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ORIGINAL ARTICLE

Novel phenanthridine (PHE-4i) derivative inhibits carrageenan-induced rat hind paw oedema through suppression of hydrogen sulfide

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Abstract This study was conducted to assess the anti-inflammatory effect of a novel synthesized phenanthridine alkaloid (PHE-4i) and to examine the possible involvement of hydrogen sulfide (H_2S) in anti-inflammatory mechanism. The synthesized phenanthridine derivative PHE-4i (2, 5, and 10 mg/kg) was administered intraperitoneally to rats. One hour following treatment, inflammation was induced by intraplantar injection of carrageenan (1 %), in the hind paw. Paw volume as the index of inflammation was measured before and after carrageenan injection. Neutrophil sequestration into the hind paw was quantified by measuring tissue myeloperoxidase (MPO) activity and was compared for the inhibition of H_2S production. Pretreatment with PHE-4i significantly reduced carrageenan-induced hind paw weight, MPO activity, leukocyte infiltration, and H_2S production in a dose-dependent manner ($p < 0.001$). These results indicate that the anti-inflammatory effect of PHE-4i on carrageenan-induced rat paw edema could be via the inhibition of the gaseous mediator H_2S.

Keywords Hydrogen sulfide · Myeloperoxidase · Phenanthridine alkaloid · Carrageenan · Hind paw oedema

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Introduction

Inflammation is a complex pathophysiological process, which include the production of inflammatory mediators by infiltrating macrophages that can result in oedema (Vogt et al. 2005). Recent studies have implied that hydrogen sulfide (H_2S) plays a crucial role in several inflammatory conditions.

H_2S, together with nitric oxide and carbon monoxide, belongs to a family of endogenous signaling mediators termed "gasotransmitter." In higher animals, H_2S is synthesized by cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS). Among these two, CSE appears to be more important in synthesizing H_2S in the vascular system and heart (Moore et al. 2003). Recent studies suggest that H_2S levels increase in the pathogenesis of variety of inflammatory conditions, such as acute pancreatitis (Tamizhselvi et al. 2008), sepsis (Zhang et al. 2006), endotoxemia (Li et al. 2005), and hind paw oedema (Bhatia et al. 2005a). Therefore, the level of H_2S may reflect the degree of inflammation, and provides an indicator to assess inflammatory processes. Inhibition of H_2S production with endogenous H_2S synthesis inhibitor, DL-propargylglycine (Tamizhselvi et al. 2008), and with a CSE gene silencer, small interfering RNA (siRNA) (Badiei et al. 2013) has been shown to ameliorate inflammation, suggesting that endogenous H_2S has an important role in the pathophysiology of inflammation. As a gaseous signaling molecule, H_2S diffuse freely across cell membranes in a receptor-independent manner and activate various cellular targets. Its effects on the cell viability, proliferation, activation, cytokine secretion, and cell adhesion have been investigated in many different cell types (Szabó 2007). In addition, H_2S activates the transcription factor nuclear factor-κB (NF-κB), essential for the activation of most

proinflammatory genes in inflammation (Zhang et al. 2007; Badiei et al. 2014).

Neutrophils play a crucial role during inflammation. Mechanism involved in activated neutrophils has been shown to amplify inflammation via release of proinflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL1-β) (Wright et al. 2010). An in vitro study suggests that H_2S stimulates the secretion of cytokines, indicating the important role for H_2S in neutrophil recruitment and the pro-inflammatory signaling (Zhi et al. 2007).

Quaternary benzo[c]phenanthridine alkaloids (QBA) widely distributed in Papaveraceae, Fumariaceae, Rutaceae, Ranunculaceae, and Meliaceae families have been used in folk medicine due to its pronounced biological properties (Slavik et al. 1968). Sanguinarine, Chelerythrine, and Nitidine are the principal representatives of QBAs. They possess an extensive range of biological activities, including antimicrobial, antifungal, anti-inflammatory, and cytotoxicity against different cancer cell lines (Zdarilova et al. 2006; Slaninová et al. 2014), with efficient DNA binding capability (Urbanová et al. 2009). Recently, it has been reported that QBA can induce the formation of human telomeric G-quadruplex structures that enhance their ability to inhibit telomerase activity and, thereby, halt tumor cell proliferation (Bai et al. 2014). They possess remarkable antimicrobial activity against a wide range of Gram-negative, Gram-positive bacteria, yeasts, fungi, and virus (Tumir et al. 2014). These alkaloids represent an important group of pharmaceuticals possessing a significant anti-inflammatory activity, the extent of which is not yet commercially exploited. Sanguinarine, a representative benzo-phenanthridine alkaloid, is found to be a potent inhibitor of NF-κB (Chaturvedi et al. 1997) as well as it suppressed the activation of mitogen-activated protein kinases, which altered inflammatory mediator synthesis and release in vitro (Niu et al. 2012). Sanguinarine also induced apoptotic pathway in colon cancer cells through reactive oxygen species-mediated Egr-1 activation and mitochondrial dysfunction (Han et al. 2013). QBA inhibited the cell surface expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 surface molecules involved in the regulation of immune response and inflammation (Tanaka et al. 2001).

In clinical settings, non-steroidal anti-inflammatory drugs (NSAIDs) are used in the treatment of various inflammatory diseases, such as rheumatism and arthritis. Although widely used, NSAIDs have several adverse effects (Ong et al. 2007) which stimulated the search for new compound possessing potent anti-inflammatory activity with minimal side effects. Phenanthridine (PHE) group derivatives, such as ethidium bromide and propidium iodide, were recognized for their usage as the fluorescent

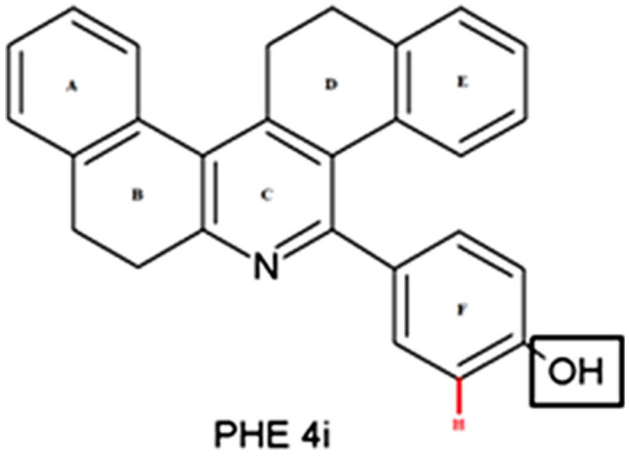

Fig. 1 PHE-4i with hydrophilic group (OH) at the (*para*) position on the 'F' phenyl ring

marker for both ds-DNA and ds-RNA (ethidium bromide) or as probes for cell viability studies (propidium iodide) (Tumir et al. 2014) because of their DNA binding ability. Recent reports on antitumor activity of phenanthridine alkaloid analogs resulted in enhanced interest toward these moieties for their pharmaceutical applications. Karthikeyan et al. showed that PHE-4i possess anticancer activity against various human tumor cell lines (Karthikeyan et al. 2012). With this regard, in identifying the functional role of benzophenanthridine compounds, we have explored a synthetic phenanthridine derivative PHE-4i, polycyclic nitrogen heterocycle with a planar structure that possesses a hydrophilic group at the *para* position on the 'F' phenyl ring structurally similar to phenanthridine (PHE) alkaloids (Fig. 1). In this study, we examined the anti-inflammatory effect of PHE-4i and its inhibitory activity toward H_2S production using carrageenan-induced rat paw edema.

Methods

Measurements of carrageenan-induced hind paw oedema formation in the rat

All experimental protocols followed the guidelines approved by the CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals) and the Institutional Animal Ethics Committee (Approval no. VIT/IEAC/VIIIth/14). Animals (male, *Wistar* rats weighed 110–160 g) were maintained at 23 °C on 12 h light/dark cycle with free access to water and food (VIT University, Vellore, India). Synthesized phenanthridine derivative PHE-4i was a generous gift from the Chemistry Division, School of Advanced Sciences (SAS), VIT University, Vellore.

Carrageenan-induced paw oedema is a classical, well-studied model to assess compounds for its anti-

inflammatory activity (Winter et al. 1962). Animals received an intraplantar injection of carrageenan (150 µl, 1 % wv^{-1}) in the hind paw. In some experimental conditions, PHE-4i (2, 5, 10 mg) was injected intraperitoneally (i.p.) 1 h before intraplantar injection of carrageenan. The paw volume was measured prior to carrageenan injection and then at 1, 2, 3, and 4 h after carrageenan injection. Aspirin (anti-inflammatory agent) was the standard used for the entire study. Animals were held firmly, and the hind paw was immersed into a beaker placed over a top-pan balance containing warm water. Animals that received saline (0.9 % wv^{-1}) served as control. Paw oedema formation was determined as the difference in paw weight between the animals that received carrageenan alone and carrageenan along with the compound. After the fourth hour, animals were killed, and the hind paw was stored at −80 °C until assayed as mentioned below.

Measurement of myeloperoxidase (MPO) activity

Neutrophil infiltration into the hind paw was determined by tissue MPO activity (Bhatia et al. 1998). Briefly, subcutaneous tissue of hind paw was removed and homogenized in 20-mM phosphate buffer (pH 7.4), centrifuged (10,000×*g*, 4 °C, 10 min), and the pellets were resuspended in 0.5 % (vv^{-1}) hexadecyltrimethylammonium bromide containing 50-mM phosphate buffer (pH 6.0). The sample was then subjected to four cycles of freezing and thawing and then disrupted by sonication (40S). Samples were then centrifuged (10,000×*g*, 4 °C, 5 min), and the supernatant were processed for MPO assay. The reaction mixture consisted of tissue supernatant (50 µl), tetramethylbenzidine (1.6 mM), sodium phosphate buffer (80 mM, pH 5.4), and hydrogen peroxide (0.3 mM). The total incubation volume was 100 µl. The reaction mixture was incubated at 37 °C for 110 s, the reaction was then terminated with H_2SO_4 (0.18 M), and absorbance measured at 450 nm. Tissue MPO activity was corrected for DNA concentration (Labarca and Paigen 1980). Results are expressed as MPO activity per microgram DNA (fold increase over control).

Assay of tissue H_2S production

The effect of PHE-4i on release of H_2S was determined as described previously (Chunyu et al. 2003). Briefly, paw tissue was removed and homogenized in 50-mM ice-cold potassium phosphate buffer (pH 6.8).Homogenate was then added to microcentrifuge tubes containing 150 µl of zinc acetate (1 % w/v) to trap H_2S. After 5 min, the reaction was terminated by adding 100 µl of NNDP sulfate (light sensitive, 20 nM in 7.2 M HCl) and 100 µl of $FeCl_3$ (30 mM in 1.2 M HCl). After the mixture was kept in the dark for 20 min, 300 µl of TCA (10 % w/v) was added subsequently, and the mixture was centrifuged at 4000 rpm for 10 min. The absorbance of the solution was determined at 670 nm.

Semiquantitative polymerase chain reaction

Total RNA was extracted from rat paw with TRIzol reagent (Sigma Aldrich) according to the manufacturer's protocol (Bhatia et al. 2005b). The concentration of isolated nucleic acids was determined spectrophotometrically by measuring the absorbance at 260 nm, and the integrity was verified by ethidium bromide staining of 18S and 28S rRNA bands on a 1 % denaturing agarose gel for quality. The samples were stored at −80 °C until required. RNA (1 µg) was reverse transcribed into cDNA using verso cDNA Synthesis Kit (Thermo scientific) at 25 °C for 5 min and 42 °C for 30 min, followed by 85 °C for 5 min. The cDNA was used as a template for PCR amplification by iQSupermix (Bio-Rad). The PCR primers for detection of CSE, sense: 5′- GAC CTC AAT AGT CGG CTT CGT TTC -3′; and antisense: 5′- CAG TTC TGC GTA TGC TCC GTA ATG -3′ 22 cycles, size 618 bp, were synthesized by Sigma Aldrich. The reaction mixture was first subjected to 95 °C for 3 min for the activation of polymerase. This was followed by an optimal cycle of amplifications consisting of 95 °C for 30 s optimal annealing temperature 60° C and for 72 °C for 30 s. PCR amplification was performed in My Cycler (Bio-Rad, Laboratories). PCR products were analyzed on 1.5 % w/v agarose gels containing 0.5 µg/ml ethidium bromide and photographed using Gel Doc-It Imaging System. β-actin was used as an internal control to normalize the signal from genes of interest.

Real-time PCR

A total of 100 ng of RNA was used for each real-time PCR. It was amplified by Light Cycler real-time PCR machine (Bio-Rad) using SYBR green I master mix (Sigma). Gene expression was calculated relative to β-actin levels by the comparative ΔCT values method.

Morphological examination

Paraffin-embedded paw samples were sectioned (5 µM), stained with hematoxylin/eosin (H and E), and were examined with light microscopy.

Drugs and chemicals

Unless indicated previously, all drugs and reagents were purchased from Sigma Aldrich.

Statistical analysis

The statistical software used is graph pad prism. All data represent a minimum of six experiments and are expressed as the value ± the standard deviation (SD). The significance of changes was evaluated using ANOVA and Tukey′ s method was used as a post hoc test for the difference between groups. p value < 0.05 was taken as the level of significance.

Results

PHE-4i reduces carrageenan-induced hind paw oedema

Preliminary experiments indicated that there was no significant difference in hind paws' weight before the experiment between groups of animals, or between left and right hind paws. Accordingly, carrageenan injection was performed into either hind paw chosen at random. The weight of the non-injected hind paw did not alter throughout the experiment in rats (4 h, 0.10 ± 0.03 g; $n = 4$). Hind paw oedema in response to intraplantar carrageenan injection was confirmed at the end of the experiment by removing and weighing directly both the injected and non-injected hind paws. Intraplantar carrageenan injection in rat hind paw resulted in an increase in hind paw weight (0.35 ± 0.028 g; $n = 4$). The standard drug, aspirin, exhibited a significant inhibition of carrageenan-induced oedema formation. Likewise, the PHE-4i administration caused a dose-related inhibition of carrageenan-induced hind paw weight gain (Fig. 2). The reduction in carrageenan-induced hind paw weight followed by PHE-4i treatment at a dose of 2, 5, and 10 mg/kg (i.p. injection) was 0.195 ± 0.01, 0.275 ± 0.028, and 0.288 ± 0.025 ($n = 4$), respectively. Among the different concentrations used, carrageenan-induced paw weight was significantly reduced at the dose of 2 mg/kg.

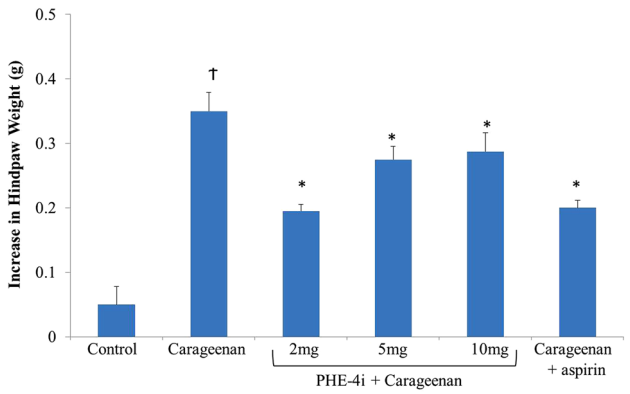

Fig. 2 Effect of carrageenan injection on hind paw oedema at 4 h and the effect of different doses of phenanthridine PHE-4i (2, 5, and 10 mg). Results show mean ± SD, $n = 4$. $^{\dagger}P < 0.001$ (F value 115.7) when compared with control, $^{*}P < 0.001$ (F value 115.7) when compared with carrageenan

PHE-4i reduces MPO activity in the carrageenan-induced hind paw oedema

In this study, we used myeloperoxidase (MPO) enzyme activity as a marker to assess neutrophil infiltration in the tissue. As expected, from the MPO activation status, it is clear that the hind paw carrageenan injection resulted in a significant increase in neutrophil infiltration (Fig. 3A). PHE-4i at a dose of 2, 5, and 10 mg administered intraperitoneally 1 h before carrageenan (intraplantar) injection reduced the MPO activity in the hind paw in a dose-dependent manner. When tested at a dose of 2 mg/kg greatly reduced the carrageenan-induced activity of MPO when compared with 5 mg and 10 mg/kg. Treatment with aspirin, reference drug used at a dosage of 1 mg/kg, exhibited prominent reduction in tissue MPO activity. Further histological examination showed no significantly reduced inflammation or tissue destruction in the paw sections of vehicle (saline)-treated mice (Fig. 3B-a). In contrast, we observed that carrageenan-caused infiltrating cells (leukocytes) populated enlarged cavities due to tissue destruction (erosion) (Fig. 3B-b). Treatment with PHE-4i (2, 5, and 10 mg) [Fig. 3B-(c–e)] clearly reduced carrageenan-induced leukocyte infiltration.

Inhibitory effect of PHE-4i on carrageenan-induced H_2S concentration

Intraplantar injection of carrageenan in the rat hind paw resulted in a significant increase in H_2S production (0.43 ± 0.003 nmol/mg protein) (approx. 51 %) in the tissue when compared with control (0.22 ± 0.0047 nmol/mg protein) animals that received intraplantar injection of saline. Pretreatment of animals with the PHE-4i, prior to carrageenan injection, reduced the increase in hind paw H_2S levels in a dose-dependent manner. H_2S concentration in the hind paw treated with 2 mg/kg was reduced to values (0.13 ± 0.0018 nmol/mg protein), even below the levels detected in control (non-carrageenan injected) animals (Fig. 4).

PHE-4i inhibits carrageenan-induced CSE expression

Based on our initial data with different dosages of PHE-4i, we decided to see if reduction in inflammation due to PHE-4i has any effect on CSE (H_2S synthesizing enzyme) expression in rat paw tissue. Supporting the H_2S synthesis, CSE expression significantly upregulated in only

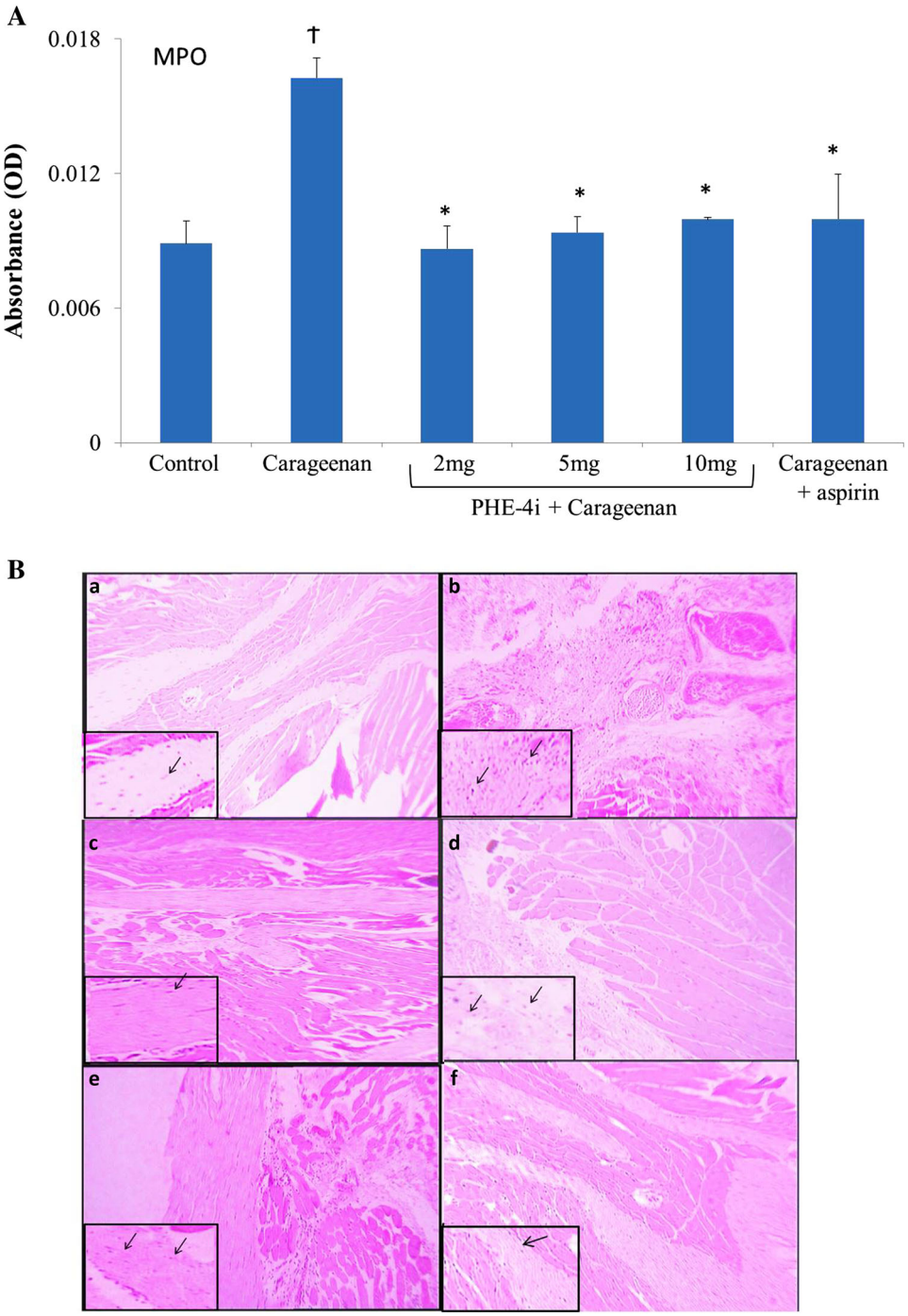

Fig. 3 **A** Effect of carrageenan injection on MPO activity and the effect of different doses of phenanthridine PHE-4i (2, 5, and 10 mg). Results show mean ± SD. $n = 4$. $^{\dagger}P < 0.001$ (F value 7.382) when compared with control, $^{*}P < 0.001$) (F valve 7.382) when compared with carrageenan. **B** Histological assessment of the effect of phenanthridine PHE-4i on carrageenan-induced paw oedema in rats. Representative sections of paw from the **a** control, **b** 1 % carrageenan, **c** 1 % carrageenan + 2 mg PHE-4i, **d** 1 % carrageenan + 5 mg PHE-4i, **e**. 1 % carrageenan + 10 mg PHE- 4i, and **f** 1 % carrageenan + aspirin groups stained with H&E stain. Arrows showing the infiltrating cells

carrageenan-treated group, whereas PHE-4i treatment significantly inhibited carrageenan-induced CSE expression in rat paw (Fig. 5).

Discussion

In this study, we evaluated the anti-inflammatory activity of novel phenanthridine derivative 4i (PHE-4i) in carrageenan-induced hind paw oedema. We found that intraplantar injection of PHE-4i remarkably reduced carrageenan-induced hind paw oedema and neutrophil sequestration at the site of inflammation, as determined by hind paw weight, myeloperoxidase activity, and histopathological studies. In this study, we also show that H_2S has a role in mediating the anti-inflammatory activity of the compound.

The intraplantar injection of carrageenan in rat results in paw oedema, the first phase of which results from the concomitant release of histamine, serotonin, and kinins,

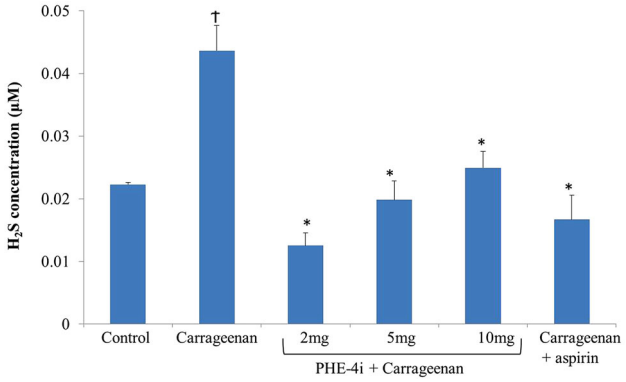

Fig. 4 Effect of intraplantar carrageenan injection and different doses of phenanthridine PHE-4i (2, 5, and 10 mg) on H_2S concentration in hind paw. Hind paws pretreated with PHE-4i were removed 4 h after carrageenan injection, and H_2S level was measured. Results show mean ± SD, $n = 4$. $^{\dagger}P < 0.001$ (F value 38.11) when compared with control, $^{*}P < 0.001$ (F value 38.11) when compared with carrageenan

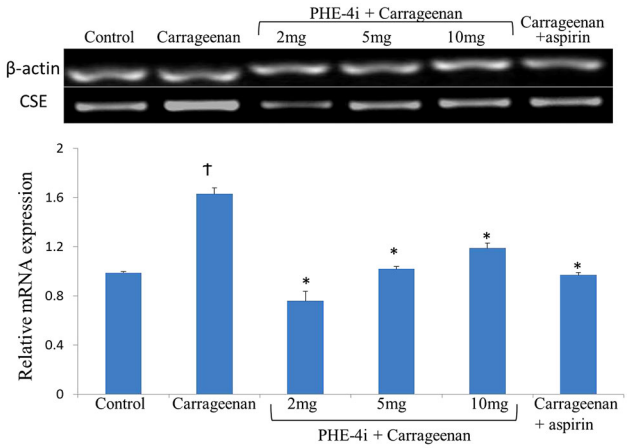

Fig. 5 Phenanthridine PHE-4i inhibits carrageenan-induced CSE enzyme activity in rat hind paw. CSE mRNA expression in control, carrageenan, and PHE-4i (10, 50, and 100 μM) treated groups was measured by **a** QRTPCR and **b** real-time PCR. CSE sample loading was normalized with β-actin internal control. Results show mean ± SD, $n = 4.^{\dagger}P < 0.001$) ($F$ value 5.616) when compared with control, $^{*}P < 0.001$ (F value 5.616) when compared with carrageenan

and the second phase is correlated with the local neutrophil sequestration (Vinegar et al. 1969; Ashok et al. 2010). Rat paw oedema, elicited by carrageenan, has been shown to be a useful model for the study of inflammation (Posadas et al. 2004; Walter 2009; Banani et al. 2012) and is increasingly used to test new anti-inflammatory drugs (Luo et al. 2010). In this study, PHE-4i derivative (2, 5, and 10 mg/kg) administered 1-h prior to carrageenan injection showed a significant reduction in carrageenan-induced paw oedema formation. In addition, we showed that intraperitoneal administration of PHE-4i derivative (2, 5, and 10 mg/kg) was an effective in vivo inhibitor of the neutrophil infiltration as determined by the MPO levels in the inflammatory paw tissue. Myeloperoxidase is an enzyme found primarily in azurophilic granules of neutrophils, and the activity of which is used as a marker for tissue neutrophil content and its reduced activity imply the presence of anti-inflammatory activity (Bradley et al. 1982). Moreover, a dose-related effect was observed with the PHE-4i derivative (2, 5, and 10 mg/kg). Interestingly, from our results, lower PHE-4i derivative (2 mg/kg) dose was more effective than the higher ones, with nearly the same efficacy as aspirin.

It has been previously reported that carrageenan-induced hind paw oedema increases H_2S biosynthesis, which resulted in a significant increase in H_2S concentration in the paw tissues (Bhatia et al. 2005a). Blockage of H_2S production by PAG, an inhibitor of CSE enzyme activity, reduced the severity of inflammation induced by carrageenan (Bhatia et al. 2005a). A similar proinflammatory effect of H_2S has also been reported in other animal models of inflammatory diseases, such as acute pancreatitis (Tamizhselvi et al. 2008), cecal ligation-induced sepsis (Zhang et al. 2006), burns (Zhang et al. 2010), and lipopolysaccharide-induced endotoxemia (Li et al. 2005). Moreover, it has been previously reported that inhibition of CSE has been shown to be effective in a number of animal models of inflammatory diseases (Li et al. 2005; Zhang et al. 2006; Tamizhselvi et al. 2008). Thus, it has been proposed that H_2S acts as a mediator of inflammation.

In this study, using carrageenan-induced rat paw oedema, PHE-4i derivative (2, 5, and 10 mg/kg) showed a significant reduction in carrageenan-induced H_2S production, with nearly the same efficacy as aspirin. Interestingly, from our results, lower PHE-4i derivative (2 mg/kg) dose was more effective than the higher ones. The phenanthridine structural features incorporate a unique set of properties related to the interaction with DNA and RNA. The variation, i.e., at low concentration (2 mg), the PHE-4i derivative is highly anti-inflammatory than that at high concentrations (5 and 10 mg), may be due to discrepancies in half-life, metabolism, and any tentative DNA, or protein binding affinity differences, which has to be studied further utilizing full array of concentrations extending both in low and high doses. This observation is in line with the variations previously reported among growth-suppressing and anti-inflammatory effects of glucocorticoid preparations in pediatric population (Allen 1996). Weerasinghe et al. (2001a, b) also found that human erythroleukemia cells treated with a low level of phenanthridine alkaloid, sanguinarine, showed the morphology of apoptosis in 96 % of cells, and the same sanguinarine at a high dose resulted in necrotic cell death morphology in over 90 % of cells. Excessive H_2S can mediate various harmful responses, including tissue injury, septic shock, and necrosis (Bhatia

2012). Thus, PHE-4i derivative demonstrating that inhibitory activity is against inflammatory H_2S production may have therapeutic potential for the treatment of inflammation accompanying overproduction of H_2S.

In this study, we provide the evidence that the synthesized PHE-4i derivative possesses noticeable anti-inflammatory activity against carrageenan-induced hind paw oedema which could be via the suppression of H_2S. Further investigations are underway to study the molecular mechanism of PHE-4i derivative in inflammation.

Conclusions

In summary, our findings demonstrate that novel synthesized phenanthridine derivative at a low dose exhibits promising anti-inflammatory activity against carrageenan-induced hind paw edema in rats through the suppression of hydrogen sulfide. Although the precise mechanism of action of phenanthridine derivative as an anti-inflammatory agent is yet to be studied, the presence of the hydrophilic group at the *para* position may contribute to its activity. The precise role of H_2S and its interaction with other inflammatory mediators involved will be the subjects for further studies.

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Compliance with ethical standards

Conflict of interest The authors report no declarations of interest.

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