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Characterization of a unique dihydropyrimidinone, ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate, as an effective antithrombotic agent in a rat experimental model

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Abstract

Objectives To evaluate the potential of a novel dihydropyrimidinone, ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate (H-DHPM), as a calcium channel blocker, endowed with the ability to inhibit platelet aggregation effectively.
Methods In-vitro and in-vivo studies were conducted for the determination of antiplatelet activity using adenosine diphosphate (ADP), collagen or thrombin as inducers. Calcium channel blocking activity and nitric oxide synthase (NOS) activity were monitored. Lipopolysaccharide (LPS)-mediated prothrombotic conditions were developed in rats to study the efficacy of H-DHPM to suitably modulate the inflammatory mediators such as inducible NOS (iNOS) and tissue factor. The cGMP level and endothelial NOS (eNOS) expression were checked in aortic homogenate of LPS-challenged rats pretreated with H-DHPM. The effect of H-DHPM on $FeCl_3$-induced thrombus formation in rats was examined.
Key findings The concentrations of H-DHPM required to give 50% inhibition (IC50) of in-vitro platelet aggregation induced by ADP, collagen or thrombin were 98.2 ± 2.1, 74.5 ± 2.3 and 180.7 ± 3.4 μM, respectively. H-DHPM at a dose of 52.0 ± 0.02 mg/kg (133 μmol/kg) was found to optimally inhibit ADP-induced platelet aggregation *in-vivo*. The level of nitric oxide was found to be up to 9 ± 0.08-fold in H-DHPM-treated platelets *in-vitro* and 8.2 ± 0.05-fold in H-DHPM-pretreated rat platelets *in-vivo* compared with control. OH-DHPM, the parent compound was found to be ineffective both *in-vitro* and *in-vivo*. H-DHPM-pretreated rats were able to resist significantly the prothrombotic changes caused by LPS by blunting the expression of iNOS, tissue factor and diminishing the increased level of cGMP to normal. H-DHPM enhanced the eNOS expression in aorta of rats treated with LPS. H-DHPM displayed synergy with antiplatelet activity of aspirin even at lower doses. H-DHPM was found to inhibit the LPS-induced platelet aggregation in younger as well as older rats. H-DHPM exhibited the ability to markedly decrease $FeCl_3$-induced thrombus formation in rats.
Conclusions H-DHPM has the attributes of a promising potent antiplatelet candidate molecule that should attract further study. H-DHPM displayed antiplatelet activity both *in vivo* and *in vitro*, which was due partially by lowering the intraplatelet calcium concentration.
Keywords calcium channel blocker; lipopolysaccharide; nitric oxide synthase; platelet aggregation

Introduction

The major function of platelets is to arrest blood loss after tissue trauma and exposure of the subendothelial matrix. However, altered platelet function can be responsible for atherothrombosis, which is the leading cause of death in the developed world.[1–3] Numerous antiplatelet agents have been developed based on their ability to block the receptors responsible for platelet activation. For instance, clopidogrel by virtue of its remarkable ability to irreversibly bind ADP receptor P2Y12, proved to be an important antiplatelet drug.[4] Aspirin

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(acetylsalicylic acid), on the other hand, inhibits prostaglandin synthesis in platelet as well as the collagen induced platelet release reaction. The mechanism of the inhibitory action of aspirin includes the involvement of acetylation of cyclooxygenase, leading to the down regulation of thromboxane A2 synthesis and concomitant inhibition of platelet aggregation.[5]

Calcium channel blockers are a class of drugs that block the entry of calcium into the muscle cells of the heart and arteries.[6] The entry of Ca^{2+} into the cells causes the heart to contract and narrowing of the arteries. Calcium channel blockers decrease the contraction of the heart and dilate the arteries by the upregulation of endothelial nitric oxide (NO) production in cells. This observation indicated that the reduction in cytosolic Ca^{2+}, inhibition of platelet aggregation, and vasodilation with calcium channel blockade were not only due to inhibition of Ca^{2+} entry but also to an NO-cGMP-mediated mechanism.[7] There are two types of calcium channel blocker: short-acting and long-acting. Short-acting calcium channel blockers such as nifedipine work quickly and their effect lasts only for a few hours.[8] The long-acting calcium channel blockers (L-type) take longer to start working and their effect persists for a prolonged period. Amlodipine, a dihydropyridine (DHP) is an L-type calcium channel blocker, a well studied, commonly used antihypertensive drug.[9]

Much less is known about the dihyropyrimidinone (DHPM) derivatives as antiplatelet drugs, most of the information available on them is the ability of DHPMs as antimicrobial and antihypertensive agents.[10,11] In this study, we have reported for the first time on ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate (H-DHPM) as an antiplatelet agent. A number of DHPM derivatives ranging from acetoxy DHPM to decanoylated DHPM, as well as the parent moiety (hydroxy derivative, OH-DHPM, Figure 1, inset) were synthesized in our laboratory.[12] Efforts were made to establish their calcium channel blocking activity. Among them H-DHPM (Figure 1, inset) was found to be a versatile calcium channel blocker endowed with antiplatelet and antithrombotic activity. A single acyl (heptanoyl) group at the C4′ position of DHPM molecule controls the calcium channel blockers and antiplatelet activity. This was evident from the fact that the parent phenolic derivative dihydropyridine (OH-DHPM) was devoid of the biological activity under consideration. The other substituents at various positions on the phenyl/pyrimidinone ring of the H-DHPM molecule were monitored and were found to hardly affect calcium channel blockers or antiplatelet activity (unpublished data). Such clear-cut structural motif of controlling calcium channel blocker activity is not encountered with other known calcium channel blockers. The precise structural requirement would help in the development of a key molecule as an effective antiplatelet agent. We have investigated the antiplatelet activity of H-DHPM in comparison with known antiplatelet drugs, like amlodipine, clopidogrel and aspirin.

Materials and Methods

Materials

Aspirin, dichlorofluorescin diacetate (DCFH-DA), L-arginine, adenosine diphosphate (ADP), arachidonic acid, collagen, histopack, horseradish peroxidase conjugated secondary antibody, endothelial NO synthase and inducible NO synthase (eNOS and iNOS, respectively) antibodies, Tween-20, Fluo-3AM (a fluorescent dye), lipopolysaccharide (LPS), L-N^G-nitroarginine methyl ester (L-NAME), the bacterial endotoxin *Escherichia coli* serotype (111:B4), and thrombin were from M/S Sigma Chemical Co., St Louis. MO, USA. Tissue factor ELISA kit and cGMP ELISA kit were purchased from Assay Pro and Cayman's Chemicals, respectively. All other chemicals used were of high purity and were obtained from local suppliers (Delhi, India).

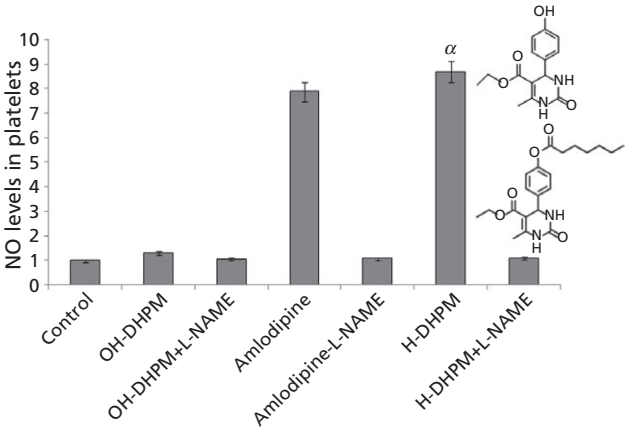

Figure 1 Enhancement of nitric oxide levels in platelets by ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate. Human platelets were incubated with OH-DHPM (parent compound), ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate (H-DHPM) or amlodipine (180 μM) along with L-arginine (100 μM) and DCFH-DA (2 μM) for 30 min at 37°C followed by the measurement of DCF fluorescence. Platelets, pretreated with L-NAME 1 h before, were further followed by addition of test compounds and assayed for nitric oxide (NO) levels. Values are mean ± SEM of five observations. $^{\alpha}P < 0.05$, compared between H-DHPM- and amlodipine-treated groups. One-way analysis of variance test followed by the Tukey multiple comparisons test were used. Inset: Structures of H-DHPM (bottom) and the hydroxy derivative of DHPM (OH-DHPM, top).

Synthesis of H-DHPM

DHPMs were synthesized as described previously.[12] The melting point, physical and spectral data of the compounds were in complete agreement with those reported earlier in the literature.[12]

Experimental animals

Male Sprague-Dawley rats (200–250 g) were housed in mesh cages in the animal house of Vallabhbhai Patel Chest Institute (University of Delhi, Delhi, India). The animal room was maintained at 25° ± 2°C and illuminated using a 12 : 12 h light/dark cycle. Before conducting experiments animals were acclimatized to laboratory conditions for seven days. All experimental procedures and protocols used in this investigation were reviewed and approved by the Institutional Animal Ethics Committee of Vallabhbhai Patel Chest Institute, University of Delhi.

Blood collection from healthy volunteers

Blood was taken from healthy volunteers ($n = 52$; age 27 ± 1.2 years) after they had received a full explanation about the

details of the experiment and their consent had been given. Approval was obtained from the Ethical Committee of Vallabhbhai Patel Chest Institute, University of Delhi. Venous blood (9 ml) was collected with 1.0 ml 3.8% trisodium citrate from healthy volunteers, who had abstained from medication including aspirin, paracetamol, and alcohol. The citrated blood was used for the preparation of platelet-rich plasma (PRP) as described previously.[13] Platelet count was determined in PRP using an electronic cell counter, SYSMEX Model no. FA 20 (M/S Sysmex Corporation, Kobe, Japan) and was adjusted to 250×10^6 cells/ml with platelet-poor plasma (PPP).

Aggregometry

PRP (500 µl) was pipetted into siliconized glass cuvettes and kept at 37°C for 2 min in the aggregometer. The test compounds were dissolved in methanol, in various concentrations (40–200 µM), and were added to the reaction cuvette to obtain the concentration–response curves. Individual samples were incubated at 37°C for 10 min. After this time platelet aggregation was induced by the addition of ADP (15 µM), collagen (10 µg/ml), or thrombin (0.2 U/ml) and assessed by using a Platelet Aggregation Profiler (Bio/Data Corporation, Hatboro, PA, USA, Model No.Pap-4). The results were expressed as the maximum percentage of light transmittance change (% max) from the baseline at the end of the recording time, using PPP as a reference. Platelet aggregation curves were recorded for 6 min and analysed according to internationally established standards.

Nitric oxide estimation by flow cytometry

The method outlined by Imrich and Kobzik[14] was followed for the assay of nitric oxide synthase (NOS) in platelets by flow cytometry. Human PRP was incubated with OH-DHPM/H-DHPM or amlodipine (180 µM) for 10 min at 37°C and the NOS activity was triggered by the addition of ADP (15 µM) for 5 min. PRP was centrifuged and platelets were isolated, washed twice with phosphate buffered saline (PBS) and resuspended in the standard buffer (in mM: 137 NaCl, 2.8 KCl, 1 $MgCl_2$, 12 $NaHCO_3$, 0.4 Na_2HPO_4, 0.35% BSA, 10 HEPES, 5.5 glucose, pH 7.4) containing 1 µl DCF-DA (dissolved in CH_3OH) in a total volume of 1 ml to make the final concentration 2 µM and kept at 37°C for 30 min. One set of samples were pre-incubated with L-NAME (100 µM), inhibitor of NOS, and was followed by further addition of the test compounds and processed in a similar manner for the measurement of NO activity.

Briefly NO was measured in platelets labelled with the NO fluorescent dye DCFH-DA using a Becton-Dickinson FACScan Flow Cytometer (BD Biosciences, Franklin lakes, NJ, USA). The FACScan was equipped with a 5 W argon laser operating at 15 mW power with an excitation wavelength of 488 nm. The baseline fluorescence was measured immediately by applying 1 ml suspension to the flow cytometer and acquiring 1000 events. The median fluorescence was measured on a log scale using the fluorescein isothiocyanate (FITC) detector (525 nm).

Ca^{2+} measurement by fluorometry

Platelets isolated from human blood were washed twice with PBS and resuspended in the standard buffer containing 2 mM $CaCl_2$. In experiments performed in the absence of external Ca^{2+}, 500 µM EGTA was added to chelate extracellular Ca^{2+}. The cells were incubated with OH-DHPM or H-DHPM (180 µM) for 10 min at 37°C, and then ADP (15 µM) was added to evoke the Ca^{2+} response.[15] The cells were centrifuged and washed twice with PBS and resuspended in the standard buffer containing 1 µl Fluo-3 (dissolved in dimethyl sulfoxide (DMSO)) in a total volume of 1 ml to make the final concentration to 2 µM and kept at 37°C for 30 min. Amlodipine-treated platelets were taken as positive controls.

Ca^{2+} was measured in platelets labelled with the Ca^{2+} fluorescent dye Fluo-3AM using a Spectrofluorometer (Shimadzu, Model RF-5301 Pc). The samples were assessed for Ca^{2+} levels by fluorescent microscopy (Nikon, Melville, New York, USA).

Per oral administration of the test compound

A known amount of the test compound OH-DHPM (MW = 276 g/mol) or H-DHPM (MW = 388 g/mol) was suspended in an appropriate volume of normal saline, sonicated for 30 s and the preparation was administered to rats (n = 3–4 per group) orally at a dose ranging from 5 to 60 mg/kg. The animals were sacrificed by diethyl ether. Blood samples were taken by cardiac puncture, followed by the assay for platelet aggregation. The dose–response curve was drawn to evaluate the optimum concentration of H-DHPM where the inhibitory effect on platelet aggregation was maximum below the saturating concentration. The so obtained optimum concentration of H-DHPM was compared with the same concentration of amlodipine besylate (MW = 567.05 g/mol); aspirin (MW = 180 g/mol) and clopidogrel bisulphate (MW = 420 g/mol).

Platelet aggregation ex vivo

The blood was centrifuged and PRP was prepared according to the above mentioned procedure and then analysed for the assessment of ADP-, collagen- or thrombin-induced platelet aggregation.

Persistence of inhibition of platelet aggregation *in vivo*: effect of a single dose

Four groups of rats (n = 3 each) were taken and administered H-DHPM (52 mg/kg or 133 µmol/kg, p.o) aspirin or clopidogrel separately for 24, 48, 72 or 96 h, respectively. Group 1, 24 h only; group 2, 48 h only; group 2, 72 h only; and group 4, 96 h only. The animals were killed as per the time interval mentioned above. ADP-induced platelet aggregation was assessed.

Measurement of nitric oxide levels in platelets in H-DHPM-treated rats

PRP obtained from rats (which had been administered orally H-DHPM or amlodipine, 133 µmol/kg), were incubated separately at 37°C for 2 min and then activity was triggered with ADP (15 µM) for 5 min. Platelets were isolated, washed twice with PBS and resuspended in the standard buffer containing 1 µl DCF-DA (dissolved in DMSO) in a total volume of 1 ml to make the final concentration 2 µM and kept at 37°C for 30 min. Samples were analysed for NO activity, as mentioned above, by flow cytometry.

Ca^{2+} measurement in rat platelets

Platelets isolated from blood of rats that had been administered with H-DHPM were washed twice with PBS and

resuspended in the standard buffer containing 2 mM $CaCl_2$. In experiments performed in the absence of external Ca^{2+}, 500 μM EGTA was added to chelate extracellular Ca^{2+}. The platelet suspension were kept for 2 min at 37°C and then Ca^{2+} response was evoked by adding ADP (15 μM).[15] The cells were centrifuged and washed twice with PBS and resuspended in the standard buffer containing 1 μl Fluo-3 (dissolved in DMSO) in a total volume of 1 ml to make the final concentration to 2 μM and kept at 37°C for 30 min. Amlodipine-treated rat platelets were taken as positive controls.

Synergistic effect of H-DHPM with aspirin

The synergistic effect of H-DHPM with a known drug aspirin was assessed. For this purpose the minimum effective dose of H-DHPM was taken i.e. 25.7 μmol/kg (10 mg/kg). An equimolar dose of aspirin (25.7 μmol/kg or ~5 mg/kg) was taken to study the synergy with H-DHPM. Four groups of animals were treated as follows: group 1, control (placebo); group 2, aspirin (25.7 μmol/kg); group 3, H-DHPM (25.7 μmol/kg); and group 4, H-DHPM + aspirin (12.8 μmol/kg, each). Platelet aggregation was analysed by the above mentioned procedure.

Lipopolysaccharide-induced thrombotic prone condition

Three groups of rats were administered a daily single dose of the following for three days: group 1, ($n = 6$) placebo (normal saline); group 2, ($n = 3$) amlodipine (133 μmol/kg); and group 3, ($n = 3$) H-DHPM (133 μmol/kg). On the third day groups 2 and 3 received an injection of LPS (2 mg/kg, i.p). Three of the rats from group 1 were randomly picked, injected with LPS, and became group 4 (LPS alone). The period of treatment was chosen as per the time dependent studies carried out with H-DHPM 133 μmol/kg to overcome the LPS insult, as reflected by the pro-inflammatory mediators such as tissue factor or iNOS. Six hours after LPS injection, the animals were anesthesized with diethyl ether and blood samples were taken for the assessment of cGMP, tissue factor and iNOS. Later, aorta from the rats of each group was separately excised and aortic homogenate was prepared to analyse the expression of eNOS and level of cGMP.

Preparation of aortic homogenates

The aorta from iliac bifurcation to the heart was removed from the above treated rats, washed in isotonic saline and were carefully freed of any adventitial fat tissue. The aortas were sectioned by scissors and immersed in iced Tris buffer (5 mmol/l, pH 7.4) containing the protease inhibitors leupeptin, benzamidine, aprotinin and phenylmethylsulfonyl fluoride. The tissues were homogenized for 30 s in a Potter-Elvehjem homogenizer. The homogenates were centrifuged for 10 min at 100*g* to remove particulate matter and unbroken cells. The supernatant obtained were designated as 'aortic homogenates'. All the operations were carried out at 0–4°C.

Immunoblotting of eNOS

The rat aortic homogenates were used for the estimation of eNOS expression in the similar way as described above. Equal amounts of proteins (20 μg/lane) were denatured and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the separated protein on the gel was transblotted to a nitrocellulose membrane (Axiva) at 300 mA for 3.5 h at 4°C. Membrane was kept in blocking reagent (5% blotto) to block the nonspecific sites. The primary antibody was diluted (1 : 2000) in Tris-buffer saline (TBS) containing 1% bovine serum albumin (BSA) and was incubated with the blot for 1 h at 4°C with moderate agitation. The membrane was extensively washed with 0.05% Tween-20 detergent in TBS (TBST) and TBS, followed by incubation with horseradish peroxidase, conjugated secondary antibody, for the same time duration at room temperature. The membrane was washed extensively with TBST/TBS and the transblotted protein bands were visualized by treating with diaminobenzidene (DAB) and hydrogen peroxide.

Measurement of cGMP

Aortic homogenates obtained were used for the determination of cGMP levels by cGMP ELISA kit from M/s Cayman's Chemicals as per manufacturer's instruction.

Immunodetection of iNOS expression

The lymphocytes (1×10^8 cells/ml) were lysed and used for Western blot to detect the LPS-induced iNOS expression.

To compare NOS expression with the expression of another protein, we analysed the expression of β-actin by Western blot using a β-actin monoclonal antibody (M/S Sigma Chemical Co., St Louis. MO, USA). For this purpose, a parallel gel with identical samples was run and after blotting onto nitrocellulose, the Western blot analysis was performed with the β-actin monoclonal antibody (1 : 5000).

Analysis of tissue factor

The lymphocytes (1×10^8 cells/ml) were prepared from the blood withdrawn from rats administered with H-DHPM or amlodipine by density gradient using Histopack and lysed in 100 μl lysis buffer (50 mmol/l Tris, 150 mmol/l NaCl, 10 mmol/l EGTA, 1% Triton X-100, 1% sodium deoxycholate, 1 mmol/l sodium vanadate, 50 mmol/l NaF, 2 mmol/l EDTA (pH 8.0), 1 mmol/l phenylmethylsulfonyl fluoride, and 5 g/ml leupeptin, pepstatin A or aprotinin) centrifuged at 2500*g* for 10 min at 4°C. The supernatant obtained was used for the assay for tissue factor using a tissue factor ELISA Kit from M/s Assay Pro kit as per the manufacturer's instructions.

Effect of age on platelet aggregation

To check the effect of age on platelet aggregation, two groups of rats were taken: group 1 ($n = 12$, 6-weeks old) and group 2 ($n = 12$, 20-weeks old). Among each group, four animals were taken as control (normal saline treated), four animals were treated orally with H-DHPM (133 μmol/kg) once daily for three days and administered with LPS (2.5 mg/kg, i.p.) on day 3, while four animals were only injected with LPS on day 3. After 6 h of LPS treatment, rats were anesthesized by diethyl ether and blood was withdrawn and platelet aggregation was analysed.

$FeCl_3$-induced arterial thrombosis

A total of 18 rats were included in this parallel study design. The animals were divided into three main groups and six animals were used in each group.

Group A, vehicle ($n = 6$); group B, H-DHPM (133 μmol/kg) was given orally for 10 days to six rats ($n = 6$); and group C, aspirin (133 μmol/kg)-treated group ($n = 6$). $FeCl_3$ was used to induce arterial thrombosis in adult male rats. The thrombus produced with this method in the carotid arteries of rats was composed of platelets and red blood cell enmeshed in a fibrin network. This model was used as a simple and reproducible test for evaluation of antithrombotic and profibrinolytic action of test compounds.[16,17] Rats (250–300 g) were anaesthetized with chloral hydrate and polyethylene catheter was inserted into the trachea via tracheostomy to facilitate breathing. A midline incision was made on the skin and after separating fascia and muscles, the right carotid artery was exposed. A small piece of parafilm 'M' was placed under the vessel to isolate it from surrounding tissues throughout the experiment. Vascular injury was induced by placing a Whatman 1 filter paper (2 × 5 mm) saturated with 25% $FeCl_3$ under the right carotid artery. The paper was allowed to remain on the vessel for 10 min before removal. Similar blunt dissection was done on the left carotid artery. After the rats were anaesthetized, the neck was exposed to surgery and right and left carotid arteries were exposed and weighed blotting paper was arranged. The blotting paper was removed after 10 min and weighed again. The weight taken after 10 min was compared in all treated groups. The thrombus weight after blotting the thrombus on filter paper was used for evaluation.

Calculation and statistics

Calculations and statistics were performed using the Graph Pad Prism 3.02 software. The one-way analysis of variance tests followed by the Tukey multiple comparisons/Post Hoc Tukey test were used. Data were expressed as mean ± standard error. Statistical significance was calculated using the Student's *t*-test, *P*-values less than 0.05 ($P < 0.05$) were considered to be statistically significant.

Results

Inhibition of human platelet aggregation by H-DHPM

The inhibitory effect of H-DHPM on human platelet aggregation was examined (see supplementary information Figure S1a). H-DHPM was found to cause concentration-dependent inhibition of platelet aggregation induced by ADP, collagen or thrombin (Table 1). The parent moiety, OH-DHPM was found to exhibit negligible activity. The concentrations of H-DHPM required to give 50% inhibition (IC50 values) for ADP-, collagen- and thrombin-induced platelet aggregation were 98.2 ± 2.1, 74.5 ± 2.3 and 180.7 ± 3.4 μM, respectively; whereas the IC50 values of amlodipine for the same were 107.4 ± 2.5, 84.6 ± 2.7 and 189.2 ± 3.1 μM, respectively. H-DHPM appeared to inhibit platelet aggregation to a similar extent as compared with amlodipine. Aspirin and clopidogrel were found to be ineffective *in vitro* (see supplementary information Figure S1b). Thrombin-induced platelet aggregation was found to be partially inhibited by H-DHPM.

Table 1 Inhibition of platelet aggregation in-vitro by ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate: comparison with standard drugs

Compounds	IC50 values (μM)		
	ADP*	Collagen$^{\alpha}$	Thrombin**
OH-DHPM	Nil	Nil	Nil
H-DHPM	98.2 ± 2.1	74.5 ± 2.3	180.7 ± 3.4
Amlodipine	107.4 ± 2.5	84.6 ± 2.7	189.2 ± 3.1
Aspirin	Nil	151 ± 2.8	Nil
Clopidogrel	Nil	Nil	Nil

Platelet rich plasma was incubated with test compound (40–200 μM) for 10 min at 37°C followed by the addition of adenosine diphosphate (ADP; 15 μM), collagen (10 μg/ml) or thrombin (0.2 U/ml) and platelet aggregation was monitored by aggregometry. Values are mean ± SEM of five observations. *$P < 0.05$, ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate (H-DHPM) vs amlodipine; $^{\alpha}P < 0.05$, H-DHPM vs amlodipine; $^{\alpha}P < 0.001$, H-DHPM vs aspirin; **$P < 0.05$, H-DHPM vs amlodipine. One-way analysis of variance tests followed by the Tukey multiple comparisons test were used.

Enhancement of nitric oxide level: effect of H-DHPM

H-DHPM was found effective *in vitro* in enhancing the NO levels in platelets where as OH-DHPM was found to be ineffective. H-DHPM pretreatment resulted in the profound increase of NO in platelets of up to 9-fold. Amlodipine on the other hand showed an 8-fold increase of NO levels in platelets. L-NAME was found to reduce the NO production to the basal level (Figure 1).

Effect of H-DHPM on Ca^{2+} mobilization in activated platelets

Platelets obtained from human blood, resuspended in the presence of Ca^{2+} and EGTA containing standard buffer were incubated with OH-DHPM or H-DHPM (180 μM) and triggered with the addition of ADP (15 μM). H-DHPM blocked the biphasic entry of Ca^{2+} into the cytosol like amlodipine, whereas the Ca^{2+} concentration in the cytosol of the OH-DHPM and control samples were high as measured on the basis of the fluorescence. Ca^{2+} measurement by fluorescence microscopy clearly depicted the Ca^{2+}-channel blocking activity of H-DHPM (Figure 2).

Effect of H-DHPM *in vivo* on platelet aggregation in rats

The dose-dependent effect of H-DHPM on the inhibition of ADP-, collagen- or thrombin-induced platelet aggregation *in vivo* is shown in Figure 3. The optimum dose obtained for H-DHPM in ADP-induced platelet aggregation was 52 ± 0.02 mg/kg (133 μmol/kg) for maximum inhibitory activity. Higher doses were found to be saturating. The parent moiety OH-DHPM was found to be ineffective with all the inducers. As shown in Table 2, amlodipine and clopidogrel appeared to cause inhibition of platelet aggregation to a lesser extent when administered orally to rats at the dose

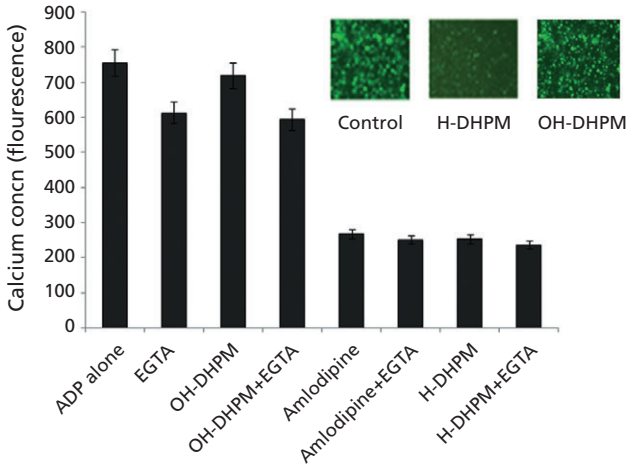

Figure 2 Ca^{2+}-channel blocking activity of ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate in washed platelets. Washed platelets were incubated with OH-DHPM (parent compound) or ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate (H-DHPM) (180 μM) for 10 min followed by incubation with adenosine diphosphate (ADP) (15 μM) for 10 min. The samples were washed and resuspended in suitable buffer, further incubated with Fluo-3AM for 30 min at 37°C and analysed by fluorimetry. $P < 0.01$, compared between ADP alone and H-DHPM-treated groups. $P < 0.01$, compared between EGTA alone and H-DHPM + EGTA treated groups. One-way analysis of variance test followed by the Tukey multiple comparisons test were used. Inset: Platelets were incubated with OH-DHPM or H-DHPM (180 μM) for 10 min followed by incubation with ADP (15 μM) for 10 min. Samples were washed and resuspended in the suitable buffer, and further incubated with Fluo-3AM for 30 min at 37°C and visualized by fluorescence microscopy.

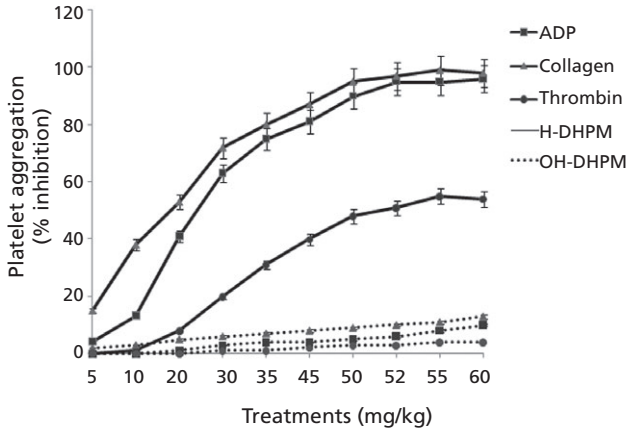

Figure 3 Dose-dependent effect of ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate or OH-DHPM *in vivo* on platelet aggregation in rats. Rats were administered test compounds (5–60 mg/kg, p.o.) and killed after 24 h. Blood was drawn, platelet rich plasma prepared and adenosine diphosphate (ADP)-, collagen- or thrombin-dependent platelet aggregation was assessed. Values are mean ± SEM of five observations. One-way analysis of variance test followed by the Tukey multiple comparisons test were used.

133 μmol/kg (see supplementary information Figure S1c). In addition, H-DHPM was found to be more effective in the inhibition of collagen-induced platelet aggregation. Clopidogrel was not effective with collagen-induced platelet aggregation. Aspirin and clopidogrel completely failed to inhibit thrombin-induced platelet aggregation, whereas H-DHPM as well as amlodipine were able to inhibit partially the thrombin-induced platelet aggregation.

Persistence of inhibition of platelet aggregation *in vivo*: effect of a single dose

The data presented in Table 3 revealed that a single dose of H-DHPM significantly inhibited platelet aggregation, even after 96 h. The effect of clopidogrel on inhibition of platelet aggregation was much less comparatively and aspirin was least effective among the three antiplatelet drugs after 96 h.

Enhancement of nitric oxide level: effect of H-DHPM

H-DHPM was found effective *in vivo* in enhancing the NO level in platelets. Accordingly, the administration of H-DHPM (133 μmol/kg) to rats resulted in the profound increase of NO in platelets up to 8.2-fold. Amlodipine on the other hand showed a 6.9-fold increase in NO level in platelets.

Effect of H-DHPM on Ca^{2+} mobilization in activated platelets

H-DHPM blocked the biphasic entry of Ca^{2+} into the cytosol *in vivo* like amlodipine, whereas the Ca^{2+} concentration in the cytosol of the control samples were high as measured on the basis of the fluorescence.

Effect of H-DHPM on lipopolysaccharide-induced tissue factor expression in rats

The administration of LPS to rats resulted in the enhancement of tissue factor level in platelets (Figure 4). Pretreatment of rats with H-DHPM led to a significant decrease in LPS-induced elevation of tissue factor. The concentration of tissue factor was 0.075 pg/100 mg protein in LPS-treated rats. Tissue factor levels dropped to 0.016 and 0.022 pg/100 mg protein in rats treated with LPS plus H-DHPM and LPS plus amlodipine, respectively, as compared with the control (0.01 pg/100 mg).

Modification of LPS induced iNOS expression

Immunodetection of LPS-induced iNOS expressions were performed in peripheral blood mononuclear cells (PBMCs) by Western blotting. As clearly shown in Figure 5a, the samples treated with LPS alone had intense iNOS expression (lane 3) whereas there was a diminished expression of iNOS in LPS plus H-DHPM-treated samples (lane 4).

H-DHPM-mediated expression of eNOS in lipopolysaccharide-induced rats

Aortic homogenates were subjected to immunodetection of eNOS was performed by using eNOS antibody. Rat aorta treated with LPS alone showed a sharp decline in the

Table 2 Effect of ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate on in-vivo platelet aggregation in rats

Inducers of platelet aggregation	Concentrations	% Inhibition of platelet aggregation *in vivo*			
		H-DHPM	Amlodipine	Aspirin	Clopidogrel
*ADP	15 μM	96 ± 4.6	85 ± 5.5	48 ± 4.2	82 ± 5.5
[α]Collagen	10 μg/ml	95 ± 4.3	88 ± 5.7	51 ± 4.5	–
[#]Thrombin	0.2 U/ml	51 ± 3.3	48 ± 3.5	–	–

Rats were administered test compound (133 μmol/kg, p.o.) and killed after 24 h. Blood was drawn, platelet rich plasma prepared and adenosine diphosphate (ADP)-, collagen- or thrombin-dependent platelet aggregation was assessed. Values are mean ± SEM of five observations. *$P < 0.05$, ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate (H-DHPM) vs amlodipine; *$P < 0.001$, H-DHPM vs aspirin; *$P < 0.05$, H-DHPM vs clopidogrel; [α]$P < 0.05$, H-DHPM vs amlodipine; [α]$P < 0.001$, H-DHPM vs aspirin; #$P > 0.05$, H-DHPM vs amlodipine. Analysis of variance tests followed by the Tukey multiple comparisons test were used.

Table 3 Persistence of inhibition of platelet aggregation in-vivo by a single dose of ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate

Time	(%) Inhibition of platelet aggregation			
	H-DHPM	Amlodipine	Aspirin	Clopidogrel
24 h	95 ± 3.11	88 ± 5.6	55 ± 4.2	82 ± 1.43
48 h	85 ± 3.32	74 ± 5.3	30 ± 4.4	60 ± 1.42
72 h	75 ± 3.13	67 ± 5.5	15 ± 4.6	43 ± 1.44
96 h	60 ± 3.02*	53 ± 5.5	8 ± 4.24	22 ± 1.43

Drugs and ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate (H-DHPM) were administered (133 μmol/kg, p.o.) to the rats and killed after 24, 48, 72 or 96 h and assessed for adenosine diphosphate-induced platelet aggregation. Values are mean ± SEM of seven observations. The one-way analysis of variance followed by Post Hoc Tukey test were used. *$P < 0.05$, antiplatelet activity of H-DHPM after 96 h vs antiplatelet activity of amlodipine, aspirin or clopidogrel after 96 h.

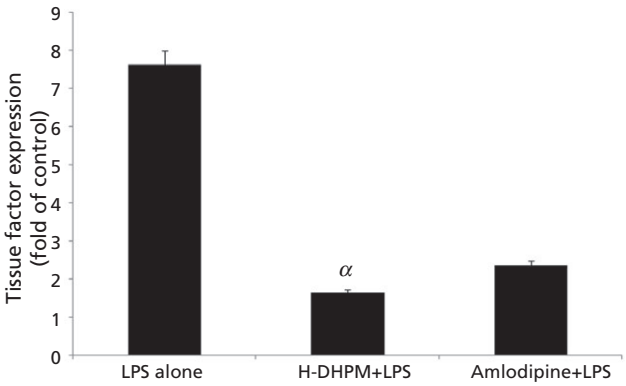

Figure 4 Effect of ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate on lipopolysaccharide-induced tissue factor expression in rats. Rats were administered test compounds (133 μmol/kg, p.o.) for three days. A group of rats treated with ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate (H-DHPM) or amlodipine were administered lipopolysaccharide (LPS; i.p., 2.5 mg/kg) 6 h before they were killed. Another group of rats were administered LPS alone. Lymphomonocytes were isolated from blood and tissue factor (TF)-expression was assayed by ELISA. Control rats were treated accordingly. Details are given under Materials and Methods. [α]$P < 0.05$ compared between H-DHPM- and amlodipine-treated groups. [α]$P < 0.001$ compared between H-DHPM- and LPS alone-treated groups. One-way analysis of variance test followed by the Tukey multiple comparisons test were used.

expression of eNOS. The enhancement of eNOS in H-DHPM-treated platelets as compared with control is presented in Figure 5b.

Modulation of aortic cGMP levels by H-DHPM in lipopolysaccharide-treated rats

Rats administered H-DHPM or amlodipine (133 μmol/kg) for three days were injected with LPS (2.5 mg/kg, i.p.) on the third day and killed after 6 h. LPS treatment caused an enhancement of cGMP levels of up to 5-fold in aorta as compared with control. H-DHPM treatment abolished the enhanced cGMP level up to 1.65-fold ($P < 0.001$), while amlodipine showed inhibition of cGMP levels of up to 1.93-fold in aorta (Figure 6). The concentration of cGMP at basal level in the control was 1.7 ± 0.2 pmol/mg protein, which was raised to 8.5 ± 0.28 pmol/mg for LPS-treated rats. H-DHPM or amlodipine pretreatment decreased the cGMP level in spite of the LPS challenge up to 2.81 ± 0.12 and 3.28 ± 0.11 pmol/mg protein, respectively, in aorta.

$FeCl_3$-induced arterial thrombosis: antithrombotic effect of H-DHPM

There was a significant increase in paper weight of H-DHPM and aspirin groups as compared with the control group. The anticoagulant effect of H-DHPM is shown significantly better than the control and aspirin groups. However, the significant difference of paper weight was found in the right carotid artery vs the left carotid artery of the H-DHPM group in comparison with that of aspirin (Figure 7).

Effect of age on platelet aggregation: anti platelet affect of H-DHPM

The extent of platelet aggregation was found to be higher in LPS-treated older rats (20 weeks) as compared with that in the LPS-treated younger ones (Figure 8). H-DHPM treatment was able to inhibit the effect of LPS on platelet aggregation effectively in both age groups.

Synergistic effect of H-DHPM with aspirin

Samples, treated with H-DHPM or aspirin alone (25.7 μmol/kg each) showed marginal antiplatelet activity, whereas the samples treated with the combination of

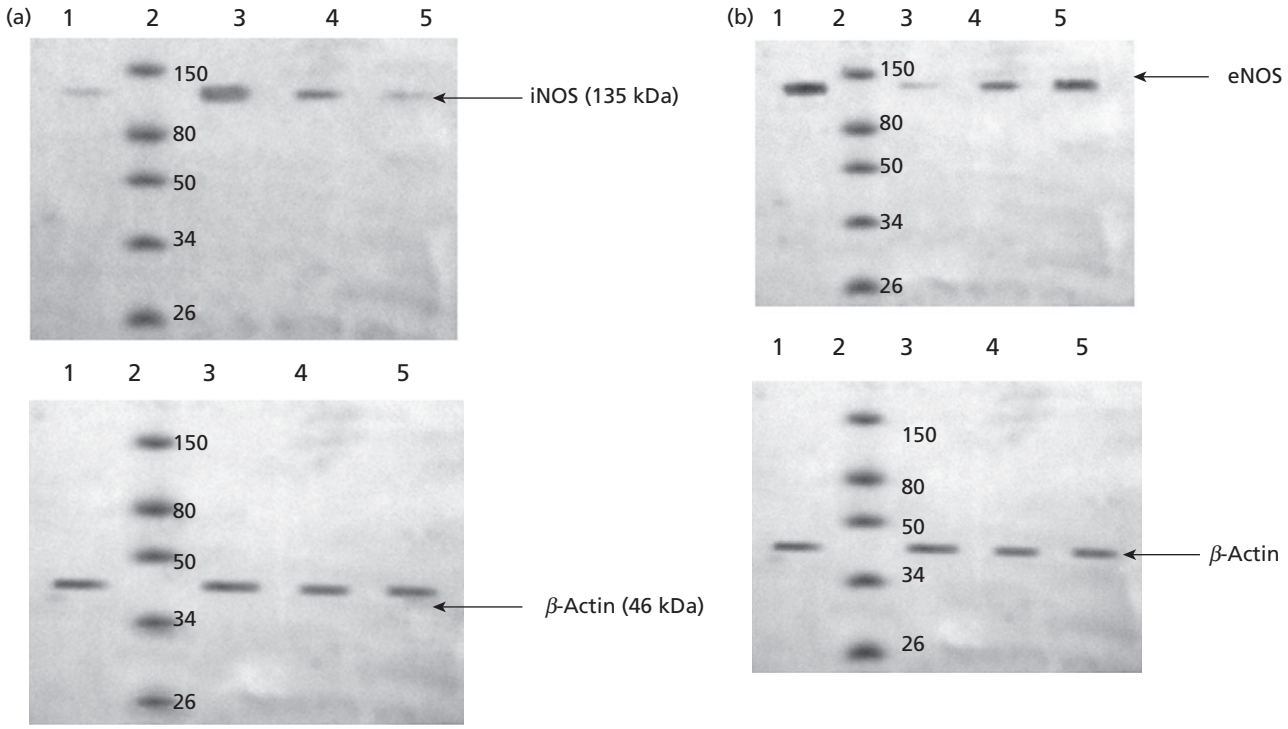

Figure 5 Modulation of inducible nitric oxide synthase and endothelial nitric oxide synthase expression by ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate in lipopolysaccharide-treated rats. Rats were administered test compounds (133 μmol/kg, p.o.) for three days. A group of rats with ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate (H-DHPM) or amlodipine were administered lipopolysaccharide (LPS; i.p. 2.5 mg/kg) on the third day, 6 h before being killed. The blood was withdrawn, PBMCs were separated and aorta was excised for the immunodetection of inducible nitric oxide synthase (iNOS) and endothelial NOS (eNOS) as described under Materials and Methods. (a) PBMCs were isolated from blood. Immunological identification of iNOS using anti-iNOS antibody (1 : 2000, v/v). Lane 1, control; lane 2, prestained molecular weight marker; lane 3, LPS treated; lane 4, amlodipine; lane 5; H-DHPM. (b) The aorta was homogenized followed by the identification of eNOS expression using anti-eNOS antibody (1 : 2000, v/v). Lane 1, aortic homogenate of control rats (untreated); lane 2, prestained molecular weight marker; lane 3, LPS-treated rat aorta; lane 4, amlodipine + LPS treatment; lane 5, H-DHPM + LPS treatment.

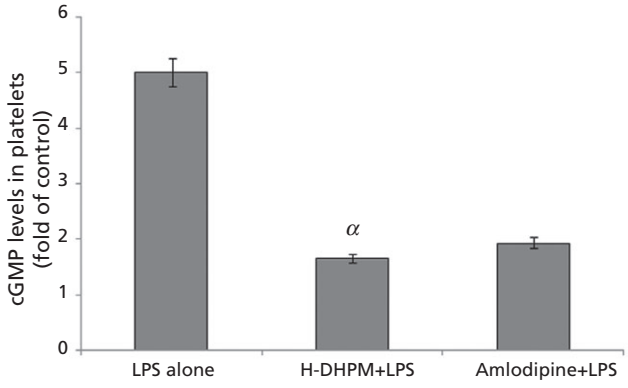

Figure 6 Enhancement of cGMP levels in aorta of lipopolysaccharide-treated rats. cGMP levels were estimated by ELISA in aortic homogenate. $^{\alpha}P < 0.05$, compared between ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate (H-DHPM)- and amlodipine-treated groups. $^{\alpha}P < 0.001$, compared between H-DHPM- and LPS alone-treated groups. One-way analysis of variance followed by the Tukey multiple comparisons test were used.

these two compounds in equimolar concentration (12.8 + 12.8 μmol/kg), inhibited the ADP-induced platelet aggregation synergistically (Figure 9).

Discussion

Cardiovascular disease is the most common cause of death in the modern world. Its risk increases with age, stress, and the irregular routine of urban societies. The clumping together of platelets in the blood causes platelet aggregation, sequentially leading to the formation of thrombus. Hence, there is a greater demand for newer antiplatelet agents.

The antiplatelet effect of our key molecule H-DHPM has been evaluated and compared with OH-DHPM, a deacylated product of H-DHPM, *in vitro* as well as *in vivo*. OH-DHPM showed no antiplatelet or Ca^{2+}-channel blocking activity as compared with the leading derivative H-DHPM. This directed our attention towards the heptanoyl group substitution at 4′ position in the aryl ring of the DHPM. The heptanoyl group plays an important role in the effectiveness of the leading derivative, H-DHPM. H-DHPM was compared with drugs like amlodipine, clopidogrel and aspirin. Although antihypertensive activity of this class of molecule has been reported,

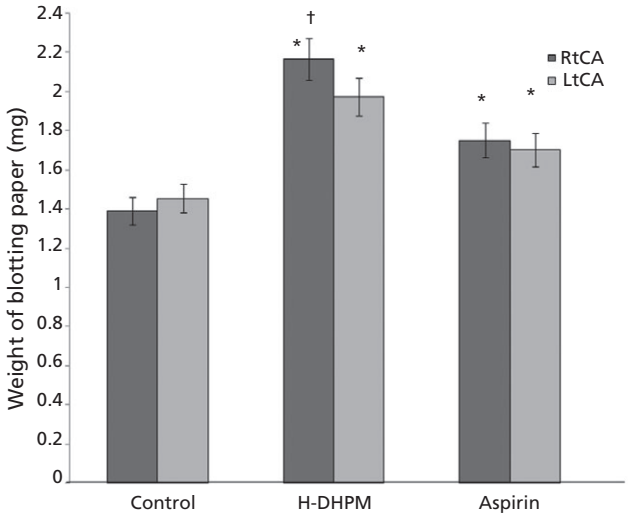

Figure 7 Antithrombotic activity of ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate. Mean plot of blotting paper weight in the intervention groups. RtCA, right carotid artery; LtCA: left carotid artery. $^{*}P < 0.05$ compared with control naïve group; $^{\dagger}P < 0.05$, compared between RtCA of ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate (H-DHPM) and aspirin group. One-way analysis of variance followed by post hoc Tukey test.

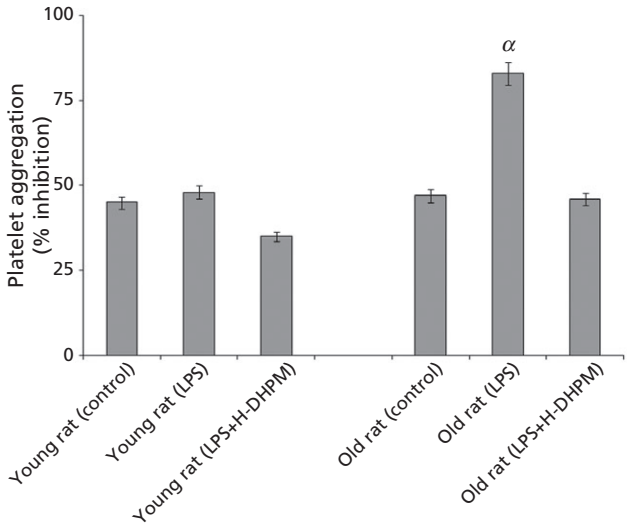

Figure 8 Effect of ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate on lipopolysaccharide-induced platelet aggregation in aged rats. Rats were administered ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate (H-DHPM; 133 µmol/kg, p.o.) for three days. A group of rats (control or drug treated) were administered lipopolysaccharide (LPS; 2.5 mg/kg, i.p.) blood was drawn and platelet aggregation was measured. Values are mean ± SEM of three observations. $^{\alpha}P < 0.01$, compared between LPS-treated young rat and older rat groups. One-way analysis of variance tests for both the groups individually followed by *t*-test were used.

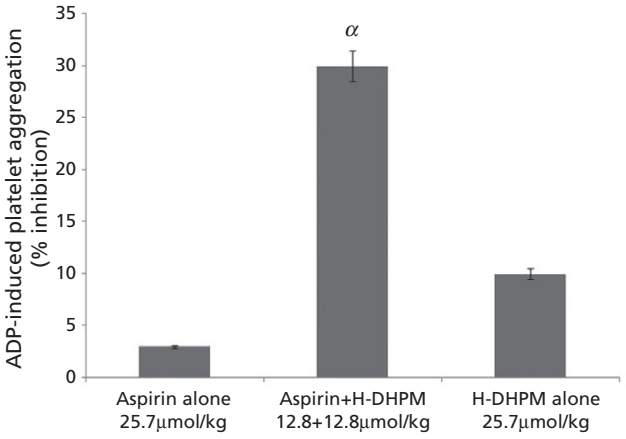

Figure 9 Synergistic effects of aspirin on antiplatelet activity of ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate. Test compounds were administered orally and rats killed after 24 h. Blood was drawn, platelet rich plasma prepared and adenosine diphosphate-dependent platelet aggregation was assessed. Values are mean ± SEM of five observations. The figures in the parenthesis represent the dose of the test compounds administered. $^{\alpha}P < 0.001$, compared between aspirin + ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate (H-DHPM)- and aspirin alone-treated groups. $^{\alpha}P < 0.01$, compared between aspirin + H-DHPM- and H-DHPM alone-treated groups. One-way analyses of variance followed by Tukey multiple comparison test were used.

nothing is known about its antiplatelet activity. Interestingly, H-DHPM was found to be very effective as an antiplatelet agent, like amlodipine, *in vivo* as well as *in vitro*, while aspirin and clopidogrel were effective only *in vivo*, and that to a lesser extent. H-DHPM, like amlodipine, effectively decreased platelet aggregation stimulated by ADP, collagen or thrombin. However, these inducers work through different mechanism of action. H-DHPM significantly enhanced the NOS activity *in vitro* and *in vivo*. Guanylate cyclase is a heterodimer formed by two polypeptides containing a haeme moiety as a prosthetic group. The interaction between its haeme group and NO appeared to be an important modulator of its activity, i.e. catalytic conversion of GTP into cGMP which is known to act by lowering the intraplatelet calcium concentration, and hence it was thought important to measure the calcium concentration in the cytosol.[18,19] Interestingly, H-DHPM, like amlodipine, was found to arrest biphasic calcium entry, thereby abolishing calcium cross talk. These observations suggested the direct stimulatory action of Ca^{2+}-channel blockers on endothelial NO production. Normally, cytosolic Ca^{2+} is kept very low (10^{-7} M) by the action of Ca^{2+} pumps in the endoplasmic reticulum, mitochondria, and plasma membrane while ADP, collagen and thrombin increase Ca^{2+} concentration in cytosol, which is obliterated by H-DHPM.[20–23] Intercellular calcium communication (ICC) is a widespread form of cell–cell communication that allows tissue coordination of cell proliferation, differentiation, and metabolism. ICC has been described in many cell types, including platelets which are involved in regulating the three-dimensional growth of developing thrombi.[24] The calcium channel blockade activity further points out the enhancement of NO generation as an additional mechanism for the vasodilatory effect.[7]

In the inflammatory conditions, an excessive amount of NO is produced, and has been shown in several experimental models of LPS-mediated inflammatory response where the augmented NO radical generation is encountered in the vascular tissues. Lymphomonocytes express the iNOS isoform after cytokine stimulation and exposure to endotoxin resulting in the generation of elevated levels of NO for prolonged periods of time. The expression of LPS-induced iNOS was measured in isolated rat PBMCs. In these models, the increased NO radical production correlated with the extent of iNOS produced leading to the vascular injury.[25] These studies purport the important role of NO radicals in the pathophysiology of cardiovascular diseases. Our results clearly depicted the inhibition of LPS-induced iNOS expression in lymphomonocytes of rats treated with H-DHPM. Rat aortic homogenates were used to assay the cGMP levels in LPS-treated rats. The results indicated a higher level of cGMP induced by LPS in rats, which was attributed to an iNOS-mediated overproduction of NO. It was found interesting that H-DHPM treatment was able to reverse the ill effects of LPS by dropping the level of cGMP to normal (Figure 6). The constitutive NOS, eNOS, expression was also monitored in aortic homogenate and was found that the LPS treatment declined the expression of eNOS, while the H-DHPM pretreatment rendered the normalization of eNOS expression even after LPS challenge. Tissue factor expression was also induced in circulating monocytes by bacterial LPS and pro-inflammatory cytokines. Binding of factor VII to the extracellular domain of tissue factor catalyses the generation of factor Xa and IXa, triggering thrombin generation. H-DHPM-treated samples showed a drastic decrease in LPS-induced tissue factor expression in PBMCs.[26] However, H-DHPM as well as amlodipine was able to overcome the inflammatory effects of LPS effectively.

It was observed that two different antiplatelet agents such as aspirin and clopidogrel, working through two different mechanisms, when combined together were proved to be an efficient drug.[27] Similarly, when lower doses of aspirin and H-DHPM were combined in equimolar quantity it resulted in higher efficacy as an antiplatelet agent. The effect of H-DHPM was found to be highly persistent even after 96 h, which was a clear advantage over drugs such as clopidogrel or aspirin which require multiple intakes to achieve optimum inhibitory activity. Age-related effects also play an important role in the physiology of platelet dysfunction: there is an increased thrombus formation during hypertension and the phenomenon of platelet adhesion and aggregation is also triggered.[28] Our results clearly showed that H-DHPM pretreatment brought the inhibition in platelet aggregation caused by LPS insult to normal in both the older and younger rats.

A model to induce vascular injury by the direct application of $FeCl_3$ at the adventitial surface of an artery is well established.[16] The result of this study led to the speculation of the fact that since the weights of the filter paper used in the H-DHPM and the aspirin groups differed significantly from the control group, both aspirin and H-DHPM showed higher weights of paper than the control group, which meant that both these groups showed antiplatelet effect due to more bleeding and slowed thrombus formation.

Similarly, Eguchi *et al*.[16] demonstrated the antiplatelet effect of nicorandil and tirofiban via this ferric chloride-induced thrombosis model, in which there was significant slowing of the growth of thrombi formation, resulting in arterial occlusion.

The new finding in this study was that the weights of the filter paper in the right carotid artery of both H-DHPM and aspirin groups were greater when compared with their left counterparts. This meant that there was more effect seen on the right side of the carotid artery in both the H-DHPM and aspirin groups. Aspirin is a well-known antiplatelet agent, acting by way of irreversible inhibition of cyclooxygenase, and the action remains for the life time of platelets.[29] This property of aspirin leads to various long-term adverse drug reactions; hence a need for new antiplatelet drugs. Amlodipine is known to cause pedal oedema and clopidogrel is reported as a causative agent for intra-abdominal bleeding through another mechanism when higher doses are taken.[30] The known antiplatelet agents amlodipine, clopidogrel and aspirin showed antiplatelet effect, but to overcome their various limitations, our key molecule, H-DHPM could be a better drug in terms of tolerability and safety. Further studies are warranted to investigate these aspects.

Conclusions

These results highlight a novel key molecule, H-DHPM as a calcium channel blocker endowed with antiplatelet property. It is well-known that disregulation of platelet aggregation sequentially leads to the formation of thrombus and can be responsible for atherothrombosis, which is a leading cause of death. Therefore, the discovery of new antithrombotic agents with promising application as a drug makes this study significant.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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References

1. White H. Contribution of platelets to thrombus formation. *Br Med Bull* 1978; 34: 123–128.

2. Mustard JF, Packham MA. Platelet aggregation and the platelet release reaction in thromboembolism. *Can Med Assoc J* 1970; 17: 859–863.
3. Tomita T *et al*. The appearance of exhausted platelets due to a duration of hypertension in stroke-prone spontaneously hypertensive rats. *Thromb Res* 1985; 37: 195–200.
4. Colman RW. Aggregin: a platelet ADP receptor that mediates activation. *FASEB J* 1990; 4: 1425–1435.
5. Vane JR, Botting RM. The mechanism of action of aspirin. *Thromb Res* 2003; 110: 255–258.
6. Conlin PR, Williams GH. Use of calcium channel blockers in hypertension. *Adv Intern Med* 1998; 43: 533–562.
7. Ding Y, Vaziri ND. Calcium channel blockade enhances nitric oxide synthase expression by cultured endothelial cells. *Hypertension* 1998; 32: 718–723.
8. Blaszak RT *et al*. The use of short-acting nifedipine in pediatric patients with hypertension. *J Pediatr* 2001; 139: 34–37.
9. Mason RP *et al*. Novel vascular biology of third-generation L-type calcium channel antagonists, ancillary actions of amlodipine. *Arterioscler Thromb Vasc Biol* 2003; 23: 2155–2163.
10. Chhillar AK *et al*. Microwave-assisted synthesis of antimicrobial dihydropyridines and tetrahydropyrimidin-2-ones: novel compounds against aspergillosis. *Bioorg Med Chem* 2006; 14: 973–981.
11. Atwal KS *et al*. Dihydropyrimidine calcium channel blockers. 3.′3-carbamoyl-4-aryl-1,2,3,4-tetrahydro-6-methyl-5-pyrimidinecarboxyli acid esters as orally effective antihypertensive agents. *J Med Chem* 1991; 34: 806–811.
12. Prasad AK *et al*. Synthesis and lipase catalysed enantioselective acylation studies on ethyl 4-aryl-3,4- dihydropyrimidine-2(1h)-ones. *Indian J Chem* 2009; 48B: 1738–1748.
13. Priya N *et al*. Characterization of 4-methyl-2-oxo-1,2-dihydroquinolin-6-yl acetate as an effective antiplatelet agent. *Bioorg Med Chem* 2010; 18: 4085–4094.
14. Imrich A, Kobzik L. Fluorescence-based measurement of nitric oxide synthase activity in activated rat macrophages using dichlorofluorescin. *Nitric Oxide* 1997; 1: 359–369.
15. Sebala P *et al*. Two subtypes of G protein-coupled nucleotide receptors, P2Y1 and P2Y12 are involved in calcium signalling in glioma C6 cells. *Br J Pharmacol* 2001; 132: 393–402.
16. Eguchi Y *et al*. Nicorandil attenuates $FeCl_3$-induced thrombus formation through the inhibition of reactive oxygen species production. *Circ J* 2009; 73: 554–561.
17. Surin WR *et al*. Optimization of ferric chloride induced thrombosis model in rats: effect of anti-platelet and anti-coagulant drugs. *J Pharmacol Toxicol Methods* 2010; 61: 287–289.
18. Shankar RP *et al*. Amlodipine, nitric oxide, and platelet aggregation. *Asian Cardiovasc Thorac Ann* 2000; 8: 357–360.
19. Ruth P *et al*. Transfected cGMP-dependent protein kinase suppresses calcium transients by inhibition of inositol 1,4,5-trisphosphate production. *Proc Natl Acad Sci USA* 1993; 90: 2623–2627.
20. Pedersen OS *et al*. Influence of human plasma or serum albumin on ADP- or vasopressin-induced calcium increases in human platelets. *Scand J Clin Lab Invest* 1994; 54: 67–74.
21. Lum H *et al*. Calcium dependence of the thrombin-induced increase in endothelial albumin permeability. *J Appl Physiol* 1989; 66: 1471–1476.
22. Daniel JL *et al*. Evidence for a role for tyrosine phosphorylation of phospholipase 2 in collagen-induced platelet cytosolic calcium mobilization. *Biochem J* 1994; 302: 617–622.
23. Ramanadham S *et al*. Arachidonic acid induces an increase in cytosolic calcium concentration in single pancreatic islet beta cells. *Biochem Biophys Res Commun* 1992; 184: 647–653.
24. Nesbitt WS *et al*. Intercellular calcium communication regulates platelet aggregation and thrombus growth. *J Cell Biol* 2003; 160: 1151–1161.
25. Loscalzo J. Inducible NO synthesis in the vasculature: molecular context defines physiological response. *Arterioscler Thromb Vasc Biol* 2000; 121: 1259–1260.
26. Toschi V *et al*. Tissue factor modulates the thrombogenicity of human atherosclerotic plaques. *Circulation* 1997; 95: 594–599.
27. Folts JD. Inhibition of platelet activity in vivo by amlodipine alone and combined with aspirin. *Int J Card* 1997; 62: S111–S117.
28. Cernadas MR *et al*. Expression of constitutive and inducible nitric oxide synthases in the vascular wall of young and aging rats. *Circ Res* 1998; 83: 279–286.
29. Feldman M *et al*. Time course inhibition of gastric and platelet COX activity by acetylsalicylic acid in humans. *Am J Physiol Gastrointest Liver Physiol* 2000; 279: G1113–G1120.
30. Lortie É *et al*. Clopidogrel induces an acute hemostatic deficit and increases intra abdominal bleeding in rabbits. *Thromb Res* 2009; 123: 869–873.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Graphical tracings of adenosine diphosphate (ADP)-induced platelet aggregation: effect of ethyl 4-(4′-heptanoyloxyphenyl)-6-methyl-3,4-dihydropyrimidin-2-one-5-carboxylate (H-DHPM), aspirin, amlodipine and clopidogrel. (a) Concentration-dependent inhibition of platelet aggregation by H-DHPM *in vitro*. (b) Platelet aggregation in presence of aspirin, clopidogrel and H-DHPM *in vitro*. (c) Aspirin, amlodipine or H-DHPM (133 μmol/kg, p.o) was administered in rats followed by platelet aggregation.

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