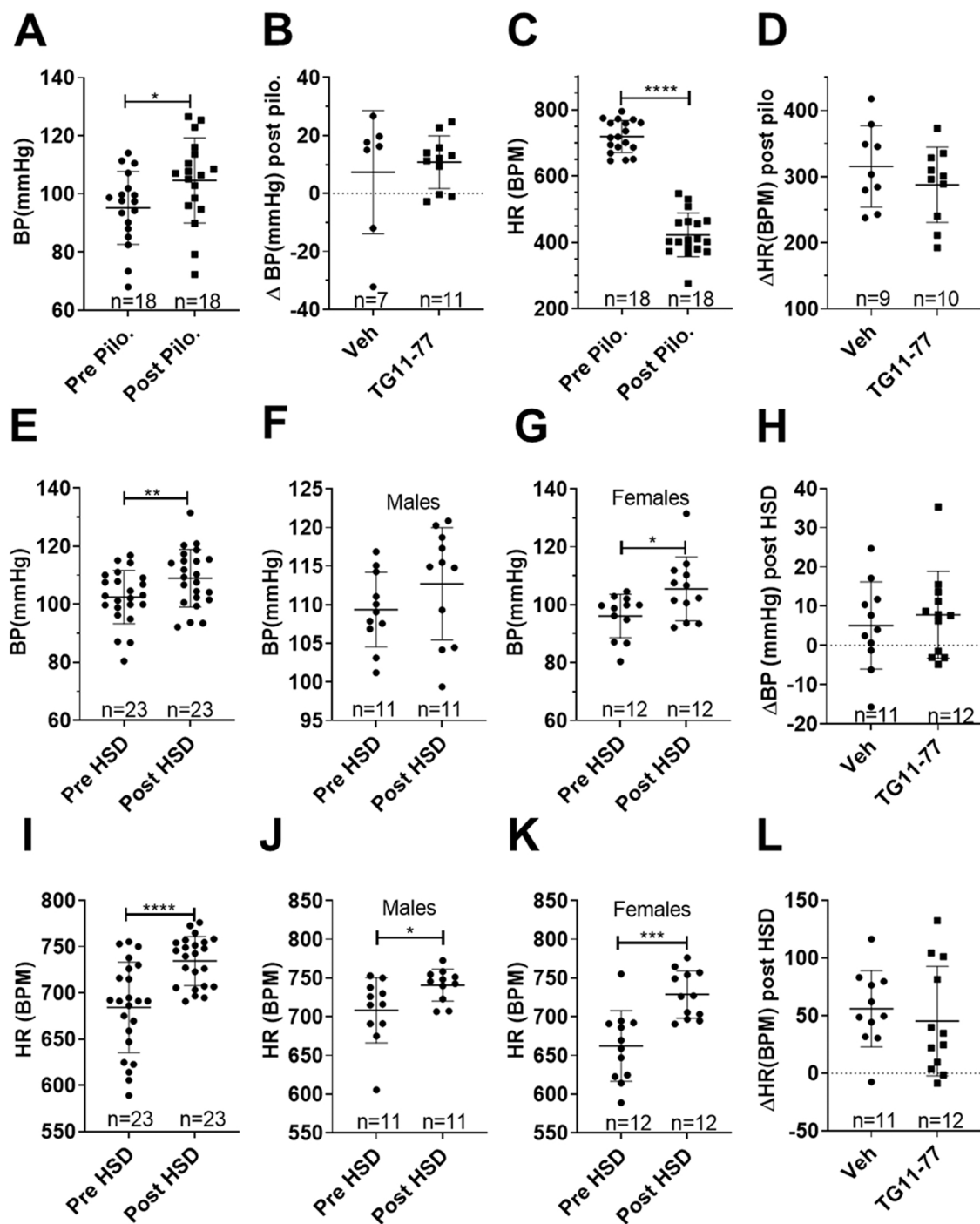
### 3.7. Acute TG6-10-1 administration did not modify respiratory function in healthy rats

To study the effect of EP2 antagonist on respiratory function, respiratory assessment was performed following single intraperitoneal injection of TG6-10-1 in plethysmograph-restrained Sprague Dawley rats (separate cohort,  $n = 32$ ) that were treated with vehicle and three different doses of TG6-10-1. There was no significant effect observed on respiratory function of rats determined by breaths per minute after single injection of TG6-10-1 at the doses 5 mg/kg, 20 mg/kg, and 50 mg/kg when compared to vehicle injected rats (see methods for details). All three parameters of respiratory function, respiratory frequency (Fig. 6A), tidal volume (Fig. 6B), and minute volume (Fig. 6C) showed similar values in 15-minute intervals at different doses of TG6-10-1 for up to 60 min after injection (Fig. 6).

### 3.8. Chronic treatment with EP2 antagonist TG11-77.HCl does not affect bone volume and bone density in naïve mice, but results in slight reduction in trabecular number only in male mice

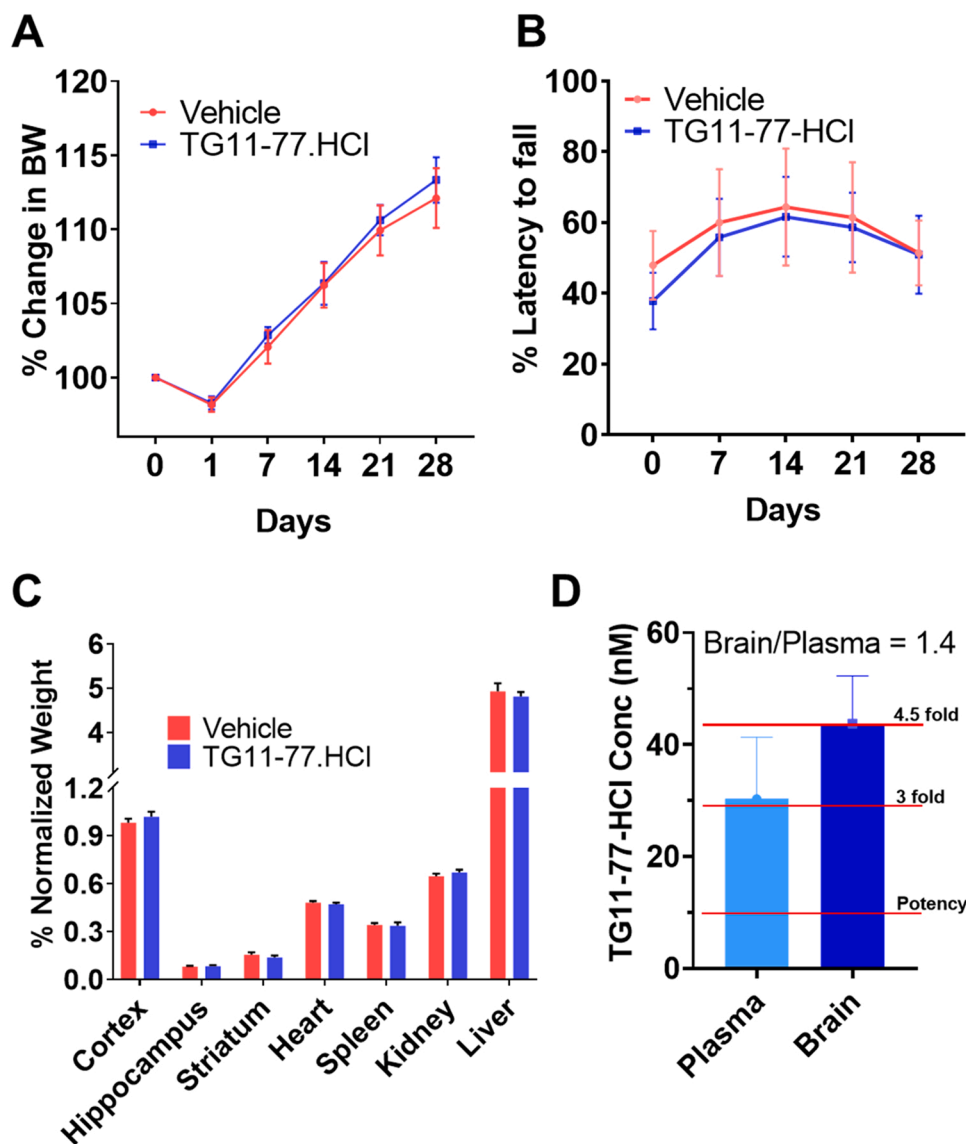
Given the beneficial effect of an EP2 agonist in a model of bone injury [32,33], we asked whether pharmacological antagonism of EP2 has any detrimental impact on bone volume and density in healthy mice. We used a separate cohort of mice (cohort 4,  $n = 30$ ) and tested the





**Fig. 2.** EP2 antagonist TG11-77.HCl, had no significant effect on mice systolic blood pressure (SBP) and heart rate (HR) on regular diet and high-salt diet. Effect of pilocarpine (pilo) injection (12 mg/kg) on blood pressure (BP) (A) of mice on D39 compared to pre-pilo (D35). Pilocarpine treatment resulted in elevated BP of mice vs pre-pilo levels (104 mmHg vs 95.2 mmHg,  $p < 0.05$ , paired t-test). Pilocarpine treatment resulted in significant reduction in heart rate (HR) (C) of mice, when compared to pre-pilo levels (419 bpm vs 287 bpm,  $p < 0.0001$ , paired t-test). Change in BP and HR of mice in vehicle vs TG11-77.HCl group did not differ significantly upon treatment with pilocarpine. Change in BP was 7.3 mmHg in vehicle group vs 10.8 mmHg in TG11-77.HCl group ( $p = 0.69$ , unpaired t-test with Welch's correction, with an anticipation of unequal variance) (B). In case of HR, in vehicle group the change after pilo was 315 vs 287 in TG11-77.HCl group ( $p = 0.32$ , unpaired t-test with Welch's correction) (D). Effect of high salt diet (HSD) on BP and HR of mice. After switching to HSD, there was a significant increase in BP of mice compared to pre-HSD levels (108 mmHg vs 102 mmHg,  $p < 0.01$ , paired t-test) (E). This effect of HSD on systolic blood pressure was observed both in male (112 mmHg vs 109 mmHg,  $p = 0.25$ , paired t-test) (F) and female (105 mmHg vs 96.1 mmHg,  $p < 0.05$ , paired t-test) (G) mice pre- and post-HSD. Effect of TG11-77.HCl on HSD induced BP change. There was no significant effect observed in change of systolic BP pre- and post-HSD in mice treated with vehicle vs

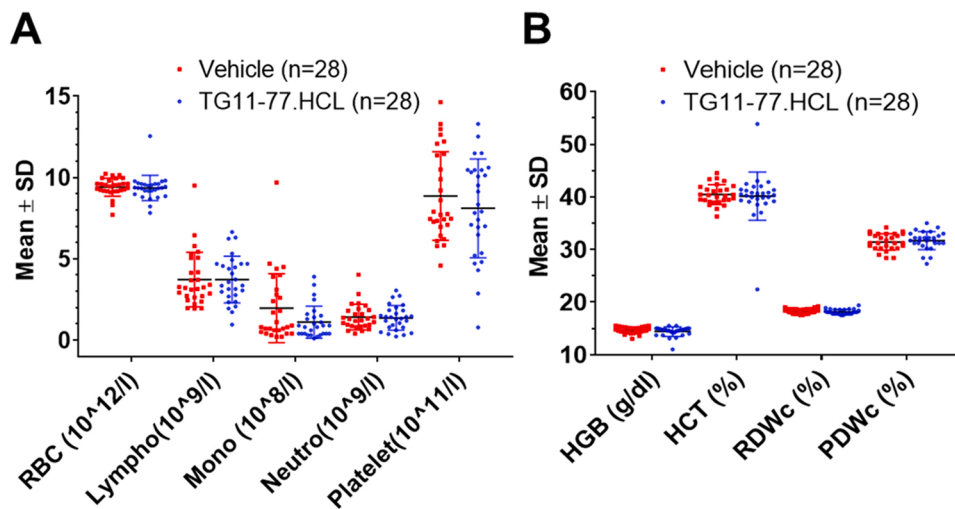
TG11-77.HCl ( $\Delta$ BP (post vs pre-HSD) 5.05 mmHg vs 7.79 mmHg,  $p = 0.56$  respectively, unpaired t-test with Welch's correction) (H). There was a significant elevation in HR of mice pre-HSD vs post-HSD (684 bpm vs 734 bpm,  $p < 0.0001$ , paired t-test) (I). This increase in post HSD HR was independent of sex of mice with significant changes observed in both male (708 bpm vs 741 bpm,  $p < 0.05$ , paired t-test) (J) and female (662 bpm vs 728 bpm mice,  $p = 0.0001$ , paired t-test) (K). Change in HR of mice treated with vehicle vs TG11-77.HCl, pre vs post HSD was not significantly different ( $\Delta$ HR post vs pre-HSD 45.3 bpm vs 55.9 bpm,  $p = 0.54$  respectively, unpaired t-test) (L). These observations show that TG11-77.HCl does not alter changes in BP and HR induced by HSD.  $p < 0.05$  was considered significant. The data are reported as mean  $\pm$  SD.



**Fig. 3.** EP2 antagonist TG11-77.HCl (62 mg/kg/day for 4 weeks), had no adverse effect on motor behavior and organ composition. (A) Body weight of mice treated with TG11-77.HCl did not differ from mice treated with vehicle. (B) Performance on Rotarod (% of total 300 s time spent on before fall) test shows no difference at any time point. (C) weight changes to vital organs- liver, heart, spleen, kidney and brain tissues also show no difference. Organ and brain region weights are normalized to body weight. Two-way repeated measure ANOVA with Sidak's multiple comparisons test was applied for Rotarod and body weight analysis. Unpaired t-test with Bonferroni correction was applied between groups for different organ weights. P values were set to be significant at  $* < 0.05$  (D) Chronic oral dosing of TG11-77.HCl in drinking water achieves 3-fold more concentration in plasma and 4.5-fold more in brain than the Schild potency ( $K_B$ ) of the molecule which is 9.7 nM [39], with a brain-to- plasma ratio 1.4. The data with  $\pm$  SEM. N = 24 mice were used in this study.

effect of chronic TG11-77. HCl treatment on bone phenotype. Both male and female C57BL/6 mice were treated with vehicle or TG11-77. HCl (134 mg/kg /day) in drinking water for 4 weeks. The tibia and femur from hind limbs were subjected to Microcomputed Tomography ( $\mu$ CT) scan to analyze the bone mass through diaphyseal scan and trabecular network through metaphyseal scan (Supplementary Fig. S2). The diaphysis of 0.6 mm volume-of-interest (VOI) at the mid shaft of tibia and femur did not show any difference in bone volume and density between TG11-77. HCl and vehicle treated mice in both males (Fig. 7A-C) and females (Fig. 7D-F). The TG11-77. HCl treatment also did not show any difference in the bone volume and density in metaphyseal scan at the growth plates of tibia and femur in both sexes, revealing there was no adverse effect of the TG11-77. HCl treatment in these mice (Fig. 7A-F). Interestingly, the metaphyseal scan of the trabecular network showed a significant decrease in trabecular numbers

upon TG11-77. HCl treatment only in males without altering the trabecular thickness and spacing (Supplementary Fig. S3). In contrast to the males, the trabecular numbers in females were found to be moderately increased but, as in males, no changes were observed in trabecular thickness and spacing (Supplementary Fig. S3). To evaluate further the effect of TG11-77HCl on trabecular network, in particular in trabecular number in male mice, we sectioned the fixed and decalcified samples of tibia and femur and analyzed by histology using H&E staining. We found no difference in cancellous bone density between the groups treated with vehicle or EP2 antagonist (supplementary Fig. S4). This result is discordant with the finding from microCT (Fig. S3 A-B). Overall, we conclude that treatment with the EP2 antagonist at high dose for up to 1 month is unlikely to have a detrimental effect on bone density of healthy animals.



**Fig. 4.** Chronic treatment with EP2 antagonist TG11-77.HCl (58.1 mg/kg/day for 12 weeks), did not have an effect on blood cell counts in mice: (A) No differences were found in RBCs, Platelets and different population of WBCs after 12 weeks of oral dosing with TG11-77.HCl compared to vehicle, suggesting no peripheral effects of the drug in blood. (B) TG11-77.HCl treatment did not have any adverse effect on the level of hemoglobin (HGB), hematocrit (HCT) and cell distribution width of RBC and platelets in these mice. Data was statistically analyzed in GraphPad Prism. Multiple unpaired t-Test with Bonferroni correction was applied between groups. The data are with  $\pm$  SD.

#### 4. Discussion

In spite of the breadth of preclinical data suggesting the value of EP2 antagonists [50], there are limited advances towards clinical development. This is in part due to two published results that indicated EP2 global deletion reduces reproductive capability and arterial blood pressure and induces salt-sensitive hypertension [30,31]. Therefore, exacerbating salt-sensitive hypertension would effectively preclude the chronic use of EP2 antagonists in humans. However, these studies could not distinguish between pharmacologically (acute or chronic) diminished EP2 activation and a developmental defect in these mice. Other studies reported that EP2 agonists promote healing of bone fractures [32], raising further doubts about potential adverse effects of EP2 antagonists. In brief we found no effects of high dose pharmacologic EP2 block on cardiovascular or respiratory parameters, bone structure or blood cell counts.

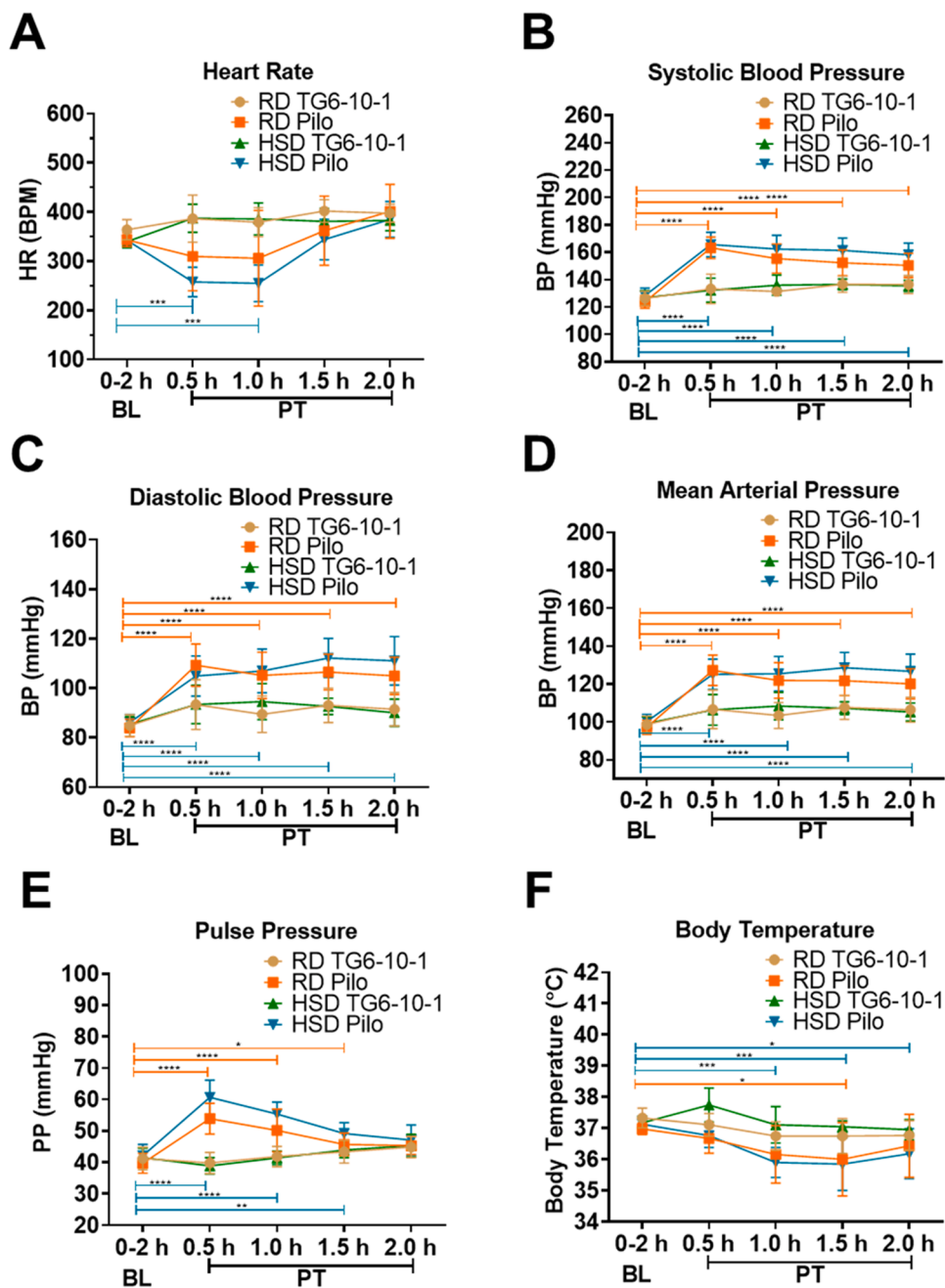
We tested two EP2 antagonists in adult EP2-expressing rodents: one antagonist (TG11-77. HCl) by chronic oral administration in mice, and another (TG6-10-1) by acute injection into rats. It is worth emphasizing that the cardiovascular and fertility adverse effects were only reported in EP2 global KO mice, and beneficial effects were reported in normal mice with the use of EP2 agonist. We wanted to monitor potential adverse effects in more than one rodent species (mouse and rat), using more than one EP2 antagonist, and by chronic and acute dosing protocols so the results can be generalized.

Chronic treatment with an EP2 antagonist showed no adverse effect on cardiovascular function in mice (Figs. 1, 2). Moreover, acute administration of an EP2 antagonist at high dose in rats was without any effect on cardiovascular (Fig. 5). These results are in discord with earlier findings, which indicated that EP2 gene KO results in reduced systolic blood pressure in mice on regular diet in one study [30], increased baseline systolic blood pressure in mice with high-salt diet in another study [31]. The discordance between our findings and previous reports using EP2 gene KO animals may be attributed to role of EP2 in development at prenatal and postnatal stage, which further complicates the co-morbidities such as cardiovascular, respiratory function and fertility efficacy. Our experimental results show that acute IP administration of an EP2 antagonist does not have any adverse effect on normal respiratory function in rats (Fig. 6). However, our study is limited to use of healthy mice and rats, therefore, further research in animals with underlying respiratory disease, such as asthma, would be a logical next step

to have a better understanding of the effects of EP2 antagonism on respiratory and cardiovascular functions.

EP2 receptor activation is associated with acute and chronic inflammation and autoimmune diseases [2]. EP2 receptor's involvement in inflammation and immune response is well established in both in-vitro [51-54] and in-vivo models [17,51]. Therefore, we intended to determine whether chronic treatment EP2 would induce any immunogenic responses in the blood. Our study showed no difference in blood composition of healthy mice treated with either vehicle or EP2 antagonist, TG11-77. HCl (Fig. 4). This observation further enhances the chance of pharmacological inhibition of EP2 to be a viable therapeutic target.

An EP2 agonist, when directly injected into the bone marrow, induced bone healing in canine model of bone fracture [32], and administration of PGE<sub>2</sub> (agonist, via EP2) prevented degeneration of articular cartilage during the early stages of osteoarthritis in rabbits [33], suggesting EP2 activation can play a role in bone repair and healing. On the other hand, activation of EP4 receptor, but not EP2, rescued the impaired bone healing [55], and EP2 deletion in mouse cultures impairs osteoclastogenesis [56], suggesting EP2 antagonism might be beneficial. Considering these mixed results, we tested an EP2 antagonist for adverse effects on bone phenotype. A high dose of EP2 antagonist TG11-77. HCl (134 mg/kg/day freebase) in drinking water for 28 days did not have any effect except number of bone trabeculae in the marrow cavity in males that was significantly reduced in the treated group. This observation can be explained by the role of prostaglandins in bone resorption and formation. It has been shown that systemic injection of prostaglandins can increase bone resorption and formation, but bone formation usually exceeds resorption; therefore, prostaglandin receptor antagonists can induce bone resorption [57]. Furthermore, prostaglandin treatment results in increased bone mass in male rats. Therefore, EP2 receptor antagonists could cause bone loss in male rats [58]. This observation must be further investigated to understand the interplay between sex hormones and TG11-77. HCl. A limitation of our study is that we did not investigate EP2 antagonist efficacy in a fractured-bone model. Therefore, our results further warrant exploration of EP2 antagonist efficacy in rheumatological models such as osteoarthritis and rheumatoid arthritis, or injured bone-models to understand whether EP2 receptor signaling will promote the activity of cells that are involved in bone remodeling process.

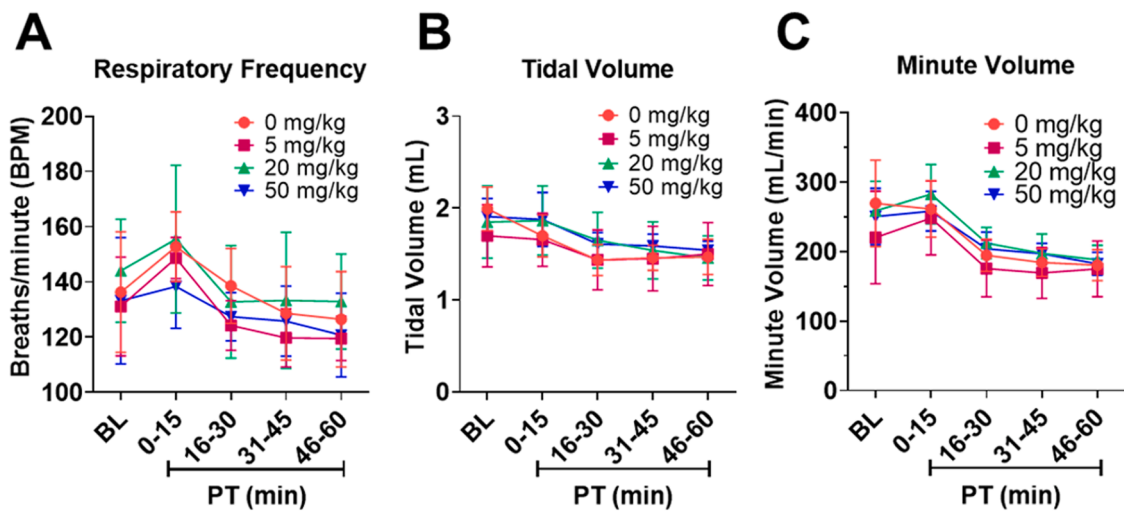


**Fig. 5.** Acute administration of EP2 antagonist TG6-10-1 (50 mg/kg), in rats does not effect the cardiovascular function. Male SD-Rats on either Regular Diet (RD) or High Salt Diet (HSD) were injected with TG6-10-1 (50 mg/kg) and their cardiovascular function was monitored for 2 h. Compared to baseline levels (BL) there was no significant effect of TG6-10-1 on Heart Rate (HR) (A), Systolic Blood Pressure (SBP) (B), Diastolic Blood Pressure (DBP) (C), Mean Arterial Pressure (MAP) (D), Pulse Pressure (PP) (E) and Body Temperature (BT) (F) of animals. Whereas pilocarpine, the positive control in the experiment, resulted in significant changes SBP, DBP, MAP, PP and BT of animals on LSD and HR, SBP, DBP, MAP, PP and BT of animals on HSD. Statistical analysis was done using 3-way ANOVA with Tukey's multiple comparison,  $p < 0.05$  was considered significant. \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ , \*\*\*\* =  $p < 0.0001$ . The data are with  $\pm$  SD.

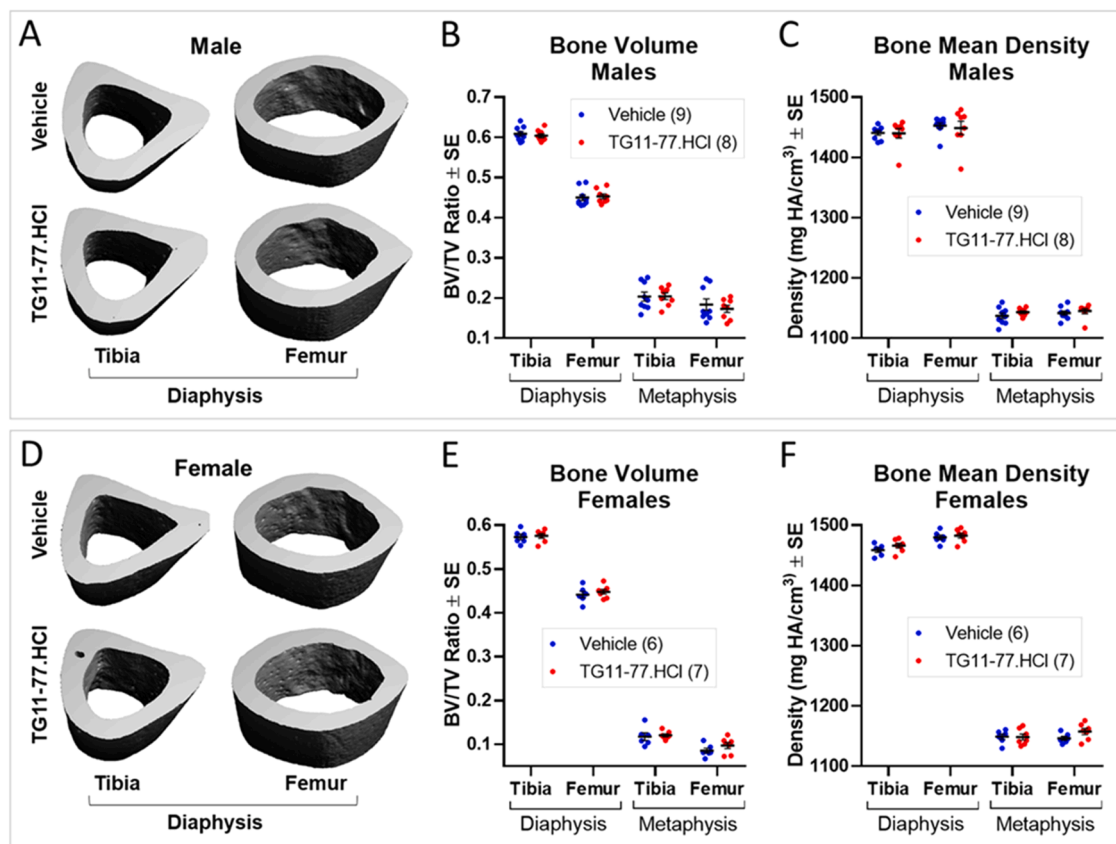
## 5. Conclusion

A number of inflammatory CNS and peripheral diseases are exacerbated by excessive PGE<sub>2</sub> induction and its actions in particular via activation of EP2 receptor. Therefore, EP2 receptor antagonism with a small molecule has a therapeutic value. However, PGE<sub>2</sub> and EP2 are also had a physiological role in relaxing the vascular, respiratory and other smooth muscles, therefore, de-risking experiments with small molecules targeting physiological EP2 actions in naïve animals were necessary to advance a small molecule antagonist for clinical use for any disease

indication. In this study, we provide experimental evidence that use of a small molecule EP2 antagonist by acute and chronic delivery should be safe for cardiovascular, respiratory, basic immune response, and bone density in healthy animals. Our results alleviate long-held reservations, which were merely derived from the studies of EP2-global knockout animals, against targeting EP2 and paving a path for EP2 antagonist to use against array of diseases. Further studies with EP2 antagonist in disease specific models are required to promote EP2 antagonist as a clinical agent.



**Fig. 6.** Acute administration of EP2 antagonist (TG6–10–1) has no effect on respiratory function in normal male rats. Respiratory Frequency (RF) (A), Tidal volume (TV) (B) and Minute volume (MV) (C). Animals injected with either vehicle (0 mg/kg) or TG6–10–1 at 5 mg/kg, 20 mg/kg and 50 mg/kg were monitored for respiratory parameters. TG6–10–1 did not have any significant effect on RF, TV or MV when compared to animals injected with vehicle, baseline (BL) values were compared to different time points post treatment (PT). Statistical analysis was done using 2-way ANOVA with Tukey’s multiple comparison,  $p < 0.05$  was considered significant. The data are with  $\pm$  SD.



**Fig. 7.** Treatment of mice with EP2 antagonist (TG11–77.HCl at 134 mg/kg/day for 4 weeks) had no adverse effect on bone mass of tibia and femur in C57BL/6 mice. 0.6 mm VOI diaphyseal scan of tibia and femur at mid shaft in male (A) and female mice (D). Quantification of bone volume and bone density between vehicle and TG11–77.HCl treatment showed no difference in male (B–C) or female (E–F) tibia and femur at diaphysis and metaphysis. Unpaired t test with Holm-Sidak correction for multiple comparisons was applied between group ( $\alpha = 0.05$ ). The data are with  $\pm$  SEM.

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## CRedit authorship contribution statement

TG, VR, AB, AR and RD designed the research. VR, AB, TN, RD and RA performed the research. VR and AB analyzed the data. TG and RD participated in data analysis. TG, VR and AB wrote the manuscript and all others contributed to the editing of the manuscript.

## Declaration of transparency and scientific rigor

All experimental procedures involving animals were conducted according to the "Principles of laboratory animal care" (NIH publication No. 86–23, revised 1985), and were approved by the Emory University School of Medicine's Animal Care and Use Committee (IACUC) and justified to the NIH and other funding agencies guidelines for animal research. This declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigor of preclinical research as stated in the British Journal of Pharmacology guidelines for Design and Analysis of the data.

## Conflict of Interests

Authors, T.G. and R.D. are the founder of, and have equity in Pyrefin Inc, which has licensed EP2 technology from Emory University in which T.G., R.D. and R.A. are inventors.

## Data availability

The data collected, analyzed and presented in this study are available from the corresponding author upon reasonable request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2022.112646](https://doi.org/10.1016/j.biopha.2022.112646).

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