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ORIGINAL RESEARCH

Design, synthesis, and antihypertensive activity of new pyrimidine derivatives endowing new pharmacophores

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Abstract

A new series of achiral pyrimidine derivatives based on nifedipine-like structure was designed and synthesized. These pyrimidyl derivatives contained hydrazine, hydrazones, acetohydrazide, differently substituted benzylidene functionalities, benzosulfohydrazine, various heterocycles such as pyrazole, pyrazolidinedione, thiazoline, and thiazolidinone rings, and fused ring systems such as triazolopyrimidine and pyrimidotriazine rings. Compounds **5a, 5b, 11b, 8b, 9b–d**, and **15b** showed a decrease in mean arterial rabbit blood pressure (MABP) ranging from 51.4 to 78.2 mmHg in rabbits in comparison with nifedipine-treated rabbits. Among these derivatives, compounds **5a, 5b, 9b**, and **9c** were found to exhibit calcium channel blockade activity on preparations of rabbit aortae. They exhibited relaxation in the range of 89.2% to 74.4% in comparison to nifedipine (57.6%) as well as a decrease in heart rate. Histopathological effect of compounds **5a,b** on the expression of endothelial nitric oxide synthase (eNOS) was also **e**xamined on rat aorta. An intense expression of eNOS immune staining in aortic endothelium was seen for compound **5b** indicating that it lowered blood pressure via activation of eNOS expression in aorta.

Keywords Nifedipine · Pyrimidines · ENOS · Antihypertensive · Histopathology · SAR

Introduction

Cardiovascular diseases (CVDs), including coronary heart diseases, hypertension, and peripheral artery diseases are the main cause of worldwide mortality (World Stroke Organization 2011). Hypertension affects billions of people all around the world despite the great effort and progress in developing new drugs targeting hypertension (European Society of Hypertension 2013). Of the several classes of antihypertensive agents that have found their way in clinical use, calcium channel blockers (CCBs) have received increasing attention in recent years. They are highly recommended and are widely used as the first line antihypertensive therapy with few contraindications in prevention and treatment of CVDs (Lopez et al. 2006; Ozawa et al. 2006; De la Sierra and Ruilope 2007). To this day, 1,4-dihydropyridines (DHPs) are still the most potent group of CCBs with nifedipine (Adalat®) (Vater et al. 1972) (Fig. 1) being the initial drug treatment alone or in combination with other classes in arterial hypertension. Nifedipine, the prototype DHP, since its introduction into clinical medicine in 1975, has been modified to numerous second, third, and fourth generation commercial products for improving duration of activity, potency, and safety profiles (Ozawa et al. 2006; Yousef et al. 2005; Catterall et al. 2003; Bossert and Vater 1989). Further structure modification of the DHP core focused on the aza-analogs of DHPs resulting in the introduction of the potent dihydropyrimidine (DHPM)

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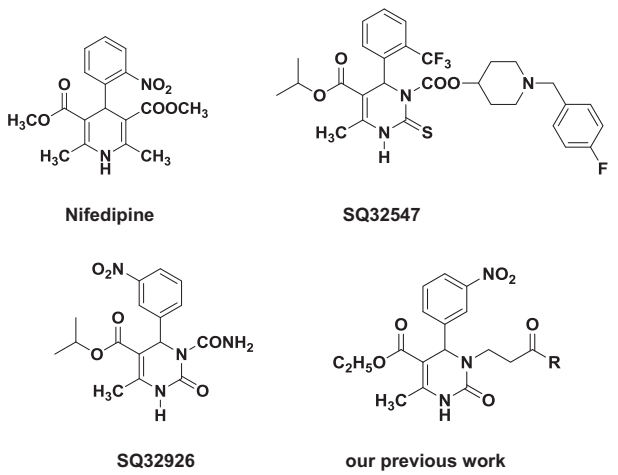


Fig. 1 Nifedipine and lead aza analogs of DHPs

derivatives, which show a very similar pharmacological profile to classical DHPs antihypertensive modulators (Kappe 1998; Grover et al. 1995; Rovnyak et al. 1995). These inherently asymmetric DHPM derivatives are not only very potent antihypertensive modulators, but have also been studied extensively to expand the existing structure–activity relationship (SAR) and to get further insight into molecular interactions at the receptor level (Kappe 1998; Grover et al. 1995; Rovnyak et al. 1995; De Fatima et al. 2015). In general, conformational features previously reported for DHP calcium channel modulators (Bikker and Weaver 1993; Gaudio et al. 1994; Palmer and Andersen 1996) were also preserved for DHPMs. The most notable difference between DHPs and DHPMs, however, is the extreme flattening of the boat-type DHPM ring around N^1 as a result of amide type bonds present in the pyrimidine core nucleus. Over the past years, several lead DHPMs have been developed among which SQ 32926 and SQ 32547 (Horovitz 1990; Negwer 1994) (Fig. 1) have been currently identified to possess antihypertensive properties. They were effective orally and found to be superior in potency and duration of antihypertensive activity to classical DHP drugs (Bikker and Weaver 1993; Kappe et al. 1997; Jain et al. 2008).

Our attention was focused on numerous pyrimidine and dihydropyrimidine derivatives bearing electron donating groups on the C4 phenyl ring that were developed and tested for biological activity (Alam et al. 2010; Kaur and Kaur 2013; Enseleit et al. 2010; Jeanneau et al. 1992) and some of them have revealed calcium channel blockade activity.

In our lab, we have explored *N*-containing pyrimidine scaffold and their activity profiles and we have reported, recently, a series of DHPM hybrids (Teleb et al. 2017a, b) (Fig. 1) as CCB for treatment of hypertension. Compounds of interest were derivatized at N^3 and were chosen to comprise various functionalities as potent and selective candidates to investigate the effect of structural variations on activity modulation (Teleb et al. 2017a, b) (Fig. 1). Based on their biological results, these moieties revealed potent (Teleb et al. 2017a, b) and selective activity against hypertension.

As an extension of our previous investigations and with the aim to generate new antihypertensive agents with desirable potency and drug-like properties, our interest was focused on novel fully aromatized pyrimidine derivatives. They were designed via replacing the pyridine core present in nifedipine by the bioisosteric pyrimidine which possesses significant and privileged bioactivity and pharmacokinetic properties. As shown in Fig. 2, pyrimidine moiety is favorably used as anchor points and served as a critical moiety responsible for H-bond interactions. According to bioisosterism and molecular hybridization (Fig. 2), substituted pyrimidine, taken as lead compound, was modified at position 2 assuming that derivatization at that position of the pyrimidine backbone does not affect the left-hand side of the molecule essential for activity. This may give rise to fruitful potentially active compounds that would be of great interest. Furthermore, substitution at C2 of the pyrimidine ring system would prevent its inactivation by hydroxylation and conjugation reactions (138–141). Therefore, it was believed that pyrimidine derivatives bearing hydrazine group at C2 would provide a valuable matrix for pyrimidine functionalized calcium channel antagonism scaffold with potential application in treatment of hypertension.

Molecular design of the compounds based on pyrimidine scaffold consisted principally of 4 pharmacophores unchanged in all synthesized compounds: 1—Pyrimidine ring essential for coordination at the receptor level. 2—Phenyl ring usually substituted with an electron donating group as OCH_3 connected to non-chiral C4 pyrimidine by one atom length NH linker. 3—Additional lipophilic group at C6 position either methyl or phenyl ring and a CN moiety introduced at C5 instead of the traditional ester functional group while retaining its electron withdrawing properties trying to provide a strong H-bonding site. Modification at C4 by substituting the aromatic *p*-substituted aniline group for *o*-nitrophenyl moiety and hydrophobic methyl/phenyl groups at C6 could be of further effect to optimize the inhibitory potency. The phenyl ring could enable the molecule to make additional π–π interaction with aromatic or heteroaromatic amino acids residues in receptor binding site. 4—Additional hydrophilic group at C2, particularly $–NHNH_2$ group, substituted or unsubstituted, is a biologically active pharmacophore that has been a part of clinically used drugs as hydralazine (Ellershaw and Gurney 2001). In order to evaluate the necessity of heterocyclic

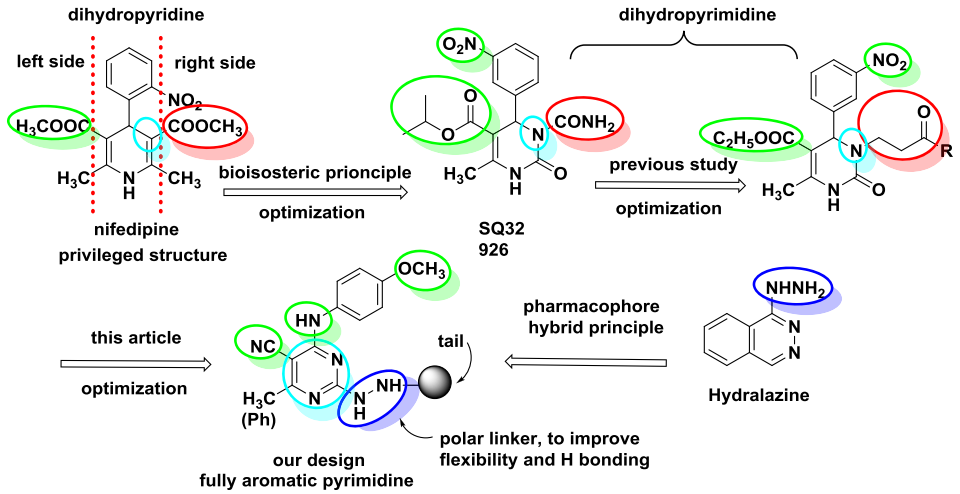


Fig. 2 Rationale for the designed compounds: bioisosterism and molecular pharmacophore hybridization

Tail = pharmacophores including hydrophobic, H-bond acceptor/donor points, heterocycles attached or fused to pyrimidine nucleus

substitution for activity, we included several fused or attached moieties as a part of the tail to establish new SAR regarding the type and size of those moieties. In the target structures, the active $-NHNH_2$ group was retained along with various functionalities at NH at the right hand side of the pyrimidine core and with either H-bond donor or acceptor properties to examine the influence of different size, polarity, electronic properties, flexibility, and conformational freedom of molecules on the inhibitory activity of the target molecules. Moreover, for further assessment of activity, we adopted a conformation restricted approach by adopting the strategy to link or fuse other heterocyclic moieties with the pyrimidine template in search of new lead molecules as potential drug candidates. Heterocyclic portions of these compounds could act as versatile building block to introduce different new functional groups, to anchor these rings into optimal space for binding, and may imply an important role in antihypertensive activity. Therefore, it was our intention to synthesize ring systems as pyrazole, pyrazolidinedione, thiazoline, and thiazolidinone rings in addition to fused ring systems as triazolopyrimidine and pyrimidotriazine rings to study the effect of increasing bulkiness, planarity, and polarity of such fused ring systems on their calcium antagonistic effects and specificity.

The principal features defining SARs in the present study are represented in Fig. 3.

Herein, we report the synthesis of rationally designed fully aromatic pyrimidine multifunctional derivatives. Antihypertensive evaluation and preliminary SAR study of these compounds are also described. Newly designed compounds were synthesized straightforward as depicted in Schemes 1–3.

Materials and methods

Chemistry

Experimental

All melting points were uncorrected and were determined in open-glass capillaries using an electrothermal melting point apparatus (Stuart Scientific, Model SMP1, UK). IR spectra were recorded (KBr) on a Shimadzu Infrared Spectrophotometer IR 470 at the Faculty of Pharmacy, Assiut University. Nuclear magnetic resonance spectra, ^{1}H NMR and ^{13}C NMR, were taken using Bruker Avance III apparatus 400 MHz; Central Laboratory Unit, Faculty of Pharmacy, Cairo and Bruker DRX 400 MHz, Central Laboratory Unit, Beni-suef Universities for ^{1}H and 101 MHz for ^{13}C NMR, Varian EM-360L NMR spectrophotometer 60 MHz, Varian, USA, Faculty of Pharmacy, Assiut University, Varian Mercury VX 300 BB Spectrophotometer; Faculty of Science, Cairo University 300 MHz for ^{1}H and 75.45 MHz for ^{13}C NMR, Bruker apparatus DRX 400 MHz; Dakota State University, Brookings, SD, USA for ^{1}H and 101 MHz for ^{13}C NMR. TMS was used as an internal reference and chemical shifts are expressed in δ units (ppm). Mass spectra (MS) were run on a gas chromatograph/mass spectrometer *Shimadzu GCMS-Qp2010* plus, single quad (70 eV), Regional Centre for Mycology and Biotechnology, Al-Azhar University, Nasr City, Cairo, and ThermoFinnigan MAT 95 XL, LR and HR work, EI, electrospray type: high resolution double-focusing magnetic sector; ESI: ESI II spray head LC/MS ThermoFinnigan LCQ Advantage Ion Trap, LR electrospray type: Ion Trap, University of South

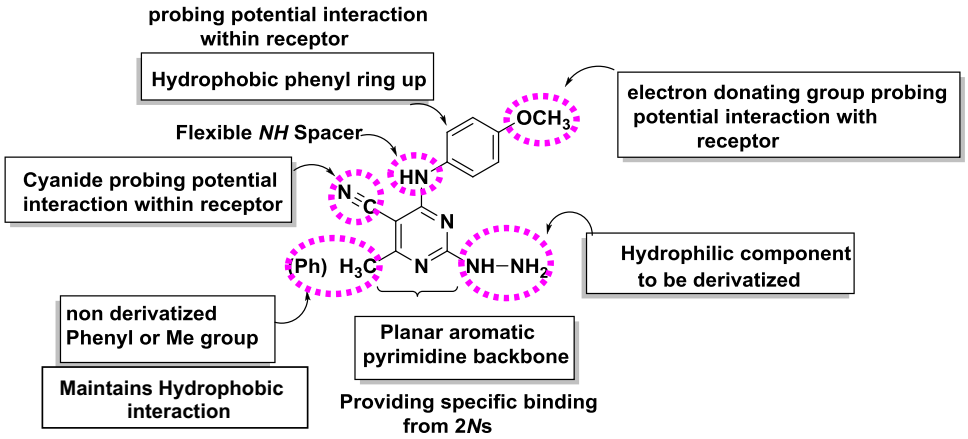


Fig. 3 Chemical target of the present research work

Dakota, USA. All the analytical data were obtained from Microanalytical Data Unit at Cairo University, Cairo, Egypt and all results were in an acceptable range.

General method for the synthesis of 2-(methylthio)-4-methyl/phenyl-6-oxo-1,6-dihydropyrimidine-5-carbonitriles (2a,b)

A mixture of ethyl 2-cyano-3-phenylacrylate (**1a**) (Hussein et al. 1985) or ethyl 2-cyano-3-ethoxybutenoate (**1b**) (Yang et al. 2012) (10 mmol), *S*-methylisothiourea sulfate (11.0 mmol), and anhydrous sodium acetate (2.0 g, 24.4 mmol) in DMF (1 ml) was heated at 70 °C for 1 h. The reaction mixture was poured onto ice-cold H_2O and the formed precipitate was filtered and crystallized from EtOH (Hussein et al. 1985; Abdel-Aziz et al. 2011).

2-(Methylthio)-4-methyl-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (2a) Yield 40%; mp 140–142 °C; IR ν/cm^{-1}: 3427 (NH), 2227 (C≡N), 1557 (C=N), 1495 (C=C–Ar); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ/ppm: 8.31 (s, *br*, 1H, N*H*), 2.25 (s, 3H, S-C*H*$_3$), 2.11 (s, 3H, C*H*$_3$) (Hussein et al. 1985).

2-(Methylthio)-4-phenyl-6-oxo-1,6-dihydropyrimidine-5-carbonitrile (2b) Yield 60%; mp 276–277 °C; IR ν/cm^{-1}: 3412 (NH), 2228 (C≡N), 1593 (C=N), 1573 (C=C–Ar) (Hussein et al. 1985; Abdel-Aziz et al. 2011).

General method for the synthesis of 4-chloro-6-methyl/phenyl-2-(methylthio)-pyrimidine-5-carbonitriles (3a,b)

A mixture of **2a,b** (1.49 mmol), $POCl_3$ (0.4 ml, 4 mmol) and *N,N*-dimethylaniline (0.2 ml, 1.58 mmol) was heated at 60 °C for 2 h. After completion of the reaction as indicated by TLC, the mixture was poured onto ice-cold H_2O and the formed precipitate was filtered and crystallized from EtOH (Vasudevan et al. 2012, 2014).

4-Chloro-6-methyl-2-(methylthio)-pyrimidine-5-carbonitrile (3a) Yield 93%; mp 88–90 °C; IR ν/cm^{-1}: 3300 (NH), 2215 (C≡N), 1614 (C=N), 1604 (C=C–Ar), 1263, 1134 (ν_{as} and ν_s C–O–C).

4-Chloro-6-phenyl-2-(methylthio)-pyrimidine-5-carbonitrile (3b) Yield 94%; mp 141–140 °C; IR ν/cm^{-1}: 3312 (NH), 2214 (C≡N), 1614 (C=N), 1572 (C=C–Ar), 1293, 1089 (ν_{as} and ν_s C–O–C).

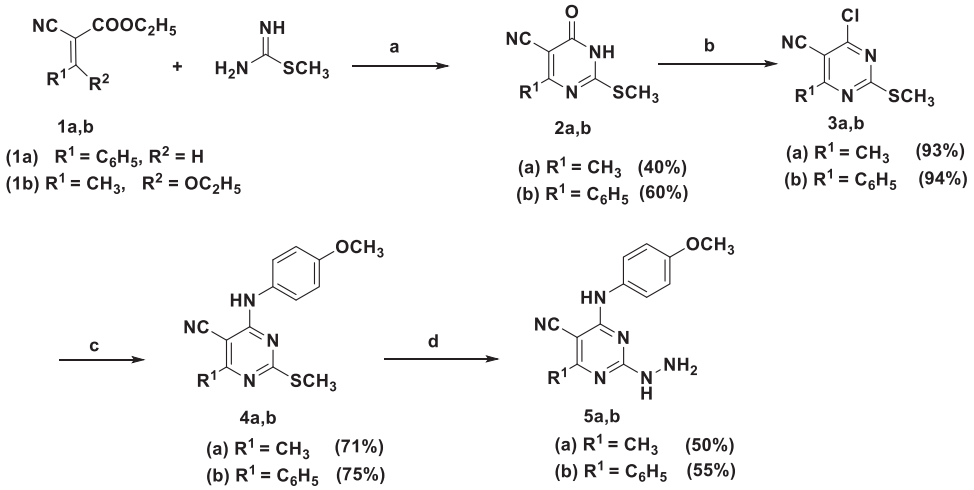
General method for the synthesis of 4-(4-methoxyanilino)-6-methyl/phenyl-2-(methylthio)-pyrimidine-5-carbonitriles (4a,b)

A mixture of **3a,b** (2.86 mmol), 4-methoxyaniline (0.36 g, 2.88 mmol) and anhydrous K_2CO_3 (0.4 g, 5.76 mmol) in EtOH (15 ml) was heated under reflux for 5 h. It was then cooled, filtered and the filtrate was concentrated to half its volume. The obtained precipitate was filtered and crystallized from EtOH-H_2O (3:1 v/v).

4-(4-Methoxyanilino)-6-methyl-2-(methylthio)-pyrimidine-5-carbonitrile (4a) Yield 71%; mp 158–160 °C; IR ν/cm^{-1}: 3300 (NH), 2214 (C≡N), 1614 (C=N), 1604 (C=C–Ar), 1293, 1134 (ν_{as} and ν_s C–O–C); ^{1}H NMR ($CDCl_3$, 60 MHz) δ/ppm: 7.49 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 12.6$ Hz), 7.21 (s, *br*, 1H, N*H*), 6.90 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 12.0$ Hz), 3.83 (s, 3H, OC*H*$_3$), 2.58 (s, 3H, S-C*H*$_3$), 2.50 (s, 3H, C*H*$_3$); EIMS *m/z* 286 ($M^{+\cdot}$, 100), 271 (33), 270 (25), 238 (13); Anal. Calcd. for $C_{14}H_{14}N_4OS$: (286.35): C, 58.72; H, 4.93; N, 19.72. Found: C, 58.97; H, 4.98; N, 19.57.

4-(4-Methoxyanilino)-6-phenyl-2-(methylthio)-pyrimidine-5-carbonitrile (4b) Yield 75%; mp 190–192 °C; IR ν/cm^{-1}: 3312 (NH), 2215 (C≡N), 1622 (C=N), 1572 (C=C–Ar), 1263, 1089 (ν_{as} and ν_s C–O–C); ^{1}H NMR ($CDCl_3$, 400 MHz) δ/ppm: 7.97 (d, 2H, C2,6-*H*s of phenyl ring, $J = 8.0$

Scheme 1 Synthetic pathway to hydrazine derivatives **5a,b**



Hz), 7.46–7.55 (m, 4H, C3,4,5-*H*s of phenyl ring, + N*H*-Ar), 7.25 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 6.0$ Hz), 6.91 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 9.2$ Hz), 3.82 (s, 3H, OCH_3), 2.50 (s, 3H,S-CH_3); Anal. Calcd. for $C_{19}H_{16}N_4OS$ (348.42): C, 65.50; H, 4.93; N, 16.08. Found: C, 65.78; H, 4.98; N, 16.34.

General method for the synthesis of 2-hydrazinyl-6-methyl/phenyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitriles (5a,b)

A mixture of **4a,b** (0.24 mmol), and hydrazine hydrate (0.012 g, 0.24 mmol) in dioxane (1 ml) was heated under reflux for 4 h. The reaction mixture was concentrated to half its volume and the obtained precipitate was filtered and crystallized from EtOH.

2-Hydrazinyl-6-methyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitrile (5a) Yield 50%; mp 190–192 °C; IR ν/cm^{-1}: 3326 (NH), 3215 (NH + NH_2), 2188 (C≡N), 1610 (C=N), 1490 (C=C–Ar), 1180, 1094 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 60 MHz) δ/ppm: 8.60 (s, *br*, 1H, C2-N*H*), 8.35 (s, *br*, 1H, N*H*-Ar), 7.63 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J =$ 11.4 Hz), 6.91 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 11.4$ Hz), 3.88 (s, 3H, OCH_3), 2.44 (s, 3H, CH_3); Anal. Calcd. for $C_{13}H_{14}N_6O$ (270.29): C, 57.77; H, 5.22; N, 31.09. Found: C, 57.96; H, 5.34; N, 31.36.

2-Hydrazinyl-6-phenyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitrile (5b) Yield 55%; mp 215–217 °C; IR ν/cm^{-1}: 3287 (NH), 3270 (NH + NH_2), 2188 (C≡N), 1612 (C=N), 1582 (C=C–Ar), 1251, 1032 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 9.12 (s, *br*, 1H, C2-N*H*, D_2O exchangeable), 8.96 (s, *br*, 1H, N*H*-Ar, D_2O exchangeable), 7.53–7.90 (m, 7H, Ar-*H*s), 6.90 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 8.92$ Hz), 4.38 (s, 2H, NH_2, D_2O exchangeable), 3.76 (s, 3H, OCH_3); Anal. Calcd. for $C_{18}H_{16}N_6O$ (332.36): C, 65.05; H, 4.85; N, 25.29. Found: C, 65.17; H, 4.91; N, 25.43.

General method for the synthesis of 8-(4-Methoxyanilino)-6-methyl-3-aryl-4*H*-pyrimido[2,1-c][1,2,4]-triazine-7-carbonitriles (6a–c)

The appropriately substituted phenacyl bromide (1 mmol) was added to a solution of the hydrazine derivative **5a** (0.27 g, 1 mmol) in absolute EtOH (5 ml). The reaction mixture was heated under reflux for 2 h, concentrated to half its volume then left to cool to RT. The obtained precipitate was filtered, washed with petroleum ether (40°–60°), dried and crystallized from EtOH.

8-(4-Methoxyanilino)-6-methyl-3-(4-bromophenyl)-4*H*-pyrimido[2,1-c][1,2,4]-triazine-7-carbonitrile (6a) Yield 60%; mp 200–203 °C; IR ν/cm^{-1}: 3565 (NH), 2223 (C≡N), 1617 (C=N), 1576 (C=C–Ar), 1254, 1026 (ν_{as} and ν_s C–O–C) ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 12.63 (s, 1H, N*H*-triazine), 10.77 (s, 1H, N*H*-Ar), 7.83 (d, 2H, Ar-*H*s, *o*- to Br, $J = 8.68$ Hz), 7.75 (d, 2H, Ar-*H*s, *m*- to Br, $J = 8.68$ Hz), 7.44 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 8.72$ Hz), 7.02 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 9.0$ Hz), 5.32 (s, 2H, C4-H_2), 3.80 (s, 3H, OCH_3), 2.82 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 101 MHz) δ/ppm: 168.6 (CN), 160.0 (C-4 of 4-bromophenyl), 159.2 (C-7), 159.0 (C-9a), 148.7 (C-6, C-1 of 4-methoxyaniline), 145.1 (C-8), 134.2 (C-3, C-5 of 4-bromophenyl), 132.8 (C-3), 130.0 (C-4 of 4-methoxyaniline), 129.3 (C-2, C-6 of 4-methoxyaniline), 126.4 (C-2, C-6 of 4-bromophenyl), 124.8 (C-1 of 4-bromophenyl), 114.0 (C-3, C-5 of 4-methoxyaniline), 55.39 (OCH_3), 44.9 (C-4), 18.9 (C6-CH_3); HRESIMS $M^+ + 1$ at *m/z* 449.0729 (100) and $M^+ + 3$ at *m/z* 451.0712 (94) corresponding to $C_{21}H_{17}{}^{79}BrN_6O$ and $C_{21}H_{17}{}^{81}BrN_6O$ radical cation fragments; calculated = 448 (450); Anal. Calcd for

$C_{21}H_{17}BrN_6O$ (449.30): C, 56.14; H, 3.81; N, 18.70. Found: C, 56.30; H, 3.78; N, 18.86.

8-(4-Methoxyanilino)-6-methyl-3-(4-chlorophenyl)-4*H*-pyrimido[2,1-c][1,2,4]-triazine-7-carbonitrile (6b) Yield 52%; mp 193–195 °C; IR ν/cm^{-1}: 3399 (NH), 2223 (C≡N), 1624 (C=N), 1509 (C=C–Ar), 1232, 1090 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ ppm: 12.64 (s, 1H, N*H*-triazine), 10.78 (s, 1H, N*H*-Ar,), 7.91 (d, 2H, Ar-*H*s, *o*- to Cl, $J = 7.6$ Hz), 7.60 (d, 2H, Ar-*H*s, *m*- to Cl, $J = 8.0$ Hz), 7.45 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 8.0$ Hz), 7.02 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 8.4$ Hz), 5.34 (s, 2H, C4-H_2), 3.79 (s, 3H, OCH_3), 2.83 (s, 3H, CH_3); Anal. Calcd. for $C_{21}H_{17}ClN_6O$ (404.85): C, 62.30; H, 4.23; N, 20.76. Found: C, 62.57; H, 4.30; N, 20.92.

8-(4-Methoxyanilino)-6-methyl-3-(4-nitrophenyl)-4*H*-pyrimido[2,1-c][1,2,4]-triazine-7-carbonitrile (6c) Yield 65%; mp 205–207 °C; IR ν/cm^{-1}: 3565 (NH), 2230 (C≡N), 1635 (C=N), 1507 (C=C–Ar), 1457, 1346 (ν_{as} and ν_s NO_2), 1254, 1032 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 12.80 (s, *br*, 1H, N*H*- triazine, D_2O exchangeable), 10.85 (s, *br*, 1H, N*H*-Ar, D_2O exchangeable), 8.36 (d, 2H, Ar-*H*s, *o*- to NO_2, $J = 8.84$ Hz), 8.14 (d, 2H, Ar-*H*s, *m*- to NO_2, $J = 8.8$ Hz), 7.44 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 8.68$ Hz), 7.03 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 8.88$ Hz), 5.38 (s, 2H, C4-H_2), 3.80 (s, 3H, OCH_3), 2.83 (s, 3H, CH_3); Anal. Calcd. for $C_{21}H_{17}N_7O_3$ (415.14): C, 60.72; H, 4.12; N, 23.60. Found: C, 60.89; H, 4.14; N, 23.84.

General method for the synthesis of 7-(4-methoxyanilino)-3,5-disubstituted-[1,2,4]triazolo[4,3-a]pyrimidine-6-carbonitriles (7a–f)

For **7a,b,d,e**: A solution of the hydrazine derivative **5a,b** (1 mmol) and the appropriate aliphatic acid (5 ml) was heated under reflux for 7 h. The reaction mixture was concentrated to a small volume and diluted with ice-cold H_2O. The obtained precipitate was filtered, washed with H_2O, dried and crystallized from EtOH/H_2O.

For **7c,f**: A solution of the hydrazine derivative **5a,b** (1 mmol), benzoic acid (0.122 g, 1 mmol) and $POCl_3$ (0.4 ml) was heated under reflux for 4 h. After cooling, the reaction mixture was poured onto ice-cold H_2O and neutralized with a saturated solution of $NaHCO_3$. The precipitate was collected by filtration, washed with cold H_2O and recrystallized from EtOH.

7-(4-Methoxyanilino)-5-methyl-[1,2,4]triazolo[4,3-a]pyrimidine-6-carbonitrile (7a) Yield 23%; mp 178–180 °C; IR ν/cm^{-1}: 3213 (NH), 2202 (C≡N), 1626 (C=N), 1581 (C=C–Ar), 1231, 1033 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 300 MHz) δ/ppm: 9.87 (s, *br*, 1H, N*H*-Ar, D_2O-exchangeable), 9.50 (d, 1H, triazole N*H*, $J = 10.5$ Hz, D_2O-exchangeable), 9.21 (s, *br*, 1H, pyrimidine N-*H*, D_2O-exchangeable), 8.02 (s, 1H, C3-*H*), 7.50 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 6.9$ Hz), 6.87 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 6.9$ Hz), 3.74 (s, 3H, OCH_3), 2.37 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 171.7 (*C*N), 167.2 (C-6), 161.5 (C-5), 160.3 (C-9), 159.8 (C-7), 131.0 (C-1 of 4-methoxyaniline), 125.2 (C-3), 123.9 (C-4 of 4-methoxyaniline), 113.5, 113.1 (C-5,C-3 of 4-methoxyaniline), 116.2, 116.1 (C-6, C-2 of 4-methoxyaniline), 55.1 (OCH_3), 23.0 (C-5-*C*H_3); Anal. Calcd. for $C_{14}H_{12}N_6O$ (280.28): C, 59.99; H, 4.32; N, 29.98. Found: C, 60.14; H, 4.39; N, 30.16.

7-(4-Methoxyanilino)-3,5-dimethyl-[1,2,4]triazolo[4,3-a]pyrimidine-6-carbonitrile (7b) Yield 32%; mp 185–187 °C; IR ν/cm^{-1}: 3315 (NH), 2216 (C≡N), 1626 (C=N), 1577 (C=C–Ar), 1254, 1022 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 9.78 (s, *br*, 1H, N*H*-Ar, D_2O-exchangeable), 9.28 (s, 1H, triazole N-*H*), 9.15 (s, 1H, pyrimidine N-*H*, D_2O-exchangeable), 7.50 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 8.8$ Hz), 6.85 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 8.4$ Hz), 3.74 (s, 3H, OCH_3), 2.36 (s, 3H, C5-CH_3), 1.85 (s, 3H, C3-CH_3); Anal. Calcd. for $C_{15}H_{14}N_6O$ (294.12): C, 61.21; H, 4.79; N, 28.55. Found: C, 61.42; H, 4.87; N, 28.17.

7-(4-Methoxyanilino)-3-phenyl-5-methyl-[1,2,4]triazolo[4,3-a]pyrimidine-6-carbonitrile (7c) Yield 40%; mp 203–202 °C; IR ν/cm^{-1}: 3340 (NH), 2212 (C≡N), 16211 (C=N), 1585 (C=C–Ar), 1249, 1042 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 10.84 (s, 1H, N*H*-Ar, D_2O-exchangeable), 8.25 (*dd*, 2H, C2,6-*H*s of C3-phenyl, $J_{AM} = 7.9$ Hz, $J_{AX} = 2.3$ Hz), 7.56–7.61 (m, 3H, C3,4,5-*H*s of C3-phenyl), 7.41 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 8.84$ Hz), 7.02 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 8.88$ Hz), 3.82 (s, 3H, OCH_3), 2.51 (s, 3H, C5-CH_3); EIMS *m/z*: 357 ($M^{+\cdot}$, 25), 356 (99), 341 (70), 185 (67), 92 (32), 77 (100), 76 (31); Anal. Calcd. for $C_{20}H_{16}N_6O$ (356.14): C, 67.40; H, 4.53; N, 23.58. Found: C, 67.64; H, 4.59; N, 23.71.

7-(4-Methoxyanilino)-5-phenyl-[1,2,4]triazolo[4,3-a]pyrimidine-6-carbonitrile (7d) Yield 20%; mp 222–220 °C; IR ν/cm^{-1}: 3199 (NH), 2212 (C≡N), 1624 (C=N), 1583 (C=C–Ar), 1248, 1042 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 9.79 (s, *br*, 1H, N*H*-Ar, D_2O-exchangeable), 9.58 (d, *br*, 1H, triazole N*H*, $J = 10$ Hz, D_2O-exchangeable), 9.30 (s, *br*, 1H, pyrimidine N*H*, D_2O-exchangeable), 8.01 (s, 1H, C3-*H*), 7.81 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 7.68$ Hz), 7.53–7.57 (m, 5H, Ar-*H*s), 6.90 (d, *dist*, 2H, Ar-*H*s, *o*- to OCH_3, $J = 7.92$ Hz), 3.76 (s, 3H, OCH_3); Anal. Calcd. for $C_{19}H_{14}N_6O$ (342.12): C, 66.66; H, 4.12; N, 24.55. Found: C, 66.81; H, 4.09; N, 24.69.

7-(4-Methoxyanilino)-3-methyl-5-phenyl-[1,2,4]triazolo[4,3-a]pyrimidine-6-carbonitrile (7e) Yield 40%; mp 213–215 °C; IR ν/cm^{-1}: 3270 (NH), 2208 (C≡N), 1618 (C=N), 1580 (C=C–Ar), 1245, 1038 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d$_6$, 400 MHz) δ/ppm: 9.77 (s, *br*, 1H, N*H*-Ar), 9.50 (s, 1H, triazole *H*), 9.30 (s, 1H, triazole N-*H*), 7.81 (d, 2H, C2,6-*H*s of C-3-phenyl, $J = 5.2$ Hz), 7.51–7.57 (m, 3H, C3,4,5-*H*s of C-5-phenyl), 7.53 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 8.8$ Hz), 6.89 (d, 2H, Ar-*H*s, *o*- to OCH$_3$, $J = 8.8$ Hz), 3.75 (s, 3H, OC*H*$_3$), 1.87 (s, 3H, C*H*$_3$); Anal. Calcd. for $C_{20}H_{16}N_6O$ (356.14): C, 67.40; H, 4.53; N, 23.58. Found: C, 67.63; H, 4.59; N, 23.74.

7-(4-Methoxyanilino)-3,5-diphenyl-[1,2,4]triazolo[4,3-a]pyrimidine-6-carbonitrile (7f) Yield 43%; mp 287–289 °C; IR ν/cm^{-1}: 3346 (NH), 2212 (C≡N), 1603 (C=N), 1573 (C=C–Ar), 1252, 1029 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d$_6$, 400 MHz) δ/ppm: 11.00 (s, *br*, 1H, N*H*-Ar), 7.83, 8.76 (2d, 4H, Ar-*H*s of C-5-phenyl, $J = 6.0$ Hz), 7.54–7.75 (m, 6H, 5 Ar-*H*s of C-3-phenyl + 1 Ar-*H* of C-5-phenyl), 7.49 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 8.0$ Hz), 7.03 (d, 2H, Ar-*H*s, *o*- to OCH$_3$, $J = 7.6$ Hz), 3.80 (s, 3H, OC*H*$_3$); Anal. Calcd. for $C_{25}H_{18}N_6O$ (418.15): C, 71.76; H, 4.34; N, 20.08. Found: C, 71.89; H, 4.41; N, 20.29.

General method for the synthesis of 2-(5-Amino-4-cyano-3-methyl-1*H*-pyrazol-1-yl)-6-methyl/phenyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitriles (8a,b)

A mixture of the hydrazine derivative **5a,b** (0.5 mmol) and 2-(1-ethoxy-ethylidene)malononitrile (**1b**) (Yang et al. 2012) (0.13 g, 0.5 mmol) in absolute EtOH (5 ml) was heated under reflux for 5 h and left to cool to RT. The separated products were filtered, washed with petroleum ether, dried, and crystallized from EtOH.

2-(5-Amino-4-cyano-3-methyl-1*H*-pyrazol-1-yl)-6-methyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitrile (8a) Yield 23%; mp 140–143 °C; IR ν/cm^{-1}: 3389 (NH), 2221 (C≡N), 1635 (C=N), 1598 (C=C–Ar), 1258, 1030 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d$_6$, 400 MHz) δ/ppm: 10.04 (s, *br*, 1H, N*H*-Ar), 9.24 (s, *br*, 2H, pyrazole-N*H*$_2$), 7.83 (m, 2H, Ar-*H*s, *o*- to NH-Ar), 6.94 (m, 2H, Ar-*H*s, *o*- to OCH$_3$), 3.76 (s, 3H, OC*H*$_3$), 2.41 (s, 3H, C6-C*H*$_3$-pyrimidine), 2.15 (s, 3H, C3-C*H*$_3$-pyrazole); Anal. Calcd. for $C_{18}H_{16}N_8O$ (360.37): C, 59.99; H, 4.48; N, 31.09. Found: C, 60.18; H, 4.57; N, 31.34.

2-(5-Amino-4-cyano-3-methyl-1*H*-pyrazol-1-yl)-6-phenyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitrile (8b) Yield 21%; mp 192–190 °C; IR ν/cm^{-1}: 3315 (NH), 2216 (C≡N), 1635 (C=N), 1578 (C=C–Ar), 1248, 1035 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d$_6$, 400 MHz) δ/ppm: 10.15 (s, *br*, 1H, N*H*-Ar), 7.88 (d, 2H, Ar-*H*s, $J = 6.80$ Hz), 7.79 (s, dist, 2H, pyrazole-N*H*$_2$), 7.55–7.79 (m, 3H, Ar-*H*s), 7.44 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 8.68$ Hz), 7.00 (d, 2H, Ar-*H*s, *o*- to OCH$_3$, $J = 8.72$ Hz), 3.81 (s, 3H, OC*H*$_3$), 2.16 (s, 3H, C3-C*H*$_3$-pyrazole); EIMS *m/z*: 422 (M$^{+\cdot}$, 49), 421 (36), 357 (54), 333 (22), 332 (100), 317 (33), 302 (30); Anal. Calcd. for $C_{23}H_{18}N_8O$ (422.16): C, 65.39; H, 4.29; N, 26.53. Found: C, 65.61; H, 4.32; N, 26.79.

General method for the synthesis of 2-(4-Cyano-5-oxo-3-aryl-4,5-dihydro-1*H*-pyrazol-1-yl)-6-methyl/phenyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitriles (9a–d)

A mixture of the hydrazine derivative **5a,b** (0.5 mmol) and ethyl 2-cyano-3-phenyl acrylate 1a or ethyl 2-cyano-3-methoxyphenylacrylate[143] 1b (0.1 g, 0.5 mmol) in absolute EtOH (5 ml) was heated under reflux for 5 h and left to cool to RT. The separated products were filtered, washed with petroleum ether dried and crystallized from EtOH.

2-(4-Cyano-5-oxo-3-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)-6-methyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitrile (9a) Yield 40%; mp 170–172 °C; IR ν/cm^{-1}: 3275 (OH), 3187 (NH), 2360, 2213 (2 × C≡N), 1606 (C=N), 1561 (C=C–Ar), 1233, 1028 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d$_6$, 400 MHz) δ/ppm: 11.60 (s, *br*, 1H, O*H*, C5-O*H*-pyrazole), 9.23 (s, *br*, 1H, N*H*-Ar), 8.16 (s, 1H, C4-*H*-pyrazole), 7.69 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 6.52$ Hz), 7.37–7.46 (m, 5H, Ar-*H*s of C-3-pyrazole), 6.93 (d, 2H, Ar-*H*s, *o*- to OCH$_3$, $J = 8.72$ Hz), 3.79 (s, 3H, OC*H*$_3$), 2.43 (s, 3H, C*H*$_3$); ^{13}C NMR (DMSO-d$_6$, 101 MHz) δ/ppm: 172.7 (2 × CN), 160.9 (C5), 159.3 (C2), 156.3 (C6), 143.7 (C4), 135.4 (C-1, C-4 of 4-methoxyaniline), 132.3 (C-3, C-5-pyrazole), 129.9 (*C*4-pyrazole), 129.2 (C-2, C-6 of 4-methoxyaniline), 127.0 (C-3, C-5 of 4-methoxyaniline), 124.4 (C-1, C-4 of phenyl at C3-pyrazole), 117.0 (C-2, C-6 of phenyl at C3-pyrazole), 113.8 (C-3, C-5 of phenyl at C3-pyrazole), 55.7 (OCH$_3$), 23.6 (C6-*C*H$_3$); EIMS *m/z* 423 (M$^{+\cdot}$, 10), 382 (16), 357 (22), 325 (24), 312 (35), 309 (20), 296 (23), 286 (100), 281 (28), 280 (63), 244 (85), 210 (35), 207 (38), 201 (40), 191 (37), 190 (38), 184 (30), 181 (47), 178 (24), 176 (25), 163 (63), 145 (35), 144 (95), 141 (38), 121 (60); Anal. Calcd. for $C_{23}H_{17}N_7O$ (423.43): C, 65.24; H, 4.05; N, 23.16. Found: C, 65.39; H, 4.11; N, 23.42.

2-(4-Cyano-5-oxo-3-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)-6-methyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitrile (9b) Yield 45%; mp 188–190 °C; IR ν/cm^{-1}: 3282 (OH), 3280 (NH), 2320, 2215 (2 × C≡N), 1619 (C=N), 1577 (C=C–Ar), 1244, 1035 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d$_6$, 400 MHz) δ/ppm: 11.60 (s, br, 1H, OH, C^5-OH-pyrazole, D$_2$O exchangeable), 9.18 (s, br, 1H, NH-Ar, D$_2$O exchangeable), 8.11 (s, 1H, C4-H-pyrazole),

7.61–7.91 (m, 4H, Ar-Hs of 4-methoxyphenyl at C3-pyrazole), 7.00 (d, 2H, Ar-Hs, o- to NH-Ar, $J = 8.36$ Hz), 6.93 (d, 2H, Ar-Hs, o- to OCH_3, $J = 8.8$ Hz), 3.78 (s, 6H, 2 × OCH_3), 2.41 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 101 MHz) δ/ppm: 172.0 (2 × CN), 160.9 (pyrazole-C4 + C5), 160.8 (C5), 159.2 (C2), 156.1 (C6), 143.8 (C4), 132.5 (C1 of 4-methoxyaniline), 128.5 (pyrazole-C3), 128.0 (C-1 of 4-methoxyphenyl at C3-pyrazole), 124.2 (C-4 of 4-methoxyaniline + C4 of 4-methoxyphenyl at C3-pyrazole), 117.1 (C-2, C-6 of 4-methoxyphenyl at C3-pyrazole), 114.7 (C-2, C-6 of 4-methoxyaniline), 113.8 (C-3, C-5 of 4-methoxyphenyl at C3-pyrazole + C-3, C-5 of 4-methoxyaniline), 55.8, 55.7 (2 × OCH_3), 23.6 (C-6-CH_3); EIMS *m/z*: 453 ($M^{+\cdot}$, 2), 390 (24), 389 (100), 388 (39), 387 (35), 386 (23); Anal. Calcd. for $C_{24}H_{19}N_7O_3$ (453.45): C, 63.57; H, 4.22; N, 21.62. Found: C, 63.80; H, 4.27; N, 21.74.

2-(4-Cyano-5-oxo-3-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)-6-phenyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitrile (9c) Yield 41%; mp 191–193 °C; IR ν/cm^{-1}: 3400 (OH), 3305 (NH), 2222, 2200 (2 × C≡N), 1621 (C=N), 1584 (C=C–Ar), 1231, 1028 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 11.79 (s, *br*, 1H, O*H*, C5-O*H*-pyrazole), 9.33 (s, *br*, 1H, N*H*-Ar), 8.21 (s, 1H, C4-*H*-pyrazole), 7.45–7.96 (m, 12H, Ar-*H*s, 2 × 5-phenyl-*H*s + 2 Ar-*H*s, *o*- to NH-Ar), 6.97 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J =$ 6.64 Hz), 3.81 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 101 MHz) δ/ppm: 180.1 (2 × CN), 160.7 (C5), 159.6 (C2), 157.5 (C6), 142.0 (C4), 138.5 (C-1 of 4-methoxyaniline), 135.5 (C-4, C-5 pyrazole), 132.0 (C4 of 4-methoxyaniline), 129.8 (C-1 of phenyl at C3-pyrazole + C3-pyrazole), 129.1 (C-2, C-6 of 4-methoxyaniline), 128.5 (C-1 of phenyl at C6-pyrimidine), 128.3 (C-2, C-6 of phenyl at C6-pyrimidine), 126.5 (C-2, C-6 of phenyl at C3-pyrazole), 124.4 (C-3, C-5 of phenyl at C6-pyrimidine), 117.1 (C-3, C-5 of 4-methoxyaniline), 113.2 (C-3, C-5 of phenyl at C3-pyrazole), 83.75 (C4 of phenyl at C3-pyrazole + C4 of phenyl at C-6-pyrimidine), 55.19 (OCH_3); EIMS *m/z*: 486 ($M^{+\cdot}$, 2), 317 (23), 316 (22), 199 (24), 175 (34), 171 (41), 158 (28), 144 (25), 141 (49), 140 (50), 131 (23), 114 (22), 111 (70), 110 (27), 109 (23), 66 (100); Anal. Calcd. for $C_{28}H_{19}N_7O_2$ (485.50): C, 69.27; H, 3.94; N, 20.20. Found: C, 69.67; H, 3.76; N, 20.52.

2-(4-Cyano-5-oxo-3-(4-methoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)-6-phenyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitrile (9d) Yield 43%; mp 201–203 °C; IR ν/cm^{-1}: 3331 (OH), 3187 (NH), 2305, 2206 (2 × C≡N), 1608 (C=N), 1586 (C=C–Ar), 1247, 1031 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 11.65 (s, *br*, 1H, O*H*, C5-O*H*-pyrazole, D_2O exchangeable), 9.28 (s, *br*, 1H, N*H*-Ar, D_2O exchangeable), 8.15 (s, 1H, C4-*H*-pyrazole), 7.56–7.96 (m, 9H, Ar-*H*s), 7.02 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 7.72$ Hz), 6.96 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 8.88$ Hz), 3.82, 3.81 (2s, 6H, 2 × OCH_3); ^{13}C NMR (DMSO-d_6, 101 MHz) δ/ppm: 172.8, 169.5 (2 × CN), 161.6 (C4-pyrazole), 160.6 (C5), 156.5 (C2), 153.1 (C6), 148.1 (C4), 131.4 (C5-pyrazole), 129.8 (C3-pyrazole), 128.5 (C1 of 4-methoxyaniline), 126.1 (C1 of phenyl at C6-pyrimidine), 124.8 (C-2, C-6 of phenyl at C6-pyrimidine), 121.0 (C4 of 4-methoxyaniline + C4 of 4-methoxyphenyl at C3-pyrazole), 120.9 (C-3, C-5 of phenyl at C6-pyrimidine), 116.5 (C1 of 4-methoxyphenyl at C3-pyrazole), 114.1 (C-2, C-6 of 4-methoxyaniline + C-2, C-6 of 4-methoxyphenyl at C3-pyrazole), 113.8 (C-3, C-5 of 4-methoxyaniline, C-3, C-5 of 4-methoxyphenyl at C3-pyrazole), 82.2 (C4 of phenyl at C6-pyrimidine), 55.7 (OCH_3), 30.4 (OCH_3); Anal. Calcd. for $C_{29}H_{21}N_7O_3$ (515.52): C, 67.56; H, 4.11; N, 19.02. Found: C, 67.74; H, 4.18; N, 19.26.

General method for the synthesis of 2-(5-Amino-4-cyano-1*H*-pyrazol-1-yl)-6-methyl/phenyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitriles (10a,b)

A mixture of the hydrazine derivative **5a,b** (0.5 mmol) and 2-(ethoxymethylene) malonitrile (Ding et al. 1987) (0.061 g, 0.5 mmol) in absolute EtOH (5 ml) was heated under reflux for 5 h and left to cool to RT. The separated product was filtered, washed with petroleum ether, dried, and crystallized from EtOH.

2-(5-Amino-4-cyano-1*H*-pyrazol-1-yl)-6-methyl-4-(4-methoxy-anilino)-pyrimidine-5-carbonitrile (10a) Yield 23%; mp 122–123 °C; IR ν/cm^{-1}: 3384 (NH), 3309 (NH_2), 2210 (C≡N), 1635 (C=N), 1564 (C=C–Ar), 1256, 1025 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 300 MHz) δ/ppm: 10.06 (s, 1H, N*H*-Ar), 7.86 (s, 2H, pyrazole-NH_2), 7.84 (s, 1H, C3-*H* pyrazole), 7.43 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 8.76$ Hz), 6.97 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 8.88$ Hz), 3.80 (s, 3H, OCH_3), 2.56 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz) δ/ppm: 172.7 (2 × CN), 160.6 (C5), 157.0 (C4-pyrazole), 155.8 (C2), 154.1 (C6), 143.0 (C-4, C-1 of 4-methoxyaniline), 129.7 (C5-pyrazole), 125.9 (C4 of 4-methoxyaniline), 114.5 (C-2, C-6 of 4-methoxyaniline), 114.0 (C-3, C-5 of 4-methoxyaniline), 72.6 (C3-pyrazole), 55.2 (OCH_3), 23.3 (C6-CH_3); EIMS *m/z*: 347 ($M^{+\cdot}$, 23), 346 (100), 331 (66), 197 (22), 196 (22), 92 (22); Anal. Calcd. for $C_{17}H_{14}N_8O$ (346.13): C, 58.95; H, 4.07; N, 32.35. Found: C, 59.18; H, 4.12; N, 32.49.

2-(5-Amino-4-cyano-1*H*-pyrazol-1-yl)-6-phenyl-4-(4-methoxy-anilino)-pyrimidine-5-carbonitrile (10b) Yield 21%; mp 200–201 °C; IR ν/cm^{-1}: 3384 (NH), 3300 (NH_2), 2223 (C≡N), 1644 (C=N), 1564 (C=C–Ar), 1247, 1040 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 10.06 (s, 1H, N*H*-Ar, D_2O-exchangeable), 7.91(d, 2H, Ar-*H*s of

C6-phenyl, $J = 6.88$ Hz), 7.87 (s, 1H, C3-*H* pyrazole), 7.84, (s, 2H, pyrazole-NH_2, D_2O-exchangeable), 7.59–7.64 (m, 3H, Ar-*H*s of C6-phenyl), 7.46 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 8.60$ Hz), 7.00 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 8.64$ Hz), 3.81 (s, 3H, OCH_3); Anal. Calcd. for $C_{22}H_{16}N_8O$ (408.42): C, 64.70; H, 3.95; N, 27.44. Found: C, 64.94; H, 3.93; N, 27.61.

General method for the synthesis of 2-(4-amino-1*H*-pyrazolo[3,4-d] pyrimidin-1-yl)-6-methyl/phenyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitriles (11a,b)

A mixture of 2-(5-amino-4-cyano-1*H*-pyrazol-1-yl)-4-(4-methoxyanilino)-6-substituted-pyrimidine-5-carbonitrile **10a,b** (1 mmol) and formamide (2 ml) was heated under reflux for 2–3 h. The solution was cooled to RT and poured onto H_2O. The obtained precipitate was filtered, dried and crystallized from EtOH.

2-(4-Amino-1*H*-pyrazolo[3,4-d]pyrimidin-1-yl)-6-methyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitrile (11a) Yield 70%; mp 199–197 °C; IR ν/cm^{-1}: 3499 (NH_2), 3294 (NH), 2213 (C≡N), 1636 (C=N), 1549 (C=C–Ar), 1248, 1033 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 8.83 (s, 1H, N*H*-Ar, D_2O exchangeable), 7.45 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 9.0$ Hz), 7.00 (s, *br*, 2H, NH_2, D_2O-exchangeable), 6.87 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 9.0$ Hz), 3.74 (s, 3H, OCH_3), 2.30 (s, 3H, CH_3); Anal. Calcd. for $C_{18}H_{15}N_9O$ (373.37): C, 57.90; H, 4.05; N, 33.76. Found: C, 58.08; H, 4.12; N, 33.94.

2-(4-Amino-1*H*-pyrazolo[3,4-d]pyrimidin-1-yl)-6-phenyl-4-(4-methoxyanilino)-pyrimidine-5-carbonitrile (11b) Yield 75%; mp 205–207 °C; IR ν/cm^{-1}: 3491(NH_2), 3296 (NH), 2211 (C≡N), 1649 (C=N), 1560 (C=C–Ar), 1242, 1034 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 8.94 (s, 1H, N*H*-Ar, D_2O exchangeable), 7.78 (d, *dist*, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 9.16$ Hz), 7.48–7.56 (m, 5H, Ar-*H*s of C6-phenyl), 7.21 (s, *br*, 2H, NH_2, D_2O-exchangeable), 6.91 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 8.92$ Hz), 3.78 (s, 3H, OCH_3); ^{13}C NMR (DMSO-d_6, 400 MHz) δ/ppm: 170.5 (CN), 163.1 (C5), 162.6 (C4 of pyrazolopyrimidine), 156.5 (C2), 137.6 (C4), 132.0 (C1 of 4-methoxyaniline), 130.8 (C6, C-1 of C6-phenyl), 129.1 (C-3a, C-7a of pyrazolopyrimidine), 128.9 (C6 of pyrazolopyrimidine), 128.8 (C-2, C-6 of C6-phenyl), 128.7 (C-3, C-5 of C6-phenyl), 125.6 (C-4 of 4-methoxyaniline + C-4 of C6-phenyl), 118.1 (C-2, C-6 of 4-methoxyaniline), 114.0 (C-3, C-5 of 4-methoxyaniline), 78.0 (C3), 55.7 (OCH_3); EIMS *m/z*: $M^{+\cdot}$ absent, 434 ($M^{+\cdot}$-1, 2), 330 (26), 300 (29), 287 (26), 243 (23), 234 (28), 230 (25), 199 (28), 198 (23), 195 (33), 181 (26), 171 (40), 169 (26), 168 (29), 167 (39), 165 (28), 158 (82), 157 (37), 156 (30), 155 (31), 153 (89), 151 (42), 148 (41), 147 (35), 142 (28), 141 (45), 137 (40), 136 (100), 134 (43), 133(38), 129 (31), 127 (36), 120 (33), 119 (56), 115 (32), 114 (34), 108 (40), 107 (409), 105 (44), 104 (93), 103 (31), 102 (48); Anal. Calcd. for $C_{23}H_{17}N_9O$ (435.44): C, 63.44; H, 3.94; N, 28.95. Found: C, 63.71; H, 3.92; N, 27.17.

General method for the synthesis of 4-(4-methoxyanilino)-6-methyl-2-[2-(substituted thiocarbamoyl)hydrazinyl]-pyrimidine-5-carbonitriles (12a,b)

The appropriate isothiocyanate derivative (1 mmol) was added to a well stirred suspension of the hydrazine derivative **5a** (0.27 g, 1 mmol) in absolute EtOH (20 ml). The reaction mixture was stirred at RT for 2 h during which time dissolution and reprecipitation occurred. The obtained precipitate was filtered, dried and crystallized from EtOH.

4-(4-Methoxyanilino)-6-methyl-2-[2-phenylthiocarbamoyl-hydrazinyl]-pyrimidine-5-carbonitrile (12a) Yield 86%; mp 130–132 °C; IR ν/cm^{-1}: 3400 (NH), 3268 (NH), 2226 (C≡N), 1700 (C=N), 1597 (C=C–Ar), 1551, 1334, 1199, 1039 (NH-C=S I, II, III, IV mixed vibrational bands), 1266, 1094 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 11.85 (s, 1H, NHN*H*C=S, D_2O-exchangeable), 10.28 (*s*, 1H, C2-N*H*, D_2O-exchangeable), 9.90 (s, 1H, N*H*C_6H_5, D_2O-exchangeable), 9.70 (s, br, 1H, C4-N*H*-Ar, D_2O-exchangeable), 7.14–7.55 (m, 5H, Ar-*H*s of phenylthiocarbamoyl), 7.54 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 7.76$ Hz), 7.15 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 7.36$ Hz), 3.33 (s, 3H, O-CH_3), 2.32 (s, 3H, CH_3); Anal. Calcd. for $C_{20}H_{19}N_7OS$ (405.14): C, 59.24; H, 4.72; N, 24.18. Found: C, 59.51; H, 4.79; N, 24.42.

4-(4-Methoxyanilino)-6-methyl-2-[2-(4-nitrophenyl)thiocarbamoylhydrazinyl]-pyrimidine-5-carbonitrile (12b) Yield 88%; mp 140–142 °C; IR ν/cm^{-1}: 3495 (NH), 3292 (NH), 2230 (C≡N), 1706 (C=N), 1640 (C=C–Ar), 1535, 1340 (ν_{as} and ν_s NO_2), 1560, 1356, 1204, 1094 (NH-C=S I, II, III, IV mixed vibrational bands), 1270, 1111 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 13.20 (s, 1H, N*H*C_6H_4-NO_2, D_2O-exchangeable), 11.62 (*s*, 1H, NHN*H*C=S N*H*C_6H_4, D_2O-exchangeable), 10.87 (s, 1H, C2-N*H*, D_2O-exchangeable), 9.60 (s, br, 1H, C4-N*H*-Ar, D_2O-exchangeable), 8.25 (d, 2H, Ar-*H*s *o*- to NO_2, $J = 7.08$ Hz), 8.23 (d, 2H, Ar-*H*s *m*- to NO_2, $J = 7.08$ Hz), 7.80 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 9.20$ Hz), 6.60 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 9.20$ Hz), 3.43 (s, 3H, O-CH_3), 2.32 (s, 3H, CH_3); Anal. Calcd. for $C_{20}H_{18}N_8O_3S$ (450.12): C, 53.32; H, 4.03; N, 24.87. Found: C, 53.59; H, 4.07; N, 25.03.

General method for the synthesis of 6-methyl-4-(4-methoxyanilino)-2-(2-(4-oxo-3-arylthiazolidin-2-ylidene) hydrazinyl)pyrimidine-5-carbonitriles (13a,b)

Ethyl bromoacetate (0.167 g, 1 mmol) and anhydrous NaOAc (0.12 g, 1.5 mmol) in EtOH (5 ml) were added to the solution of the appropriate thiosemicarbazide derivative **12a,b** and the mixture was heated under reflux for 3 h, concentrated to half its volume, allowed to attain RT and poured onto ice-cold H_2O (10 ml). The obtained precipitate was filtered, dried and crystallized from EtOH.

6-Methyl-4-(4-methoxyanilino)-2-(2-(4-oxo-3-phenylthiazolidin-2-ylidene) hydrazinyl)pyrimidine-5-carbonitrile (13a) Yield 22%; mp 199–201 °C; IR ν/cm^{-1}: 3359 (NH), 2211 (C≡N), 1742 (C=O), 1651 (C=N), 1585 (C=C–Ar), 1234, 1166 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d$_6$, 400 MHz) δ/ppm: 10.42 (s, *br*, 1H, C2-N*H*), 9.37 (s, 1H, N*H*-Ar), 7.08–7.50 (m, 5H, Ar-*H* of thiazolidinone-N-phenyl), 6.89 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 8.68$ Hz), 6.68 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 7.64$ Hz), 4.18 (d, 1H, thiazolidinone-C5-H_B, $J = 7.68$ Hz), 3.77 (d, 1H, thiazolidinone-C5-H_A, $J = 7.6$ Hz), 3.72 (s, 3H, OCH_3), 2.43 (s, 3H, CH_3); ^{13}C NMR (DMSO-d$_6$, 101 MHz) δ/ppm: 172.8 (CN), 169.5 (C=O), 161.1 (C5), 160.6 (C2), 156.6 (C1 of thiazolidinone-N-phenyl), 153.0 (thiazolidinone-C2), 148.1 (C6), 131.4 (C4), 129.8 (C1 of 4-methoxyaniline), 124.8 (C-2, C-6 of thiazolidinone-N-phenyl), 120.9 (C-2, C-6 of 4-methoxyaniline), 116.5 (C-4 of phenyl), 114.1 (C-3, C-5 of thiazolidinone-N-phenyl), 113.8 (C-3, C-5 of 4-methoxyaniline), 82.2 (C4 of thiazolidinone-N-phenyl), 55.7 (OCH_3), 30.4 (thiazolidinone-C5), 23.5 (CH_3); EIMS: 445 ($M^{+\cdot}$, 31), 240 (100), 239 (53), 225 (23), 197 (16), 196 (22), 135 (13), 108 (22), 92 (17), 77 (49); Anal. Calcd. for $C_{22}H_{19}N_7O_2S$ (445.50): C, 59.31; H, 4.30; N, 22.01. Found: C, 59.48; H, 4.38; N, 22.19.

6-Methyl-4-(4-methoxyanilino)-2-(2-(4-oxo-3-(4-nitrophenyl)thiazolidin-2-ylidene) hydrazinyl)pyrimidine-5-carbonitrile (13b) Yield 20%; mp 203–205 °C; IR ν/cm^{-1}: 3399 (NH), 2213 (C≡N), 1740 (C=O), 1651 (C=N), 1585 (C=C–Ar), 1489, 1339 (ν_{as} and ν_s NO_2), 124, 1176 (ν_{as} and ν_s C–O-C); ^{1}H NMR (DMSO-d$_6$, 400 MHz) δ/ppm: 10.54 (s, 1H, C2-N*H*), 9.44 (s, 1H, N*H*-Ar), 8.18 (d, 2H, Ar-*H*s, *o*- to NO_2, $J = 8.0$ Hz), 7.41 (d, 2H, Ar-*H*s, *o*- to NH-Ar, $J = 8.0$ Hz), 6.94 (d, 2H, Ar-*H*s, *m*- to NO_2, $J = 8$ Hz), 6.85 (d, 2H, Ar-*H*s, *o*- to OCH_3, $J = 8.0$ Hz), 4.29 (d, 1H, thiazolidinone-C5-H_B, $J = 15.0$ Hz), 3.85 (d, 1H, thiazolidinone-C5-H_A, $J = 16.0$ Hz), 3.73 (s, 3H, OCH_3), 2.43 (s, 3H, CH_3); ^{13}C NMR (DMSO-d$_6$, 101 MHz) δ/ppm: 171.3 (CN), 168.8 (C=O), 160.0 (C5), 159.8 (C4 of thiazolidinone-N-nitrophenyl), 159.1 (C2), 156.6 (C1 of thiazolidinone-N-nitrophenyl), 155.2 (thiazolidinone-C2), 142.5 (C6), 130.0 (C4), 125.2 (C-3, C-5 of thiazolidinone-N-nitrophenyl), 124.7 (C-2, C-6 of thiazolidinone-N-nitrophenyl), 124.8 (C1 of 4-methoxyaniline), 121.5 (C-2, C-6 of 4-methoxyaniline), 116.0 (C4 of 4-methoxyaniline), 113.5 (C-3, C-5 of 4-methoxyaniline), 82.2 (thiazolidinone-C5), 55.3 (OCH_3), 23.0 (CH_3); HRESIMS: $M^{+\cdot}+1$ 491.12363 (100) (calculated mass = 490.49); Anal. Calcd. for $C_{22}H_{18}N_8O_4S$ (490.49): C, 53.87; H, 3.70; N, 22.85. Found: C, 54.03; H, 3.74; N, 23.03.

General method for the synthesis of 6-methyl-4-(4-methoxyanilino)-2-(3,4-diarylthiazol-2(3*H*)-ylidenehydrazinyl)pyrimidine-5-carbonitriles (14a–c, 15a–c)

The properly 4-substituted phenacyl bromide (1 mmol) was added to a solution of the corresponding thiosemicarbazide derivative **12a,b** and sodium acetate (0.164 g, 2 mmol) in EtOH (5 ml) and the reaction mixture was heated under reflux for 2 h, concentrated to half its volume then left to cool to RT. The obtained precipitate was filtered, washed with petroleum ether (40°–60°), dried and crystallized from EtOH.

6-Methyl-4-(4-methoxyanilino)-2-(3-phenyl-4-(4-bromophenyl)thiazol-2(3*H*)-ylidene hydrazinyl)pyrimidine-5-carbonitrile (14a) Yield 22%; mp 178–180 °C; IR ν/cm^{-1}: 3320 (NH), 2215 (C≡N), 1636 (C=N), 1570 (C=C–Ar), 1230, 1114 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d$_6$, 400 MHz) δ/ppm: 9.77, 9.60 (2s, 2 × 1H, C2-N*H*), 9.31, 8.84 (2s, 2 × 1H, N*H*-Ar), 6.85–8.18 (hump, 26 H, Ar-*H*s), 6.79, 6.77 (2s, 2 × 1H, thiazoline-*H*), 3.76, 3.73 (2s, 2 × 3H, 2 × OCH_3), 2.34, 2.30 (2s, 2 × 3H, 2 × CH_3); HRESIMS: $M^+ + 1 = 584.08429$ (9), $M^+ + 3 = 586.07745$ (8); calculated mass = 583 (585); Anal. Calcd. for $C_{28}H_{22}BrN_7OS$ (584.49): C, 57.54; H, 3.79; N, 16.77. Found: C, 57.70; H, 3.82; N, 16.89.

6-Methyl-4-(4-methoxyanilino)-2-(3-phenyl-4-(4-chlorophenyl)thiazol-2(3*H*)-ylidene hydrazinyl)pyrimidine-5-carbonitrile (14b) Yield 23%; mp 150–152 °C; IR ν/cm^{-1}: 3293 (NH), 2209 (C≡N), 1636 (C=N), 1573 (C=C–Ar), 1510, 1040 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d$_6$, 400 MHz) δ/ppm: 9.77, 9.52 (2s, 2 × 1H, C2-N*H*), 9.38, 8.84 (2s, 2 × 1H, N*H*-Ar), 6.74–7.67 (hump, 26 H, Ar-*H*s), 6.50, 6.41 (2s, 2 × 1H, thiazoline-*H*), 3.76, 3.73 (2s, 2 × 3H, 2 × OCH_3), 2.34, 2.29 (2s, 2 × 3H, 2 × CH_3); ^{13}C NMR (DMSO-d$_6$, 101 MHz) δ/ppm: 172.7, 172.0 (CN), 163.0, 162.2 (C5), 161.4, 160.9 (C2), 157.0, 156.7 (C6), 155.9, 155.7 (thiazoline-C2), 151.0, 150.9 (C4 of 4-chlorophenyl), 140.8, 140.6 (C1 of thiazoline-2-(or 3-)-phenyl), 133.4, 133.3 (C4), 133.2, 131.9 (C1 of 4-methoxyaniline), 130.0,

129.5 (C-3, C-5 of 4-chlorophenyl), 129.4, 129.3 (C-2, C-6 of thiazoline-2-(or 3-)-phenyl), 128.5, 127.9 (C1 of 4-chlorophenyl), 125.7, 125.6 (C-2, C-6 of 4-chlorophenyl), 123.8, 123.7 (C-2, C-6 of 4-methoxyaniline), 121.60, 121.5 (C-3, C-5 of thiazoline-2-(or 3-)-phenyl), 116.7, 116.4 (C4 of 4-methoxyaniline), 114.5, 114.1 (C4 of thiazoline-2-(or 3-)-phenyl), 113.9, 113.7 (C-3, C-5 of 4-methoxyaniline), 92.7, 91.5 (thiazoline-C4), 82.1, 81.3 (thiazoline-C5), 55.8, 55.7 (OCH_3), 23.4 (CH_3); HRESIMS: $M^+ + 1 = 540.1364$ (100), $M^+ + 3 = 542.1340$ (39) (calculated mass = 540.04); Anal. Calcd. for $C_{28}H_{22}ClN_7OS$ (540.04): C, 62.27; H, 4.11; N, 18.16. Found: C, 62.41; H, 4.17; N, 18.34.

6-Methyl-4-(4-methoxyanilino)-2-(3-phenyl-4-(4-nitrophenyl)-2(3*H*)-ylidene-hydrazinyl)pyrimidine-5-carbonitrile (14c) Yield 25%; mp 166–167 °C; IR ν/cm^{-1}: 3411 (NH), 2213 (C≡N), 1636 (C=N), 1593 (C=C–Ar), 1415, 1347 (ν_{as} and ν_s NO_2), 1237, 1114 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 9.83, 9.43, (2s, 2 × 1H, C2-N*H*), 9.29, 8.95 (2s, 2 × 1H, N*H*-Ar), 6.53–8.38 (hump, 28 H, thiazoline-*H* + Ar-*H*s), 3.76, 3.74 (2s, 2 × 3H, 2 × OCH_3), 2.35, 2.29 (2s, 2 × 3H, 2 × CH_3); HRESIMS: $M^+ + 1 = 551.15948$ (12) (calculated mass = 550.59); Anal. Calcd. for $C_{28}H_{22}N_8O_3S$ (550.59): C, 61.08; H, 4.03; N, 20.35. Found: C, 61.35; H, 4.11; N, 20.57.

6-Methyl-4-(4-methoxyanilino)-2-(3-(4-nitrophenyl)-4-(4-bromophenyl)thiazol-2(3*H*)-ylidenehydrazinyl)pyrimidine-5-carbonitrile (15a) Yield 23%; mp 183–182 °C; IR ν/cm^{-1}: 3305 (NH), 2210 (C≡N), 1620 (C=N), 1580 (C=C–Ar), 1515, 1342 (ν_{as} and ν_s NO_2), 1235, 1040 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 10.67, 10.16 (2s, 2 × 1H, C2-N*H*), 9.41, 9.13 (2s, 2 × 1H, N*H*-Ar), 6.88–8.19 (hump, 24H, Ar-*H*s), 6.70, 6.45 (2s, 2 × 1H, thiazoline-*H*), 3.76, 3.74 (2s, 2 × 3H, 2 × OCH_3), 2.34, 2.29 (2s, 2 × 3H, 2 × CH_3); ^{13}C NMR (DMSO-d_6, 101 MHz) δ/ppm: 172.5, 172.1 (CN), 162.6, 161.9 (C5), 161.5, 161.1 (C2), 157.5, 157.3 (C6), 157.0, 156.9 (thiazoline-C2), 143.5, 143.3 (C4 of 4-bromophenyl), 140.9, 140.5 (C4 of thiazoline-2-(or 3-)-4-nitrophenyl), 131.7, 131.5 (C4), 130.9, 130.7 (C1 of 4-methoxyaniline), 129.8, 129.6 (C-3, C-5 of thiazoline-2-(or 3-)-4-nitrophenyl), 126.1, 125.8 (C-3, C-5 of 4-bromophenyl), 125.6, 125.5 (C1 of 4-bromophenyl), 122.5, 122.4 (C-2, C-6 of thiazoline-2-(or 3-)-4-nitrophenyl), 122.3, 122.2 (C-2, C-6 of 4-bromophenyl), 122.1, 121.8 (C-2, C-6 of 4-methoxyaniline), 116.7, 114.0 (C4 of 4-methoxyaniline), 114.5, 114.1 (C1 of thiazoline-2-(or 3-)-4-nitrophenyl), 114.0, 113.8 (C-3, C-5 of 4-methoxyaniline), 93.3, 92.0 (thiazoline-C4), 82.0, 81.4 (thiazoline-C5), 55.8, 55.7 (OCH_3), 23.4 (CH_3); Anal. Calcd. for $C_{28}H_{21}BrN_8O_3S$ (629.49): C, 53.42; H, 3.36; N, 17.80. Found: C, 53.69; H, 3.34; N, 18.02.

6-Methyl-4-(4-methoxyanilino)-2-(3-(4-nitrophenyl)-4-(4-chlorophenyl)thiazol-2(3*H*)-ylidenehydrazinyl)pyrimidine-5-carbonitrile (15b) Yield 25%; mp 185–186 °C; IR ν/cm^{-1}: 3293 (NH), 2209 (C≡N), 1610 (C=N), 1573 (C=C–Ar), 1415, 1338 (ν_{as} and ν_s NO_2), 1230, 1034 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 9.86, 9.45 (2s, 2 × 1H, C2-N*H*), 9.41, 9.13 (2s, 2 × 1H, N*H*-Ar), 6.96–8.21 (hump, 24H, Ar-*H*s), 6.50 (s, 1H, thiazoline-*H*), 3.73, 3.70 (2s, 2 × 3H, 2 × OCH_3), 2.60, 2.30 (2s, 2 × 3H, 2 × CH_3); EIMS *m*/*z*: 586 ($M^{+\cdot}$, 17), 584 (43), 449 (26), 448 (21), 447 (71), 417 (19), 416 (73), 401 (41), 391 (13), 389 (19), 331 (30), 286 (14), 285 (13), 284 (33), 255 (57), 254 (64), 240 (100), 239 (93), 225 (25), 224 (20), 218 (14), 214 (20), 211 (24), 197 (32), 170 (40), 168 (90), 139 (33), 134 (27), 133 (41), 108 (27), 90 (32); Anal. Calcd. for $C_{28}H_{21}ClN_8O_3S$ (584.11): C, 57.48; H 3.62; N, 19.15. Found: C, 57.62; H, 3.60; N, 19.38.

6-Methyl-4-(4-methoxyanilino)-2-(3,4-(4-nitrophenyl)thiazol-2(3*H*)-ylidenehydrazinyl)pyrimidine-5-carbonitrile (15c) Yield 26%; mp 190–191 °C; IR ν/cm^{-1}: 3411 (NH), 2213 (C≡N), 1636 (C=N), 1593 (C=C–Ar), 1509, 1347 (ν_{as} and ν_s NO_2), 1237–1230, 1114–1034 (ν_{as} and ν_s C–O–C); ^{1}H NMR (DMSO-d_6, 400 MHz) δ/ppm: 9.88, 9.73 (2s, 2 × 1H, C2-N*H*), 9.44, 9.21 (2s, 2 × 1H, N*H*-Ar), 6.97–8.24 (hump, 24H, Ar-*H*s), 6.66 (s, 1H, thiazoline-*H*), 3.74, 3.72 (2s, 2 × 3H, 2 × OCH_3), 2.33, 2.28 (2s, 2 × 3H, 2 × CH_3); Anal. Calcd. for $C_{28}H_{21}N_9O_5S$ (595.59): C, 56.47; H, 3.55; N, 21.17. Found: C, 56.59; H, 3.59; N, 21.39.

Pharmacological studies

Drugs, chemicals, and materials

Nifedipine was obtained from Amriya Pharmaceutical Industries, Alexandria-Cairo Desert Road Km 25, Amriya, Alexandria, Egypt. Other chemicals such as pentobarbital sodium, heparin, dimethylsulfoxide (DMSO), freshly mixed heparinized saline, physiological solutions were of analytical grade. i.v. Catheters were of 20GA, 22 GA, 24 GA calibers.

Animals

White New Zealand rabbits (2–2.5 kg) of either sex were obtained from Animal House, Faculty of Medicine, Assiut University. Rabbits were kept in cages in Faculty of Medicine, Al-Azhar University, Assiut Branch, Assiut, housed at ordinary RT, exposed to natural daily light-dark cycles, fed with standard laboratory diet and allowed for tap H_2O ad libitum.

In vivo experiments

Forty-two normotensive white New Zealand rabbits (2–2.5 kg) of either sex were used for this assay and six rabbits were used for each group.

In vivo assessment: hypotensive activity on normotensive anesthetized rabbits

Normotensive rabbits were anesthetized with pentobarbital sodium (30 mg/kg, i.v.). They were placed on their backs on the operating table while legs were fixed and heads pinned down. Trachea were exposed and cut and plastic cannula were inserted and firmly tied in place. The common carotid artery lying in the neck between the lateral bundle of muscle (*longus capitis*) and the trachea was exposed by separating the two muscle bundles of the sternomastoid and the sternothyroid. The carotid artery was separated from the nerves and veins and carefully freed of connective tissue. A venous cannula filled with heparin to prevent thrombus formations was carefully inserted and a thread was tied on it. The cannula was connected to a pressure transducer via universal oscillograph (Harvard apparatus, ser. No. K10542) for blood pressure records. On the opposite side, a venous cannula was inserted into the external jugular vein and tied with thread for injection of 29 tested compounds followed by heparinized saline (0.90% w/v NaCl). The mean arterial blood pressure was calculated using the formula: MAP = DBP + 1/3 (SBP − DBP), *where* MDP = mean arterial blood pressure, DBP = diastolic blood pressure, and SBP = systolic blood pressure.

In vitro experiments

Seven white New Zealand rabbits (2–2.5 kg) of either sex were starved and allowed free access to H_2O for 24 h prior to experiments then slaughtered at the day of experiment to isolate the heart and aorta.

Calcium channel blockade activity

Calcium channel blockade activity procedure was carried out in accordance to the previously reported technique (Kaur and Kaur 2013; Girgis et al. 2006, 2008). Compounds **5a, 5b, 9b**, and **9c** which elicited high hypotensive activity were used for assessment of the calcium channel blockade activity. In order to check for antagonistic effects, contractions were induced with potassium chloride (67 mmol/L). After thorough washing out, this process was repeated until the amplitude of the contraction became constant. The substances to be tested were investigated using the single-dose technique. KCl contractions were induced after addition of the substances at 10^{-4} M concentration and 10 min exposure time. Between administrations of the individual substances, the preparation was washed until the initial situation had been re-established and the potassium chloride contractions were induced. The contractions were enrolled by FTO3 force–displacement transducer, and recorded on a polygraph (Kaur and Kaur 2013; Girgis et al. 2006, 2008). When response reached its plateau, response of aorta rings to solutions of **5a, 5b, 9b**, and **9c** was done and each ring was serially washed after obtaining the maximum response to reach the baseline and equilibrated. At this point, another compound solution was added.

Effect of test compounds on the rabbits heart rates

Hearts of rabbits were prepared according to the previously reported method (Spáčilová 2007; Burn 1952). Aorta was dissected off, freed from all other connecting vessels, carefully tied and cut. Threads were attached to the ventricle by means of a small clip and connected to a special transducer via universal oscillograph (Harvard apparatus, ser. No. K10542) for heart activity records. Test compounds **5a**, **5b, 9b**, and **9c** were added to the cannulae containing the perfusion fluid and entered aorta to reach the coronaries. Aorta was tied into the glass cannula of the perfusion apparatus. Oxygenated Locke solution at 37 °C was allowed in from a reservoir maintained at a constant pressure. That keeps the aortic valve closed while the fluid can pass through the coronary vessels and exit from the inferior vena cava to be collected in a container (Spáčilová 2007; Burn 1952).

In vitro preliminary screening of eNOS activation in aorta and histopathological changes

Materials and method

Chemicals

Endothelial nitric oxide synthase antibody (eNOS) was purchased from Thermo Scientific Co.

Animals

Eight adult male rats weighing about 220–250 g were used in the present work. They were purchased from Assiut University Animal Breeding Unit. Animals were kept in a controlled light room with photoperiod of 12 h dark and 12 h light (dark–light cycle 12:12) with lights ON from 6:00 to 18:00 h at a temperature of 28 + 2 °C. All animals were given free access to standard laboratory chow and tap water.

Rats were randomly divided into 4 groups, 2 rats each. Group (I) served as control group and used for hematoxylin and eosin staining and eNOS immunostaining while Group (II) and (III) received compounds **5a** and **5b** respectively by intra-peritoneal injection in a dose of 30 mg/kg body weight for eNOS immunostaining. Finally, Group (IV) served as positive control and received L-NAME an inhibitor for eNOS synthesis in a dose of 30 mg/kg body weight by intra-peritoneal injection for eNOS immunostaining.

Tissue preparation

Animals were anesthetized with ether, fixed by intracardiac perfusion with 10% formaldehyde. Aorta specimens were rapidly dissected immediately, and immersed into 10% formaldehyde solution to continue fixation 2 more days (pH 7.2). Specimens were processed to prepare paraffin blocks through dehydration in ascending series of EtOH, cleared in methyl benzoate and embedded in paraffin wax. Paraffin sections (5 µm) were cut using a microtome mounted on glass slides, for histological examination with hematoxylin–eosin stain and immunohistochemical staining.

Histological and histopathological examinations

Immunohistochemistry

For eNOS immunohistochemistry: After fixation in 10% neutral formalin for 2 days, dehydration, clearing, and embedding in paraffin soon followed. Paraffin sections were cut at 5 µm and stained with avidin–biotin peroxidase technique. Sections underwent deparaffinization and rehydration by descending grades of EtOH (100, 90, and 70%). They were treated with 0.01 M citrate buffer (pH 6.0) for 10 min to unmask antigen and incubated in 0.3% H_2O_2 for 30 min to abolish endogenous peroxidase activity before blocking with 5% horse serum for 1–2 h. Slides were incubated with the primary antibody (1:100 monoclonal mouse anti-eNOS) at 4 °C for 18–20 h, then washed and incubated with biotinylated secondary antibodies and then with avidin–biotin complex. Finally, sections were developed with diaminobenzidine (DAB) chromogen. Slides were counter stained with Mayer's hematoxylin, dehydrated, cleared, and mounted. eNOS positive cells appeared with brown cytoplasm while nuclei appeared blue.

Results and discussion

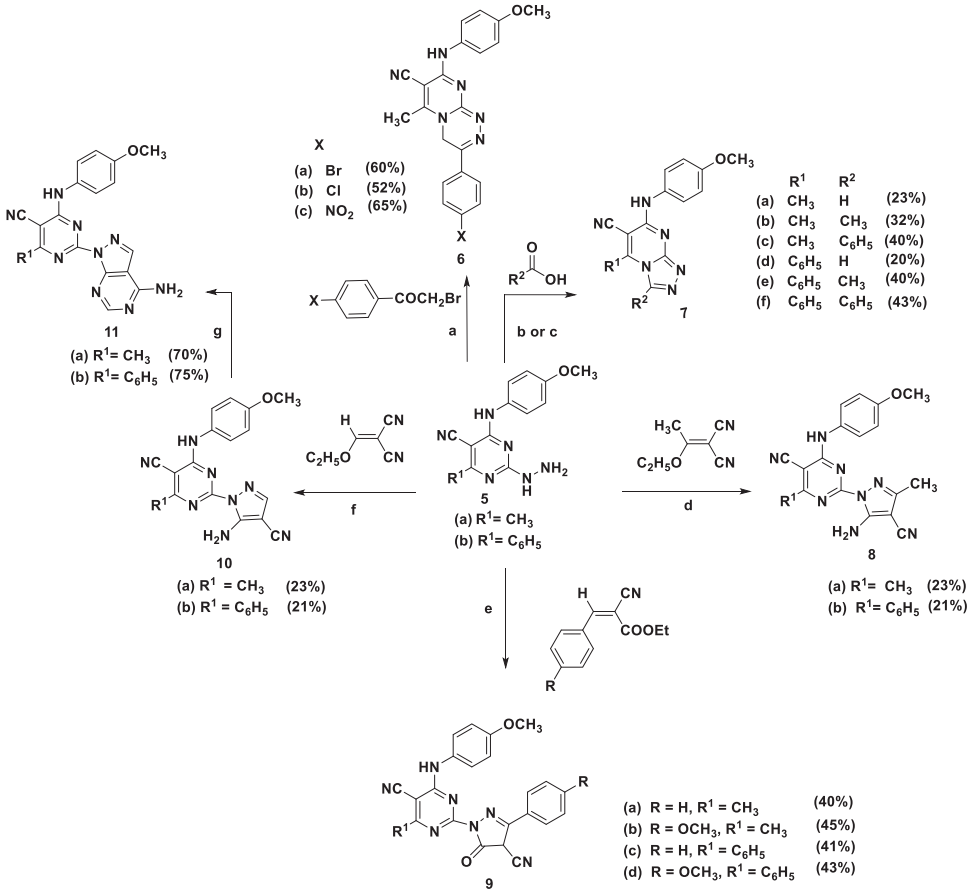
Chemistry

The designed pyrimidine derivatives were obtained through the synthesis of hydrazinyl pyrimidine derivatives **5a**,**b** as described (Scheme 1). 2-Methylthio derivatives **2a,b** were prepared by heating a mixture of compounds **1a** or **1b** (Jones 1952), *S*-methylthiourea sulfate and anhydrous AcONa in DMF. Heating compounds **2a,b** with 2.5 molar equivalents of $POCl_3$ at RT in presence of *N*,*N*-dimethylaniline furnished compounds **3a,b**. This modified method was better than the reported one (Ding et al. 1987) avoiding the use of excess of $POCl_3$. Compounds **4a,b** were prepared by heating under reflux the corresponding 4-chloro derivatives **3a,b** with 4-methoxyaniline in EtOH in presence of K_2CO_3 (Atwal et al. 1987). Hydrazine hydrate was allowed to react with the *S*-methyl derivatives **4a,b**, where upon the good leaving sulfanyl group was successfully displaced (Spáčilová et al. 2007) furnishing the 2-hydrazinyl derivatives **5a**,**b** (Scheme 1).

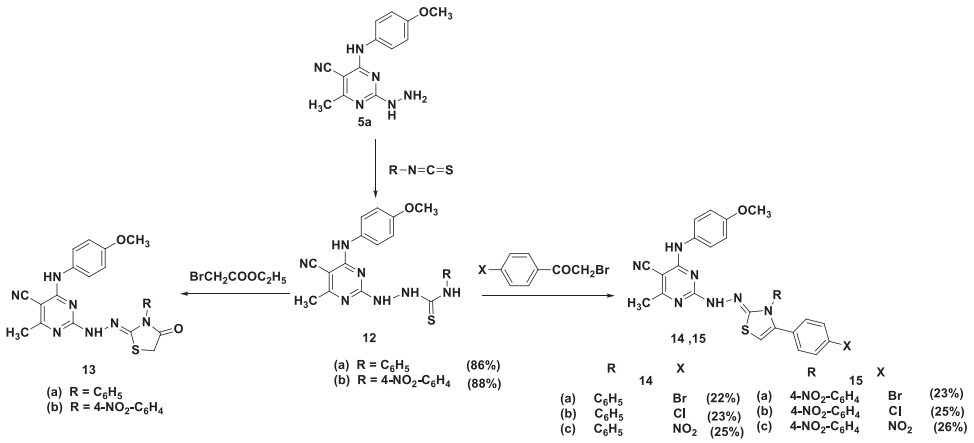
It was of interest to extend the scope of such valuable transformation allowing the introduction of considerable functionalities at C2 of Biginelli derived pyrimidine core. In this context, hydrazine derivatives **5a**,**b** reacted with different reagents to install a variety of heterocycles linked with pyrimidine system as illustrated in Schemes 2 and 3. Addition of phenacyl bromides to the key intermediate **5a** in EtOH furnished the desired compounds **6a–c**, Scheme 2. Triazolopyrimidine derivatives **7a–f** were successfully prepared by heating the hydrazines **5a**,**b** with aliphatic carboxylic acids where in situ cyclization occurred. In case of the less reactive benzoic acid, $POCl_3$ was necessary to catalyze the reaction, Scheme 2. Compounds **8a**,**b** and **10a**,**b** were obtained in a fairly good yield upon treatment of the hydrazines **5a,b** with appropriate acceptors as 2-(1-ethoxyethylene)malononitrile (Jones 1952) or 2-(ethoxymethylene)malononitrile (Ding et al. 1987), in boiling EtOH, Scheme 2. Heating under reflux compounds **10a**,**b** with excess formamide gave the corresponding 2-4-amino-1*H*-pyrazolo[3,4-d]pyrimidine derivatives **11a,b**, Scheme 2. A mixture of the hydrazine derivatives **5a**,**b** and ethyl 2-cyano-3-arylacrylates (Hussain et al. 1985) were reacted in boiling EtOH, however, unlike previously evidenced, the second attack of N^1 of hydrazine took place on the carbonyl ester rather on the nitrile C giving rise to the postulated pyrazolonenitrile product **9a–d** instead of the pyrazole ester initially reported in literature (Aggarwal et al. 2011; Bakr and Kamal 2012). Confirmatory evidence for structures **9a–d** was proved by spectral data (IR, ^{1}H NMR and ^{13}C NMR and MS).

Under neutral conditions, the hydrazine derivatives **5a**,**b** were allowed to react with phenyl or *p*-nitrophenyl isothiocyanate affording products **12a**,**b** in good yields (Scheme 3). Condensation of **12a**,**b** with ethyl bromoacetate in boiling EtOH containing anhydrous NaOAc afforded the corresponding thiazolidinone derivatives **13a**,**b**, Scheme 3. It was postulated that reaction proceeded via *S*-alkylation followed by elimination of H_2O and cyclization. Reacting

Scheme 2 Synthetic pathways to pyrimidotriazines **6a–c**, triazolopyrimidines **7a–f**, pyrazolylpyrimidines **8a,b**, **9a–d**, and **10a,b** and pyrazolopyrimidinylpyrimidines **11a,b**



Scheme 3 Synthetic pathways to thiosemicarbazide derivatives **12a,b**, thiazolidinone derivatives **13a,b**, thiazoline derivatives **14a–c** and **15a–c**



12a,b with various phenacyl bromides provided the thiazoline derivatives **14a–c** and **15a–c**. However, 2 positional isomers might be possible (Bonde and Gaikwad 2004). ^{1}H NMR analysis confirmed the presence of a mixture of the 2 isomers as indicated by duplication of many of ^{1}H and ^{13}C NMR signals.

Pharmacological screening: calcium channel blockade activity

Several in vitro and in vivo techniques are available for evaluating calcium channel blocking activity (Vogel 2008).

Table 1 Effect of nifedipine and selected test compounds (mg/kg, i.v.) in normotensive anesthetized rabbits represented by change in MAP (mmHg)

Cpd No.	ABP (mmHg) as mean ± SE	Cpd No.	ABP (mmHg) as mean ± SE
Normal	92.2 ± 0.86	**9c**	51.8 ± 0.37*#
Nifedipine	84.2 ± 0.58*	**9d**	56 ± 0.70*#
5a	51.4 ± 0.74*#	**10c**	91.6 ± 0.81#
5b	51.4 ± 0.6*#	**11b**	71.8 ± 0.37*#
7b	91.6 ± 0.81#	**14c**	91.8 ± 0.66#
7f	91.4 ± 0.8#	**15b**	78.2 ± 0.66*#
8b	66.6 ± 0.55*#	**15c**	92.0 ± 0.54#
9b	55.2 ± 0.86*#		

Each value represents the mean ± SE (standard error) of 6 experiments
Nifedipine (0.01 M) caused 8 mmHg drop in arterial blood pressure
Results were significant at $P < 0.01$ according to Tukey–Kramer test
*Significant difference from the normal rabbits ($P < 0.01$)
#Significant difference from rabbits treated with nifedipine ($P < 0.01$)

In vitro studies are considered rapid useful tools for primary screening and various smooth muscle preparations such as rabbit jejunum (Taqvi et al. 2006), rat ileum (Taqvi et al. 2006), rat colon (Hadizadeh et al. 2008), and guinea pig trachea (Yu et al. 1994) were utilized as testing models. Moreover, whole animal experiments provide reliable assessment of their various in vivo effects. For evaluation of their cardiovascular activity, the main therapeutic value of CCBs, different models were employed, of which monitoring their effect on arterial blood pressure in hypertensive or normotensive dogs (Guarneri et al. 1997) or rabbits (Jeanneau et al. 1992).

In the current pharmacological assessment, three phases were established for in vitro and in vivo evaluation of the newly synthesized compounds: (1) Thirteen selected compounds were investigated for preliminary evaluation of hypotensive activity in normotensive anesthetized rabbits. (2) Active compounds from the previous screening were tested for calcium channel blockade on preparations of rabbit aortae. (3) Further, the same active compounds were evaluated for their effect on the rabbits heart rate.

Effect of tested compounds on mean arterial blood pressure (MABP)

Preliminary results of biological evaluation for synthesized compounds on mean arterial blood pressure (MABP) were examined and presented in Table 1 and Fig. 4. Among the tested compounds, results showed that MABP in rabbits treated with nifedipine decreased (84.2 ± 0.58 mmHg) significantly ($P < 0.01$) in comparison with normal rabbits (92.2 ± 0.86 mmHg). Compounds **5a**, **5b**, **8b**, **9b–d**, **11b**, and **15b** showed a decrease in MABP in the range

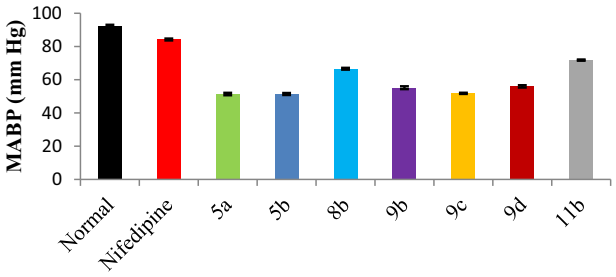


Fig. 4 Effect of nifedipine and compounds **5a**, **5b**, **8b** and **9b–d**, **11b** on MABP

51.4–78.2 mmHg in rabbits in comparison with nifedipine-treated rabbits. Among the compounds with hypotensive effects, we selected compounds **5a**, **5b**, **9b**, and **9c** for further assessment as CCBs.

Calcium channel blockade activity

As previously reported by other investigators (Zorkun et al. 2006), smooth muscles depend on calcium influx for contraction and although their underlying mechanism is somewhat different, inhibition of calcium channel influx into smooth muscles by CCBs leads to relaxation. Therefore, compounds **5a, 5b, 9b**, and **9c** which elicited high hypotensive activity were further evaluated for their calcium channel blockade activity as indicated by their effects on KCl-stimulated rabbit aortae. Nifedipine was included in all tests as the reference drug (Table 2 and Fig. 5). Results obtained from nifedipine, **5a, 5b, 9b**, and **9c**, indicated that the relaxant response of the aorta of the rabbits treated with test compounds increased significantly ($P < 0.01$) in comparison to nifedipine-treated rabbits aortae, as shown in Table 2 and Fig. 5. As shown, the maximum relaxation values of all tested compounds were much more superior when compared with that of nifedipine. In particular, the hydrazine derivatives **5a** and **5b** exhibited the highest activities showing 89.2% relaxation while all other tested compounds **9b** and **9c** showed 77–74.4% relation in comparison with nifedipine (57.6% relaxation).

Effect of nifedipine and compounds 5a, 5b, 9b, and 9c on heart rates

Active candidates that elicited significant lowering of blood pressure in normotensive rabbits in particular compounds **5a**, **5b**, **9b**, and **9c** were further evaluated for their effects on heart rates. Results of this study showed that, there is no significant difference ($P > 0.05$) between heart rates of nifedipine-treated rabbits and normal rabbits, but there were a significant difference ($P < 0.01$) on the heart rates between normal and nifedipine treated rabbits from one side and rabbits treated with test compounds from the other side. The

Table 2 Effect of nifedipine and compounds **5a, 5b, 9b**, and **9c** on the relaxant response of rabbit aortae

Groups	Nifedipine	5a	5b	9b	9c
Relaxation%	57.6 ± 3.20	89.2 ± 2.4[#]	89.2 ± 2.41[#]	77.4 ± 3.12[#]	77.2 ± 3[#]

Each value represents the mean ± SE (standard error) of 6 experiments

[#]Significant difference from rabbits treated with nifedipine ($P < 0.01$)

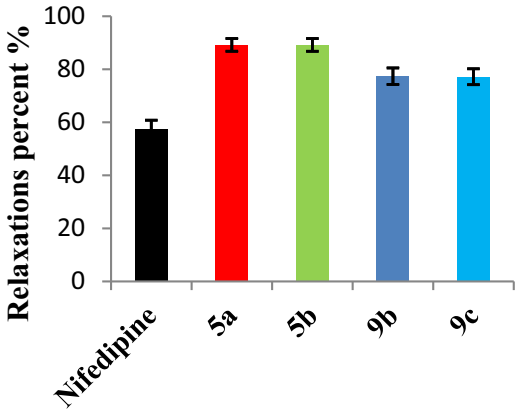


Fig. 5 Effect of nifedipine and compounds **5a, 5b, 9b**, and **9c** on the relaxant response of rabbit aortae

effect of the test compounds on rabbit heart rate indicated that all compounds possessed a significant mean percentage decrease on rabbit's heart rate as shown in Table 3 and Fig. 6.

Correlation between in vivo hypotensive activity in normotensive anesthetized rabbits (Table 1) and in vitro calcium channel blockade activity as indicated by relaxant response of rabbit aortae (Table 2) indicated that compounds **5a**, **5b**, **9b**, **9c** exhibited promising lowering on MABP, calcium channel blocking activity accompanied by decrease in heart rate while compounds **8b**, **8d** elicited good lowering on MABP. In addition, compounds **7b**, **8a**, **11b**, **15b** showed moderate lowering on MABP. Such compounds could be further investigated to constitute a special class of compounds for treatment of hypotension. Meanwhile, **6c**, **7b**, **7f**, **14c**, **15b**, **15c** could be considered as inactive compounds.

Histopathological studies

The endothelium of blood vessels including aorta plays a modulator role in the basal and dynamic regulation of blood vessel diameter by releasing endothelium-derived nitric oxide (NO) (Zhao et al. 2015). In the blood vessel wall, eNOS has a protective function in the cardiovascular system attributable to NO production. Once NO is produced in endothelial cells, it diffuses across the vascular smooth muscle cell membranes and, through a special mechanism, promotes vascular relaxation (Zhao et al. 2013). In addition, endothelium-derived NO may be involved in preventing the progression of age-related vascular diseases (Moncada and Higgs 2006, Pacher et al. 2007, Vanhoutte 2009; Vanhoutte et al. 2009).

Immuno-staining of the aortic sections of the four groups showed the followings changes.

Light microscopic examination of aortic section of the control rat group stained with hematoxylin and eosin showed the three layers of the aortic wall (Fig. 7). Control group showed mild immune-positive staining in the aortic endothelial cells of the intima and very few in adventitia (Fig. 8). In this study, compounds **5a** and **5b** were evaluated upon modulation blood pressure in an animal model of hypertension through its effect upon eNOS expression in the endothelium of aorta. Compounds **5a** and **5b** showed dense staining of the aortic endothelium in comparison to control group (Figs 9 and 10) respectively. The adventitia of **5a** and **5b** treated groups had immune-positive staining in the endothelial lining of blood vessels but seems to be more intense in the phenyl **5b** treated group (Figs 9 and 10). Compound **5b** induced increase in eNOS expression in the aorta as confirmed histologically in this study (Fig. 10). Intense eNOS expression localized to the aortic endothelium was observed with compound **5b** more than compound **5a** (Fig. 9). L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride) treated group as inhibitor of eNOS (positive control) showed no immunostaining of eNOS in the endothelial cells or other parts of the aorta (Fig. 11).

Structure–activity relationship (SAR) studies

As can be seen in the most active compounds, **5a, 5b**, the most favorable structural elements contributing favorably to binding $-NHNH_2$ is capable of acting as H-bond acceptor/donor bound in a complementary region of the receptor containing amino acid residues with opposite features. Compound bearing a C6-methyl group **5a** exhibited a slight increase in inhibition activity. These results implied that electron donating groups at this position seemed to be beneficial to inhibitory activity (more than phenyl group). Thus, it was reasonable to presume that size of substituent at C6 is important to the activity of the target compounds. Besides, the promising activity of some compounds containing attached heterocyclic pyrazoline ring as **9b, 9c** indicates that, while steric effects are important characteristics for activity, electronic and lipophilic effects are

Table 3 Effect of nifedipine and compounds **5a, 5b, 9b**, and **9c** on heart rates

Groups	Normal	Nifedipine	5a	5b	9b	9c
Heart rate/min	76 ± 1.30	74.6 ± 1.07	54 ± 0.70*#	52 ± 0.70*#	61.2 ± 0.58*#	60 ± 0.70*#

Each value represents the mean ± SE (standard error) of 6 experiments

*Significant difference from the normal rabbits ($P < 0.01$)

#Significant difference from rabbits treated with nifedipine ($P < 0.01$)

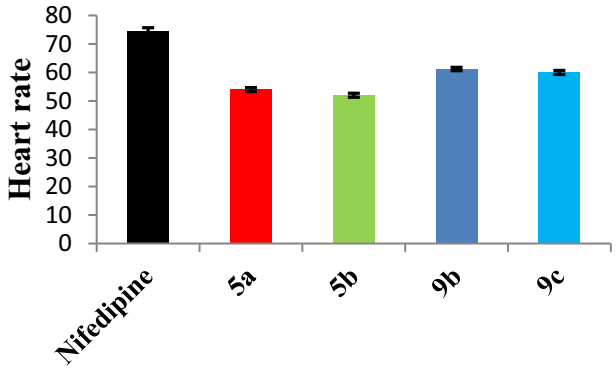


Fig. 6 Effect of nifedipine and compounds **5a, 5b, 9b**, and **9c** on the heart rates

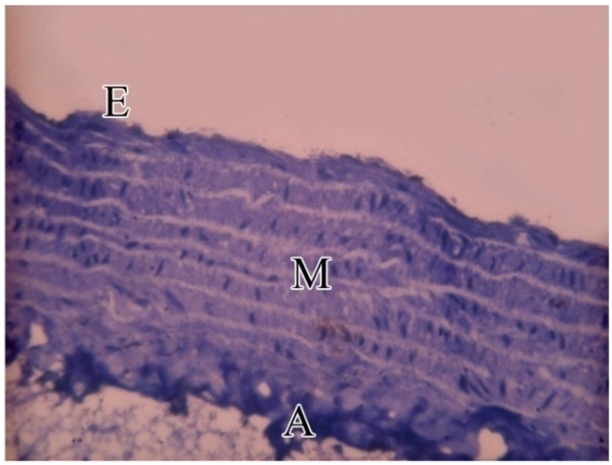


Fig. 7 Photomicrograph of a control adult rat aorta showing intimal endothelium (E) layer, the media (M), and the adventitia (A). H&E (400)

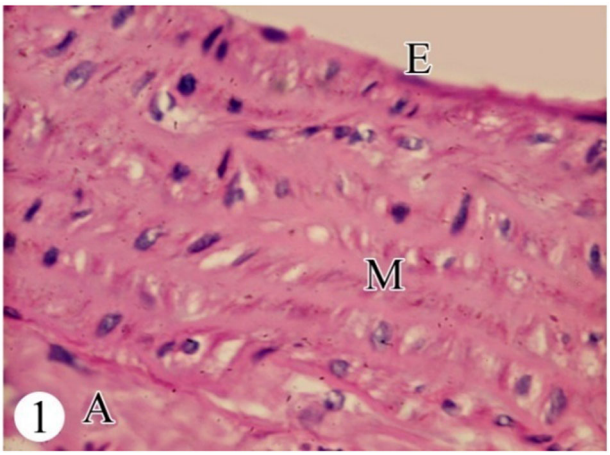


Fig. 8 Photomicrograph of a control adult rat aorta immunostained with anti-eNOS, a marker for e-NOS enzyme, showing immuno-positive staining of the endothelium (eNOS immunostaining ×400). Inset: few immune-positive stained endothelial cells of adventitial blood vessels (↑) (eNOS immunostaining ×1000)

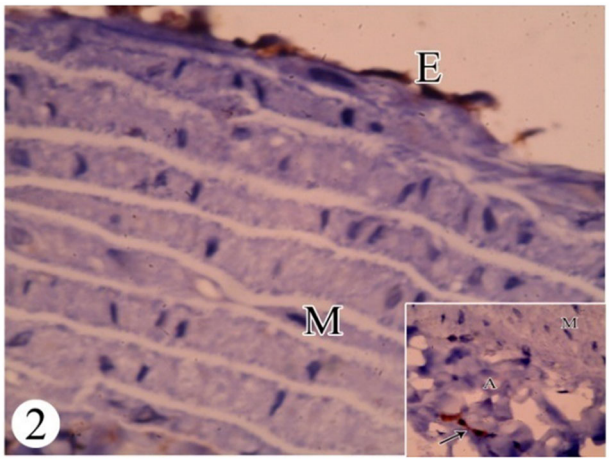


Fig. 9 Photomicrograph of adult rat aorta treated with **5a** and immunostained with anti-eNOS showing immune-positive staining of endothelium (E) more or less similar to control rat section (eNOS immune-staining ×400). Inset: more immune-positive stained endothelial cells of adventitial blood vessels (brown color) (eNOS immunostaining ×1000)

consistent also to contribute well for activity as the heterocyclic moiety might play a favorable role in overall binding and therefore in recognition area on the receptor. Such compounds, bearing a non-chiral planar pyrimidine ring would adopt the same total volume and polar surface area present in similar DHPs and DHPMs known to exhibit antihypertensive activity and indicated that introducing polar substituents at this position (NH) near the vicinity of C4 may lead to even better activity. Since all compounds have a common scaffold in their structures, observed SAR is mainly due to the variation of other functionalities on this scaffold. Variation of the tail on the main scaffold at C2 is responsible for variation in activity potential although all structural features are taking part in the activity. Derivatives including versatile functionalities at position 2 varied

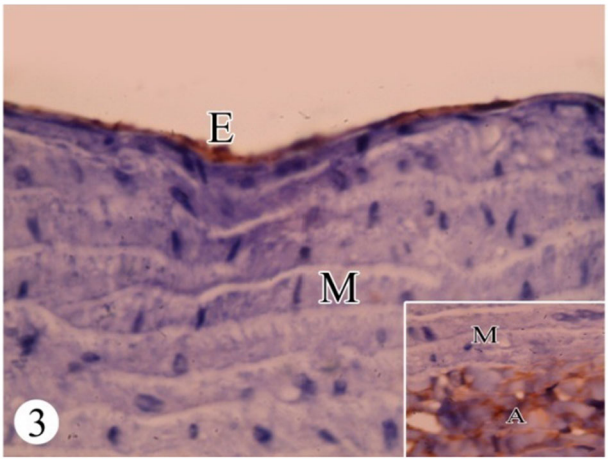


Fig. 10 Photomicrograph of adult rat aorta-treated with **5b** and immunostained with anti-eNOS showing dense immune-positive staining of endothelium (E) than control rat section (eNOS immunostaining ×400). Inset: dense immune-positive stained endothelial cells of adventitial blood vessels (brown color) (eNOS immunostaining ×1000)

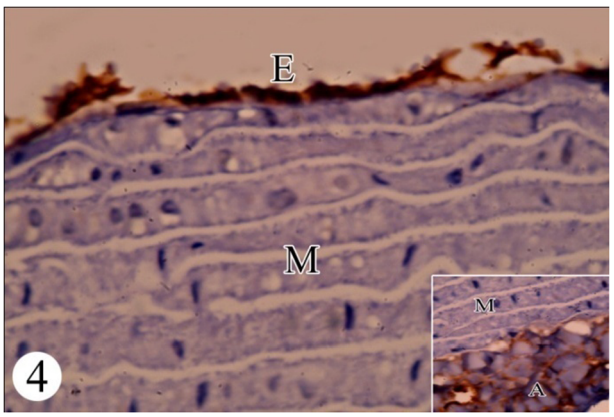


Fig. 11 Photomicrograph of adult rat aorta; a positive control (treated with L-NAME drug) immunostained with anti-eNOS, a marker for e-NOS, showing no immuno-staining (eNOS immunostaining ×400)

between simple flexible hydrazine derivatives, planar or non-planar aromatic heterocycles, and fused ring systems. Variation of substituents on C2-pyrimidine ring can achieve a host of biological actions, through subtle electron density changes in the substituent framework. Other factors including absorption, transport, and site of intracellular action might also be of importance.

This study also demonstrates that compounds **5a** and **5b** are NO-mediated vascular relaxation via aortic eNOS activation. This may lead to decreased BP in a hypertensive animal model. The 2 compounds exhibited excellent bioactivity profiles indicating that they might be multi-functional lead compounds for the purpose of improving activity. Correlating the significant hypotensive and vasodilating activities of compounds **5a,b** bearing a hydrazine moiety at C2, led to the investigation of the mechanism of vasodilatation of other antihypertensive hydrazines. In particular, Hydralazine (Apresoline®), has been in clinical use as an anti-hypertensive agent for nearly 5 decades, but its mechanism of action remained poorly understood. It causes vasodilation as a result of a direct action on vascular smooth muscle cells at an intracellular site to cause vasorelaxation (Ellershaw and Gurney 2001). It was previously shown to be a directly acting vasodilator, evoking a slowly developing, long-lived relaxation in rabbit aorta rings. It causes vasodilation as a result of a direct action on vascular smooth muscle cells at an intracellular site to cause vasorelaxation (Ellershaw and Gurney 2001). It does not act as a calcium antagonist, because it is a poor inhibitor of vasoconstriction caused by elevated extracellular K^+ (Khayyal et al. 1981; Higashio and Kuroda 1988; Gurney and Allam 1995) and its effectiveness at inhibiting phenylephrine-induced contraction is little affected by removing extracellular Ca^{2+} (Higashio and Kuroda 1988; Gurney and Allam 1995; Orallo et al. 1991).

Conclusion

In summary, the present work is an extension of an ongoing effort toward the development and identification of new molecules with antihypertensive activity. In the present investigation, bioisosterism, and hybrid pharmacophore-based drug design led to the identification of multi-functional non-chiral C4-pyrimidine derivatives with anti-hypertensive potency. Compounds **5a, 5b, 9b, 9c** exhibited promising lowering on MABP, calcium channel blocking activity, and decrease in heart rate. It could be concluded clearly that, synthesized pyrimidine derivatives containing C5-CN, C6-methyl (or phenyl) and C4-*p*-methoxyanilino moieties, showed variable activities. The impact of structural variation on activity was examined leading to that changes in C5-, C6-, and C4-positions could be important in receptor binding.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval All procedures performed involving animals were in accordance with the ethical standards. The experimental protocol was approved by Animal Care and Use Committee, Faculty of Pharmacy, Alexandria University (ACUC Project Number 16/1).

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References

Abdel-Aziz SA, Hussein MA, Abdel-Raheem IT (2011) Design, synthesis and antidiabeti activity of some new 4-amino (or 6-oxo)-2- methyl/benzylthio (or substituted amino)pyrimidine derivatives. Bull Pharm Sci, Assiut Univ 34:149–158

Aggarwal R, Kumar V, Kumar R, Singh SP (2011) Approaches towards the synthesis of 5-aminopyrazoles. Beilstein J Org Chem 7:179–107

Alam O, Khan SA, Siddiqui N, Ahsan W, Verma SP, Giliani SJ (2010) Antihypertensive activity of newer 1,4-dihydro-5-pyrimidine carboxamides, synthesis and pharmacological evaluation. Eur J Med Chem 45:5113–5119

Atwal KS, O'Reilly BC, Gougoutas JZ, Malley MF (1987) Synthesis of substituted 1,2,3,4-tetrahydro-6-methyl-2-thioxo-5-pyrimidinecarboxylic acid esters. Heterocycles 26:1189–1192

Bakr FA, Kamal MD (2012) Synthesis and applications of bipyrazole systems Arch Org Chem 1:491–545

Bikker JA, Weaver DF (1993) Theoretical studies applicable to the design of novel anticonvulsants: Part 2. A comparison of AM1, MNDO, and PM3 semi-empirical molecular orbital conformational analyses of dihydropyridine calcium channel blockers. J Mol Struct: THEOCHEM 281:173–184

Bonde CG, Gaikwad NJ (2004) Synthesis and preliminary evaluation of some pyrazine containing thiazolines and thiazolidinones as antimicrobial agents. Bioorg Med Chem 12:2151–2161

Bossert F, Vater W (1989) 1,4-Dihydropyridines—a basis for developing new drugs. Med Res Rev 9:291–324

Burn JH (1952) Pharmacology, vol 25. Blackwell Scientific Publications Ltd. Oxford, p 30–31

Catterall WA, Striessnig J, Snutch TP, Perez-Reyes E (2003) International Union of Pharmacology. XL. Compendium of voltage-gated ion channels: calcium channels. Pharmacol Rev 55:579–581

De Fatima A, Braga TC, Neto LDS, Terra BS, Oliveira BGF, Da Silva DL, Modolo LV (2015) A mini-review on Biginelli adducts with notable pharmacological properties. J Adv Res 6:363–373

De la Sierra A, Ruilope LM (2007) Are calcium channel blockers first-line drugs for the treatment of hypertension and cardiovascular disease? Curr Hypertens Rev 3:9–13

Ding R, He Y, Jingli X, Liu H, Wang X, Feng M, Qi C, Zhang J, Peng C, Atwal S, Oreilly BC, Gougoutas JZ, Malley MF (1987) Synthesis of substituted 1,2,3,4-tetrahydro-6-methyl-2-thioxo-5-pyrimidinecarboxylic acid esters. Heterocycles 26:1189–1192

Ellershaw DC, Gurney AM (2001) Mechanisms of hydralazine induced vasodilation in rabbit aorta and pulmonary artery. Br J Pharmacol 134:621–631

Enseleit F, Luscher TF, Ruschitzka F (2010) Therapeutic advances in cardiovascular diseases. Ther Adv Cardiovac Dis 4:231–240

European Society of Hypertension–European Society of Cardiology (2013) Guidelines for the management of arterial hypertension. Eur Heart J 34:2159–2219

Gaudio AC, Korolkovas A, Takahata Y (1994) Conformational analysis of the 1,4-dihydropyridines linking the structural aspects to the biological binding event a study of the receptor-site conformation. J Mol Struct: THEOCHEM 303:255–263

Girgis AS, Kalmouch A, Ellithey M (2006) Synthesis of novel vasodilatory active nicotinate esters with amino acid function. Bioorg Med Chem 14:8488–8490

Girgis AS, Mishriky N, Farag AM, El-Eraky WI, Farag H (2008) Synthesis of new 3-pyridinecarboxylates of potential vasodilation properties. Eur J Med Chem 43:1818–1825

Grover GJ, Dzwonczyk S, McMullen DM, Normadinan CS, Sleph PG, Morel SJ (1995) Pharmacologic profile of the dihydropyrimidine calcium channel blockers SQ 32,547 and SQ 32,946. J Cardiovasc Pharmacol 26:289–294

Guarneri L, Sironi G, Angelico P, Ibba M, Greto L, Colombo D, Leonardi A, Testa R (1997) In vitro and in vivo vascular selectivity of lercanidipine and its enantiomers. J Cardiovasc Pharmacol 29:S25–S32

Gurney AM, Allam M (1995) Inhibition of calcium release from the sarcoplasmic reticulum of rabbit aorta by hydralazine. J Pharmacol 114:238–244

Hadizadeh F, Fatehi M, Fatehi-Hassanabad Z, Zandieh M (2008) Effects of 4-(2-Alkylthio-1-benzyl-5-imidazolyl)dihydropyridines on the isolated rat colon and right atrium contractility. Iran J Basic Med Sci 11:159–165

Higashio T, Kuroda K (1988) Effects of cadralazine on contractions induced by Ca^{2+} and norepinephrine in isolated rabbit aortic strips. Arzneim-Forsch 38:346–349

Horovitz ZP (1990) European Patent Application EP400665A2

Hussain SM, El-Barbary AA, Mansour SA (1985) A one-step synthesis of 2-methylthio-6-oxopyrimidine derivatives: preparation of fused pyrimidinones. J Heterocycl Chem 22:169–171

Jain KS, Bariwal JB, Kathiravan MK, Phoujdar MS, Sahne RS, Chauhan BS, Shah AK, Yadav MR (2008) Recent advances in selective α_1-adrenoreceptor antagonists as antihypertensive agents. Bioorg Med Chem 16:4759–4800

Jeanneau NE, Benoit-Guyod M, Namil A, Leclercq G (1992) New thiazolo[3,2-a] pyrimidine derivatives, synthesis and structure–activity relationships. Eur J Med Chem 27:115–120

Jones RG (1952) Reactions of orthoesters with active methylene compounds. J Am Chem Soc 74:4889–4891

Kappe CO, Fabian WM, Semones MA (1997) Conformational analysis of 4-aryl-dihydropyrimidine calcium channel modulators: a comparison of ab initio, semi-empirical and X-ray crystallographic studies. Tetrahedron 53:2803–2816

Kappe CO (1998) 4-Aryldihydropyrimidines via the Biginelli condensation: aza-analogs of nifedipine-type calcium channel modulators. Molecules 3:1–9

Kaur R, Kaur B (2013) Design, synthesis and study of fused 1,4-dihydropyrimidines of biological interest—a review. J Appl Chem 2:1102–1115

Khayyal M, Gross F, Kreye VA (1981) Studies on the direct vasodilator effect of hydralazine in the isolated rabbit renal artery. J Pharmacol Exp Ther 216:390–394

Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJ (2006) Systematic analysis of population health data. Lancet 367:1747–1757

Moncada S, Higgs EA (2006) Nitric oxide and the vascular endothelium: the vascular endothelium I. In: Handbook of Experimental Pharmacology. Springer Berlin Heidelberg, p 213–254

Negwer M (1994) Organic-Chemical Drugs and their Synonyms. Akademie Verlag, Berlin, p 2558

Orallo F, Gilongo J, Bardon B, Calleja JM (1991) Comparison of the effects of hydralazine and nifedipine on contractions and Ca influx in rat aorta. J Pharm Pharmacol 43:356–359

Ozawa Y, Hayashi K, Kobori H (2006) New generation of calcium channel blockers in hypertensive treatment. Curr Hypertens Rev 2:103–111

Pacher P, Beckman JS, Liaudet L (2007) Nitric oxide and peroxynitrite in health and disease. Physiol Rev 87:315–424

Palmer RB, Andersen NH (1996) Conformational dynamics of 4-aryl-1,4-dihydropyridine calcium channel antagonists. 1. Quantitation of C4–C1′ bond rotational barriers. Tetrahedron 52:9665–9675

Rovnyak GC, Kimball SD, Beyer B, Cucinotta G, DiMarco JD, Gougoutas J, Hedberg A, Malley M, McCarthy JP, Zhang R, Morel SJ (1995) Calcium entry blockers and activators: conformational and structural determinants of dihydropyrimidine calcium channel modulators. J Med Chem 38:119–129

Spáčilová L, Džubák P, Hajdúch M, Křupková S, Hradil P, Hlaváč J (2007) Synthesis and cytotoxic activity of various 5-[alkoxy-(4-

nitrophenyl)methyl]uracils in their racemic form. Bioorg Med Chem Lett 17:6647–6650

Taqvi SI, Aftab MT, Ghayur MN, Gilani AH, Saify ZS (2006) Synthesis and pharmacological screening of 1-(2',4'-dimethoxyphenacyl)-4-hydroxy-4-phenyl-piperidinium bromide. Int J Pharmacol 2:146–155

Teleb M, Zhang F-X, Farghaly AM, AboulWafa OM, Fronczek FR, Zamponi GW, Fahmy H (2017a) Synthesis of new N^3-substituted dihydropyrimidine derivatives as L-/T-type calcium channel blockers. Eur J Med Chem 134:52–61

Teleb M, Zhang F-X, Huang J, Gadotti VM, Farghaly AM, Aboul-Wafa OM, Fronczek FR, Zamponi GW, Fahmy H (2017b) Synthesis and biological evaluation of novel N^3-substituted dihydropyrimidine derivatives as T-type calcium channel blockers and their efficacy as analgesics in mouse models of inflammatory pain. Bioorg Med Chem 25:1926–1938

Vanhoutte PM (2009) How we learned to say NO. Arterioscler Thromb Vasc Biol 29:1156–1160

Vanhoutte PM, Shimokawa H, Tang EH, Feletou M (2009) Endothelial dysfunction and vascular disease. Acta Physiol 196: 193–222

Vasudevan A, Penning TD, Chen H, Liang B, Wang S, Zhao ZQ, Chai D, Yang L, Gao YX (2012) Abbott Laboratories Trading (Shanghai) Company, Ltd. WO 2012/97683

Vasudevan A, Penning TD, Chen H, Liang B, Wang S, Zhao ZQ, Chai D, Yang L, Gao YX, Pliushchev M (2014) Abbvie Inc. US 2014/171429

Vater W, Kroneberg G, Foffmeiser F, Kaller H, Meng K, Oberdorf A, Puls W, Schiossmann K, Stoepel K (1972) Arzneim-Forsch 22:1–14

Vogel HG (2008) Drug Discovery and Evaluation: Pharmacological Assays. Springer-Verlag, Berlin, Heidelberg, New York, NY, p 280–289

World Stroke Organization (2011) Heart disease and stroke statistics—2015 update: A report from the American Heart Association. Circulation 27:131 (2015 e29-322, Global Atlas on Cardiovascular Disease Prevention and Control; Policies, Strategies and Interventions, World Health Organization: World Heart Federation)

Yang LL, Li GB, Yan HX, Sun QZ, Rong SZ, Feng WS, Zou J, Yang SY (2012) Discovery of N^6-phenyl-1*H*-pyrazolo[3,4-d]pyrimidine-3,6-diamine derivatives as novel CK1 inhibitors using common-feature pharmacophore model based virtual screening and hit-to-lead optimization. Eur J Med Chem 56:30–38

Yousef WM, Omar AH, Morsy MD, El-Wahed MMA, Ghanayem NM (2005) The mechanism of action of calcium channel blockers in the treatment of diabetic nephropathy. Int J Diabetes Metab 13:76–82

Yu S-M, Chen CC, Huang Y-L, Tsai CW, Lin C-H, Teng C-M (1994) Cinnamophilin, a novel thromboxane A_2 receptor antagonist, isolated from *Cinnamomum philippinense*. Eur J Pharmacol 256:85–91

Zhao Y, Vanhoutte PM, Leung SW (2013) Endothelial nitric oxide synthase-independent release of nitric oxide in the aorta of the spontaneously hypertensive rat. J Pharmacol Exp Ther 344:15–22

Zhao Y, Vanhoutte PM, Susan WS (2015) Vascular nitric oxide, beyond eNOS. J Pharmacol Sci 129:83–94

Zorkun İS, Saraç S, Çelebi S, Erol K (2006) Synthesis of 4-aryl-3,4-dihydropyrimidin-2(1H)-thione derivatives as potential calcium channel blockers. Bioorg Med Chem 14:8582–8589