Discovery of Michael acceptor containing 1,4-dihydropyridines as first covalent inhibitors of L-/T-type calcium channels

Hande Aygün Cevhera, David Schallerb, Maria A. Gandinic, Ozan Kapland, Eder Gambetac, Fang Xiong Zhangc, Mustafa Çelegie, Muhammad Nawaz Tahir, Gerald W. Zamponic, Gerhard Wolberb, Miyase Gözde Gündüzd,⁎

a Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Hacettepe University, 06100 Sıhhiye, Ankara, Turkey
b Department of Pharmaceutical and Medicinal Chemistry, Institute of Pharmacy, Freie Universität Berlin, Königin-Luise-Str. 2 + 4, 14195 Berlin, Germany
c Department of Physiology & Pharmacology, Hotchkiss Brain Institute and Alberta Children’s Hospital Research Institute, University of Calgary, 3330 Hospital Drive NW, Calgary T2N 4N1, Canada
d Department of Analytical Chemistry, Faculty of Pharmacy, Hacettepe University, 06100 Sıhhiye, Ankara, Turkey
e Department of Physics, University of Sargodha, Sargodha, Pakistan

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ABSTRACT

1,4-Dihydropyridines (DHPs) are an important class of blockers targeting different calcium channel subtypes and have great therapeutic value against cardiovascular and neurophysiologic conditions. Here, we present the design of DHP-based hexahydroquinoline derivatives as either selective or covalent inhibitors of calcium channels. These compounds were synthesized via a modified Hantzsch reaction under microwave irradiation and characterized by IR, 1H NMR, 13C NMR and mass spectra. Additionally, the proposed structure of HM12 was resolved by single crystal X-ray analysis. The abilities of the target compounds to block both L- and T-type calcium channels were evaluated by utilizing the whole-cell patch clamp technique. Our results identified covalent inhibitors of calcium channels for the first time, which could be achieved by introducing a Michael acceptor group into the ester side chain of the compounds. The proposed covalent binding between the compounds and the cysteine amino acid (Cys1492) within the DHP binding pocket of L-type calcium channel was supported by docking and pharmacophore analysis as well as a glutathione reactivity assay.

1. Introduction

Voltage-gated calcium channels are crucial regulators of calcium influx into excitable cells of heart, brain, and smooth and skeletal muscle. Different subtypes of calcium channels have been identified and shown to mediate critical physiological processes varying from neurotransmitter release to muscle contraction and gene transcription [1]. Consequently, they are considered as therapeutic targets for the treatment of various pathologies ranging from cardiovascular to neurologic and psychiatric diseases [2]. Many current therapeutic agents, particularly 1,4-dihydropyridines (DHPs), target the L-type calcium channel Ca1.2 isomorph and are widely prescribed for the treatment of hypertension [3]. Subsequently, T-type calcium channels have emerged as suitable pharmacological targets for the therapeutic intervention into neurophysiologic disorders such as pain and epilepsy [4] and can also be targeted by DHPs [5–8].

As a consequence of these distinct physiological roles, the identification of new agents targeting calcium channels is of utmost importance. DHPs have been the most studied scaffold by medicinal chemists so far for this purpose. Ongoing efforts to modify this privileged structure led to a new generation of compounds with a condensed ring system (hexahydroquinoline) possessing selective or equipotent activity on different calcium channel subtypes [6,7,9,10]. Within these DHP-based hexahydroquinoline (HHQ) derivatives, modification of the ester moiety determined the blocking affinity for both L- and T-type channels [6]. Exemplarily, 3-pyridylmethyl moiety as the alkyl group in the ester function provided the development of DHPs, represented by N10, with high selectivity for T-type calcium channels over L-type [7]. These findings suggested that focusing on the modifications of the ester side chain is a rational approach to identify more efficient calcium channel blockers.

Bioisosteric replacement is still one of the most commonly used drug design strategies and is considered not only to improve the potency but also to maintain the original biological activity [11]. As bioisosters...
possess near-equal molecular volumes and shapes in their biological environment, swapping benzene with pyridine often resulted in analogues with sustained biological activity within different series of drug candidates [12].

Despite generally being avoided during the drug design process, drugs that covalently modify their target have been approved as treatments for diverse clinical indications ranging from obesity to cancer [13]. Compared to noncovalent binding drugs, covalent inhibitors possess prominent advantages such as increased potencies, prolonged duration of action, less-frequent drug dosing, and the potential to avoid some drug resistance mechanisms [14]. One of the most common structural electrophilic moieties that enable a drug to form a covalent bond to its target is the α,β-unsaturated carbonyl system, known as Michael acceptor. The addition of nucleophiles to the double bond of the Michael acceptor is called Michael addition and occurs through the critical positions of the phenyl ring for the DHP derivatives. The compounds were each achieved according to a modified Hantzsch reaction via condensation of 4,4-dimethyl-1,3-cyclohexanedione, substituted salicylaldehyde, benzyl/2-(methacryloyloxy)ethyl acetoacetate and excess ammonium acetate in absolute ethanol under microwave irradiation. Microwave activation stands among alternative synthetic strategies to improve the classical Hantzsch preparation method. It is obvious that microwave irradiation shortened the reaction time, minimized the use of organic solvent, and simplified the process compared to conventional heating in our case [16,17]. Although the syntheses of HM1, HM2 and HM10 were reported in our previous studies [18,19], these compounds were included in the present work as their effects on L- and T-type calcium channels were not investigated before.

The general synthetic route for obtaining the target compounds is presented in Fig. 2.

In this reaction, an excess amount of ammonium acetate was used as the source of ammonia. As the excess amount increases the yield and can easily be removed with water, it is a general approach in many organic reactions, including modified Hantzsch synthesis [20,21].

The selection of the type of the substituents and their substitution pattern on the phenyl ring were based on our previous study, which describes the binding mechanism of DHP derivatives to L-type calcium channel [10]. We aimed to choose substituents which can possibly interact with the channel via hydrogen bonds and hydrophobic interactions through the critical positions of the phenyl ring for the DHP binding pocket.

The structures of the title compounds and their chemical characteristics are provided in Table 1.

The molecular structures of the compounds were confirmed on the basis of their spectral data obtained from IR, 1H NMR, 13C NMR, and mass spectra. In IR spectra, characteristic stretching bands due to the presence of O-H, N-H, and C=O bonds were observed. In the 1H NMR spectra, the signals of H-4 and N-H protons of the HHQ ring were observed as singlets at 4.85–5.04 and 9.61–10.81 ppm, respectively. Due to the chiral center at C-4 of HHQ ring, the methylene protons of the benzyl moiety (HM1-8) are no longer equivalent. The methylene groups appeared as AB spin system with J values ranging between 12.8 and 13.6 Hz. The same situation counted for the vinyl protons of the methacryloyl moiety (HM9-16). These protons produced different signals as separate singlets. 13C NMR spectra of the compounds generally displayed appropriate numbers of resonance fitting the number of carbon atoms. Exceptionally, a smaller number of resonances was also observed for some compounds because of the overlaid signals in the aromatic field. The inductive and resonance effects of the nitrogen atom led to a significant difference in the chemical shifts between sp2 hybridized carbons of DHPs confirming the formation of the HHQ ring. The signals of C-3 and C-4a appeared at 100 and 110 ppm, while the more deshielded carbons (C-2 and C-8a) were observed at around 145 and 150 ppm, respectively. The mass spectra of the compounds also confirmed the structures of HM1-16 by the peaks belonging to molecular ions mostly created by the addition of a sodium ion. Additionally, isotope peaks were observed in the molecular ion region of the

2. Results and discussion

2.1. Chemistry

This study describes the synthesis of sixteen DHP-based hexahydroquinoline derivatives. The compounds were achieved according to a modified Hantzsch reaction via condensation of 4,4-dimethyl-1,3-cyclohexanedione, substituted salicylaldehyde, benzyl/2-(methacryloyloxy)ethyl acetoacetate and excess ammonium acetate in absolute ethanol under microwave irradiation. Microwave activation stands among alternative synthetic strategies to improve the classical Hantzsch preparation method. It is obvious that microwave irradiation shortened the reaction time, minimized the use of organic solvent, and simplified the process compared to conventional heating in our case [16,17]. Although the syntheses of HM1, HM2 and HM10 were reported in our previous studies [18,19], these compounds were included in the present work as their effects on L- and T-type calcium channels were not investigated before.

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Fig. 1. The design strategy of HM1-16.
compounds containing halogens; depending on the atom type (Cl and/or Br), number of the halogens and which isotope (\(^{35}\)Cl, \(^{37}\)Cl, \(^{79}\)Br, \(^{81}\)Br) the molecular ion contains.

2.2. X-ray structure determination

The three-dimensional structure of HM12, evaluated by X-ray crystallography, is shown in Fig. 3.

In 2-((2-methacryloyloxy)ethyl 4-(3,5-dibromo-2-hydroxyphenyl)-2,6,6-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (HM12), the dimethyl substituted oxocyclohexene ring is not planar or exactly chair or boat form. The A part (C2/C3/C4/O1) of this ring is planar with r. m. s. deviation of 0.0089 Å. The propane parts B (C1/C6/C5) and C (C7/C4/C8) are twisted at dihedral angles 20.36 (15)° and 74.75 (18)° with A, respectively. The 2-methyl-1,4-dihydropyridine ring D (C1/C2/C9/C16/C17/C18/N1) is roughly planar with r. m. s. deviation of 0.1051 Å. The 3,5-dibromo-2-hydroxyphenyl ring E (C10-C15/O2/Br1/Br2) is planar with r. m. s. deviation of 0.0158 Å. The dihedral angle between D/E is 75.61 (5)°. The 2-(Methacryloyloxy)ethyl moiety is the alkyl group of the ester substituent of the DHP ring. In this substitution, the parts of F (O3/C19/O4/C20/C21) and G (C22-C25/O5/O6) are roughly planar with r. m. s. deviation of 0.3441 Å and 0.0621 Å, respectively. The dihedral angle between F/G is 64.03 (21)°. In the molecular structure S (8) loop [22] is completed due to O-H...O bonding. The molecules are interlinked in the form of infinite one-dimensional chain due to N-H...O interaction along the crystallographic [1 0 0] direction. These chains are further interlinked due to C-H...O interactions (Fig. 4, Table 2).

2.3. Determination of Cav1.2 and Cav3.2 calcium channel inhibition

Calcium channel blocking activities of the synthesized compounds were identified by whole-cell patch clamp assays on tsA-201 cells expressing Cav1.2 (plus ancillary subunits) and Cav3.2. Data were represented as percent inhibition of the current after applying each test compound at a 10μM concentration, which does not cause any acute toxicity effects in isolated cells, to measure the resulting resting state block (Fig. 5).

Within the first subseries of compounds (HM1-8), designed by the replacement of the 3-pyridylmethyl group with benzyl moiety, only HM8 was found to be a selective blocker of Cav3.2 over Cav1.2. The other compounds failed to block either L- or T-type current. These data suggested that the nitrogen atom in the pyridine ring is likely to be essential for the interactions of the compounds with T-type calcium channels.

Introduction of a Michael acceptor group to the ester function in the
second subseries of the compounds (HM9-16) enabled us to identify more effective calcium channel blockers. Except for HM9 and HM11, all compounds had potent inhibitory effects on Cav1.2 currents. It is also interesting to note that non-substitution of C-5 position of the phenyl ring (HM9, HM11) was detrimental for the ability of these compounds to block L-type calcium channels. Fig. 5B shows that all test compounds were effective blockers of Cav3.2 T-type calcium channels. Among them; HM10 and HM12-14 produced almost complete inhibition (> 90%) of Cav3.2, whereas HM11, HM15, and HM16 showed a robust current inhibition (65–80%). Additionally, moderate inhibition was also obtained by HM9.

We examined the reversibility of the block for several compounds (i.e., HM9, HM12, HM13 and HM16). For these compounds, there was no appreciable recovery from the block for Cav3.2. Also, no recovery from the block was observed for HM11 and HM12 block of Cav1.2. Finally, given that HM12 produced the most potent inhibition of Cav3.2 and Cav1.2 currents, we tested the effect of this compound on a member of the Cav2 family (Cav2.2) and observed only 25.3 ± 4% inhibition (n = 3) at a concentration of 10 µM. Altogether, as the main difference between these subseries is their ester group; the type of the ester moiety plays an important role in the capacity of these compounds to block calcium current.

2.4. Docking

Michael acceptors like the methacrylate moiety of HM9-18 specifically react with the thiol group of cysteines [23]. The reaction product of Michael addition was determined in a glutathione incubation assay for the most active compounds HM12-16. Thus, the previously

![Fig. 3. ORTEP diagram of HM12 drawn at 50% probability level with H-atoms as small circles of arbitrary radii.](image)

![Fig. 4. C-H…O, N-H…O and O-H…O bonding are shown which form infinite one-dimensional polymeric network. H-atoms not involved in H-interaction are omitted for clarity.](image)

<table>
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<td>0.97</td>
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<td>3.248</td>
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Symmetry codes: (i) x + 1, y, z; (ii) –x + 1, –y + 1, –z; (iii) –x, –y, –z.
The first step of the Michael addition reaction is the nucleophilic attack on the β-position of the polarized double bond by the deprotonated form of the thiol group. The next steps of the reaction to obtain the adduct involve the enolate intermediate and the keto-enol tautomerization [15] (Fig. 7).

Although the reaction course including 1,2-addition is also possible, in most cases the 1,4-addition remains the favorable reaction over direct addition, as the activation energy barrier of the 1,4-addition is generally lower than the corresponding one for the direct addition [28]. In the present study, the 1,4-addition adduct formed by the reaction between the active L-type calcium channel blocker derivatives and GSH was confirmed by mass spectrometry (Fig. 8). For this purpose; GSH and the molecules were kept in HPLC vials to perform the reaction. When the $t=0$ min, the first injections were performed. The molecular ion peak [M + H]$^+$ in the MS spectrum for the GSH was at 308.1 $m/z$ value for the injection of each vial. Molecular ion peaks of the molecules were observed at 598.1, 554.1, 563.2, 565.2, and 530.2, as [M + H]$^+$ for HM12, HM13, HM14, HM15, and HM16 respectively. When $t=120$ min, second injections from the same vials were carried out, and the formation of new peaks for each spectrum was detected. The molecular weights of the newly existing peaks indicated the reaction of GSH with the molecules. The MS spectrum of the mixture of GSH with HM12 is provided as an example (Fig. 9) of this application. The other spectra for GSH with HM13, HM14, HM15, and HM16 were presented as the supplementary data.

3. Conclusion

In this study, we designed and synthesized DHP based-hexahydroquinolines as either selective or covalent inhibitors of L- and T-type calcium channels. These compounds, obtained under microwave irradiation, were tested on Cav1.2 and Cav3.2 using patch clamp assays. Introducing a Michael acceptor group into the ester side chain of the compounds enabled us to identify very effective calcium channel blockers as the covalent inhibitors of calcium channels. Molecular modeling studies and glutathione reactivity assays supported the proposed covalent binding between the active compounds and the cysteine amino acid within the DHP binding pocket of L-type calcium channel. Observing no appreciable recovery from the block for Cav1.2 and Cav3.2 currents for the selected compounds, where recovery from block was examined, stands as a complement for our binding hypothesis. With these covalent inhibitors of calcium channels, not only increased potencies but also prolonged duration of action and less-frequent drug dosing can be obtained. As a result, our data provide a new approach for the design of future calcium channel blockers for the treatment of cardiovascular and neurological diseases.

4. Experimental

4.1. Chemistry

4.1.1. Materials and methods

All chemicals and solvents were purchased from commercial sources and were used without further purification. Reactions to obtain the target compounds were carried out in a Discover microwave apparatus (CEM, USA). Thin layer chromatography (TLC) was performed on silica gel 60 F254 precoated aluminium sheets (Merck, Germany) using mobile phase ethyl acetate-hexane (6:4) to initially check the purity of the compounds. UV absorbing spots were visualized under short-wavelength (254 nm) light (Camag UV Cabinet, Germany). Melting points were determined using Thomas Hoover Capillary Melting Point Apparatus (Philadelphia, PA, USA) without calibration. Infrared spectra (IR) were recorded using Perkin Elmer Spectra BX FT-IR (Beaconsfield, UK) equipped with the MIRacle ATR accessory (Pike Technologies) and were reported in cm$^{-1}$. $^1$H NMR (400 MHz) and $^{13}$C NMR (100 MHz) spectra were obtained on a Varian Mercury 400,
400 MHz High Performance Digital FT-NMR Spectrometer (Palo Alto, CA, USA) in dimethylsulfoxide (DMSO-$d_6$) solutions. Chemical shifts are reported in parts per million (ppm) relative to the internal standard tetramethysilane (TMS) and spin multiplicities are characterized as s (singlet), d (doublet), dd (doublet of doublets) and m (multiplet). Coupling constant ($J$) values are reported in hertz (Hz). The ESI-MS spectra were carried out on a micromass ZQ-4000 single quadrupole mass spectrometer (Waters, Eschborn, Germany). Elemental analyses (C, H, N) were performed on a Leco CHNS-932 Elemental Analyzer (Philadelphia, PA, USA).

### 4.1.2. Synthesis

The general procedure for the synthesis of DHP derivatives was as follows: 1 mmol 4,4-dimethyl-1,3-cyclohexanedione, 1 mmol substituted salicylaldehyde, 1 mmol appropriate alky acetoacetate (benzyl acetoacetate or 2-(methacyryloyloxy)ethyl acetoacetate) and excess amount of ammonium acetate were dissolved in a 35-ml microwave pressure vial in 5 mL absolute ethanol and subjected to microwave irradiation (power 100 W) for 10 min. After completion of the reaction, monitored by TLC, the reaction mixture was cooled, poured into ice-water. The obtained precipitate was filtered and this crude solid was purified by recrystallization from ethanol-water.

#### 4.1.2.1. Benzyl 4-(5-chloro-2-hydroxyphenyl)-2,6,6-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (HM1)

Yellowish solid, yield: 59%. IR ($\nu$, cm$^{-1}$): 3432 (O-H), 3316 (N-H), 1704 (C=O, ester), 1656 (C=O, ketone). $^1$H NMR (δ, DMSO-$d_6$): 0.94 (3H; s; 6-CH$_3$), 1.02 (3H; s; 6-CH$_3$), 1.67–1.73 (2H; m; H-7), 2.36 (3H; s; 2-CH$_3$), 4.38 (1H; br s; H-5), 5.16 (1H; d; J = 2 Hz; H-3), 5.69 (1H; d; J = 2 Hz; H-2), 7.28 (1H; d; J = 2 Hz; H-8), 7.42 (1H; d; J = 2 Hz; H-1), 7.98 (1H; d; J = 2 Hz; H-4), 8.12 (2H; s; H-6).
2.49–2.55 (2H; m; H-8), 4.93 (1H; s; H-4), 5.05 (2H, AB system, J<sub>AB</sub>=13.6Hz, -COOCH<sub>2</sub>C<sub>6</sub>H<sub>5</sub>), 6.72 (1H; d; J = 8.4 Hz; Ar-H<sub>4</sub>), 6.79 (1H; d; J = 2.8 Hz; Ar-H<sub>6</sub>), 7.02 (1H; dd; J = 8.4/2.8 Hz; Ar-H<sub>3</sub>), 7.04–7.25 (5H; m; -CH<sub>2</sub>-C<sub>6</sub>H<sub>5</sub>), 9.49 (1H; s; O-H), 9.82 (1H; s; N-H).

13C NMR (δ, DMSO-d<sub>6</sub>): 18.1 (2-CH<sub>3</sub>), 23.1 (6-CH<sub>3</sub>), 24.1 (6-CH<sub>3</sub>), 25.2 (C-8), 31.3 (C-7), 33.5 (C-4), 39.6 (C-6), 64.6 (-COOCH<sub>2</sub>-), 102.4 (C-3), 107.9 (C-4a), 118.6, 122.9, 126.9, 127.0, 127.4, 127.5, 128.1, 128.2, 128.3, 135.8, 136.6 (phenyl carbons), 146.1(C-2), 152.6 (C-3,5-C6), 152.7 (phenyl carbon), 166.2 (-COOCH<sub>2</sub>-), 202.4 (C-5).


4.1.2.2. Benzyl 4-(3,5-dichloro-2-hydroxyphenyl)-2,6,6-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (HM2). Yellowish solid, yield: 65%. IR (ν, cm<sup>-1</sup>): 3442 (O-H), 3312 (N-H), 1704 (C=O, ester), 1651 (C=O, ketone).<sup>1</sup>H NMR (δ, DMSO-d<sub>6</sub>): 0.98 (3H; s; 6-CH<sub>3</sub>), 1.05 (3H; s; 6-CH<sub>3</sub>), 1.68–1.73 (2H; m; H-7); 2.41 (3H; s; 2-CH<sub>3</sub>), 2.53–2.59 (2H; m; H-8), 4.94 (1H; s; H-4), 4.88, 5.11 (2H, AB system, J<sub>AB</sub>=13.6 Hz, -COOCH<sub>2</sub>C<sub>6</sub>H<sub>5</sub>), 6.74 (1H; d; J = 2.8 Hz; Ar-H<sub>6</sub>), 6.97–7.25 (5H; m; -CH<sub>2</sub>-C<sub>6</sub>H<sub>5</sub>), 7.29 (1H; d; J = 2.8 Hz; Ar-H<sub>4</sub>), 10.64 (1H; s; O-H), 10.72 (1H; s; N-H).<sup>13</sup>C NMR (δ, DMSO-d<sub>6</sub>): 18.1 (2-CH<sub>3</sub>), 23.3 (6-CH<sub>3</sub>), 24.1 (6-CH<sub>3</sub>), 25.2 (C-8), 31.3 (C-7), 33.5 (C-4), 39.6 (C-6), 64.7 (-COOCH<sub>2</sub>-), 102.7 (C-3), 107.7 (C-4a), 122.4, 123.3, 126.6, 126.7, 126.8, 127.1, 127.4, 128.1, 136.4, 137.7 (phenyl carbons), 146.8 (C-2), 148.6 (C-8a), 154.1 (phenyl carbon), 165.7 (-COOCH<sub>2</sub>-), 203.8 (C-5). ESI-MS (m/z): 508/510/512 [M + Na]<sup>+</sup> (100%)/[M + 2 + Na]<sup>+</sup>/[M + 4 + Na]<sup>+</sup>. Anal. Calcd. for C<sub>26</sub>H<sub>26</sub>ClNO<sub>4</sub>; C, 64.20; H, 5.18; N, 2.88. Found: C, 64.15; H, 5.12; N, 2.86.

4.1.2.3. Benzyl 4-(5-bromo-2-hydroxyphenyl)-2,6,6-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (HM3). Yellowish solid, yield: 72%. IR (ν, cm<sup>-1</sup>): 3438 (O-H), 3317 (N-H), 1704 (C=O, ester), 1657 (C=O, ketone).<sup>1</sup>H NMR (δ, DMSO-d<sub>6</sub>): 0.94 (3H; s; 6-CH<sub>3</sub>), 1.02 (3H; s; 6-CH<sub>3</sub>), 1.67–1.72 (2H; m; H-7); 2.35 (3H; s; 2-CH<sub>3</sub>), 2.49–2.55 (2H; m; H-8), 4.93 (1H; s; H-4), 4.94, 5.05 (2H, AB system, J<sub>AB</sub>=13.2 Hz, -COOCH<sub>2</sub>C<sub>6</sub>H<sub>5</sub>), 6.67 (1H; d; J = 8.4 Hz; Ar-H<sub>4</sub>), 6.92 (1H; d; J = 2.4 Hz; Ar-H<sub>6</sub>), 7.04–7.07 (2H; m; -CH<sub>2</sub>-C<sub>6</sub>H<sub>5</sub>), 7.14 (1H; dd;
J = 8.4/2.4 Hz; Ar-H), 7.23–7.26 (3H; m; -CH2-C6H5), 9.47 (1H; s; O-H), 9.84 (1H; s; N-H). 13C NMR (δ, DMSO-d6): 18.1 (2-CH3), 23.1 (6-CH3), 24.1 (6-CH3), 25.2 (C-8), 31.4 (C-7), 33.4 (C-4), 39.5 (C-6), 64.6 (-COOCH2-), 102.3 (C-3), 107.9 (C-4a), 110.6, 119.1, 126.9, 127.4, 128.1, 129.9, 131.1, 136.6 (phenyl carbons), 146.1 (C-2), 152.6 (C-8a), 162.2 (-COOCH2-), 202.3 (C-5). ESI-MS (m/z): 518/520 [M+Na]+/M+2+Na]+ (100%). Anal. Calcd. for C26H26BrNO4; C, 62.91; H, 5.28; N, 2.82. Found: C, 62.95; H, 5.31; N, 2.86.

4.1.2.4. Benzyl 4-(3,5-dibromo-2-hydroxyphenyl)-2,6,6-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (HM4). Pale yellow solid, yield: 45%. IR (ν, cm⁻¹): 3440 (O-H), 3314 (N-H), 1703 (C=O, ester), 1646 (C=O, ketone). 1H NMR (δ, DMSO-d6): 0.95 (3H; s; 6-CH3), 1.02 (3H; s; 6-CH3), 1.67–1.71 (2H; m; H-7); 2.40 (3H; s; 2-CH3), 2.53–2.57 (2H; m; H-8), 4.91 (1H; s; H-4), 4.86, 5.10 (2H, AB system, JAB=13.2Hz, -COOCH2C6H5), 6.90 (1H; d; J=2.4Hz; Ar-H6), 6.94–7.23 (5H; m; -CH2-C6H5), 7.51 (1H; d; J=2.4Hz; Ar-H4), 9.71 (1H; s; H-5), 10.84 (1H; s; O-H). 13C NMR (δ, DMSO-d6): 18.1 (2-CH3), 23.3 (6-CH3), 24.1 (6-CH3), 25.2 (C-8), 31.4 (C-7), 33.1 (C-4), 39.5 (C-6), 64.7 (-COOCH2-), 102.9 (C-3), 107.7 (C-4a), 111.0, 125.2, 126.7, 127.4, 128.1, 130.0, 132.5, 136.4, 137.9 (phenyl carbons), 146.8 (C-2), 149.9 (C-8a), 154.2 (phenyl carbon), 165.7 (-COOCH2-), 203.9 (C-5). ESI-MS (m/z): 596/598/600 [M + Na]+/[M + 2 + Na]+ (100%)/[M + 3 + Na]+. Anal. Calcd. for C26H25BrNO4; C, 58.83; H, 4.75; N, 2.64. Found: C, 58.97; H, 4.88; N, 2.63.

4.1.2.5. Benzyl 4-(3-bromo-5-chloro-2-hydroxyphenyl)-2,6,6-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (HM5). Yellow-orange solid, yield: 52%. IR (ν, cm⁻¹): 3446 (O-H), 3314 (N-H), 1703 (C=O, ester), 1657 (C=O, ketone). 1H NMR (δ, DMSO-d6): 0.99 (3H; s; 6-CH3), 1.06 (3H; s; 6-CH3), 1.69–1.73 (2H; m; H-7); 2.42 (3H; s; 2-CH3), 2.55–2.60 (2H; m; H-8), 3.42 (1H; s; O-H), 4.95 (1H; s; H-4), 4.88, 5.12 (2H, AB system, JAB=13.2Hz, -COOCH2C6H5), 6.77 (1H; d; J=2.4Hz; Ar-H4), 6.96–7.25 (5H; m; -CH2-C6H5), 7.24 (1H; d; J=2.4Hz; Ar-H4), 10.81 (1H; s; N-H). 13C NMR (δ, DMSO-d6): 18.1 (2-CH3), 23.3 (6-CH3), 24.1 (6-CH3), 25.2 (C-8), 31.4 (C-7), 33.1 (C-4), 39.7 (C-6), 64.7 (-COOCH2-), 102.9 (C-3), 107.7 (C-4a), 112.1, 123.7, 126.7, 127.2, 127.4, 128.1, 129.9, 136.4, 137.4 (phenyl carbons), 146.8 (C-2), 149.5 (C-8a), 154.2 (phenyl carbon), 165.7 (-COOCH2-), 203.9 (C-5). ESI-MS (m/z): 552/554/556 [M + Na]+/[M + 2 + Na]+ (100%)/[M + 4 + Na]+. Anal. Calcd. for C26H25BrClNO4; C, 58.83; H, 4.75; N, 2.64. Found: C, 58.97; H, 4.88; N, 2.63.

4.1.2.6. Benzyl 4-(5-bromo-2-hydroxy-3-nitrophenyl)-2,6,6-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (HM6). Yellowish solid, yield: 38%. IR (ν, cm⁻¹): 3439 (O-H), 3318 (N-H), 1703 (C=O, ester), 1651 (C=O, ketone). 1H NMR (δ, DMSO-d6): 0.95 (3H; s; 6-CH3), 1.02 (3H; s; 6-CH3), 1.67–1.72 (2H; m; H-7); 2.38 (3H; s; 2-CH3), 2.53–2.58 (2H; m; H-8), 4.88, 5.09 (2H, AB system, JAB=13.2Hz, -COOCH2C6H5), 4.98 (1H; s; H-4), 7.02–7.05 (2H; m; -CH2-C6H5), 7.17 (1H; d; J=2.0Hz; Ar-H4), 7.22–7.24 (3H; m; -CH2-C6H5), 7.84 (1H; d;
1.4.1.2. Benzyl 4-(3-bromo-2-hydroxy-5-nitrophenoxy)-2,6,6-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (HM7). Yellowish solid, yield: 41%. IR (ν, cm⁻¹): 3435 (O-H), 3322 (N-H), 1704 (C=O, ketone), 1657 (C=O, ketone). 1H NMR (δ, DMSO-d₆): 8.99 (3H, s; 6-CH₃), 7.62 (1H; d; J = 2.4 Hz; Ar-H4), 7.03 (1H; d; J = 2.4 Hz; Ar-H5), 7.07 (1H; d; J = 2.4 Hz; Ar-H6), 7.46 (1H; d; J = 2.4 Hz; Ar-H7), 6.92 (1H; d; J = 2.4 Hz; Ar-H8), 4.98 (1H; s; H-5), 5.62 (1H; s; C=CH₂). 13C NMR (δ, DMSO-d₆): 177.0 (C=CH₂), 119.0 (C=CH₂), 130.5 (C=CH₂), 130.8 (2-CH₃), 136.4 (2-CH₃), 140.1 (2-CH₃), 140.2 (C-13), 147.2 (2-CH₃), 151.2 (2-CH₃), 151.4 (2-CH₃), 169.1 (2-CH₃), 210.5 (2-CH₃). ESI-MS (m/z): 563/565 [M + Na]⁺ / [M + 2 + Na]⁺ (100%). Anal. Calcld. for C₂₆H₂₆BrNO₈; C, 57.80; H, 4.65; N, 5.17. Found: C, 57.80; H, 4.70; N, 5.22.

1.4.1.2.8. Benzyl 4-(2-hydroxy-3,5-dinitrophenyl)-2,6,6-trimethyl-5-oxo-1,4,5,6,7,8-hexahydroquinoline-3-carboxylate (HM8). Yellow-orange solid, yield: 68%. IR (ν, cm⁻¹): 3443 (O-H), 3297 (N-H), 1708 (C=O, ketone). 1H NMR (δ, DMSO-d₆): 8.99 (3H, s; 6-CH₃), 7.62 (1H; d; J = 2.4 Hz; Ar-H4), 7.03 (1H; d; J = 2.4 Hz; Ar-H5), 7.07 (1H; d; J = 2.4 Hz; Ar-H6), 6.92 (1H; d; J = 2.4 Hz; Ar-H7), 4.98 (1H; s; H-5), 5.62 (1H; s; C=CH₂). 13C NMR (δ, DMSO-d₆): 177.0 (C=CH₂), 130.5 (C=CH₂), 130.8 (2-CH₃), 136.4 (2-CH₃), 140.1 (2-CH₃), 140.2 (C-13), 147.2 (2-CH₃), 151.2 (2-CH₃), 151.4 (2-CH₃), 169.1 (2-CH₃), 210.5 (2-CH₃). ESI-MS (m/z): 563/565 [M + Na]⁺ / [M + 2 + Na]⁺ (100%). Anal. Calcld. for C₂₆H₂₆BrNO₈; C, 57.80; H, 4.65; N, 5.17.
Table 3

Crystallographic data HM12.

<table>
<thead>
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<th>Crystal data</th>
<th>HM12</th>
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CCDC 1,912,160
Chemical formula C25H27BrN2O6
M, 597.29
Crystal system, space group Triclinic, Pı
Temperature (K) 296
α, β, γ (Å) 8.4756 (8), 12.6735 (17), 12.8388 (18)
α, 8.0368 (3), β, 84.159 (4), γ (°) 80.440 (4)
V (Å³) 1260.1 (3)
Z 2
Density (calculated) 1.574 Mg/m³
F(000) 604
Radiation type Mo Kα
Wavelength (Å) 0.71073 Å
µ (mm⁻¹) 3.257
Crystal size (mm) 0.36 x 0.24 x 0.22
No. of measured, independent and observed (I > 2σ(I)) reflections 15227, 5786, 3412
Rint 0.048
Theta range for data collection 2.798 to 27.626°
Index ranges −16 ≤ h ≤ 16, −14 ≤ k ≤ 16, −14 ≤ l ≤ 14
Rmax 0.98 (3H; s; -C(CH3)=CH2), 1.06 (3H; s; 6-CH3), 1.67–1.75 (2H; m; H-7); 1.76 (3H; s; -C(CH3)=CH2), 2.38 (3H, s, 2-CH3), 2.53–2.61 (2H; m; H-8), 4.03–4.31 (4H; m; -OCH2CH2O-), 5.03 (1H; s; H-4), 5.55 (1H; s; -C=CH2A), 5.81 (1H; s; -C=CH2B), 7.64 (1H; d; J = 2.8 Hz; Ar-Ha), 9.84 (1H; s; N-H), 11.8 (1H; s; -OCH2-), 129.8 (phenyl carbons), 135.3 (-C(CH3)=CH2), 137.3, 146.6 (phenyl carbons), 102.5 (C-3), 107.2 (C-4a), 111.9, 123.7, 125.8, 127.0 (phenyl carbons), 154.1 (C-5). ESI-MS ([m/z]: 585/587 [M+Na]⁺(100%)/[M+2+Na]⁺.

103.1 

1708 (C=O, ester), 1648 (C=O, ketone). 1H NMR (δ, DMSO- 6): 17.6 (C(CH3)=CH2), 18.2 (2-CH3), 23.2 (6-CH3), 24.1 (6-CH3), 24.9 (C-8), 32.1 (C-7), 33.4 (C-4), 39.5 (C-6), 61.1 (–OCH2CH2O–), 62.5 (–OCH2CH2O–), 102.5 (C-3), 107.2 (C-4a), 111.9, 123.5 (phenyl carbons), 126.5 (C = CH2), 135.3 (–C(CH3)=CH2), 135.8, 140.2 (phenyl carbons), 137.4, 140.2 (phenyl carbons), 149.4 (C-2), 153.9 (C-8a), 165.9 (–COOCH2–), 166.0 (–COOC=CH2), 203.8 (C-5). ESI-MS (m/z): 585/587 [M + Na]⁺(100%)/[M + 2 + Na]⁺.

1709 (C=O, ester), 1647 (C=O, ketone). 1H NMR (δ, DMSO- 6): 17.6 (C(CH3)=CH2), 18.2 (2-CH3), 23.2 (6-CH3), 24.1 (6-CH3), 24.9 (C-8), 32.1 (C-7), 33.4 (C-4), 39.5 (C-6), 61.1 (–OCH2CH2O–), 62.5 (–OCH2CH2O–), 102.5 (C-3), 107.2 (C-4a), 111.9, 123.5 (phenyl carbons), 126.5 (C = CH2), 135.3 (–C(CH3)=CH2), 135.8, 140.2 (phenyl carbons), 149.4 (C-2), 153.9 (C-8a), 165.9 (–COOCH2–), 166.0 (–COOC=CH2), 203.8 (C-5). ESI-MS (m/z): 585/587 [M + Na]⁺(100%)/[M + 2 + Na]⁺.

4. X-ray structure determination

Single crystal data collection of HM12 was performed at 296 K on a Bruker Kappa APEXII CCD diffractometer equipped with a four-circle goniometer and using MoKα graphite mono-chromated radiation. The structure was solved using SHELXS97 [30] suit, and refinement was carried out using SHELXL-2014/7 [31]. PLATON was used for molecular graphics ORTEP-3 for Windows [32] and PLATON [33]. Crystal data and details of the data collection are summarized in Table 3.

4.3. Calcium channel inhibition

Human embryonic kidney tsA-201 cells were cultured and trans-fected using the calcium phosphate method as described previously [34]. Cells were plated on glass coverslips and transfected with either 3 μg of human Cav3.2 cDNA, or 3 μg of each cDNA for the rat Cav1.2 or Cav2.2, Cavβ1b and Cavα2δ1. pEGFP (0.5μg) was included into the transfection mix as a transfection marker.

4.3.2. Electrophysiology

Electrophysiological recordings were performed according to the whole cell configuration of the patch-clamp technique 72h after transfection at room temperature. The external recording solution contained (in mM): 20 BaCl2, 1 MgCl2, 40 TEACl, 65 CsCl, 10 HEPES, 2Na-ATP, and 0.3Mg-GTP, pH 7.3. Drugs were prepared daily in external solution and were applied locally to cells. The effects of the compounds (10μM) were assessed by normalizing the current amplitude in the presence of the compound to that observed in control conditions.

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One of the most active compounds (HM12) against Cav1.2 was built in MOE 2018 [35] and conformations for docking were generated with Corina 3.00 [36]. The proposed covalent bond formation between Michael acceptor and cysteine results in a second chiral center (Fig. 6B, highlighted in red). Thus, conformations for docking were generated for all four possible isomers of the reaction product. The prepared isomers of HM12 were docked into a homology model of rCav1.2 that was reported previously [10]. Docking was performed with GOLD 5.6.3 [24] with default setting using the following modifications: The genetic algorithm was set ‘very flexible’ and 30 poses were generated per isomer with enabled ring corner flipping, early termination was disabled and substructure-based covalent docking was specified towards the sulfur atom of CYS1492. Residues defining DHF binding to Cav1.2 in mutational studies [37–42] were selected as active site residues (GLN1069, MET1134, PHE1138, SER1141, TYR1178, TYR1489 and MET1490) as well as CYS1492. LigandScout 4.2 was used to systematically analyze docking results [25].

4.5. Glutathione incubation assay

20 mM stock solutions of molecules entitled HM12 (MW:597 g/mol), HM13 (MW:553 g/mol), HM14 (MW:563 g/mol), HM15 (MW:563 g/mol) and HM16 (MW:529 g/mol) were prepared in 200 µL DMSO (Sigma Aldrich, USA) separately. 20 mM stock solution of glutathione (Sigma Aldrich, USA) (GSH) was prepared in 10 mL Milli-Q water. 50 µL from the stock solutions of the molecules and 450 µL from the GSH solution were transferred into clean HPLC vials. These solutions were prepared for each of the five molecules respectively. Final concentrations of each vial consist of 2 mM of the molecule and 18 mM of GSH.

Mass spectrometry (MS) system consisted of a Shimadzu LC-20AXR (Shimadzu Corp, Kyoto, Japan) liquid chromatography coupled with a Shimadzu 8030 electrospres ionization (ESI) triple quadrupole tandem mass spectrometer (Shimadzu Corp, Kyoto, Japan). MS detection was operated in the positive electrospres ionization at 100–1000 m/z scan mode. Data acquisition and processing were carried out using LC Solution Software. Direct infusion mass spectrometry method used for injections and some important parameters for system were as follows: mobile phase consists of water and acetonitrile (50% water [0.1% v/v formic acid] and 50% acetonitrile [0.1% v/v formic acid]), 0.25 mL/min flow rate, 5 µL of injection volume, 4500 V ESI voltage. Consecutive injections were applied at t = 0 min and t = 120 min from same vials, respectively.

Author contributions

MGG designed the compounds, HAC and MGG carried out the synthesis and structure elucidation of DHPs. DS performed computational studies. MAG, EG and FXZ carried out electrophysiology experiments. MNT accomplished the X-ray structure elucidation. OK and MÇ were responsible for the glutathione reactivity assay. GWZ and GW reviewed and revised the manuscript.

Declaration of Competing Interest

The authors declared no potential conflicts of interest.

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