Original Article

Novel Azabicyclo [3.3.1] Nonan-9-One (ABN-5d) Derivative Inhibits Carrageenan-Induced Rat Hind Paw Edema

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DOI: 10.47750/pnr.2022.13.S08.556

Abstract

Background: 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, this novel 2,4-Diaryl-6,7 -benzo azabicyclo [3.3.1] nonan-9-ones (azabicyclononane derivative-ABN-5d) compound that has similar structure of curcumin and piperdine derivative hence in this we unravelled the mechanism of anti-inflammatory effect in carrageenan-induced (1% intraplantar injection) hind paw inflammation in rat model.

Methods and Results: The synthesized azabicycle derivative ABN-5d (2.5, 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. Carrageenan-induced peripheral inflammation in the rat. ABN-5d administration significantly decreased in histopathological changes, myeloperoxidase (MPO) activity, gaseous mediators such as hydrogen sulphide (H_2S), Nitric oxide (NO) level, inflammatory mediators such as TNF-α, CSE, and iNOS gene expression ($p < 0.001$). We also demonstrated that ABN-5d significantly reduced carrageenan-induced NF-κB activation and IκBα degradation in the inflamed paw tissue. These results indicate that the anti-inflammatory effect of ABN-5d on carrageenan-induced rat paw edema could be through the inhibition of the iNOS/NO-CSE/ H_2S -NFκB pathway.

Conclusion: ABN-5d doses has the potent of anti-inflammatory effect near to that of control and standard anti-inflammatory drug.

Keywords: Azabicyclononane derivative, carrageenan, hind paw edema, Myeloperoxidase, gaseous mediators, inflammatory mediators, iNOS/NO-CSE/ H_2S -NFκB pathway.

INTRODUCTION

Inflammation is a process associated with infiltrating neutrophils and macrophages which are involved in the production and regulation of proinflammatory mediators [1] including cytokines, chemokines as well as the gaseous mediators nitric oxide (NO) and hydrogen sulphide (H_2S) [2]. 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_2S 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 [3]. 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_2S in inflammation [4–6]. Also, the inhibition of H_2S and NO production by gene silencing [7] using pharmacological inhibitors [8] , and natural compounds [9] resulted in the reduction of TNF-α and the accompanying pro-inflammatory signalling pathways including nuclear factor kappa B (NF-κB) [10].

Scientific evidence supports that naturally derived phytochemicals are effective anti-inflammatory agents used as therapeutic agents for various inflammatory conditions [11]. 3-azabicyclononane (ABN) group of heterocyclic alkaloids are naturally occurring in *aconitum*, *delphinium*, *consolida*, and *thalictrum* plant species [12]. These are widely distributed among the family Ranunculaceae. These compounds are under scrutiny due to their multiple biological roles as analgesics, anaesthetics [13], antimicrobial [14], and herbicidal, insecticidal, anti-inflammatory, and recently discovered to play a role as anticancer agents [15]. 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 [12].

Amongst the synthesized azabicycle products, it was recently reported that the derivatives ABN- 5d possesses anti-proliferative activity against various human tumor cell lines [16]. 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 (Figure 1). Though the cancer-specific anti-proliferation was proved, and Like ABN derivatives, many mannich based compounds piperdine derivatives found to have anti-inflammatory activity[17] and the anti-inflammatory effect specifically the molecular mechanism of ABN-5d synthesised by us has to be explored. In this study, we, therefore, examined whether the anti-inflammatory effect of the alkaloid ABN-5d is through H_2S /NO linked NF-κB activation using the carrageenan-induced inflammatory hind paw edema model in rats. ABN-5d compound showed ~~to~~ no toxic effect in our *in vitro* studies and hence we proceeded further with *invivo* model.

EXPERIMENTAL SECTION

Structure of azabicyclononane (ABN)

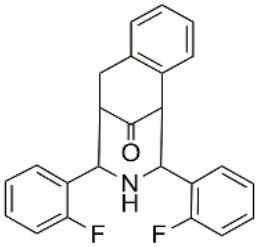

Figure 1. ABN 5d with fluoro (O-F) at the ortho position electron withdrawing group in the phenyl ring.

Compound 2,4-Diaryl-6,7-benzo-3-azabicyclo [3.3.1] nonan-9-ones (azabicyclononane) was synthesized by Research group, SAS-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 Figure 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.

Carrageenan induced paw edema model

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 [18].

Measurement of myeloperoxidase (MPO) activity

Measurement of the myeloperoxidase (MPO) activity was determined as described by Leema et al [18]. 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 H_2SO_4 (0.18 M), and absorbance was measured at a wavelength of 450 nm.

Morphological examination

Paw biopsies were taken after 4 h of carrageenan injection. Paw tissues were fixed with 10% formalin solution then embedded with Paraffin and sectioned (5 µM). Sectioned tissues were stained with haematoxylin/eosin (H and E), were examined with light microscopy. All tissues were photographed with a Canon camera connected to an optical microscope [19].

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-5d (2.5 mg/kg, 0.5 mg/kg and 1 mg/kg). Samples were collected after 4th hr induction of carrageenan and then homogenized in 1X Phosphate buffered saline (PBS) solution containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 (Merck) and centrifuged at 1200 g for 10 mins at 4˚C [20]. Then the supernatant was taken for measuring TNF-α using ELISA kit (R&D systems) according to the manufacture instructions.

Assay of tissue H_2S production

Measurement of hydrogen sulphide (H_2S) was determined as described by Leema et al [18]. 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 sulphate (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 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 production was measured at its breakdown products nitrite (NO2-) and nitrate (NO3-) using a Griess method [21, 22]. Briefly, hind paw tissue samples were first homogenized and centrifuged. To the supernatant, 100 µl of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride) has added, and the absorbance at 540 nm was measured. The absorbance of the samples was compared with the standard curve of sodium nitrite.

Real time PCR

Total RNA was extracted from rat paw with TRIzol reagent (Sigma Aldrich) according to the manufacturer's protocol [23]. 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 analysed 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.

Immunoblot Analysis

Western blot analysis was performed to evaluate the IκBα protein levels in hind paw edema induced Rats and the assay was performed by I.F. Florentino et al 2017 with slight modifications [24]. 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 3 thrice with 0.1% buffer solution (Tween 20/PBS-buffered saline). Alkaline phosphatase-conjugated antibody anti-mouse IgG as the 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.

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.

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, 0.10 g ± 0.02(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.

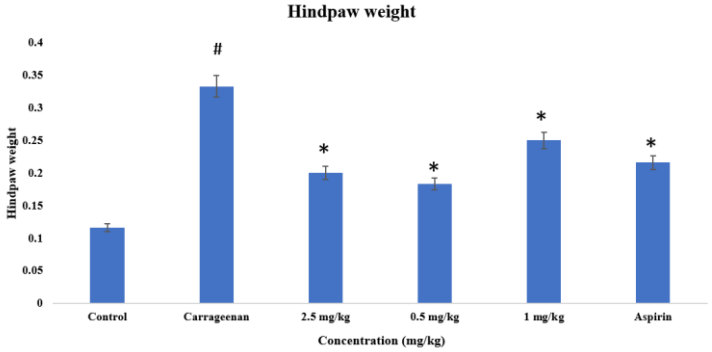

Figure 2. Effect of carrageenan injection on hind paw edema at 4th hour and the effect of different doses of azabicyclononane ABN-5d (2.5, 0.5 and 1 mg/kg). Results show mean ± SD, n = 6. #p<0.001 when compared with control, *p<0.001 when compared with carrageenan.

Intraplantar carrageenan injection in rat hind paw resulted in a visual and quantifiable increase in hind paw weight (0.33 g ± 0.02 (n =6). Whereas, the ABN-5d administration caused a significant dose-dependent inhibition of the carrageenan-induced increase in hind paw weight (Figure 2). The reduction in carrageenan-induced hind paw weight followed by ABN-5d treatment at a dose of 2.5, 0.5 and 1 mg/kg (*i.p*. injection) was 0.11 g ± 0.066, 0.18 g ± 0.058 and 0.25 g ± 0.05 (n =6), respectively. The standard drug aspirin exhibited significant inhibition of carrageenan-induced edema formation (0.22 g ±0.03) in rat hind paw.

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 [25]. As expected, the elevated MPO activity in the carrageenan injected rat hind paw resulted in a significant increase in neutrophil infiltration (p<0.001) (Figure 3a) when compared with control. Whereas ABN-5d at a dose of 2.5, 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 whereas 2.5 mg/kg showed 0.11 g ± 0.02, 0.5 mg/kg concentration showed 0.09 g ± 0.010 and 1mg/kg showed 0.15 g ± 0.01. Here 1 mg/kg concentration showed similar activity as standard drug Aspirin whereas 0.5 mg/kg showed prominent reduction nearly to control. Furthermore, histological examination showed no inflammation or tissue destruction in the paw sections of saline-treated rats (Figure 3a).

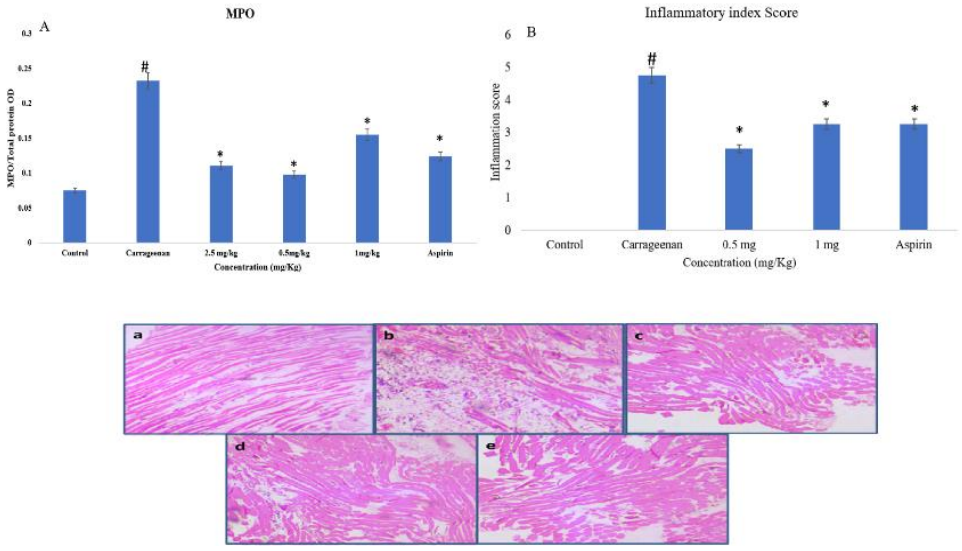

Figure 3a. Effect of carrageenan injection on MPO activity and the effect of different doses of azabicyclononane ABN-5d (2.5 mg, 0.5, and 1 mg/kg). Results show mean ± SD. n = 6. #p<0.001 when compared with control, *p<0.001 when compared with carrageenan. b. scoring of inflammation in hind paw edema 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.

In contrast, we observed that carrageenan caused increased leukocyte infiltration and enlarged cavities due to tissue destruction (erosion) (Figure 3b). Whereas treatment with ABN-5d (0.5 and 1.0 mg/kg) (Figure 3c and d) clearly decreased carrageenan-induced neutrophil infiltration. Aspirin administration also reduced leukocyte infiltration in the paw of carrageenan treated rat (Figure 3e). Correspondingly, with carrageenan administration significant increases in paw edema and inflammatory cell infiltration were confirmed through assessment of histological sections, reflected with 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 (Figure 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 (Figure 3a and b).

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.

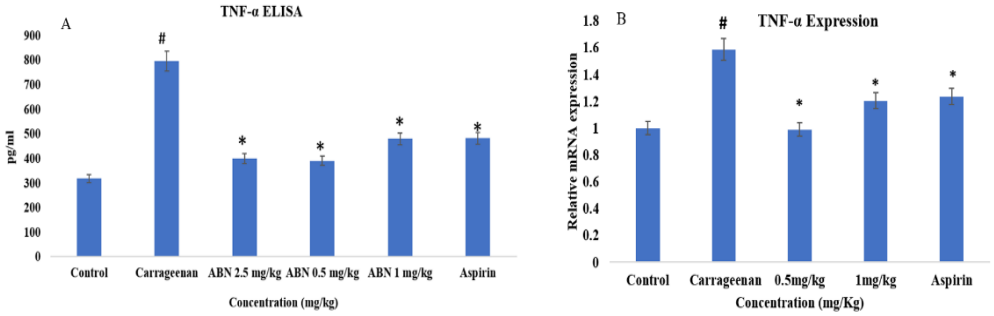

Figure 4a. Effect of intraplantar carrageenan injection and different dosage of azabicyclononane ABN-5d on TNF-α level. Hind paws pre-treated with ABN 5d were removed at 4th hour after carrageenan injection, and TNF-α level was measured. The data are the mean ± S.D. of three experiments. The symbols 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<0.001when compared with control, *p< 0.001 when compared with carrageenan

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) (Figure 4a and b). The inhibitory effect in 2.5 mg/kg was little higher 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.

Inhibitory effect of ABN-5d on carrageenan-induced H_2S level and CSE gene expression

The increase in H_2S production (0.44 ± 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 (2.5, 0.5 and 1 mg/kg) or aspirin significantly reduced the H_2S levels in carrageenan-induced inflammation in rat paw (Figure 5a). As H_2S production is found to be increasing in carrageenan treated hind paw, we evaluated the gene expression of the H_2S synthesizing enzyme CSE. Supporting the increased burst of H_2S level, CSE gene expression significantly upregulated in carrageenan treated paw when compared with control rat paw (Figure 5b).

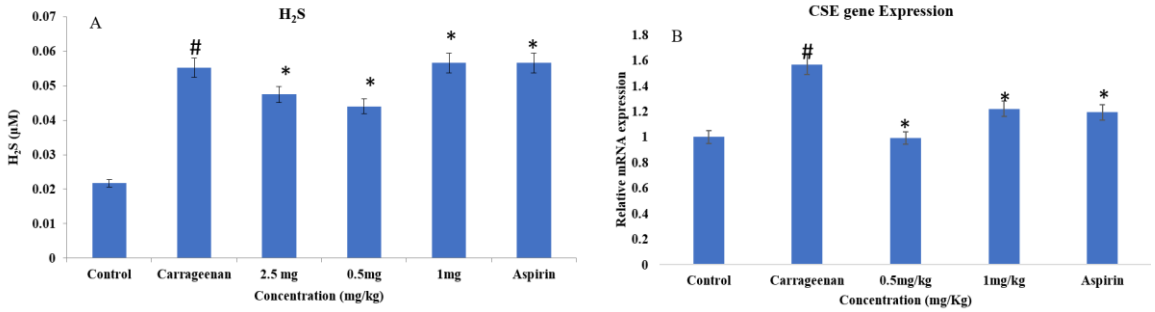

Figure 5a. Effect of intraplantar carrageenan injection and different doses of azabicyclononane ABN 5d on H_2S concentration in hind paw. Hind paws pre-treated with ABN 5d were removed at 4th hour after carrageenan injection, and H_2S level was measured. Results show mean ± SD, n =6. #p<0.001 when compared 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 1mg/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.

However, ABN-5d and aspirin significantly blocked the CSE gene expression in rat paw edema. Both the doses of ABN-5d inhibited H_2S level and CSE gene expression but the inhibition was greater when treated with 0.5 mg/kg (Figure 5a). CSE gene expression in low dose 0.5 mg/kg prominently reduced than 2.5 mg/kg and 1mg/kg. Here the results indicate the 1mg concentration was effectively similar to that of standard drug Aspirin. (Figure 5b). Here 2.5 mg/kg showed slight increase than 0.5 mg/kg.

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.

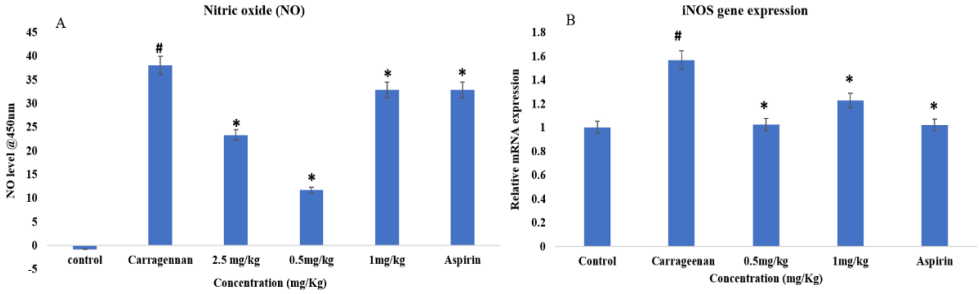

Figure 6a. Effect of intraplantar carrageenan injection and azabicyclononane ABN-5d on Nitric oxide production. Different dosages of azabicyclononane ABN 5d Nitrite/Nitrate measured by Griess method. Vertical bars represent mean ± SEM (n=6)). *P<0.01 and **P<0.001 (compared control group). b. Azabicyclononane 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.

As shown in figure 6a and b, NO production and iNOS expression significantly increased carrageenan-induced inflammation when compared with control animals. However, the treatment with dose of ABN-5d decreased the production of NO and the expression of iNOS, while 2.5 mg/kg increased the production of NO than 0.5mg/kg of ABN-5d 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.

ABN-5d suppresses IκBα 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.

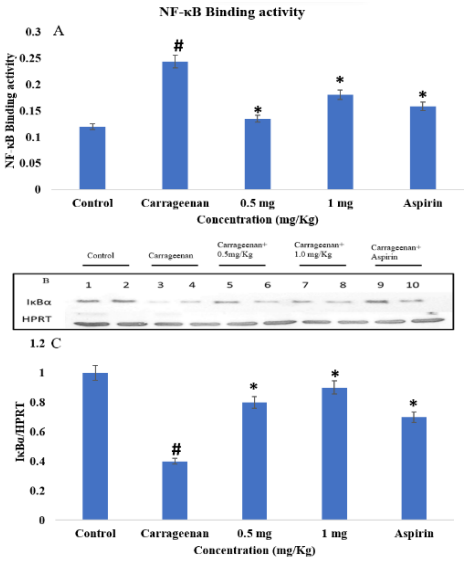

Figure 7a. NF-κB binding activity at different concentration of ABN-5d (0.5 and 1mg/kg) is shown. Data are presented as mean ± SEM of groups. *p<0.001 versus the control group (Saline and received carrageenan). b. Representative

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.

Our results exhibited that ABN-5d could significantly suppress NF-κB translocation (Figure 7a). As NF-κB translocation is associated with IκBα degradation, we further evaluated if ABN-5d influences IκBα degradation using Western blot analysis. As shown in figure 7b and c, carrageenan injection led to markedly reduced IκBα degradation in the inflamed paws of rats. Remarkably, pre-treatment with ABN-5d was able to prevent IκBα 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. As 2.5 mg/kg has slight increase in inflammatory markers. Overall, 0.5 mg/kg values taken as potent concentration for anti-inflammatory.

DISCUSSION

Our study focused on the anti-inflammatory activity of newly synthesized azabicyclononane derivative-5d (ABN-5d) in carrageenan-induced hind paw edema. This hind paw edema induction 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. Anti-inflammatory activity was observed with ABN-5d derivative (2.5, 0.5 and 1 mg/kg) when administered 1 hr before the inducer carrageenan injection.

Usually, the first phase of inflammatory response in the 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 [26, 27].

Intraplantar injection of carrageenan leads to increase in the size of the paw edema volume, whereas ABN-5d significantly reduced carrageenan-induced paw edema formation (Figure 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 (Figure 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 the sequestration of neutrophils at the site of inflammation [18, 28]. Among the two optimized doses tested, a dose-related reduction in edema was observed with the ABN-5d derivative (2.5, 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 2.5 mg/kg show similar effect like 0.5 mg/kg and 1.0 mg/kg was similar to the anti-inflammatory effect of the standard drug aspirin [18].

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 [29], and blocking TNF-α would be more effective in attenuating the inflammation [23, 30]. In the present study, ABN-5d significantly downregulated the pro-inflammatory cytokine TNF-α, in carrageenan-induced inflammation. Our present findings are also consistent with the results, of other workers reported [31, 32].

In this study, we have also found that H_2S 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 H_2S by CSE are the mediator of inflammation on paw edema [2]. Blockage of H_2S production by PAG, an inhibitor of CSE enzyme activity, and nitric oxide by L-NAME, an inhibitor of iNOS reduced the severity of inflammation induced by carrageenan [33]. Our results showed ABN-5d derivative (2.5, 0.5 and 1 mg/kg) inhibited the carrageenan-induced H_2S and NO production significantly accompanied with reduced CSE and iNOS gene expression, that too with nearly the same efficacy as aspirin. Interestingly it was observed here that, ABN-5d derivative at 0.5 mg/kg inhibited the pro-inflammatory mediators TNF-α, H_2S, and NO relatively stronger than 2.5 mg/kg and 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 H_2S and NO production, besides inhibition of TNF-α in paw edema. Dominant with suppression of NO, H_2S, and TNF-α results indicate that ABN-5d regulates the second phase of an inflammatory response in edema.

H_2S upregulated iNOS expression and NO, whereas NO enhanced CSE expression and H_2S 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 wide variety of proinflammatory genes and is implicated as the major regulator in the progression of inflammatory diseases[34]. 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, it is likely that 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 [35]. 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. Hence we took 0.5 mg/kg as the potent dosage for effective anti-inflammatory effect so further studies in gene expression and immunoblot analysis we used 0.5 mg/kg and 1.0 mg/kg.

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_2S, and NO that triggers the inflammatory responses. Our finding of inhibition of TNF-α as well as H_2S and NO production which acts as a prominent inflammatory mediator, 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 azabicyclononane derivative at a low dose exhibit 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 azabicyclononane also showed a protective effect against the NF-κB pathway, and the reason for this activity may be due to the presence of the hydrophilic group at the para position of the moiety. The precise role of H_2S and nitric oxide and its interaction with other inflammatory mediators involved will be the subjects for further studies.

ACKNOWLEDGMENTS

We thank VIT University, Vellore, Tamilnadu, and India for providing support for this study. The authors would like to extend the appreciation to the Vellore Institute of Technology (VIT), India for providing seed fund and research scholar fund to carry out this research work.

FUNDING

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

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Ethical Declarations

Conflict of interest

Authors declare that none of them has any conflict of interest or financial arrangements that could potentially influence the described research.

Ethical approval

The maintenance and ethical treatment of laboratory animals was done in accordance with 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/ 13)**.

Research involving human and animal participants

The studies were performed in Male Wistar Rats and did not involve any human participants, human data or human tissues.

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