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Monocyclic and Dicyclic Hydrocarbons: Structural Requirements for Proximal Giant Axonopathy

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Abstract

The chromogenic and neurotoxic γ -diketone 1,2-diacetylbenzene (1,2-DAB), but not its isomer 1,3-DAB, induces blue discoloration of tissues and urine, clustering of axonal microtubules and proximal neurofilament-filled axonal swellings in rodents. The remarkable chromogenic property of 1,2-DAB, a monocyclic aromatic hydrocarbon, arises from reaction with lysine residues of proteins and formation of dimeric and polymeric derivatives. Tetralin, a dicyclic solvent with structural relations to acetyl ethyl tetramethyl tetralin, a chromogenic and neurotoxic agent, reportedly induces excretion of green urine, and causes neurological disturbances in humans. Monocyclic aromatic 1,2,4-Triethylbenzene (1,2,4-TEB), but not its isomer 1,3,5-TEB, is also reportedly chromogenic and induces neurophysiological deficits in rodents consistent with axonal neuropathy, but without neuropathological confirmation. We treated 12 week-old C57Bl/6 mice by gavage with 300, 600, or 900 mg/kg/d 1,2,4-TEB, or equivalent doses of 1,3,5-TEB, 3 days/week, for up to 12 weeks, or intraperitoneally with 400 mg/kg/d tetralin, or 50 or 100 mg/kg/d of its α -tetralol analogue, 5 days a week, for up to 5 weeks. Animals treated with 1,2,4-TEB, but not 1,3,5-TEB, tetralin or α -tetralol, developed hind limb weakness, excreted greenish urine, and showed 1,2-DAB-like neuropathology. These findings support the hypothesis that 1,2-spaced ethyl (or acetyl) moieties on a benzene ring of hydrocarbons are required for hydrocarbons to induce chromogenic changes and proximal giant neurofilamentous axonopathy. Key molecular targets of these compounds likely reside in the axon where they serve to maintain normal cytoskeletal organization.

Keywords

Acetyl ethyl tetramethyl tetralin; clustered microtubules; γ -diketone neuropathy; giant axonopathy; tetralin; α -tetralol; triethylbenzene isomers

1. Introduction

The aromatic hydrocarbon 1,2-diacetylbenzene (1,2-DAB), an *ortho* diacetyl metabolite of the solvent 1,2-diethylbenzene (1,2-DEB), reacts as a γ -diketone with ϵ -amino groups of lysines in proteins to form a blue pigment, causes blue-greenish discoloration of urine and tissues, and induces proximal giant neurofilamentous axonopathy in rodents; by contrast, its isomer 1,3-

DAB (which lacks an *ortho* diacetyl structure) fails to react with proteins, is non-chromogenic, and does not induce axonopathy [14,15,27]. Properties comparable to those of 1,2-DAB and 1,3-DAB have been reported for 2,5-hexanedione (2,5-HD), the neurotoxic aliphatic γ -diketone metabolite of *n*-hexane, and its non-neurotoxic isomer 2,4-HD, respectively. Neurotoxic 2,5-HD reacts with amino acids and ϵ -amino groups of lysines in proteins to form pyrrole polymers with an orange hue; with prolonged incubation in vitro, these form blue-purple isoindole compounds comparable to the blue chromogen produced by 1,2-DAB and proteins [6,14,22,26]. These findings demonstrate a relationship between the protein-reactive, chromogenic, and neurotoxic properties of aromatic (1,2-DAB) and aliphatic (2,5-HD) γ -diketones, both of which are oxidation products of the corresponding solvents 1,2-DEB and *n*-hexane, respectively [6,8].

1,2-DEB and 1,3-DEB appear on a 1960 list of commercially important monocyclic and dicyclic hydrocarbon solvents that induced the excretion of greenish urine by rats treated with large, single subcutaneous doses [11] (Table 1). The listed agents may have been impure in the 1950s when the studies were performed. Indeed, studies published in the 1990s demonstrated that a pure sample of 1,2-DEB, but not of 1,3-DEB, was chromogenic and neurotoxic in repeatedly treated rats, presumably because the 1,2-DEB metabolite (1,2-DAB) but not the corresponding 1,3-DEB metabolite reacts with proteins to form blue-colored pigments [9,14,29]. Our previous work with aromatic hydrocarbons has shown a direct association between protein reactivity, chromogenicity, and neurotoxicity of the axonopathy type [14,15,22,27].

We have continued our series of studies to define the structural requirements for chromogenicity and neurotoxicity of the monocyclic and dicyclic hydrocarbon solvents described in Gerarde's 1960 list (Table 1 and Figure 1). The present experimental murine studies examine the neurotoxic potential of individual monocyclic triethylbenzenes (TEB), namely 1,2,4-TEB and 1,3,5-TEB, and the dicyclic hydrocarbon solvent tetralin (and its metabolite α -tetralol), reportedly a compound that in native or derivatized form is chromogenic and acutely neurotoxic in both rodents and humans [5,20,21]. These studies demonstrate that 1,2,4-TEB alone, the only one of the four tested agents with potential to form a γ -diketone metabolite (1,2,4-TAB), has chromogenic and neurotoxic properties, and induces 1,2-DAB-like giant proximal axonopathy.

2. Materials and Methods

2.1. Chemicals

1,2,4-TEB (99.4%), 1,3,5-TEB (99.8%), tetralin (99%), and α -tetralol (97%) were purchased from Aldrich Chemical Co. (Madison, WI). Compounds were stored at room temperature (tetralin, α -tetralol), desiccated at room temperature (1,2,4-TEB), or stored at 4°C (1,3,5-TEB). Purity of stock samples was checked by gas chromatography-mass spectrometry (GC-MS). Impurities could not be readily identified, but 1,3,5-TEB was found to be free of 1,2,4-TEB and vice-versa, and tetralin was free of α -tetralol and vice-versa.

2.2. Animals

These were male 12-week-old C57 Bl/6 mice and 9-week-old Sprague-Dawley rats (Charles River, CA) each weighing ~ 25 and 300 g on arrival, respectively. Mice were singly housed in cages supplied with soft and absorbent bedding whereas rats were individually housed in metabolic cages. Stacked cages were held in a room provided with a 12-h/12-h light-dark cycle and maintained at the temperature of 20°C. Animals were given a rodent chow (PMI® Nutrition International, NJ) and water *ad libitum*; they were acclimated for up to 5 days prior to the

treatment with the test articles. All aspects of this study were conducted in accordance with the institutional guide for care and use of laboratory animals.

2.3. Dosing regimens

2.3.1. Tetralin and α -tetralol

2.3.1.1. Rat study: To reproduce Gerarde's 1950 experiments [11], rats ($n = 2$) were treated subcutaneously (s.c.) with a single injection of neat tetralin at the dose of 5 ml/kg body weight and observed for physical and behavioral changes, and changes in the colour of urine. Animals were individually placed in metabolic cages and 24-Hour urine collected and subjected to GC-MS analysis for the presence of the test article and/or its metabolites according to the method of Serve and colleagues [19]. After 24 hours, the animals were severely ill; they were anesthetized with 4% isoflurane (0.5 l oxygen/min) and their internal organs examined for any tissue discoloration prior to euthanasia by decapitation.

2.3.1.2. Mice study: A preliminary study was performed to estimate the toxic potency of tetralin and α -tetralol in mice ($n = 8$) equally divided into two groups. Animals were treated intraperitoneally (i.p.) with either 800 mg/kg/d ($n = 2$) or 1600 mg/kg/d ($n = 2$) of each test article for up to 7 days. Injectate was prepared daily by dissolving the test article in HPLC-grade acetone followed by the addition of saline to a concentration of 2 % acetone; this produced an emulsion that was vortexed immediately prior to animal treatment. Mice with severe illness were terminated by decapitation and their internal organs examined for discoloration. All animals treated with α -tetralol showed severe illness on the first day and were subsequently terminated.

A formal experiment was conducted with mice treated with 50 mg/kg/d ($n = 2$) or 100 mg/kg/d ($n = 4$) α -tetralol, 400 mg/kg/d ($n = 4$) tetralin, or Vehicle (8 μ l/g saline containing 2% acetone, $n = 4$), 5 days a week, for up to 5 weeks. Test articles were administered via the intraperitoneal route with a 1-ml disposable plastic syringe equipped with a 27-gauge needle. Injection sites were rotated around the abdomen to minimize discomfort, and care was taken to minimize leakage from the injection site.

2.3.2. Study of TEB isomers—Mice were dosed by gastric intubation with 300 mg/kg ($n = 3$), 600 mg/kg ($n = 3$), or 900 mg/kg ($n = 3$) 1,2,4-TEB or its isomer 1,3,5-TEB, or Vehicle (8 μ l/g olive oil), 3 days a week (i.e. days 2, 4, & 6), for up to 12 weeks. The solution for gavage was prepared daily by mixing the test article with olive oil in varying proportions according to the dosage to be administered. The test article was given in a 1-ml disposable plastic syringe equipped with a 21-gauge gavage needle that was inserted into the lower oesophagus.

2.4. Animal observation

Animals were daily observed in their home-cages for position, movement, and activity level. Each animal was placed in an open field (uncovered plexiglass box 45 inches square, 10 inches height) and observed for ability to walk, locomotion pattern, and reaction to tail pinch. Locomotion was rated semi-quantitatively as follows: 1 = normal; 2 = slightly affected (walking on tip-toes or hunched-back gait, broad-based gait, abnormal outward rotation of the hind limbs), 3 = moderately affected (hind limb dragging), 4 = severely affected ("duck-walk", inability to walk). Digital video recordings were made of selected animals for further analysis of their locomotion and/or other physical signs. Animals were weighed and treated with the test article immediately thereafter.

2.5 Tissue preparation and examination

For morphological study, animals were deeply anesthetized with 4% isoflurane (0.5 l oxygen/min) prior to systemic perfusion with tissue fixatives. The abdomen and chest were opened and the heart injected with 50 µl of heparin (1,000 USP units/ml) solution. Immediately thereafter, the left atrium and ventricular apex were excised, a cannula spurring perfusate introduced and clamped into the ascending aorta, and the animal perfused with 4% paraformaldehyde followed by 5% glutaraldehyde, each in 0.2 M sodium cacodylate buffer (pH 7.4). Tissues were sampled from the central nervous system (CNS) and peripheral nervous system (PNS). CNS tissues included: frontal cortex, hippocampus, basal ganglia, cerebellum, medulla oblongata, lumbar and cervical spinal cords. PNS tissues included: lumbar dorsal and ventral spinal roots, lumbar dorsal root ganglia, sciatic, tibial, peroneal, and sural nerves. Peripheral nerves were excised and cut into proximal, medium, and distal segments. All tissues were post-fixed in excess cacodylate-buffered 1% osmium tetroxide (pH 7.4), dehydrated, and embedded in epoxy resin. Cross sections (~900 nm thick) were stained with 1% toluidine blue and screened by bright-field microscopy. Thin sections (~90 nm) of regions of special interest were stained with 2% uranyl acetate followed by 1% lead citrate for examination by transmission electron microscopy.

2.6. Statistical methods

The weekly body weight for each animal was calculated as the median of five daily measurements for each week; this yielded weekly body-weight profiles for 13 animals; three animals had data from only the first week and were excluded from further analysis. Profiles from each animal were adjusted so that all had the same average first-week body weight. The remaining time points (weeks 2–13) were used for analysis. A linear mixed-effect model [17] was used to analyze the changes in body weight over time (slope), compare differences between Vehicle and TEB-treated animals and compare differences between the two forms of TEB within a given dose. Daily locomotion scores from the same 16 animals were transformed to weekly scores in the same manner as for body weight. All computations were performed using version 2.0.1 of R [18].

3. Results

3.1. Animal observations

3.1.1. Study of tetralin and α -tetralol—Tetralin-treated rats (5 ml/kg) and mice that received α -tetralol at a dose ≥ 800 mg/kg were terminated within 24 hours as they became severely ill. Animals showed treatment-related changes consisting of drowsiness, acute respiratory distress, and lethargy with greater acute toxicity for α -tetralol. Rats excreted dark brown urine. Mice showed no urine or tissue discoloration. During the formal 5-week experiment, mice showed decreased reactivity to touch and tail pinch, wasting, and generalized weakness in proportion to the total dose of the test article. There was no evidence to suggest specific drug-related neuromuscular (γ -diketone-like) weakness.

3.1.2. Study of TEB isomers—Mice treated with 1,2,4-TEB, but not 1,3,5-TEB or Vehicle, displayed blue-green discoloration of urine and CNS/PNS tissues, including the brain, spinal cord, and peripheral nerves. Blue-green urine was first seen after 5 days of treatment with 900 mg/kg of TEB.

Animals receiving 300 mg/kg of 1,2,4-TEB or Vehicle gained body weight at a similar rate through the entire experiment (~0.4 g/week for both treatments). Animals that received 600 mg/kg failed to gain significant weight (~0.01 g/week); those that received 900 mg/kg lost weight at a rate of ~0.11 g/week (Table 2). In comparison to the Vehicle-treated animals, the highest dose of 1,2,4-TEB (900 mg/kg/d) induced significant changes ($p < 0.05$) in body weight

only later during the course of the experiment, i.e. after 10 weeks of treatment (Table 3). Animals developed limb weakness in proportion to the total dose of the chemical (Figure 2). Animals treated with 1,3,5-TEB showed a constant increase in body weight (between 0.24–0.57 g/week) regardless of the dosage (Table 2, Figure 3).

Mice treated with 1,2,4-TEB, but not 1,3,5-TEB or Vehicle, developed muscle spasms, limb weakness, and walking difficulties in the following sequence: tip-toe walking, outward rotation of hindlimbs, dragging of hind limbs, duck-walk and, eventually, complete inability to walk. Signs of neuromuscular compromise in animals treated with 600 mg/kg or 900 mg/kg 1,2,4-TEB (but not 300 mg/kg) were observed after treatment periods of ~7 weeks and 2 weeks, respectively. Animals treated with 300 mg/kg 1,2,4-TEB developed slight walking difficulties (tip-toe and broad-based gait) noticeable after ~8 weeks of treatment. These signs remained unchanged until the termination of the study (12 weeks).

3.2. Morphological findings

Mice treated with 1,2,4-TEB, but not 1,3,5-TEB or Vehicle, showed two major types of abnormality. First, microscopic examination of distal sciatic nerves revealed disorganization of the axonal cytoskeleton with clustering of microtubules and organelles (Figure 4). Microtubule clustering was seen in the presence or absence of the second type of change, namely the development of time and dose-dependent giant axonal swellings in lumbar ventral roots, anterior horns, and dorsal root ganglia. Mice treated with 300 or 600 mg/kg 1,2,4-TEB showed giant axonal swellings most prominently in lumbar ventral roots and to a lesser extent in dorsal root ganglia (Figure 5). Nerve fibers within lumbar ventral roots and dorsal root ganglia also exhibited myelin bubbles containing shrunken axons. Treatment with 900 mg/kg 1,2,4-TEB was associated with these changes in lumbar ventral roots, dorsal root ganglia, and spinal anterior horns (Figure 6). Electron microscopy of intraspinal swollen axons revealed tightly packed and maloriented 10-nm neurofilaments (NFs) (Figure 7). Lumbar dorsal horns and distal segments of afferent dorsal roots were usually unremarkable. Axonal swellings were also seen in the cervical anterior horns, and the lower medulla oblongata, proximal to the hypoglossal nucleus (not shown). No noticeable changes were seen in the cerebellum, hippocampus, basal ganglia, or frontal cortex. CNS and PNS tissues from animals treated with 1,3,5-TEB, tetralin, α -tetralol, or Vehicle were unremarkable.

4. Discussion

These findings show that 1,2,4-TEB, but not 1,3,5-TEB, is chromogenic and axonopathic. The respective neurotoxic and non-neurotoxic properties of these benzene derivatives are shared with those of 1,2-DEB and 1,3-DEB [9], 1,2-DAB and 1,3-DAB [8,15,27], and 2,5-HD and 2,4-HD [23]. Tetralin and α -tetralol lacked chromogenicity and neurotoxicity in contrast to acetyl ethyl tetramethyl tetralin (AETT), the diketone metabolite of which is also chromogenic but untested for neurotoxic properties [21,24]. The prominent spinal root demyelination reported after prolonged (30 week) treatment of rats with AETT is consistent with the late effects (secondary demyelination) of a 1,2-DAB-like or 1,2,4-TEB-like axonopathy — not a myelinopathy as previously surmised [24,25]. Taken in concert, therefore, the present findings support the hypothesis that monocyclic and dicyclic aromatic hydrocarbons lack chromogenicity and neurotoxicity unless they have a side chain that can pose as or be metabolized to a γ -diketone-like structure [10,21,22]. This would exclude several commercially important compounds listed in Table 1, and it would also exclude trimethylbenzene mixtures provided they lack an impurity such as 1,2,4-TEB.

1,2,4-TEB induces a 1,2-DAB-like neuropathology consisting of proximal giant axonal swellings filled with 10-nm NFs. Segregation of cytoskeleton elements, with clustering of microtubules and organelles, is another important early feature of this axonopathy. That axonal

microtubule clustering was detected prior to and concurrent with the development of axonal swellings suggests it is an early event in the pathological process. Indeed, previous studies demonstrated that microtubule clustering takes place within minutes of direct application of a gamma-diketone (2,5-HD) to the living rat sciatic nerve (30). This observation is of paramount importance because it appears to be a common feature of both 1,2,4-TEB — which is likely to be metabolized into active γ -diketones such as 1,2-diacetyl-4-ethylbenzene (1,2-DA-4-EB), 1,3,4-triacetylbenzene (1,2,4-TAB), and/or 1,2-diacetyl-4(1'-hydroxy)-ethylbenzene (1,2-DA-4(1'-OH)-EB) — 1,2-DAB, and 2,5-HD neuropathies [27,28]. It is therefore likely that both aliphatic and aromatic γ -diketones induce axonopathy by common molecular mechanisms that involve attack on proteins required for maintenance of axon cytoskeletal integrity.

Mechanisms of axonal swelling resulting from the time-dependent accumulation of 10-nm NFs are not understood. Proximal γ -diketone-like neurofilament-filled axonal swellings have been reported in other neurological disorders including amyotrophic lateral sclerosis in humans, for which the pathogenetic mechanism remains enigmatic [1,7,13,16] and experimental IDPN-(β,β' -iminodipropionitrile) induced neuropathy, where arrest of slow but not fast axonal transport has been demonstrated [12]. Whereas NFs accumulate in distal regions of axons after repeated systemic treatment with the less protein-reactive 2,5-HD, stepwise-more potent 3-methyl-2,5-hexanedione and 3,4-dimethyl-2,5-hexanedione (DMHD) cause the development of mid-level and 1,2-DAB-like proximal neurofilament-filled axonal swellings, respectively [2,3,4,15,27]; of which the latter are also inducible with 1,2,4-TEB. The differential ability of γ -diketones to react with proteins according to their total lysine content has been proposed to explain how these compounds may induce proximal or distal accumulation of NFs depending on their ability to disrupt the transport of axonal cargo via crosslinking of motor and/or other structural proteins within the axon [6,14,22]. Further studies on axonal transport in γ -diketone-induced neuropathy are needed to confirm this hypothesis.

Neither tetralin nor its metabolite α -tetralol induced chromogenic changes or neuropathology. Both compounds induced lethargy, drowsiness, and respiratory distress with greater acute toxicity for α -tetralol. GC-MS analysis confirmed that α -tetralol was the most abundant metabolite of tetralin. It is possible that tetralin exerts an acute neurotoxic effect via its α -tetralol metabolite that acts ultimately as CNS depressant [20]. Long-term dosing of mice with these two compounds resulted in wasting, diminished reactivity to touch and tail pinch, lethargy, and progressive generalized weakness. These signs suggest non-specific systemic toxicity that could be explained by the indirect effect (metabolic) that tetralin and/or its α -tetralol metabolite would cause after inducing hemolysis and/or kidney damage [5,19]. Rats excreted dark brown urine presumably reflecting hemolysis after treatment with a high dose of tetralin [5]. A clear distinction should be made between urine (and/or tissue) discoloration induced by the presence of neurotoxic chromophores and that reflecting the presence of hemoglobin by-products after hemolysis.

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Figure 1.

(A) Chemical structures of neurotoxic solvents (parent compounds, their putative active γ -diketone metabolites, and their non-neurotoxic isomers. Note structural similarities (1,4 spacing of ethyl groups) between the neurotoxic solvents 1,2-DEB and 1,2,4-TEB and between 2,5-HD and 1,2-DAB (1,4 spacing of acetyl groups), the γ -diketone neurotoxic metabolites of *n*-hexane and 1,2-DEB, respectively. 1,2,4-TAB, a putative γ -diketone neurotoxic metabolite of 1,2,4-TEB shares the same similarity. (B). Chemical structures of neurotoxic AETT (tetralin derivative), its putative γ -diketone metabolite keto-AETT, tetralin and α -tetralol.

Figure 2.
Mean weekly body weight for animals treated with Vehicle or varying doses of 1,2,4-TEB. The size of each plotting symbol is proportional to the average weekly locomotion score (smallest, within normal limits; larger symbols indicate greater impairment).

Figure 3. Mean weekly body weight for animals treated with Vehicle or varying doses of 1,3,5-TEB. All locomotion scores remained at 1 for all treatments throughout the course of the study.

Figure 4.

Distal sciatic nerve of a mouse treated with 900 mg/kg 1,2,4-TEB 3/week for 12 weeks.

Electron micrograph shows clustering of axonal microtubules and mitochondria (box and inset) in a myelinated fiber. This animal also had giant intraspinal axonal swellings filled with 10-nm neurofilaments. Epoxy-embedded tissues stained with 2% uranyl acetate followed by 1% lead citrate.

Figure 5. Lumbar ventral spinal roots (A) and dorsal root ganglion (B) of a mouse treated with 600 mg/kg 1,2,4-TEB 3d/week for 12 weeks. Swollen axons (*) within the myelin sheath are evident in both tissues. Epoxy-embedded tissues stained with 1% toluidine blue.

Figure 6. Lumbar anterior horns of animals treated with (A) vehicle, (B) 900 mg/kg 1,2,4-TEB, or (C) an equivalent dosage of 1,3,5-TEB 3d/week for 12 weeks. Axonal swellings (*) are evident in anterior horns of mice treated with 1,2,4-TEB, but not with 1,3,5-TEB or vehicle. Epoxy-embedded tissues stained with 1% toluidine blue.

Figure 7.

(A) Electron micrograph of intraspinal swollen axons in anterior horns of animals treated with 900 mg/kg 1,2,4-TEB 3d/week for 12 weeks. (B) Enlarged view of box in A, showing densely packed and maloriented 10-nm NFs associated with a mitochondrion (*m*). Epoxy-embedded tissues stained with 2% uranyl acetate followed by 1% lead citrate.

Table 1

List of aromatic hydrocarbons reportedly associated with production of colored urine [11].

Monocyclic	Dicyclic
Benzene	Indane
<i>o</i> -Xylene	Indene
<i>o</i> -Ethyltoluene	Tetralin *
<i>o</i> -Diethylbenzene (1,2-DEB)	Diphenyl
<i>m</i> -Diethylbenzene (1,3-DEB)	Diphenylmethane
<i>o</i> -Diisopropylbenzene	1-Methylnaphthalene
Triethylbenzene (mixture)	2-Methylnaphthalene
Diethyldisopropylbenzene	1-Ethylnaphthalene
	2-Ethylnaphthalene

* Rats, single subcutaneous dose of 5 ml/kg body weight. Humans may also excrete colored (green) urine following exposure to tetralin [5]. Reproduced from Gerarde [11]

Table 2

Estimated slopes (b) describing the rate of change in body weight (g/week) for each treatment. The standard error for each estimated slope is given in parentheses; two-sided *p*-values and 95 % confidence intervals for the slope are also provided.

Treatment	<i>b</i> (std. error)	<i>p</i> -value	95% CI
Vehicle	0.37 (0.04)	<0.001	(0.29, 0.45)
1,2,4-TEB			
300 mg/kg	0.40 (0.03)	<0.001	(0.34, 0.46)
600 mg/kg	0.01 (0.04)	0.749	(−0.07, 0.09)
900 mg/kg	−0.11 (0.04)	0.003	(−0.19, −0.03)
1,3,5-TEB			
300 mg/kg	0.57 (0.04)	<0.001	(0.49, 0.65)
600 mg/kg	0.24 (0.03)	<0.001	(0.18, 0.30)
900 mg/kg	0.24 (0.04)	<0.001	(0.16, 0.32)

Table 3

Estimated mean body weight (weeks 10-13) for animals treated with either vehicle or varying doses of 1,2,4-TEB. The standard error for each estimated mean or difference between vehicle and a given dose of 1,2,4-TEB is given in parentheses. The two-sided *p*-value tests significance of the difference between vehicle and the indicated dose of 1,2,4-TEB.

Treatment	Week			
	10	11	12	13
(0) Vehicle	29.1 (1.20)	29.5 (1.20)	29.8 (1.20)	30.2 (1.21)
(1) 300 mg/kg	29.2 (0.98)	29.6 (0.98)	30.0 (0.98)	30.4 (0.99)
(2) 600 mg/kg	26.6 (1.00)	26.6 (1.01)	26.6 (1.02)	26.6 (1.03)
(3) 900 mg/kg	25.1 (1.20)	25.0 (1.20)	24.9 (1.20)	24.8 (1.21)
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(1)–(0)	0.16 (1.54)	0.18 (1.55)	0.21 (1.55)	0.23 (1.56)
<i>p</i> -value	0.92	0.91	0.89	0.88
(2)–(0)	−2.48 (1.56)	−2.84 (1.57)	−3.20 (1.58)	−3.56 (1.59)
<i>p</i> -value	0.14	0.10	0.07	0.05
(3)–(0)	−3.97 (1.70)	−4.45 (1.70)	−4.93 (1.70)	−5.41 (1.71)
<i>p</i> -value	0.04	0.03	0.02	0.01