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Synthesis of novel calcium channel blockers with ACE2 inhibition and dual antihypertensive/anti-inflammatory effects: A possible therapeutic tool for COVID-19

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1. Introduction

Hypertension, a long-term medical condition in which the blood pressure in the arteries is persistently elevated, has been recognized as one of the most frequent comorbidities and risk factors for the seriousness and adverse consequences in COVID-19 patients [1]. Several drug classes have been used for the treatment of high blood pressure such as angiotensin converting enzyme (ACE)-inhibitors, diuretics, β-blockers, calcium channel blockers (CCBs) and others [2]. However, hyponatremia caused by diuretic therapy is considered a negative prognostic factor in COVID-19 patients [3]. On the other hand, ACE-inhibitors, β-blockers, angiotensin II (Ang II) receptor blockers and CCBs, depend on the synthesis of vasodilating prostaglandins to produce their effects [2]. CCBs were originally developed as potent vasodilators because of their ability to bind to and block calcium channels. This reduced calcium influx into the smooth muscle cells results in smooth muscle relaxation and vasodilation. They also alter heart rate to prevent peripheral and cerebral vasospasm and reduce chest pain caused by angina pectoris. Several studies have been performed to analyze the potential use of CCBs for treatment of a broad range of diseases from angina pectoris to different forms of dementia [4]. Nifedipine is one of the most common and classic calcium channel blockers. It is a first generation dihydropyridine and often used to reduce systemic vascular resistance and arterial pressure [4]. However, concerns on nifedipine focusing on its short half-life [5] and the rapid unpredictable fall in blood pressure. Consequently, precipitation in ischemic events, which led to the development of other CCB generations. In clinical practice, ACE inhibitors or Ang II type 1 receptor blockers are often used in combination with CCBs...
to reach a sufficient antihypertensive effect [6], to prevent developing cardiovascular diseases or renal failure as blood pressure increases [7]. ACE inhibitors have been considered the first line treatment of hypertension, through inhibiting the hydrolysis of Ang I to the biologically active Ang II, as central regulators of the renin-angiotensin system (RAS). Ang II is the main vasoconstrictor, pro-inflammatory, pro-fibrotic, and anti-diuretic agent through its receptor AT1R. Subsequently, inhibition of the production of Ang II and its receptor-induced signaling, via AT1R blockers, have been highly effective therapies in hypertension [8].

On the other hand, ACE2 also is crucial in the modulation of blood pressure having an opposite effect to ACE [9]. ACE increases blood pressure by increasing the level of Ang II, while ACE2 decreases blood pressure and plays a critical physiologic role in the homeostasis of tissue microcirculation and inflammation [8,10,11]. ACE2 catalyzes the conversion of Ang II to Ang 1–7 and has direct effects on cardiac function along with several organs via counter-regulation of the RAS by lowering Ang II [10]. ACE2 is expressed on the plasma membranes of numerous cell types, like the alveolar and intestinal epithelia, cardiac and renal vascular endothelial cells, and on macrophages [8,11,12]. Unfortunately, membrane-bound ACE2 may also be targeted by virus like in severe COVID-19 cases, as it may act as a binding site for the virus spike proteins of SARS-CoV-1 and SARS-CoV-2 [13–16]. SARS-CoV-2 invasion unbalances the RAS, since viral cellular invasion and replication via ACE2, especially under conditions of enhanced ACE2 expression like hypertension, can lead to reduction of cell membrane-bound ACE2 through degradation of membranal ACE2 and increasing circulating ACE2. Thus, resulting in unbalanced paracrine action of Ang compounds, along with a local depletion of Ang 1–7, leaving Ang II activity unopposed, which leads to altered regional microcirculation, hypoxia, reactive oxygen species generation, endothelial damage, severe inflammation, hypercoagulability, tissue damage, and fibrosis [12]. Furthermore, unopposed Ang II activates NF-κB and proinflammatory cytokines release [17,18], along with activation of TNF-α/IL-6/STAT-3 pathways [19]. Thus, inhibitors of ACE2 binding to SARS-CoV-2 Spike RBD may offer some protection against the viral infection and the inflammatory organ damage sequela [20].

Therefore, in this study, we hypothesized that CCBs with ACE2 inhibitory effect might exert an antihypertensive activity with protective effect against COVID-19 infection by suppression of ACE2 binding to SARS-CoV-2 Spike RBD.

The dihydropyridines (DHPs), and their prototype nifedipine, are still the most potent group of CCBs, used for the treatment of arterial hypertension. Nifedipine has been modified to improve its potency, safety, and duration of activity. Among structure modifications of DHPs, the bioisosters aza-analogs of DHPs, which gave rise to the
dihydropyrimidines (DHPMs) [21]. DHPMs are privileged heterocyclic scaffolds due to their biological and pharmaceutical activities as anti-inflammatory, anti-oxidant, anti-hypertensive, anti-filarial and anti-SARS [22-24], Fig. 1.

Moreover, recently some pyrimidinethiol compounds and their thioglycoside derivatives showed moderate in vitro anti-SARS-CoV-2 [25] and anti-avian influenza H5N1 virus [26] activities.

Motivated by the aforementioned discoveries, some bioisosteres of DHPMs were prepared and screened for their antihypertensive potential and ACE2 inhibition. Furthermore, their anti-inflammatory activity on LPS-stimulated THP-1 cells were evaluated. Here, we developed a pro

2. Results and discussion
2.1. Chemistry

As depicted in Scheme 1, the target compounds (4-9) were developed using a short and cost-effective route. Key considerations in the development of Dihydropyrimidine (DHPM) analogues were the ability to easily introduce structural variation at the C5 substituent. The target compounds were designed in such a way as to create diversity around the core skeleton using the easiest possible synthetic steps. Therefore, aldehydes and amines were chosen based upon their chemical character (i.e., electron-withdrawing and -polar groups).

Biginelli condensation protocol [27-30] is a one pot, three component coupling reaction of commercially available starting material thiourea, ethyl acetoacetate and selected aromatic aldehydes namely, p-fluoroaniline, o-chloro and o-bromobenzaldehyde in acid medium to afford the corresponding 1,4-dihydropyrimidine esters 4a-c. Hence, saponification of the ethyl ester furnished the sodium salt of the 1,4-dihydropyrimidine corresponding 1,4-dihydropyrimidine esters (4a-c). Hence, saponification of the ethyl ester furnished the sodium salt of the 1,4-dihydropyrimidine esters (4a-c). Meanwhile, when the later compounds were chlorinated by thionyl chloride, they gave acyl chlorides (6a-c). These acyl chlorides served as a template for the next reaction, in which the acyl chlorides were coupled with three aromatic amines namely, aniline, p-toluidine and p-fluoroaniline to produce the target 1,4-

2.2. Biological evaluation
2.2.1. Evaluation of the antihypertensive activity

Pyrimidines and DHPMs, the important lead compounds for treatment of hypertension [32], have been developed as for SQ 32926, SQ 32547 and some pyrimidines analogues (Fig. 2 & Fig. 3). These compounds were proven to be orally active, with more potency and longer duration over the DHP antihypertensive drugs [33]. In the present study, nifedipine and the test compounds have similar bioisosteric nucleus, as revealed in (Fig. 2). Thus, all test compounds (4-9) were screened for their potential antihypertensive and calcium channel blocking (CCB) activities, using nifedipine as standard reference drug.

As presented in Table 1, all synthesized compounds; except compound 6e; caused significant reduction in the mean arterial blood pressure (BP) compared to the control group. Compounds 7a and 8a displayed the most potent antihypertensive activity; almost equal to that of the standard nifedipine; with % reduction in BP reaching up to 29%, followed by 9a (27%). While compounds 4a, 4b, 7b, 7c, 8b, and 8c showed moderated antihypertensive activity with % reduction in BP ranging from 22 to 23% along with compounds 4c and 9c, which demonstrated % decrease in BP about 21%. On the other hand, the rest of the compounds had lower antihypertensive potency.

SAR studies revealed that the 5-ethoxycarbonyl-2-thiopyrimidines derivatives (4a-c) showed comparable activity relative to nifedipine, while their hydrolysis decreased the activity as exemplified by carboxylic acid derivatives (5a-c). Meanwhile, when the later compounds were chlorinated by thionyl chloride, they gave acyl chlorides (6a-c) of weak antihypertensive activity. Conversely, amide derivatives (7a-c, 8a-c, and 9a-c) retained the activity especially when bearing a free phenyl moiety as in compounds 7a, 8a and 9a, which had promising activity compared to nifedipine, over the amidic group bearing p-tolyl substitution as in compounds 7b, 8b and 9b, or 4-florophenyl substitution as in compounds 7c, 8c and 9c.

Fig. 2. DHP and its aza analogues bioisosteres. DHP: Dihydropyridine, DHPM: Dihydropyrimidine.
2.2.2. Calcium antagonism blocking activity in the isolated rat ileum.

In order to investigate the CCB blocking effect, the first approach was to study the effect of different synthetic derivatives on $K^+$ induced contracting isolated rat ileum. $K^+$ activates voltage-dependent $Ca^{2+}$ channels to trigger this ion influx [34]. These changes in intracellular $Ca^{2+}$ concentration regulate the contractility of the gastrointestinal tract.

Scheme 1. Synthesis of dihydropyrimidines (DHPMs) (4–9).

Fig. 3. Some potent antihypertensive agents containing pyrimidine moiety.

2.2.2. Calcium antagonism blocking activity in the isolated rat ileum.

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Table 1
Screening of Antihypertensive Activity.

| Compound | Mean arterial BP (mm Hg) |
|----------|--------------------------|
| Control  | 29.46 ± 0.54             |
| 4a       | 22.51 ± 1.11***           |
| 4b       | 22.68 ± 0.94***           |
| 4c       | 23.04 ± 0.97              |
| 5a       | 24.70 ± 1.23***           |
| 5b       | 24.81 ± 0.94***           |
| 5c       | 23.82 ± 0.89***           |
| 6a       | 26.98 ± 1.44***           |
| 6b       | 25.37 ± 1.34***           |
| 6c       | 27.58 ± 0.74***           |
| 7a       | 20.71 ± 0.68***           |
| 7b       | 22.84 ± 0.95***           |
| 7c       | 22.44 ± 0.96***           |
| 8a       | 20.92 ± 0.64              |
| 8b       | 22.81 ± 1.13              |
| 8c       | 22.86 ± 0.92***           |
| 9a       | 21.34 ± 1.34***           |
| 9b       | 23.47 ± 0.73***           |
| 9c       | 23.07 ± 0.87              |
| Nifedipine| 20.82 ± 0.58             |

Data are expressed as mean ± SD. *, ** Significant from control group at p < 0.05 and 0.001, respectively. ### Significant from Nifedipine group at p < 0.01 and 0.001, respectively. BP: Blood pressure.

Table 2
Screening of Ca\(^{2+}\) channel blocking activity of different synthetic compounds and nifedipine on K\(^+\) (80 mM)-induced contractions of rat ileum.

| Compound | IC\(_{50}\) (µg/ml) |
|----------|--------------------|
| 4a       | 23.07 ± 1.53       |
| 4b       | 24.69 ± 2.35       |
| 4c       | 23.62 ± 2.33       |
| 5a       | 24.37 ± 1.45       |
| 5b       | 25.25 ± 1.39       |
| 5c       | 24.81 ± 1.94       |
| 6a       | 26.84 ± 1.51       |
| 6b       | 30.78 ± 2.52       |
| 6c       | 29.73 ± 2.44       |
| 7a       | 19.65 ± 1.60       |
| 7b       | 22.81 ± 2.46       |
| 7c       | 22.85 ± 1.11       |
| 8a       | 20.23 ± 1.79       |
| 8b       | 22.91 ± 2.59       |
| 8c       | 22.63 ± 1.99       |
| 9a       | 21.45 ± 2.55       |
| 9b       | 22.43 ± 1.83       |
| 9c       | 22.57 ± 2.39       |
| Nifedipine| 21.00 ± 1.20       |

Data are expressed as mean ± SD for three independent experiments.

Table 3
Effect of synthesized derivatives on ACE2:SARS-CoV-2 Spike (RBD) inhibition.

| Compound | IC\(_{50}\) (nM) |
|----------|----------------|
| 4a       | 8.50 ± 0.92   |
| 4b       | 12.50 ± 1.13  |
| 4c       | 23.00 ± 1.00  |
| 5a       | 9.80 ± 1.06   |
| 5b       | 12.50 ± 1.35  |
| 5c       | 18.40 ± 1.05  |
| 6a       | 17.50 ± 1.31  |
| 6b       | 23.70 ± 1.35  |
| 6c       | 16.50 ± 1.08  |
| 7a       | 11.60 ± 0.94  |
| 7b       | 10.80 ± 0.75  |
| 7c       | 20.10 ± 1.10  |
| 8a       | 10.60 ± 0.61  |
| 8b       | 19.20 ± 1.11  |
| 8c       | 12.50 ± 1.08  |
| 9a       | 13.70 ± 1.37  |
| 9b       | 15.50 ± 1.37  |
| 9c       | 21.60 ± 0.92  |

Data are presented as mean ± SD of three independent experiments.

ACE2: Angiotensin-converting enzyme 2, SARS-CoV-2: severe acute respiratory syndrome coronavirus, RBD: Receptor-binding domain.

smooth muscles. All test compounds produced a decrease in the tone of ileal contractions in a dose dependent manner (0.5–0.1 mL), (Table 2). As the relaxation of K\(^+\) induced contractions by the tested compounds was similar to that caused by the standard drug nifedipine, thus the observed spasmylytic effect might be mediated through Ca\(^{2+}\) channel inhibition. The most active compounds were 7a, 8a and 9a (IC\(_{50}\), 19.65 ± 1.60, 20.23 ± 1.79, 21.45 ± 2.55 µg/mL, respectively), which was consistent with the potent antihypertensive activity produced by those compounds.

2.2.3. In vitro screening of ACE2: spike RBD (SARS-CoV-2) inhibitory effect

Overwhelmed by the serious outcomes of the coronavirus disease 2019 (COVID-19) pandemic, finding a potential tool to inhibit viral invasion and virulence is of greatest value. Antiviral treatment for COVID-19 is still a big challenge and investigations have not yet been able to find an adequately potent antiviral drug for SARS-CoV-2 infection. So far, many molecules have been explored to discover an efficient treatment [35]. ACE2 receptor; a part of the dual renin-angiotensin system (RAS) [36]; was found to be a key component in COVID-19 infection, and it is expressed on cell membranes of pulmonary and intestinal host cells. ACE2 serves as a receptor for initial viral homing, binding to COVID-19 spike-protein domains enabling viral entry into cells and subsequent replication [20]. ACE2 produces Ang I–7, which plays a crucial role in counterbalancing the vasoconstrictive, pro-inflammatory, and pro-coagulant consequences of ACE-induced Ang II. Consequently, Ang I–7 may drop in the tissues infected by COVID-19, leading to unrestrained deleterious effects of Ang II [37].

Thus, in this study all the synthesized compounds were screened for their inhibitory effect on ACE2 aiming to find antihypertensive compounds with inhibitory ACE2 potency thus, block the binding anchor for COVID-19 spike-protein domains. The results showed that out of 18 derivatives, nine showed promising inhibitory activity against ACE2, (Table 3). The most active ACE2 inhibitory compounds were 4a and 5a (IC\(_{50}\), 8.5 ± 0.92 and 9.8 ± 1.06 nM, respectively). Compounds 8a, 7b, and 7a showed strong inhibitory effects (IC\(_{50}\), 10.6 ± 0.61, 10.8 ± 0.75 and 11.6 ± 0.94 nM, respectively), followed by 4b, 5b, 8c (IC\(_{50}\) ~12.5 nM) and 9a (IC\(_{50}\), 13.7 ± 1.37 nM). Hence, we can recognize the effect of the electron withdrawing group 4-(2-Cyanophenyl) in C4, as in compounds 4a (ester), 5a (acid) and 7a (amide), which exhibited the highest ACE2 inhibitory activity, over 2-haloenyl as in compounds 4c, 5c, 7c, 8c and 9b,c, which showed moderate to no activity. Thus, the activity among compounds 4a, 5a, 7a and 7b could be attributed to the more polar cyano group over the electron withdrawing effects of the less polar halo groups (chloro and bromo) in compounds 7c, 8b,c and 9b,c.

Regarding the anti-SARS-CoV-2 activity of the synthesized DHPMs analogues shown in our study, Abu-Zaied et al. revealed that some pyrimidinethiol compounds and their thioglycoside derivatives showed moderate in vitro anti-SARS-CoV-2 activity [25]. Also, Abu-Zaied and coworkers, assessed some synthesized cytosine thioglycoside analogues for their in vitro activity against avian influenza H5N1 virus and those derivatives displayed high to moderate activity [26].

The THP-1 cell line has been widely used to study immune responses [38] as an in vitro model of macrophages in studies of macrophage involvement in inflammatory responses. It is differentiated by using Phorbol 12-myristate 13-acetate (PMA) and activated by bacterial...
lipopolysaccharides (LPS). Activated THP-1 cells secrete inflammatory cytokines as a result of cell signaling cascade events stimulated by LPS. Cytokines expression levels are considered valuable physiological readouts for cell-based models of inflammation [39] that could help to assess the activities of anti-inflammatory compounds. Since COVID-19 pandemic, many anti-viral drugs have been tested for COVID-19 management; however, none were proven to be fully effective. On the other hand, management of the complications caused mainly by inflammation has shown to be the potential key for better survival rates and shorter hospitalization period for COVID-19 patients. Therefore, several FDA approved drugs for different diseases were tested as current or potential therapies for the treatment or management of COVID-19 through their direct or indirect anti-inflammatory actions. In addition to the investigation of their ability to interact with ACE2 [40].

In the present study, we assessed some of the synthesized derivatives for their anti-inflammatory effects using THP-1 cells stimulated with LPS.

2.2.4. Cytotoxic effect of different compounds against PMA-differentiated THP-1 Cells

The most active compounds with potential ACE2 inhibitory activity viz 4a, b, 5a, b, 7a, b, 8a, c and 9a were tested for their cytotoxic effect on PMA-differentiated THP-1 cells. Results are shown in (Table 4) and demonstrated that compound 7b (amidic moiety bearing polar methyl group) was the most toxic with IC50 value of 61 µM. Compounds 5a (bearing both cyano and acidic moities), 7a, 8a, and 9a (amidic moiety bearing hydrophobic phenyl group) showed less cytotoxic effect on THP-1 cells. While, compounds 5b (bearing polar acidic moiety) and 4a (ester group) gave the lowest toxicity at 342 and 533 µM, respectively. Compounds 5a, 7a, b and 9a were the final selected compounds used for cytokine production assessments to study their activity as anti-inflammatory agents and were used below their corresponding IC50 values (1/4 IC50).

2.2.5. Effect of the selected compounds on pro-inflammatory cytokine IL-6 and CRP production

Macrophages differentiate into various subpopulations with diverse functions in response to various microbial and environmental signals. These cell subpopulations are vital for the inflammatory process and the defence mechanism against infections via the secretion of inflammatory cytokines, such as TNF-α, and IL-6 [41]. However, over-secretion of these mediators has been noticed in several inflammatory diseases including the recent pandemic COVID-19 in which hyper-inflammatory response prompted by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a foremost cause of disease severity and mortality [42,43]. On the other hand, elevated C-reactive protein (CRP), which is an acute-phase protein synthesized in the liver in response to IL-6 and a widely available biomarker of inflammation [44], is associated with cardiovascular disease [45], pneumonia [46], inflammatory rheumatic diseases as well as severe H1N1 influenza [47]. CRP was reported as a procoagulant with implications for atherothrombosis [48]. Recently, several studies have reported an association between higher CRP level and greater disease severity in COVID-19 patients [49,50]. Moreover, higher CRP concentrations were associated with mortality [51]. From the classic drugs that have been screened for their effect to control COVID-19, the old known antimalarial drugs, chloroquine, and hydroxychloroquine. They showed to possess direct anti-inflammatory effect via inhibition of IL-1 and IL-6 production by monocytes [52]. Moreover, when applied to RBD–ACE-2 using molecular docking studies, they showed a potential ability to interfere with the initial attachment of virus particles to the respiratory tract epithelium [53]. Also, hydroxychloroquine in combination with azithromycin has been suggested for COVID-19 treatment through decreasing viral replication along with the anti-inflammatory effect [35]. Moreover, combination of umifenovir with lopinavir/ritonavir showed good antiviral activity against SARS-CoV-2 [43].

Regarding the use of CCBs in COVID-19 patients, a small retrospective study was performed on elderly hospitalized COVID-19 patients with hypertension treated or not treated with either nifedipine or amlodipine. Treatment with either CCBs was found to be significantly correlated with an improved mortality, and a reduced risk for mechanical ventilation in those patients. However, levels of CRP and IL-6 were not significantly different between the two groups. Therefore, the vasodilatory CCBs mentioned in the referred study may improve clinical outcomes in COVID-19 patients. Nevertheless, they should be accompanied with anti-inflammatory agents [54].

Hence, our biological evaluation journey of the synthesized compounds ended by assessing the selected compounds 5a, 7a, b, and 9a for their potential in vitro anti-inflammatory activity in LPS-stimulated THP-1 cells through measuring the levels of IL-6 and CRP.

Results revealed that the secretion of IL-6 by LPS-stimulated THP-1 cells in response to all selected test compounds was significantly lower than in the cells stimulated with LPS (positive control), (Fig. 4).

| Table 4 |
|---|
| Effect of different derivatives against THP-1 cells viability. |
| **Compound** | **IC50, pg/mL (IC50 µM)** |
| 4a | 1551.50 ± 134.30 (533) |
| 4b | 697.60 ± 65.60 (231) |
| 4c | ND |
| 5a | 496.10 ± 38.70 (118) |
| 5b | 967.10 ± 79.20 (342) |
| 5c | ND |
| 6a | ND |
| 6b | ND |
| 6c | ND |
| 7a | 433.70 ± 32.90 (123) |
| 7b | 222.40 ± 10.10 (61) |
| 7c | ND |
| 8a | 464.20 ± 29.70 (125) |
| 8b | ND |
| 8c | 790.60 ± 58.60 (197) |
| 9a | 484.90 ± 3.24 (116) |
| 9b | ND |
| 9c | ND |

Data are presented as mean ± SD of three independent experiments.

Value between ( ) is IC50 in µM. ND: Not determined.

THP-1: Human monocytic cell line.

![IL-6 production in THP-1 cells](image)
Compounds 5a and 9a showed the greatest effect on the cytokine level with a reduction reaching about 56% and 79%, respectively, followed by 7b (47%). However, compound 7a had the lowest effect on the level of this cytokine.

Regarding the level of CRP in LPS-stimulated THP-1 cells in response to the treatment by compounds 5a, 7a, 7b and 9a, it was significantly lower when compared to its level in the cells stimulated with LPS (positive control), (Fig. 5). The greatest effect on CRP level was exhibited by compound 9a, which caused about 95% decrease in the level of CRP, while treatment with compounds 5a and 7b showed moderate decrease in CRP production. However, 7a showed no significant effect on the level of this prototypic marker of inflammation.

Based on the cytokine production and anti-inflammatory performance, these remarkable results highlight the spectacular promising activity of compounds 5a (free acidic group) and 9a (amidic group bearing hydrophobic phenyl and aryl group in C4 bearing 2-Bromo group).

The biological activity exhibited by the test compounds, suggested that the amidic group is a key element for CCBs activity as for compounds 7a, 8a and 9a. In meanwhile, the ester in 4a and acid in 5a is a predominant factor for ACE2 inhibitory activity, where both bearing an electron withdrawing group (2-CN) in C4 aryl. Yet, amidic group in 7a, 8a and 9a showed strong inhibitory activity against ACE2, but less than acidic and ester. For anti-inflammatory activities, acid in 5a and amidic in 7b, 8a and 9a showed the highest effect. The 4-flouro group in the amidic moieties of compounds 7c, 8c and 9c masked the activity of these tested compounds, as revealed in Fig. 6.

3. Conclusion

In this study, we have presented novel DHPMs analogues that can act as both CCBs and ACE2 inhibitory agents, for the treatment of elevated BP. In addition, some of these scaffolds showed potential anti-inflammatory activities via reduction of IL-6 and CRP production in LPS-stimulated THP-1 cells, with a much potent profile. The outcomes of this study may pave the way to find an effective therapeutic tool for hypertensive patients with a protective effect against COVID-19 infection coupled with anti-inflammatory activities.

4. Experimental

4.1. Chemistry

4.1.1. Material and methods

All the reagents and solvents were purchased from Merck (Darmstadt, Germany) and used without further purification. All melting points were uncorrected and measured using Electro-thermal IA 9100 apparatus (Shimadzu, Japan). 1H NMR spectra were recorded on Bruker AMX400 and Bruker Current AV400 Data spectrometer (400 MHz), Bruker BioSpin GmbH, Germany. Spectra and chemical shifts (δ) were expressed as ppm against TMS as internal reference. ESI mass spectra with a Finnigan Thermo Quest MAT 95XL spectrometer and FAB high-resolution (HR) mass spectra with a VG Analytical 70-250S spectrometer; Palmer, USA; using an MCA method and polyethylene glycol as a support. The reactions were monitored by thin layer chromatography (TLC) analysis using silica gel (60 F254 –coated aluminium plates (Merck) which were visualized by UV irradiation (254 nm) and iodine vapors. Column chromatography was performed by using silica gel (60–120 mesh). All reactions were carried out under dry nitrogen.

Compounds 4b [55–57, 4c [58], 5b [59], 8a [60] 8c [61] were previously prepared. Their melting points and characterization data are in agreement with the published references. Compounds 9a-c are commercially available [62–64].

Ethyl-6-methyl-4-(2-substituted phenyl)-2-thioxo-1,2,3,4-tetrahydro pyrimidine-5-carboxylate 4a-c:

A mixture of thiourea (7.6 g, 0.1 mol), ethyl acetoacetate 2 (13 mL, 0.1 mol) and the appropriate aromatic aldehyde 3 (0.1 mol) in 50 mL of 10% alcoholic NaOH was refluxed for 2 h. Then was cooled and acidified with conc. HCl, the precipitate was filtered off, washed with water, dried under suction, and recrystallized from ethanol.

Ethyl-4-(2-cyanophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro pyrimidine-5-carboxylate 4a:

Yield: 76%; m.p.: 252–254 °C; IR ν (KBr cm⁻¹): 3357 (NH), 3173 (CH, aromatic), 2978 (CH, aliphatic), 1724 (C=O), 1683 (C=O), 1600 (C=O), 1455 (C=O), 1277 (C=O), 1220(C–O). 1H NMR (DMSO-d6, 400 MHz): 1.5 (t, 3H, CH3), 1.9 (q, 2H, CH2), 4.1 (t, 3H, CH), 7.3–7.5 (m, 4H, aromatic), 7.6–7.6 (m, 1H, CH), 2.2–7.6 (m, 6H, aromatic), 13C NMR: (DMSO-d6, 400 MHz) δ (ppm) 174.75, 167.75, 160.62, 145.52, 133.75, 132.29, 127.66, 111.65, 105.79, 59.59, 54.4, 18.2, 14.5; MS (EI) m/z: 301.11 (M+, 16.5%); Anal. Calcd., for C15H13N2O2S: C, 59.78; H, 5.02; N, 13.94. Found: C, 59.68; H, 5.17; N, 13.86.

Ethyl-4-(2-chlorophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro pyrimidine-5-carboxylate 4b:

Yield: 69%; m.p.: 220–222 °C.

Ethyl-4-(2-bromophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro pyrimidine-5-carboxylate 4c:

Yield: 70%; m.p.: 202–204 °C.

6-Methyl-4-(2-substituted phenyl)-2-thioxo-1,2,3,4-tetrahydro pyrimidine-5-carboxylic acid 5a-c:

A solution of any 4a-c (3.6 g, 0.01 mol) in 50 mL of 10% alcoholic NaOH was refluxed for 2 h. Then was cooled and acidified with conc. HCl, the precipitate was filtered off, washed with water, dried under suction, and recrystallized from ethanol.

4-(2-cyanophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro pyrimidine-5-carboxylic acid 5a:

Yield: 77%; m.p.: 241–243 °C; IR ν (KBr cm⁻¹): 3451 (OH), 3334 (NH), 2979 (CH, aromatic), 2224 (CN), 1683 (C=O), 1270 (C=O), 1225 (C=O). 1H NMR (DMSO-d6, 400 MHz) δ (ppm) 2.5 (s, 3H, CH3), 5.3 (s, CH, pyrimidine), 7.3–7.5 (m, 4H, aromatic), 7.8, 10.00 (2 s, 2H, NH (D2O exchangeable)), 12.00 (s, 1H, COOH (D2O exchangeable)). 13C NMR: (DMSO-d6, 400 MHz) δ (ppm) 178.12, 169.70, 154.70, 147.57, 139.03, 129.30, 128.40, 126.03, 125.49, 116.84, 114.96, 79.70, 15.09; MS (EI) m/z: 273.13 (M+, 14.7%); Anal. Calcd., for C13H11N2O2S: C, 57.13; H, 4.06; N, 15.37. Found: C, 57.09; H,
4.17; N, 15.45.

4-(2-chlorophenyl)-6-Methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid 5b:
Yield: 67%; m.p.: 210–212 °C.

4-(2-bromophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid 5c:
Yield: 65%; m.p.: 239–241 °C; IR $\nu$ (KBr cm$^{-1}$): 3372 (OH), 3247 (NH), 3173 (CH, aromatic), 2973 (CH, aliphatic), 1687 (C–O), 1271 (C–S), 1229 (C–O).

$^{1}$HNMR (DMSO-d$_6$, 400 MHz) $\delta$: 2.5 (3H, CH$_3$, pyrimidine), 5.1 (s, 1H, CH, pyrimidine), 7.3–7.4 (m, 4H, aromatic), 9.7, 10.2 (2 s, 2H, 2NH (D$_2$O exchangeable)), 11.5 (s, 1H, COOH (D$_2$O exchangeable)).

$^{13}$C NMR: (DMSO-d$_6$, 400 MHz) $\delta$ (ppm) 179.34, 168.76, 160.43, 145.32, 131.75, 129.30, 128.40, 121.49, 106.45, 56.05, 19.05; MS (EI) m/z: 325.82 (M$^+$, 34.9%), 327.91 (M$^+$+2, 33.1%) Anal. Calcd., for C$_{12}$H$_{11}$BrN$_2$O$_2$: C, 44.05; H, 3.39; N, 15.45. Found: C, 44.12; H, 3.53; N, 15.45.

6-Methyl-4-(2-substituted phenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonyl chloride 6a-c:
A mixture of any of 5a-c (3.6 g, 0.01 mol) and 15 mL thionyl chloride was refluxed for 40 min. Unreacted thionyl chloride was removed by heating the reaction mixture on water bath. The produced acid chlorides 6a-c were rapidly dried under suction and used as a crude for subsequent work.

6-Methyl-4-(2-cyanophenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonyl chloride 6a:
Yield: 61%; m.p.: 276–278 °C; IR $\nu$ (KBr cm$^{-1}$): 3340 (NH), 3185 (CH, aromatic), 2983 (CH, aliphatic), 2250 (CN), 1775 (C–O), 1270 (C–S), $^{1}$HNMR (DMSO-d$_6$, 400 MHz) $\delta$: 2.5 (3H, CH$_3$, pyrimidine), 5.2 (s, 1H, CH, pyrimidine), 7.3–7.4 (m, 4H, aromatic), 9.7, 10.2 (2 s, 2H, 2NH (D$_2$O exchangeable)), 11.5 (s, 1H, COOH (D$_2$O exchangeable)).

$^{13}$C NMR: (DMSO-d$_6$, 400 MHz) $\delta$ (ppm) 179.34, 168.76, 160.43, 145.32, 131.75, 129.30, 128.40, 121.49, 106.45, 56.05, 19.05; MS (EI) m/z: 291.75 (M$^+$, 16.0%), (M$^+$+2, 5.3%) Anal. Calcd., for C$_{13}$H$_{10}$ClN$_3$OS: C, 53.52; H, 3.45; N, 14.40. Found: C, 53.19; H, 3.47; N, 14.37.

6-Methyl-4-(2-chlorophenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonyl chloride 6b:
Yield: 71%; m.p.: 263–265 °C; IR $\nu$ (KBr cm$^{-1}$): 3297 (NH), 3181 (CH, aromatic), 2985 (CH, aliphatic), 1772 (C=O), 1720 (C=S), $^{1}$HNMR (DMSO-d$_6$, 400 MHz) $\delta$: 2.4 (3H, CH$_3$), 2.4 (3H, CH$_3$), 5.2 (s, 1H, CH, pyrimidine), 7.5–7.8 (m, 4H, aromatic), 8.9, 9.3 (2s, 2NH, D$_2$O exchangeable). MS (EI) m/z: 301.19 (M$^+$, 22.8%), (M$^+$+2, 7.6%) Anal. Calcd., for C$_{12}$H$_{10}$ClN$_2$OS: C, 47.85; H, 3.49; N, 9.30. Found: C, 47.67; H, 3.47; N, 9.30.

Fig. 6. SAR for the most potent compounds. DHPMs: Dihydropyrimidines, CCBs: calcium channel blockers, ACE2: Angiotensin-converting enzyme 2, THP-1: Human monocytic cell line, IL-6: Interleukine-6, LPS: Lipopolysaccharide, CRP: C-Reactive Protein.
6-Methyl-4-(2-bromophenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid: Yield: 77%; m.p.: 250–252 °C; IR (KBr cm⁻¹): 3343, 3230 (NH), 3179 (CH, aromatic), 2973 (CH, aliphatic), 1671 (C=O), 1270 (C=S).¹HNMR (DMSO-d₆, 400 MHz): δ: 2.3 (s, 3H, CH₃), 5.2 (s, 1H, CH=pyrimidine), 7.1–7.7 (m, 8H, aromatic), 7.9, 9.5 (s, 2H, 2NH (D₂O exchangeable)), 9.1 (s, 1H, CONH (D₂O exchangeable)).¹³C NMR: (DMSO-d₆, 400 MHz) δ (ppm): 173.12, 164.56, 157.56, 144.45, 138.22, 131.44, 130.96, 128.34, 127.64, 125.16, 118.75, 113.90, 106.42, 53.45, 19.53; Anal. Calcd., for C₂₉H₂₉BrCIN₂O: C, 62.28; H, 4.13; N, 15.29. Found: C, 62.39; H, 4.32; N, 15.24.

4-(2-chlorophenyl)-6-methyl-N-(4-fluorophenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxamide 9a:

Yield: 76%; m.p.: 281–283 °C.

4.2. Biological evaluation

4.2.1. Evaluation of the antihypertensive activity

4.2.1.1. Animals. Male Wistar albino rats (200–250 g), were purchased from VACSERA (Helwan, Cairo, Egypt).

Animals were housed in plastic cages with free access to food and water under standard conditions of temperature and humidity with an alternating 12 h light and dark cycle. The study protocol was approved by the Animal Ethics Committee of the Faculty of Pharmacy, Helwan University and was conducted according to the guidelines of the EC, directive 86/609/EEC for animal experiments.

4.2.2. Direct arterial pressure measurement. Rats were fasted overnight and anesthetized by i.p. injection of Pentothal sodium (80 mg/Kg). For each animal, the reflexes were checked, and the rat was placed on a proper rodent surgical table. The ventral side of the neck, right hind leg, and chest were cautiously disinfected then shaved. A small incision (1.5–2 cm) was made in the neck of the rats for carotid artery cannulation. The carotid artery was cannulated using a cannula pre-filled with heparinized normal saline (0.5 IU/mL) and attached to blood pressure transducer to record the arterial blood pressure. Three hours prior to cannulation, animals were treated with the test drugs, nifedipine or normal saline in the control group at a dose of 0.3 mL. Following cannulation, the sensor was connected to the Power Lab instrument and the blood pressure was recorded and analyzed [65].

4.2.2. Calcium antagonism in the isolated rat ileum

4.2.2.1. Ileum segment preparation and recording of the contraction. Tissue segments’ isolation and ileal spasmytic activity estimation were achieved as previously described [66]. After overnight fasting, rats were sacrificed by cervical dislocation and the terminal ileum was dissected out and stored in Tyrode’s solution (136.89 mM NaCl, 2.68 mM KCl, 1.05 mM MgCl₂, 1.80 mM CaCl₂, 0.42 mM NaH₂PO₄, 11.09 mM NaHCO₃ and 5.55 mM glucose; pH 7.4). The mesentery of ileum was removed. Each 2-cm-long segment was suspended in a 25-mL tissue bath containing Tyrode’s solution at 37 °C and constantly aerated with 5% (v/v) CO₂ in oxygen. One end of the isolated ileum was attached to the bath bottom, while the other one to an isotonic force transducer (TSZ-200, Easylab Instruments Ltd.). The transducer to record the arterial blood pressure. Three hours prior to cannulation, animals were treated with the test drugs, nifedipine or normal saline in the control group at a dose of 0.3 mL. Following cannulation, the sensor was connected to the Power Lab instrument and the blood pressure was recorded and analyzed [65].

To assess the possible Ca²⁺ channel blocking effects of the test compounds, a solution containing K⁺ (80 mM) was added to the bath with rat ileum to produce a sustained contraction. The test compounds (0.1–0.5 mL) were cumulatively added to the tissue bath. To assess the possible Ca²⁺ channel blocking effects of the test compounds, a solution containing K⁺ (80 mM) was added to the bath with rat ileum to produce a sustained contraction. The test compounds (0.1–0.5 mL) were cumulatively added to the tissue bath.
compounds and standard (Nifedipine). IC50 dose was calculated by dose response inhibitory curve using GraphPad Prism version 5 for Windows (GraphPad Inc., USA) and the conc of the test compound causing 50% inhibition was calculated according to the following equation:

$$IC_{50} (\mu g/mL) = \text{dose for 50\% inhibition (mL)} \times \text{conc. (1000 \mu g/mL)}$$

4.2.4.1. Cell culture and differentiation.

serum (FCS), 2 mmol/L L-glutamine and 100 IU/100 C and 5% CO
incubated at 37
without phenol red then 10
were determined by the MTT test. Briefly, the culture medium was
blank, respectively. After incubation for 24 h, the number of viable cells
removed from the wells, and 50
THP-1 cells were seeded in 96-well plates at a density of 1
evidence of differentiation.

4.2.4.3. Detection of pro-inflammatory cytokine IL-6 and CRP production.

5.00, GraphPad Inc, CA, USA) was used to obtain dose
GraphPad Prism for Windows (version 5 for Windows (GraphPad Inc., USA).

4.2.5. Statistical analysis

Mean and SD values were calculated for each group, and the com-
comparison between the groups was performed by one way ANOVA followed
post-hoc test, using GraphPad InStat software version 3.05 (GraphPad
Inc., La Jolla, CA, USA). A probability value of P < 0.05 was considered
significant.

Declaration of Competing Interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

Herein, we extend our profound appreciation to our colleagues in
department of Pharmacology and Toxicology, Faculty of Pharmacy,
Helwan University for their help during the experimental part involving
the animals.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.
.org/10.1016/j.bioorg.2021.105272.

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