Novel Azabicyclononane (ABN-5d) Derivative Inhibits Carrageenan-Induced Rat Hind Paw Edema

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Abstract

**Purpose:** Recent reports confirm the anti-inflammatory role of 3-azabicyclononane ring-based phytochemicals derived from a variety of plant alkaloids. We recently synthesized and established preliminary cytotoxic activities on different cancer cell lines. Further to characterize the azabicyclononane compounds, in this study we unravelled the mechanism involved in the anti-inflammatory effect of a novel azabicyclononane derivative (ABN-5d).

**Methods:** Carrageenan-induced (1% intraplantar injection) rat hind paw edema model was used for the induction of inflammation. The synthesized azabicycle derivative ABN-5d (0.5, and 1.0 mg/kg) was administered intraperitoneally to rats 1hr before the injection of carrageenan. Paw volume as the index of inflammation was measured before and after the administration of carrageenan.

**Results:** Carrageenan-induced peripheral inflammation in the rat. ABN-5d administration significantly decreased carrageenan-induced paw histopathological changes, myeloperoxidase (MPO) activity, H$_2$S, NO level, TNF-α, CSE, and iNOS gene expression in a dose-dependent manner (p < 0.001). We also demonstrated that ABN-5d significantly reduced carrageenan-induced NF-κB activation and IκBα degradation in the inflamed paw tissue.

**Conclusion:** These results indicate that the anti-inflammatory effect of ABN-5d on carrageenan-induced rat paw edema could be via the inhibition of the iNOS/NO-CSE/ H$_2$S -NFκB pathway.

Introduction

Inflammation is a process associated with infiltrating neutrophils and macrophages which are involved in the production and regulation of proinflammatory mediators (Vogt et al. 2005) including cytokines, chemokines as well as the gaseous mediators' nitric oxide (NO) and hydrogen sulphide (H$_2$S) (Li et al. 2005). These inflammatory mediators play a major role in deciding the initiation and extent of progression of inflammatory processes. TNF-α is one of the most important pro-inflammatory mediators involved in edema formation. NO is produced by inducible nitric oxide synthase (iNOS) and H$_2$S is generated by cystathionine gamma-lyase (CSE), acts as the regulatory signalling molecules, and turns out to be the pathogenic mediators while there is excessive production (Bhatia 2012). It was reported previously that increase in TNF-α is associated with the upregulation of iNOS, CSE gene expression, and signalling through their products NO and H$_2$S in inflammation (Sanders et al. 2001; Soufli et al. 2016; Basu et al. 2017) Also the inhibition of H$_2$S and NO production by gene silencing (Choy et al. 2019) using pharmacological inhibitors (Asimakopoulou et al. 2013), and natural compounds (Berenyiova et al. 2020) resulted in the reduction of TNF-α and the accompanying pro-inflammatory signalling pathways including nuclear factor kappa B (NF-κB) (Badiei et al. 2014).

Scientific evidence reports that naturally derived phytochemicals are effective anti-inflammatory agents used as therapeutic agents for various inflammatory conditions (Kemp 1994). 3-azabicyclononane (ABN)
group of heterocyclic alkaloids are naturally occurring in aconitum, delphinium, consolida, and thalictrum plant species (Arias-Pérez et al. 1997). These are widely distributed among-the family Ranunculaceae. These compounds are under scrutiny due to their multiple biological roles as analgesics, anesthetics (Mazimba and Mosarwa 2015), antimicrobial (Rajesh et al. 2014), and herbicidal, insecticidal, anti-inflammatory, and recently discovered to play a role as an anticancer agent (Parthiban et al. 2010). Thus, we are interested in synthesizing them chemically for larger medical applications. Azabicycle derivatives were synthesized using Mannich reaction where 2-tetralone is used to produce 3-ABN reacting cyclohexanone with 4-ethoxybenzaldehyde at 35˚C (Arias-Pérez et al. 1997).

Azabicyclononane (ABN) was synthesized and its anti cancer activity studied against a panel of tumor cell lines (Karthikeyan et al. 2012). Amongst the synthesized azabicycle products, it was recently reported that the derivatives ABN-5d possesses anti-proliferative activity against various human tumor cell lines (Karthikeyan et al. 2012). ABN-5d compound is a polycyclic nitrogen heterocycle with a planar structure that possesses a fluoro substituted group at the para position on the benzene phenyl ring (Fig. 1). Though the cancer-specific anti-proliferation was proved, the anti-inflammatory effect and the overall molecular mechanism of ABN-5d are yet to be explored. In this study, we, therefore, examined whether the anti-inflammatory effect of the alkaloid ABN-5d is through H$_2$S /NO linked NF-$\kappa$B pathway using the carrageenan-induced inflammatory hind paw edema model in rats.

**Methods**

**Structure of azabicyclononane (ABN)**

Compound 2,4-Diaryl-6,7-benzo-3-azabicyclo [3.3.1] nonan-9-ones (azabicyclononane) was synthesized by Sathiyanarayanan K I Research group, chemistry division, VIT University Vellore. This compound is synthesized based on Mannich based approach. The molecular weight of the compound is 399.09; the chemical structure is shown in Fig. 1.

**Drugs and chemicals**

All drugs and reagents were purchased from Sigma Aldrich. Synthetic drug ABN was dissolved in 10% (w/v) DMSO in saline, and all other drugs were dissolved in 0.9% (w/v) saline.

**Animals**

All experiments were performed with adult male Wistar rats (weighing 110-160 g) obtained from the animal house VIT University, Vellore, India. The animals were maintained at constant room temperature (23 °C) under a 12-h light/dark cycle with free access to standard food and water. Experimental groups of rats (n=6) were used in this study. All experimental protocols were developed according to the guidelines approved by CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals). Animals were approved by Institutional Animal Ethics Committee (Approval no. VIT/IEAC/13/Feb13/24).
Animals received an intraplantar injection of carrageenan (150 μl, 1 % wv−1) in the hind paw. In some experimental conditions, ABN-5d (0.5 and 1.0 mg/kg) was injected intraperitoneally (i.p.) 1 hr before intraplantar injection of carrageenan. The paw volume was measured before carrageenan injection and then at 1, 2, 3, and 4 hr after carrageenan injection. 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 edema 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 hr, animals were killed, and the hind paw was stored at −80 °C until assayed as mentioned below (George et al. 2016). [ESM 1]

Measurement of myeloperoxidase (MPO) activity

Measurement of the myeloperoxidase (MPO) activity was determined as described by Leema et al (George et al. 2016). Briefly, the homogenized hind paw tissue samples in 20 mM phosphate buffer (pH 7.4) were centrifuged (10,000g, 4 °C, 10 mins), and the pellets were resuspended in 0.5 % (vv−1) hexadecyltrimethylammonium bromide containing 50-mM phosphate buffer (pH 6.0). Samples were subjected to freeze-thaw cycles and sonicated for 40 S. 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). Samples were then centrifuged (10,000g, 4 °C, 5 mins). The reaction mixture consisted of tissue supernatant (50 μl), tetramethylbenzidine (1.5 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, the enzyme reaction was then stopped with H₂SO₄ (0.18 M), and absorbance was measured at a wavelength of 450 nm. [ESM 1]

Morphological examination

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

TNF-α ELISA assay

The concentration of TNF-α in the hind paw samples was assessed using an enzyme-linked immunosorbent assay (ELISA) commercial kit (R&D Systems) available according to the manufacturer’s instructions. Each group of rats (n=6) were pre-treated with and without ABN (0.5 mg/kg and 1 mg/kg). The concentration of TNF-α in hind paw samples was determined by ELISA using a commercial kit (R&D). Samples were collected after 4th hr induction of carrageenan and then centrifuged at 1200 g for 10 mins at 4°C and stored at -80°C until the assay. [ESM 2].

Assay of tissue H₂S production

Measurement of hydrogen sulphide (H₂S) was determined as described by Leema et al (George et al. 2016). Briefly, paw tissue was removed and homogenized in 50-mM ice-cold potassium phosphate buffer
Homogenate was then added to microcentrifuge tubes containing 150 μl of zinc acetate (1 % w/v) to trap H₂S. After 5 min, the reaction was terminated by adding 100 μl of NNDP sulphate (light-sensitive, 20 nM in 7.2 M HCl) and 100 μl of FeCl₃ (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 mins. The absorbance of the solution was determined at 670 nm. The absorbance of the samples was compared with the standard curve of NaHS.

**Assay of nitric oxide production**

Nitric oxide was measured at its breakdown products nitrite (NO₂⁻) and nitrate (NO₃⁻) using a Griess method. Briefly, hind paw tissue samples were first homogenized and centrifuged. To the supernatant, 100 μl of Griess reagent has added, and the absorbance at 490 nm was measured. The absorbance of the samples was compared with the standard curve of sodium nitrite. [ESM 1]

**Real time PCR**

Total RNA was extracted from rat paw with TRIzol reagent (Sigma Aldrich) according to the manufacturer’s protocol (Aggarwal et al. 2013). Isolated RNA was quantified the absorbance at 260 nm using a spectrophotometer. RNA (1 μg) was reverse transcribed into cDNA using verso cDNA Synthesis Kit (Thermo Scientific) at 25 °C for 5 mins and 42 °C for 30 mins, followed by 85 °C for 5 mins. The cDNA was used as a template for PCR amplification by iQSupermix (Bio-Rad). The reaction mixture was first subjected to 95 °C for 3 mins for the activation of the polymerase. This was followed by an optimal cycle of amplifications consisting of 95 °C for 30 s optimal annealing temperature 60° C and 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. Positive control is GAPDH. [ESM 3]

**Immunoblot Analysis**

Western blot analysis was performed to evaluate the IκBα protein levels in hind paw edema induced Rats and the assay was performed as described by I.F. Florentino et al 2017 with slight modifications (Florentino et al. 2017). The protein concentration was determined by the Bradford protein assay using a standard curve of BCA and was subjected to western blot analysis. Each protein extract was separated on 12% SDS-PAGE gels and was stained with Coomassie brilliant blue or was transferred to nitrocellulose membranes (Ge Healthcare®). HPRT (cytosolic protein) was used as the housekeeping endogenous control. The membranes were incubated in 0.05% (v/v) Tween-20 plus Tris-buffered saline (TBS) containing 1% (w/v) skim milk and then were incubated with primary antibodies to HPRT (1:5000 dilution), IκBα-mouse, Polyclonal (1:2000 dilution - Cayman Chemical Company, USA). The blots were washed thrice with 0.1% buffer solution (Tween 20/PBS-buffered saline). Alkaline phosphatase-conjugated antibody anti-mouse IgG was used as secondary antibody (1:10000 dilution). Then, membranes were washed as the reactions occurred with BCIP-T (5-bromo-4-chloro-3-indolyl phosphate)
and NBT (nitro blue tetrazolium). The protein levels were evaluated through densitometry, were quantified using Image-J Software, and were expressed as arbitrary units of the ratio to HPRT. [ESM 4]

NF-κB Binding activity

Each group of rats (n=6) were pre-treated with and without ABN (0.5 mg/kg and 1 mg/kg). Samples were taken and nuclear extracts were prepared and stored at -80°C until the assay. The assay was performed using a commercial nuclear extraction kit (Cayman chemicals, Bangalore, India) for binding activity of NF-κB according to manufacturer instructions. Carrageenan-induced paw edema tissues were washed and resuspended in ice-cold PBS in the presence of phosphatase inhibitors and centrifuged at 300g for 5 min. The pellets were resuspended in hypotonic buffer and centrifuged at 14,000g for few seconds. The supernatant containing cytoplasmic fraction was removed, and the pellet contained nuclei were lysed with lysis buffer which contains protease inhibitors, and extracts were solubilized in lysis buffer. Nuclear extract (10 µg of protein) was prepared according to the manufacturer’s instructions. Further, these nuclear extracts were added to a 96-well plate coated with a specific DNA sequence with the NF-κB response element. Absorbance read at 450 nm. [ESM 4]

Statistical analysis

All data represent minimum experiments are expressed as the mean value ± the standard deviation (SD). The significance of change groups was evaluated by using ANOVA with a post-hoc Tukey's test for the difference between groups. p value<0.001 was taken as the level of significance.

Results

ABN-5d reduces carrageenan-induced hind paw edema

The rat hind paws were weighed before the experiment between animals as well as between the left and right hind paws. For hind paw edema, induction carrageenan was administered into the randomly chosen hind paw of each animal. The weight of the non-injected hind paw (control) did not alter throughout the experiment (4 h, 0.5 ± 0.01 g: n = 6). After 4 hrs, both injected and non-injected hind paws were amputated from the sacrificed animals, weighed, and statistically quantified for edema formation. Intraplantar carrageenan injection in rat hind paw resulted in a visual and quantifiable increase in hind paw weight (0.813 ± 0.025 g: n =6). Whereas, the ABN-5d administration caused a significant dose-dependent inhibition of the carrageenan-induced increase in hind paw weight (Fig. 2). The reduction in carrageenan-induced hind paw weight followed by ABN-5d treatment at a dose of 0.5 and 1 mg/kg (i.p. injection) was 0.55 g ± 0.05 and 0.525 g ± 0.03 (n =6), respectively. The standard drug aspirin exhibited significant inhibition of carrageenan-induced edema formation (0.6 g ± 0.03) in rat hind paw. [ESM 1].

ABN-5d reduces MPO activity in the carrageenan induced hind paw edema

The enhanced myeloperoxidase (MPO) enzyme activity is used as an inflammatory marker to assess neutrophil infiltration into the inflamed paw tissue (Khan et al. 2018). As expected, the elevated MPO
activity in the carrageenan injected rat hind paw resulted in a significant increase in neutrophil infiltration (p<0.001) (Fig. 3a) when compared with control. Whereas ABN-5d at a dose of 0.5 and 1.0 mg/kg injected (i.p) 1 hr before the intraplantar injection of carrageenan reduced the MPO activity in the hind paw in a dose-dependent manner (p<0.001). Treatment with the reference drug aspirin, at a concentration of 1 mg/kg, showed a prominent reduction in carrageenan induced tissue MPO activity. Furthermore, histological examination showed no inflammation or tissue destruction in the paw sections of saline-treated rats (Fig. 3a). In contrast, we observed that carrageenan caused increased leukocyte infiltration and enlarged cavities due to tissue destruction (erosion) (Fig. 3b). Whereas treatment with ABN-5d (0.5 and 1.0 mg/kg) (Fig. 3c and d) clearly decreased carrageenan-induced neutrophil infiltration. Aspirin administration also reduced leukocyte infiltration in the paw of carrageenan treated rat (Fig. 3e). Correspondingly, with carrageenan administration, significant increases in paw edema and inflammatory cell infiltration were confirmed through assessment of histological sections, reflected in a marked increase in the overall histology (inflammation) score. There was a significant reduction in histology score because of ABN-5d (0.5 and 1 mg/kg) and aspirin treatment in carrageenan-induced paw animals (Fig 3B). Further in terms of neutrophil content of tissue sections, pre-treatment of 0.5 mg of ABN-5d had a greater effect on reducing the neutrophil infiltration in rat paw edema (Fig 3a and b). [ESM 1].

**ABN-5d reduces TNF-α expression in carrageenan-induced rat hind paw**

TNF-α is a prominent pro-inflammatory cytokine that is involved in the pathophysiological process of inflammation in all clinical conditions. Hence, we decided to check whether ABN-5d-induced reduction in inflammation and any effect on TNF-α in the rat paw edema. As expected, TNF-α protein and mRNA expression increased in carrageenan-induced rat paw edema (p<0.001). However, ABN-5d pre-treatment exhibited a significant reduction in TNF-α expression. (p < 0.001) (Fig. 4a and b). The inhibitory effect was greater when treated with 0.5 mg/kg ABN-5d. The standard drug, aspirin, exhibited significant inhibition of carrageenan-induced TNF-α expression in rat hind paw. [ESM 2].

**Inhibitory effect of ABN-5d on carrageenan-induced H₂S level and CSE gene expression**

The increase in H₂S production (0.43 ± 0.003 nmol/mg protein) was observed after 4 hrs of carrageenan administration when compared with control rats. However, intraperitoneal injection of ABN-5d at either dose (0.5 and 1 mg/kg) or aspirin significantly reduced the H₂S levels in carrageenan-induced inflammation in rat paw (Fig. 5a). As H₂S production is found to be increasing in carrageenan treated hind paw, we evaluated the gene expression of the H₂S synthesizing enzyme CSE. Supporting the increased burst of H₂S level, CSE gene expression significantly upregulated in carrageenan treated paw when compared with control rat paw (Fig.5b). However, ABN-5d and aspirin significantly blocked the CSE gene expression in rat paw edema. Both the doses of ABN-5d inhibited H₂S level and CSE gene expression but the inhibition was greater when treated with 0.5 mg/kg (Fig. 5a and b). [ESM 1&3].

**Inhibitory effect of ABN-5d on carrageenan- induced NO production and iNOS expression**
The anti-inflammatory effect of ABN-5d was also evaluated by measuring the levels of NO and its synthesizing enzyme iNOS in the paw edema. As shown in figures 6a and b, NO production and iNOS expression significantly increased carrageenan-induced inflammation when compared with control animals. However, the treatment with either dose of ABN-5d decreased the production of NO and the expression of iNOS, while 0.5mg/kg of ABN-5d decreased NO and iNOS more than the higher dose 1.0 mg/kg. Interestingly, pre-treatment with 1.0 mg/kg was comparable with the effect of the anti-inflammatory drug aspirin. [ESM 1&3].

**ABN-5d suppresses IkBα degradation and NF-κB translocation in paw edema induced by carrageenan**

During an inflammatory response, the production of pro-inflammatory mediators is regulated by the activation of NF-κB. In this connection, we further examined whether ABN-5d could mediate the inflammatory response through the NF-κB pathway. The results exhibited that ABN-5d could significantly suppress NF-κB translocation in a dose-dependent manner (Fig 7a). As NF-κB translocation is associated with IkBα degradation we further evaluated if ABN-5d influences IkBα degradation using Western blot analysis. As shown in fig 7b and c, carrageenan injection led to markedly reduced IkBα degradation in the inflamed paws of rats. Remarkably, pre-treatment with ABN-5d was able to dose-dependently prevent IkBα degradation in the inflamed paw tissue. Pre-treatment with 1.0 mg/kg ABN-5d had more degradation than 0.5 mg/kg dose and was comparable with the anti-inflammatory effect of the reference drug aspirin. [ESM 4].

**Discussion**

Our study focused on the anti-inflammatory activity of newly synthesized azabicyclononane derivative-5d (ABN-5d) in carrageenan-induced hind paw edema. It is a well-known acute model of inflammation used to screen the anti-inflammatory compounds. We found that intraplantar injection of a rat with ABN-5d markedly reduced carrageenan-induced hind paw edema and is confirmed by histopathological observations. Here the anti-inflammatory activity of the ABN-5d derivative (0.5 and 1 mg/kg) is observed when administered 1 hr before carrageenan injection.

Usually, the first phase of an inflammatory response in this rat paw edema model involves the simultaneous release of histamine, serotonin, and kinins, and the second phase is observed with the local neutrophil sequestration and release of pro-inflammatory mediators (Bhukya et al. 2009; Ashok et al. 2010).

Intraplantar injection of carrageenan leads to an increase in the size of the paw edema volume, whereas ABN-5d significantly reduced carrageenan-induced paw edema formation (Fig 2). Also, we showed that intraperitoneal administration of ABN-5d derivative (0.5 and 1.0 mg/kg) effectively inhibited the infiltration of neutrophils as determined by the levels of the marker enzyme myeloperoxidase (MPO) in the paw edema (Fig 3a). Myeloperoxidase is an enzyme found primarily in azurophilic granules of neutrophils, and the activity of which is used as a marker for tissue neutrophil level and its reduced activity imply the presence of anti-inflammatory activity due to reduction in sequestration of the
neutrophils at the site of inflammation (Bradley et al. 1982; George et al. 2016). Among the two optimized doses tested, a dose-related reduction in edema was observed with the ABN-5d derivative (0.5 and 1 mg/kg). Interestingly, from our results, a lower ABN-5d derivative (0.5 mg/kg) dose was more effective than the high dose and the effect of 1.0 mg/kg was similar to the anti-inflammatory effect of the standard drug aspirin (George et al. 2016).

While exploring the anti-inflammatory role, we further showed the possible protective effect arising due to the inhibition of the pro-inflammatory cytokine TNF-α. Previously, researchers have proposed that TNF-α plays a pivotal role in the inflammatory process. For instance, TNF-α can stimulate the macrophages and secrete other inflammatory cytokines (Liao et al. 2013), and blocking TNF-α would be more effective in attenuating the inflammation (Aggarwal et al. 2013; Ma et al. 2014). In the present study, ABN-5d significantly downregulated the pro-inflammatory cytokine TNF-α, in carrageenan-induced inflammation. Our present findings are consistent with the results of other workers reported (Min et al. 2011; Rose et al. 2017).

In this study, we have also found that H₂S and NO levels have a role in regulating the anti-inflammatory activity in presence of ABN-5d. It has also been reported previously that the individual and combined increased production of NO by iNOS and H₂S by CSE are the mediator of inflammation on paw edema (Li et al. 2005). Blockage of H₂S production by PAG (DL-propargylglycine), an inhibitor of CSE enzyme activity, and nitric oxide by L-NAME, an inhibitor of iNOS reduced the severity of inflammation induced by carrageenan (Pan et al. 2012). Our results showed ABN-5d derivative (0.5 and 1 mg/kg) inhibited the carrageenan-induced H₂S and NO production significantly accompanied with inhibition of CSE and iNOS gene expression, that too with nearly the same efficacy as aspirin. Interestingly, from our results, ABN-5d derivative at 0.5 mg/kg inhibited the pro-inflammatory mediators’ TNF-α, H₂S, and NO relatively stronger than 1.0 mg/kg. From these results, it is suggestive that the mechanism of anti-inflammatory action of the derivative could be via the suppression of H₂S and NO production, besides the inhibition of TNF-α in paw edema. Dominant with suppression of NO, H₂S, and TNF-α results indicate that ABN-5d regulates the second phase of an inflammatory response in edema.

H₂S upregulated iNOS expression and NO, whereas NO enhanced CSE expression and H₂S generation. These two signalling molecules showed interplay between each other by regulating their synthesis via the activation of the NF-κB pathway. The nuclear transcription factor NF-κB regulates the transcription of a wide variety of proinflammatory genes implicated as the major regulator in the progression of inflammatory diseases (Liu et al. 2017). It has also been reported that inactivation of NF-κB and degradation of IκBα using genetic models and pharmacological studies have attenuated the disease severity. In the present study, ABN-5d may attenuate the inflammatory response by inhibiting the nuclear translocation of NF-κB and cytoplasmic degradation of IκBα in carrageenan-induced rat paw edema. Previous research works showed that carrageenan-induced inflammatory response is mediated through the activation of NF-κB (Borthakur et al. 2012). Hence, our observed inhibition of NF-κB activation may also contribute to the protective effect of ABN-5d on paw edema. The present results suggest that ABN-5d
suppresses both the first and second phase of carrageenan-induced paw edema, thus, confirming the NSAID-like property.

Extensive effort over recent years in better understanding the pathogenesis of inflammation has led to the development of novel therapeutic approaches in targeting the cytokines, H$_2$S, and NO that triggers the inflammatory responses. Our finding of inhibition of TNF-α, as well as H$_2$S and NO production which act as prominent inflammatory mediators, may regulate the stage and the progression during the pathogenesis of the disease. Hence, a better understanding and targeting the underlying mechanisms of the inflammation may give rise to new alternative therapeutic strategies.

**Conclusion**

In summary, our findings demonstrate that novel synthesized azabicyclanonane derivative at a low dose exhibits promising anti-inflammatory activity against carrageenan-induced hind paw edema in rats through the suppression of hydrogen sulphide and nitric oxide. As an anti-inflammatory agent azabicyclanonane also showed a protective effect against the NF-κB pathway. The precise role of H$_2$S and nitric oxide and its interaction with other inflammatory mediators involved will be the subjects for further studies.

**Declarations**

**Ethical approval**

All experimental protocols were developed according to the guidelines approved by CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals). Animals were approved by Institutional Animal Ethics Committee (Approval no. VIT/IEAC/13th/Feb13/24).

**Consent to publish**

Human patients/biological tissue are not involved in this study.

**Authors contributions**

BR performed the work, analyzed the data, and wrote the manuscript; VM and TR also conceived and designed the research, supervised the study provided critical feedback and helped to shape the research work manuscript; SKI and VR contributed reagents and synthesized the chemical compound azabicyclanonane. All authors read and approved the manuscript and all data were generated in-house and that no paper mill was used.

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Competing Interests

Authors declare there are no competing interests.

Availability of data and materials

The data used to support the findings of the study are included within the article. The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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**Table**

**Table 1.** Real time PCR Primer Sequences
| Primer Name | Accession number | Primers                  |
|-------------|------------------|--------------------------|
| iNOS        | AY211532         | sense: 5′-CACCACCCTCCTTGTTCAAC-3’<br>antisense: 5′-CAATCCACAACTCGCTCCAA-3’ |
| TNF-α       | XM_032888689     | sense: 5′-CCACGCTCTTCTGTCTACTGA-3’<br>antisense: 5′-GGCCATGGAACTGATGAGAGG-3’ |
| CSE         | NM_017074        | sense: 5′-GACCTCAATAGTCGGCTTCGTTTC-3’<br>antisense: 5′-CAGTTCTGCGTATGCTCCGTAATG-3’ |
| GAPDH       | XM_032910454     | sense: 5′-ACTCCACTCAGGCAATTC-3’<br>antisense: 5′-GTCATGAGCCCTTCCACAAT-3’ |

**Figures**

**Figure 1**

ABN 5d with fluoro (O-F) at the ortho position electron withdrawing group in the phenyl ring.
Figure 2

Effect of carrageenan injection on hind paw edema at 4th hour and the effect of different doses of azabicyclononane ABN-5d (0.5, and 1 mg/kg). Results show mean ± SD, n = 6. #p<0.001 when compared with control, *p<0.001 when compared with carrageenan.
Figure 3

a Effect of carrageenan injection on MPO activity and the effect of different doses of azabicyclononane ABN-5d (0.5, and 1 mg/kg). Results show mean ± SD. n = 6. #p<0.001 when compared with control, *p<0.001 when compared with carrageenan. b. scoring of inflammation in hind paw edema based on H&E staining, c. Histological assessment of the effect of azabicyclononane ABN 5d on carrageenan-induced paw edema in rats. Representative sections of paw from the a) control, b) 1 % carrageenan, c) 1 % carrageenan + 0.5 mg ABN 5d and d) 1 % carrageenan + 10 mg ABN-5d groups stained with H&E stain, e) 1% carrageenan+aspirin.
a. Effect of intraplantar carrageenan injection and different dosage of azabicyclononane ABN-5d on TNF-α level. Hind paws pre-treated with ABN 5d were removed at 4th hour after carrageenan injection, and TNF-α level was measured. The data are the mean ± S.D. of three experiments. The symbols represent statistical significance at: *p<0.05. b. Azabicyclononane ABN 5d inhibits carrageenan-induced TNF-α activity in rat hind paw. TNF-α mRNA expression in control, carrageenan, and ABN-5d (0.5 and 1mg/kg) treated groups was measured by real-time PCR. TNF-α sample loading was normalized with GAPDH
internal control. Results show mean ± SD, n = 6. #p<0.001 when compared with control, *p<0.001 when compared with carrageenan.

Figure 5

a. Effect of intraplantar carrageenan injection and different doses of azabicyclononane ABN 5d on H2S concentration in hind paw. Hind paws pre-treated with ABN 5d were removed at 4th hour after carrageenan injection, and H2S level was measured. Results show mean ± SD, n = 6. #p<0.001 when compared with control, *p<0.001 when compared with carrageenan.

b. Azabicyclononane ABN 5d inhibits carrageenan induced CSE enzyme activity in rat hind paw. mRNA expression in control, carrageenan, and ABN-5d (0.5mg and 1 mg/kg) treated groups was measured by real-time PCR. CSE sample loading was normalized with GAPDH internal control. Results show mean ± SD, n = 6. #p<0.001) when compared with control, *p<0.001 when compared with carrageenan.

Figure 6

a. Effect of intraplantar carrageenan injection and different doses of azabicyclononane ABN 5d on NO concentration in hind paw. Hind paws pre-treated with ABN 5d were removed at 4th hour after carrageenan injection, and NO level was measured. Results show mean ± SD, n = 6. #p<0.001 when compared with control, *p<0.001 when compared with carrageenan.
Figure 6

a. Effect of intraplantar carrageenan injection and azabicyclononane ABN-5d on Nitric oxide production. Different dosages of azabicyclononane ABN 5d Nitrite/Nitrate measured by Griess method. Vertical bars represent mean ± SEM (n=6). *P<0.01 and **P<0.001 (compared control group). b. Azabicyclononane ABN 5d inhibits carrageenan-induced iNOS enzyme activity in rat hind paw. mRNA expression in control, carrageenan, and ABN-5d (0.5 and 1mg/kg) treated groups was measured by real-time PCR. iNOS sample loading was normalized with GAPDH internal control. Results show mean ± SD, n =6. #p<0.001 when compared with control, *p<0.001 when compared with carrageenan.

Figure 7
a. NF-κB binding activity at different concentration of ABN-5d (0.5 and 1mg/kg) is shown. Data are presented as mean ± SEM of groups. *p<0.001 versus the control group (Saline and received carrageenan). b. Representative images of immunoblot analysis for IκBα. The graphical shown the effect of pre-treatment with saline or ABN-5d (0.5 and 1mg/kg), c. Represent IκBα & HPRT. Protein levels were expressed in arbitrary units, as the ratio of signal intensity for the target protein relative to HPRT, which represented the relative values among all samples.

**Supplementary Files**

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