

STRUCTURE-ACTIVITY RELATIONSHIP OF 1,4-DIHYDROPYRIDINES AS POTENTIATORS OF THE CFTR CHLORIDE CHANNEL

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Abbreviations: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; YFP, yellow fluorescent protein; FRT, Fischer rat thyroid; DHP, 1,4-dihydropyridines; NBD, nucleotide binding domain; VDCC, voltage-dependent Ca²⁺ channels; HTS, high-throughput screening

ABSTRACT

Mutations occurring in the CFTR gene, encoding for the cystic fibrosis transmembrane conductance regulator chloride channel, cause cystic fibrosis (CF). Mutations belonging to class II, like $\Delta F508$, give rise to a protein with both a defective maturation and altered channel gating. Mutations belonging to class III, like G551D and G1349D, cause only a gating defect. We have previously identified anti-hypertensive 1,4-dihydropyridines (DHPs), a class of drugs which block voltage-dependent Ca^{2+} channels, as effective potentiators of CFTR gating, able to correct the defective activity of CFTR mutants (Pedemonte et al., Mol Pharmacol 68: 1736-1746, 2005). However, optimization of potency for CFTR vs Ca^{2+} channels is required to design selective compounds for CFTR pharmacotherapy. In the present study, we have established DHP structure-activity relationship (SAR) for both CFTR potentiation and Ca^{2+} channel inhibition using cell-based assays for both types of channels. A panel of 333 felodipine analogs was studied to understand the effect of various substitutions and modifications in the DHP scaffold. Our results show that alkyl substitutions at the *para*-position of the 4-phenyl ring lead to compounds with very low activity on Ca^{2+} channels and with a strong effect as potentiators on the $\Delta F508$, G551D, and G1349D CFTR mutants.

Cystic fibrosis (CF), one of the most common and severe genetic diseases, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a cAMP-dependent chloride channel expressed in the apical membrane of epithelial cells in the airways, intestine, pancreas, and sweat glands (Pilewski and Frizzell, 1999; Sheppard and Welsh, 1999). CFTR belongs to the ATP-binding cassette transporter superfamily, and is composed by five distinct parts: two membrane-spanning domains, two nucleotide-binding domains (NBDs), and a regulatory region (Hyde et al., 1990). ATP binding and hydrolysis at the NBDs are responsible for CFTR pore gating. However, cAMP-dependent phosphorylation of the regulatory domain is also needed to allow channel activity (Sheppard and Welsh, 1999).

To date, more than 1400 mutations have been described, distributed along the entire aminoacidic sequence. CF mutations have been grouped into five classes, according to the mechanism through which they cause loss of function (Welsh and Smith, 1993). A pharmacological approach aimed to correct the basic defect appears particularly appropriate for mutations belonging to class II and III. Class II mutations cause a defect in CFTR maturation and targeting to the plasma membrane, that provokes the confinement of the mutant protein inside the endoplasmic reticulum and its subsequent degradation through the proteasome-dependent pathway. Conversely, mutations belonging to class III severely impair CFTR function, decreasing channel openings (“gating defect”), without interfering with protein maturation and membrane targeting. The most frequent CF mutation is the deletion of phenylalanine 508 ($\Delta F508$), that is present in at least one allele in about 50 – 90% of CF patients (Bobadilla et al., 2002). The $\Delta F508$ mutant presents both a maturation (class II) and a gating (class III) defect (Dalemans et al., 1991; Denning et al., 1992; Haws et al., 1996; Kopito, 1999), the latter being evident when the $\Delta F508$ protein is allowed to reach the plasma membrane by cell incubation at low temperature or with chemical chaperones. The most common class III mutation is G551D (glycine-to-aspartic acid

change at position 551), with a worldwide frequency of 3.1% of CF alleles (Hamosh et al., 1992). Other class III mutations, like G1349D, are much rarer. However, the total number of missense mutations causing a gating defect may be present in a significant fraction of all CF patients.

Various chemical compounds are known to stimulate the activity of CFTR mutants affected by altered channel gating. For example, high concentrations of flavonoids such as genistein can improve $\Delta F508$ - and G551D-CFTR channel gating (Hwang et al., 1997; Illek et al., 1999; Zegarra-Moran et al., 2002). In addition, high-throughput screening (HTS) has allowed the identification of new classes of CFTR potentiators with improved potency (Yang et al., 2003; Pedemonte et al., 2005a). Some potentiators, like tetrahydrobenzothiophenes and sulfonamides, are active only on the $\Delta F508$ mutant. Conversely, another class of compounds identified by HTS, phenylglycines, are effective also on G551D and G1349D channels. In a recent screening of a set of compounds including drugs and natural compounds, we have identified anti-hypertensive 1,4-dihydropyridines (DHPs) as an effective family of CFTR potentiators (Pedemonte et al., 2005b). Among them, felodipine was the most potent compound, having activity on $\Delta F508$ - and G551D-CFTR. DHPs act as anti-hypertensive drugs by blocking L-type voltage-dependent Ca^{2+} channels (VDCCs) and therefore causing the relaxation of arterial smooth muscle cells (Harrold, 2002).

We have previously shown that activation of mutant CFTR by DHPs occurs through a mechanism not involving the inhibition of Ca^{2+} channels, but possibly by direct interaction with the CFTR protein itself (Pedemonte et al., 2005b). Therefore, DHPs represent an interesting family of compounds for the development of effective drugs for CF therapy. Indeed, they have been extensively studied and a large amount of information is available on their medicinal chemistry properties (Harrold, 2002). However, optimization of potency for CFTR vs Ca^{2+} channels is required to minimize possible side effects. In fact, felodipine activates CFTR at

concentrations that are considerably higher than those effective on Ca^{2+} channels. A better understanding of structural features required for CFTR activation may help in the development of more selective and effective DHP-based potentiators. In the present study, we have established DHP structure-activity relationship (SAR) for both CFTR activation and Ca^{2+} channel blockade. To this purpose, we screened a set of 333 felodipine analogs using cell-based assays to determine their activity on three CFTR mutants (ΔF508 , G551D, and G1349D) and on DHP-sensitive Ca^{2+} channels. Our results show that modifications of the DHP scaffold, particularly in the 4-phenyl ring, may lead to compounds with highly improved selectivity for CFTR.

MATERIAL AND METHODS

Cell Culture. Fischer rat thyroid (FRT) cells, were stably transfected with Δ F508, G551D, or G1349D-CFTR and the halide-sensitive yellow fluorescent protein mutant YFP-H148Q/I152L (Galiotta et al., 2001a). The cells were cultured on plastic in Coon's modified F-12 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. For fluorescence assays of CFTR activity, cells were plated (100,000 cells/well) on clear-bottom black 96-well microplates (Corning Life Sciences, Acton, MA). For Ussing chamber experiments, FRT cells were seeded into Snapwell permeable supports (Corning Life Sciences) at 500,000 cells per insert.

H9C2 cells are a clonal cell line derived from embryonic BD1X rat heart tissue which exhibits many of the properties of skeletal muscle. H9C2 cells present endogenous expression of DHP-sensitive L-VDCC, mainly of cardiac subtype, i.e. with prevalence of α_{1C} subunits (Hescheler et al., 1991). H9C2 cells were grown in DMEM/F-12 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. For fluorescence assays of L-VDCC activity, cells were plated (100,000 cells/well) on clear-bottom black 96-well microplates. After 24 hours, cells received fresh medium containing *all-trans* retinol (10 nM) to increase α_{1C} L-VDCC expression (Menard et al., 1999). The functional assay was performed after additional 24 hours.

Compounds. A set of 333 felodipine analogs was purchased from ChemDiv. The analogs were selected browsing the ChemDiv database with the ChemoSoft software version 2.1. A similarity search was performed using felodipine as a template and setting similarity (as determined by Tanimoto coefficient) to 90%. Compounds were prepared as 10 mM stock solutions in dimethyl sulfoxide. Secondary plates were prepared for screening at 1 mM

concentration in dimethyl sulfoxide using a Biomek 2000 liquid handling workstation (Beckman Coulter, Fullerton, CA). All plates were stored at - 70°C.

Fluorescence Assay for CFTR activity. Measurements of CFTR activity were carried out on FRT cells expressing mutant CFTR and the halide-sensitive YFP, 24 hours (G551D and G1349D) or 48 hours (Δ F508) after plating on microplates. Before the assay, Δ F508-CFTR expressing cells were incubated at 27°C for 20 to 24 h to allow rescue of the mutant protein to the plasma membrane. At the time of assay, cells were washed with PBS (containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1 mM CaCl₂, and 0.5 mM MgCl₂) and stimulated for 20 min with forskolin and test compounds at the desired concentration. Then, cells were transferred to a microplate reader (FluoStar Galaxy; BMG Labtech GmbH, Offenburg, Germany) for CFTR activity determination. The plate reader was equipped with high-quality excitation (HQ500/20X: 500 ± 10 nm) and emission (HQ535/30M: 535 ± 15 nm) filters for YFP (Chroma Technology Corp., Brattleboro, VT). Each assay consisted in a continuous 14-s fluorescence reading with 2 s before and 12 s after injection of an iodide-containing solution (PBS with Cl⁻ replaced by I⁻; final I⁻ concentration 100 mM). Data were normalized to the initial background-subtracted fluorescence. To determine I⁻ influx rate, the final 11 s of the data for each well were fitted with an exponential function to extrapolate initial slope (dF/dt). To obtain the activation constant (K_a) for each compound, dose-response relationships were fitted with the following equation:

$$dF/dt = (dF/dt)_{\max} / (1 + (K_a / [\text{compound}])^n).$$

Fluorescence Assay for VDCC activity. Cells were incubated with a loading solution containing the fluorescent probe Fluo-4 AM (4 μM), glucose (10 mM) and probenecid (2 mM). After 1 hour, cells were washed with PBS and then incubated for 20 min in the presence of test compounds or known VDCC modulators (probenecid and glucose were maintained in the

solution). Then, cells were transferred to the microplate reader (excitation: 485 nM; emission: 520 nM). Each assay consisted in a continuous 8-s fluorescence reading after which a solution containing high-potassium (PBS with Na⁺ replaced by K⁺) was injected into the well. The fluorescence was continuously monitored for additional 27 sec. The activity of VDCC channels was determined as the increase in Fluo-4 fluorescence upon injection, normalized to the initial background-subtracted fluorescence.

Transepithelial Current Measurements. Experiments on FRT cells were performed on days 7-9. Snapwell inserts were mounted in a self-contained Ussing chamber system (Vertical diffusion chamber; Corning Life Sciences). Transepithelial currents were measured using a transepithelial Cl⁻ gradient. Accordingly, the basolateral solution contained 130 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM Na-HEPES (pH 7.3) and 10 mM glucose. For the apical side, this solution was modified by replacing half of NaCl with sodium gluconate and increasing CaCl₂ to 2 mM to compensate for calcium buffering caused by gluconate. The basolateral membrane was permeabilized with 250 µg/ml amphotericin B. During experiments, solutions in both chambers were continuously bubbled with air. The hemichambers were connected to DVC-1000 voltage clamps (World Precision Instruments, Inc., Sarasota, FL) via Ag/AgCl electrodes and 1 M KCl agar bridges. Transepithelial currents were digitized using PowerLab 4/25 data acquisition systems and stored on Macintosh computers. All measurements were done at 37°C. ΔF508 cells were previously incubated at 27°C for 24 hours to allow rescue of the mutant protein from the endoplasmic reticulum

RESULTS

To determine the structural features required by a DHP to activate CFTR and to block VDCCs, we selected a series of felodipine analogs from a commercial library. The set of compounds was designed to explore the effects of: 1) various chemical groups in different positions of the 4-phenyl ring; 2) substitutions of the whole phenyl core with other cyclic groups; 3) substitutions of the pyridine ring with other cyclic scaffolds; 4) various alkyl esters bound to the carboxyl residues of the pyridine ring. To assess the activity of compounds on CFTR, we used the functional assay based on the halide-sensitivity of yellow fluorescent proteins (Galiotta et al., 2001b). To assess the effect on Ca^{2+} channels, we developed a cell-based assay in a high-throughput format. For this purpose, we used H9C2 cells, a cell line derived from rat myocardium, which express endogenously L-type VDCCs (Menard et al., 1999). The assay was done using the fluorescent Ca^{2+} -sensitive probe Fluo-4. After reading the background cell fluorescence, a rapid addition of a high- K^+ solution triggered membrane potential depolarization and therefore Ca^{2+} channel activation. Representative traces showing the increase in Fluo-4 fluorescence due to Ca^{2+} influx are presented in Fig. 1A. In the presence of felodipine or R-(+)-BayK-8644 (the enantiomer of BayK-8644 which acts as a VDCC blocker), the Ca^{2+} increase evoked by K^+ addition was abolished. On the contrary, treatment with S-(-)-BayK-8644 (the enantiomer of BayK-8644 acting as a VDCC potentiator) caused a more pronounced fluorescence increase (Fig. 1A). Chronic treatment for 24h with all-trans-retinol (10 nM), previously described to increase Ca^{2+} channels expression (Menard et al, 1999), determined an improvement of the signal-to-noise ratio, without altering substantially the pharmacology of the channel (Fig. 1B). We decided to take advantage of the increased response to depolarizing stimulus, observed upon incubation with S-(-)-BayK-8644, to design an assay in which test compounds are

examined in the presence of the potentiator. Fig. 1C and D show representative traces recorded with test compounds in the presence of 50 nM or 100 nM S(-)-BayK-8644, respectively. The high affinity drug felodipine applied at 0.25 and 1 μ M fully blocked Ca^{2+} channels at both concentrations of the VDCC potentiator. The less potent compound nitrendipine caused instead a partial reduction of the Ca^{2+} influx. Fig. 1E shows dose-response relationships established for felodipine in the presence of 50 or 100 nM S(-)-BayK-8644. It can be noted that by increasing the concentration of the potentiator we observed a shift in the apparent affinity of felodipine with an increase in K_i from 4 to 21 nM and no change in maximal effect. This behavior is consistent with a competitive interaction between inhibitory DHPs and S(-)-BayK-8644 as previously described (Freedman and Miller, 1984; Spedding, 1985; Wei et al., 1986). The sensitivity of the assay allowed us also to discriminate between the two enantiomers of isradipine, the (+)-form being much more potent as Ca^{2+} channel blocker (Fig. 1F). Such results demonstrated that we could use our assay to evaluate the Ca^{2+} -channel blocking activity of felodipine analogs.

The panel of compounds was tested on Fischer rat thyroid (FRT) cells coexpressing the halide-sensitive mutant H148Q/I152L of the fluorescent protein YFP and the CFTR mutants Δ F508, G551D, and G1349D. Cells expressing Δ F508-CFTR were incubated at 27 °C for 24 hours before the screening to enhance the amount of the mutant protein in the plasma membrane. Each compound was tested at different concentrations in the presence of maximal forskolin (20 μ M) to allow full phosphorylation of the CFTR channel. For each compound, and at each concentration, activity was determined as the increase in the rate of I influx with respect to forskolin alone and corresponding dose-response relationships were generated. In parallel, the same compounds were tested for their ability to block VDCCs, on H9C2 cells chronically treated with retinoic acid and using the fluorescence assay described above (with 50 nM S(-)-BayK-8644). Compounds were tested at 1 μ M, a concentration at which all anti-hypertensive DHPs

cause complete blockade of Ca^{2+} channels. For the most interesting compounds, a dose-response curve was generated by testing concentrations in the 1 nM to 30 μM range.

Selected sets of compounds are shown in Fig. 2 – 7 and Tables 1 – 3 to demonstrate the effect of specific chemical modifications of the basic scaffold. For each set of DHPs, a reference compound having activity on both CFTR and VDCCs is compared to others having substitutions and modifications at a specific position. In general, conclusions arising from the examples shown in Fig. 2 – 7 and Tables 1 – 3 are supported also by data obtained from other DHPs present in the library. Data for all compounds are reported in the Supplementary Material.

The first important observation obtained from the evaluation of felodipine analogs is that both rings composing the DHP structure (the phenyl ring and the pyridine ring) are necessary for CFTR potentiation as well as for VDCC block (Fig. 2). Indeed, compounds like DHP-001 and DHP-285, lacking the 4-phenyl group, are essentially inactive. On the other hand, the presence of a substituted phenyl ring (see DHP-005 in Fig. 2) linked to a structure different from the pyridine ring, does not provide activity. The second major point is that modification of the pyridine ring affects heavily the ability of compounds to activate CFTR (Fig. 3). Substitution of the pyridine ring with a pyrimidine ring (DHP-035) completely abolishes activity on both CFTR and VDCC. Other modifications of this part of the molecule, like substitutions of the pyridine core with a piperidin-2-one scaffold (DHP-079), or conjugation of the pyridine ring with a cyclohexanone ring (DHP-058), results in the inability of the compounds to stimulate CFTR while maintaining part of the activity as VDCC inhibitors. Substitutions of the hydrogen bound to the nitrogen at position 1 of the pyridine ring also impair activity on CFTR (DHP-270 and DHP-271).

The methyls at positions 2 and 6 of the pyridine ring represent critical groups for activity. Replacement of either methyl with another group inactivated the compound as CFTR potentiator

(compare DHP-008 with DHP-279 in Table 1 and Fig. 4). Regarding the positions 3 and 5, our data clearly indicate that compounds with a free acidic carboxyl group are not active on CFTR (see DHP-050, Fig. 4 and Table 1). On the other hand, the esterification of the carboxyl group with different types of alkyls preserves the activity. Among the active compounds, we found methyl, ethyl, isopropyl, benzyl, propenyl, and methoxyethyl esters (Fig. 5A, Table 2).

Interestingly, potency data for CFTR activation demonstrated that there is a ranking order for the size of the alkyl groups, that need to be not too small, but not too bulky either. Accordingly, the ethyls are better than the methyl groups, and the esterification with propenyls causes a further increase in the potency of the analog. Many examples of this behavior can be found in the library of DHPs, as shown in Fig. 5B. Esterification with very bulky groups like benzyls causes instead a decrease in compound affinity for CFTR. In parallel, we observed that ability to block Ca^{2+} channels was not dramatically altered by these types of modification.

An interesting finding was that the phenyl ring of DHPs is a very adaptable site. Indeed, activity of DHPs is maintained despite the presence of none or multiple substitutions at this position. Halogen atoms, methoxy or ethoxy groups, nitro groups, various alkyls, methoxycarbonyls or amino groups are widely represented at various positions in the subset of compounds that are active on CFTR (Table 3, Fig. 6). Interestingly, we found one very strict constriction: the presence of a hydroxyl at position 4 (R_3) of the phenyl ring severely impairs the ability to potentiate CFTR (see DHP-004, Table 3 and Fig. 6). Hydroxyls were deleterious for CFTR activity also in other positions like position 3 (see for example Supplementary Materials, DHP-013, DHP-014 and DHP-263). Multiple halogen substitutions are well tolerated at every position of the phenyl ring. Particularly interesting is also the presence of alkyl groups at position 4. Actually, substitutions at this position (*para*-substitutions) with a methyl, an isopropyl or a tert-butyl, preserve activity on CFTR (see for example DHP-179, Table 3 and Fig.

6). On the contrary, looking at the activity on Ca^{2+} channels, we found that a general rule is that the *para*-position of the phenyl ring must not be substituted, as this kind of modification causes a marked decrease of the inhibition of VDCC (Table 3, Fig. 6). More extensive changes in the chemical structure involving the phenyl ring do not influence dramatically the ability of the compounds to potentiate CFTR (Fig. 7). For example, substitution of the phenyl ring with a benzodioxole group (DHP-110) does not change the activity of the compound as a CFTR potentiator, as compared to the phenyl ring without any substitution, but significantly decrease its ability to inhibit Ca^{2+} channels. Furthermore, the presence of a styryl group (DHP-134) instead of the phenyl ring also preserves activity on CFTR. However, when the phenyl ring is substituted with very bulky groups, we observed loss of activity on both CFTR and Ca^{2+} channels (e.g. DHP-182, Fig. 7).

The comparison between the sensitivity to DHP analogs of the three CFTR mutants is shown in Fig. 8. We found consistently that G551D is the most refractory to potentiation, the concentrations needed to stimulate this mutant being always 10 – 20 times higher than those active on ΔF508 (Fig. 8A, see also Supplemental Data). Conversely, the G1349D mutant displayed a sensitivity that is between that of ΔF508 and G551D (Fig. 8B and C). The comparison between the potency data for ΔF508 activation and the extent of VDCC inhibition at 1 μM demonstrates the existence of a subset of compounds that can activate the mutant CFTR at nanomolar concentration, with negligible effect on Ca^{2+} channels (Fig. 8D).

Among the DHPs that were the most interesting as CFTR potentiators, we paid particular attention to compounds DHP-106, DHP-194, DHP-229, and DHP-256 (Fig. 9A). As shown in Fig. 9B, these compounds show a dramatic decrease in activity on Ca^{2+} channels. In particular, the K_i for three of these compounds is in the range 1 – 2 μM , a more than 250-fold decrease in potency with respect to felodipine ($K_i = 4 \text{ nM}$). On the other hand, the same DHPs maintain a

good activity on CFTR channels with an apparent K_a for the $\Delta F508$ and G1349D mutants in the range 100 – 700 nM (Fig. 9C, E). In agreement with all previous observations, activity on G551D required higher concentrations with K_a values of 1 – 6 μM (Fig. 9D). These results demonstrate that the structure of DHPs can be modified to obtain selective CFTR potentiators with minimal activity on Ca^{2+} channels.

The activity of these compounds was confirmed in measurements of transepithelial CFTR Cl^- currents in FRT cells (Fig. 10). Compared to genistein, the selected DHPs showed a marked ability to elicit CFTR activity at significantly lower concentrations. DHP-194 was the most potent compound, with an apparent K_a of approximately 0.11 μM and 1.2 μM on $\Delta F508$ - and G551D-CFTR, respectively, in agreement with the values deriving from the fluorescence assay (0.11 and 2.0 μM). Also other compounds showed in short-circuit current recordings a potency comparable to that calculated from fluorescence experiments. For example, DHP-229 displayed K_a values of 0.32 and 2.6 μM for $\Delta F508$ and G551D channels, respectively, in fluorescence experiments and of 0.31 and 4.0 μM in short-circuit current recordings. In contrast, genistein had a reduced potency for $\Delta F508$ (K_a of 18 μM in fluorescence assays and 14 μM in short-circuit current experiments) and for G551D (K_a of 94 μM in fluorescence assays and 124 μM in short-circuit current experiments). The maximal response to the compounds in $\Delta F508$ -CFTR cells was comparable to that to genistein, while in G551D-CFTR cells the activity elicited by DHPs was at least 2-fold larger than that evoked by genistein, as already described for anti-hypertensive DHP drugs (Pedemonte et al., 2005b). In all cases, currents elicited by DHPs were fully abolished by the selective CFTR blocker CFTR_{inh}-172 (Ma et al., 2002).

DISCUSSION

Identification of selective activators of the CFTR Cl⁻ channel is a key step for the development of new drugs for cystic fibrosis patients. In particular, CFTR potentiators are needed to restore activity in those CFTR mutants affected by impaired channel gating (class III mutants), like G551D and G1349D. For these mutants CFTR potentiators could constitute a relevant monodrug therapy. Potentiators may be also useful to enhance the activity of the Δ F508 mutant, once the trafficking defect is corrected, at least partially, by pharmacological chaperones, like bisamimethylbitiazoles and quinazolinones (Pedemonte et al., 2005c; Van Goor et al., 2006).

In a previous study, we described the effect of anti-hypertensive 1,4-dihydropyridines as potentiators of Δ F508 and G551D-CFTR (Pedemonte et al., 2005b). This activity was not mediated by block of Ca²⁺ channels but probably by direct interaction with CFTR itself. However, activation of CFTR occurred at concentrations well above those effective on Ca²⁺ channels. Therefore, it was important to verify whether more selective and effective DHPs can be developed for CFTR. To this aim, we have analyzed a library of felodipine analogs to determine structural requirements for CFTR activation and VDCC block. The set of analogs was chosen to explore the importance of various regions of DHP chemical structure, as the pyridine and the phenyl rings, and the alkyl esters.

For the present study, we have developed a high-throughput assay to measure effect of compounds on voltage-dependent Ca²⁺ channels (VDCCs). This assay, based on the Ca²⁺-sensitivity of the Fluo-4 fluorescent probe and the cells H9C2, can detect with high sensitivity and accuracy the inhibition of VDCC by DHPs. In parallel, we have used the functional assay based on the halide-sensitive yellow fluorescent proteins (YFPs) to measure activity of compounds on Δ F508, G551D, and G1349D CFTR mutants. We have analyzed the set of 333

felodipine analogs using both assays. Our first result is that DHP-based structures activate also G1349D-CFTR, besides Δ F508 and G551D mutants. This finding indicates that DHPs represent a class of CFTR activators that similar to phenylglycines, and in contrast to sulfonamides and tetrahydrobenzothiophenes (Yang et al., 2003; Pedemonte et al., 2005a), are effective on a wide panel of CFTR mutations causing channel gating defect. However, the potency of DHPs changes in relation with the type of mutation. Δ F508 and G1349D are activated at nanomolar concentrations by the most potent compounds. In contrast, G551D requires consistently higher concentrations, as already found for other CFTR activators (Zegarra-Moran et al., 2002, Pedemonte et al., 2005a; Zegarra-Moran et al., 2007). This may indicate that G551 is located near a common binding site for potentiators, and that mutation to aspartate alters dramatically this site (Moran and Zegarra-Moran, 2005; Moran et al., 2005; Zegarra-Moran et al., 2007). Our results also establish the structural criteria required by DHPs for CFTR activation. For example, the esterification of the carboxyl groups with ethyls or propenyls, instead of methyls, can increase potency on CFTR. In addition, substitutions at the various positions of the phenyl ring preserve activity, with the only exception of hydroxyls.

The use of the Ca^{2+} channel assay has allowed us to establish whether changes in DHP structure may favor selectivity for CFTR. Indeed, we have found that substitutions at position 4 of the phenyl ring strongly reduce VDCC block while preserving activity on CFTR. The improvement in selectivity is demonstrated by the more than 200-fold decrease in potency for Ca^{2+} channels shown by some of the most interesting compounds compared to felodipine. Therefore, the result of our screening is the identification of DHP structures (i.e. DHP-106, DHP-194, DHP-229 and DHP-256) that are more potent on CFTR than on Ca^{2+} channels, an inversion of selectivity with respect to anti-hypertensive DHPs. Such compounds represent a promising starting point for the development of potentiators useful for class III CF mutations and, in

combination with pharmacological chaperones (Pedemonte et al., 2005c; Van Goor et al., 2006), for the treatment of $\Delta F508$.

Many of the active DHPs identified in our screening are characterized by a phenyl ring at position 4 of the pyrimidine ring, in which substitutions with hydrophobic groups seem to increase potency. Such a behavior suggests that this part of the molecule interacts with a hydrophobic pocket in the CFTR protein as indicated also by the loss of activity caused by substitutions with strongly polar hydroxyls. A donor hydrogen at position 1 and two acceptor oxygens in the carboxyl substitutions in positions 3 and 5 of the pyrimidine ring must also be present. These carboxyls are esterified with hydrophobic chains. Increase in hydrophobicity seems to favor the activity of the molecules, although excessive bulkiness represent a negative factor. These observations are consistent with the hypothesis of a binding site for CFTR potentiators in a cavity of the NBD1, where the ligand would interact with the protein by hydrogen bonds and close hydrophobic interactions, and to the surface of the NBD2, where the interaction would be mostly hydrophobic (Moran et al, 2005; Zegarra-Moran et al, 2007). In conclusions, the best DHPs discovered in our study are highly selective for the CFTR channel with potencies comparable or even better than those of previously described potentiators (Illek et al., 1999; Yang et al., 2003; Pedemonte et al., 2005a; Pedemonte et al., 2005b; Van Goor et al., 2006). They represent promising potential drugs for the pharmacotherapy of CF and interesting research tools to investigate the structure-function correlation in the CFTR protein.

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FIGURE LEGENDS

Figure 1. Assay for the determination of VDCC activity in H9C2 cells. **(A, B)** representative fluorescence traces showing the response to K^+ addition in the absence (saline) or in the presence of indicated DHPs (500 nM). Cells were untreated **(A)** or treated **(B)** for 24h with 10 nM retinoic acid. Note the block of VDCC activity by felodipine and R-(+)-BayK-8644, and potentiation of KCl effect by S-(-)-BayK-8644. **(C, D)** representative fluorescence traces showing the block of VDCC by nitrendipine and felodipine (dashed line, 200 nM; solid line, 1 μ M). Assays were performed in the presence of 50 nM **(C)** or 100 nM **(D)** S-(-)-BayK-8644. **(E)** dose-response relationships obtained for indicated DHPs in the presence of 100 nM (filled symbols) or 50 nM (empty symbols) S-(-)-BayK-8644. **(F)** dose-response relationships obtained for isradipine enantiomers and racemate in the presence of 50 nM S-(-)-BayK-8644. Data in **E** and **F** are the mean \pm SEM of five experiments.

Figure 2. DHP structural requirements for CFTR activation and VDCC block: DHP core structure. **(A)** chemical structure of tested compounds. **(B)** representative fluorescence traces showing the response to I^- addition in FRT cells expressing G1349D-CFTR under resting conditions (saline) or upon stimulation with forskolin alone (20 μ M) or in the presence of test compounds. **(C)** bar graph showing DHP analogs activity on Δ F508-CFTR (filled bars) and on VDCC (empty bars). The activity on Δ F508-CFTR is expressed as the reciprocal of the K_a value obtained from dose-response relationships of fluorescence experiments (mean \pm SD, n = 3). Activity on VDCC is reported as the percentage block obtained at a single concentration (1 μ M) on H9C2 cells (mean \pm SD, n = 3).

Figure 3. DHP structural requirements for CFTR activation and VDCC block: modification of the pyridine ring. **(A)** chemical structure of tested compounds. **(B)** bar graph showing DHP analogs activity on Δ F508-CFTR (filled bars) and on VDCC (empty bars). Data reported as described in Figure 2C.

Figure 4. Determination of DHP structural requirements for CFTR activation and VDCC block: substitutions at the pyridine ring. Bar graph showing DHP analogs activity on Δ F508-CFTR (filled bars) and on VDCC (empty bars). Data reported as described in Figure 2C. Chemical structures of tested compounds are shown in Table 1.

Figure 5. Determination of DHP structural requirements for CFTR activation and VDCC block: alkyl esters at the carboxyl groups of the pyridine ring. **(A)** bar graph showing DHP analogs activity on Δ F508-CFTR (filled bars) and on VDCC (empty bars). Data reported as described in Figure 2C. Chemical structures of tested compounds are shown in Table 2. **(B)** Role of alkyls esterified to carboxyl groups of DHPs on Δ F508-CFTR activity. Data points report activity ($1/K_a$) of the various DHPs. Lines connect data from DHPs having same basic structure and symmetric methyls, ethyls, or propenyls as esters.

Figure 6. Determination of DHP structural requirements for CFTR activation and VDCC block: substitutions at the phenyl ring. Bar graph showing DHP analogs activity on Δ F508-CFTR (filled bars) and on VDCC (empty bars). Data reported as described in Figure 2C. Chemical structures of tested compounds are shown in Table 3.

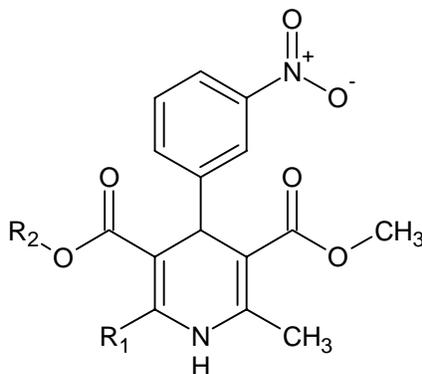
Figure 7. Determination of DHP structural requirements for CFTR activation and VDCC block: modification of the phenyl ring. **(A)** chemical structure of tested compounds. **(B)** bar graph showing DHP analogs activity on Δ F508-CFTR (filled bars) and on VDCC (empty bars). Data reported as described in Figure 2C.

Figure 8. Correlation between DHP analogs activity on CFTR and VDCC. Each symbol represents the activity of a single DHP on mutant CFTR versus another CFTR mutant (A – C) or versus VDCC (D). Activities are reported as $1/K_a$ (CFTR) or percentage of VDCC block. Note that the concentrations needed to stimulate the G551D mutant are always 10 – 20 times higher than those effective on Δ F508 or G1349D. Arrows outline compounds having good activity on CFTR mutant and negligible effect on VDCC.

Figure 9. Validation of selected DHP analogs. **(A)** chemical structure of compounds. **(B – E)** dose-response relationships obtained for indicated compounds on VDCC (B), Δ F508-CFTR (C), G551D-CFTR (D) and G1349D-CFTR (E). Felodipine dose-responses are shown for comparison. Data are the mean \pm SEM of five experiments.

Figure 10. Activation of CFTR Cl^- currents by DHPs. Graphs report transepithelial Cl^- currents in FRT cells stimulated with forskolin (20 μ M) followed by genistein or DHPs at the indicated concentrations. At the end of the experiments, CFTR currents were blocked with CFTR_{inh}-172 (10 μ M). **(A)** Data from FRT cells expressing Δ F508-CFTR. Cells were pre-incubated at low temperature to rescue the processing defect. **(B)** Data from FRT cells expressing G551D-CFTR. Data are representative of three similar experiments for each compound.

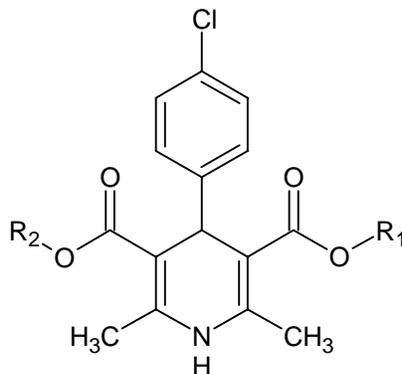
Table 1.
DHPs with different substitutions at the pyridine ring



	R ₁	R ₂
DHP-008	CH ₃	CH ₃
DHP-279	CH ₂ COOCH ₃	CH ₃
DHP-050	CH ₃	H

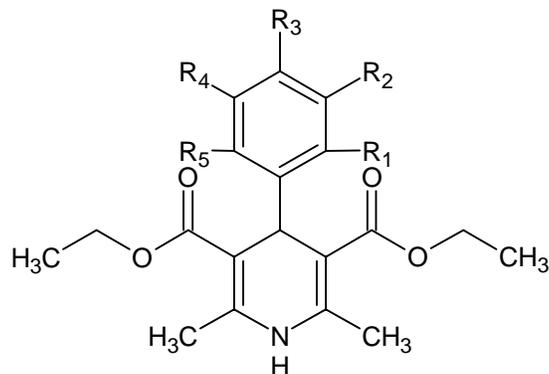
Table 2.

DHPs with different alkyl esters at the carboxyl groups of the pyridine ring



	R ₁	R ₂
DHP-051	CH ₃	CH ₃
DHP-052	CH ₂ CH ₃	CH ₂ CH ₃
DHP-181	CH(CH ₃) ₂	CH(CH ₃) ₂
DHP-138	CH ₂ C ₆ H ₅	CH ₂ C ₆ H ₅

Table 3.
 DHPs with different substitutions at the phenyl ring



	R ₁	R ₂	R ₃	R ₄	R ₅
DHP-002	H	H	H	H	H
DHP-052	H	H	Cl	H	H
DHP-153	Cl	H	H	H	Cl
DHP-009	H	H	F	H	H
DHP-012	F	F	F	F	F
DHP-027	H	OCH ₃	OCH ₃	OCH ₃	H
DHP-038	H	NO ₂	H	H	H
DHP-291	H	H	NO ₂	H	H
DHP-245	H	H	CH ₃	H	H
DHP-179	H	H	CH(CH ₃) ₂	H	H
DHP-132	H	H	C(CH ₃) ₃	H	H
DHP-003	H	H	N(CH ₃) ₂	H	H
DHP-004	H	H	OH	H	H

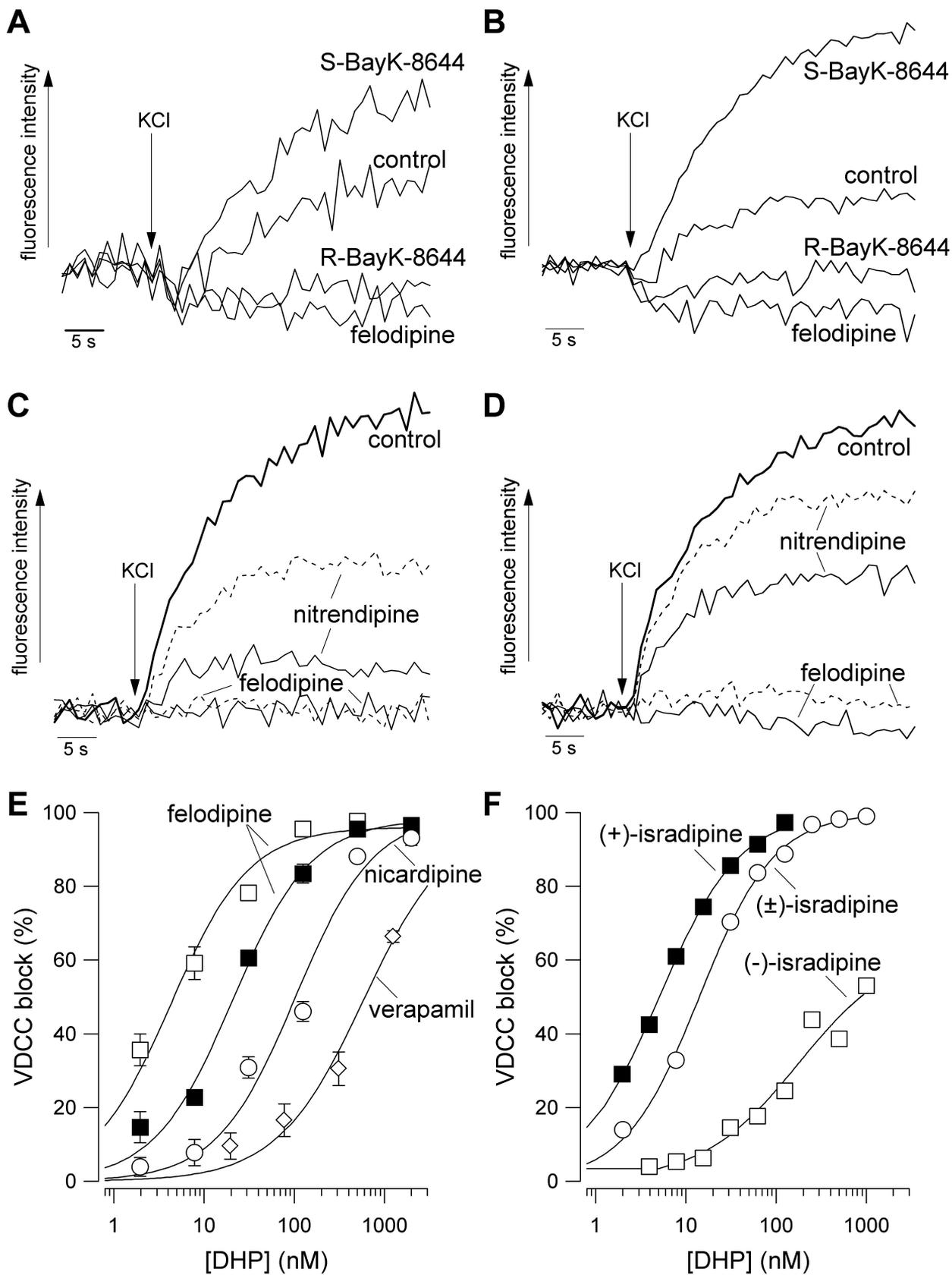


Figure 1

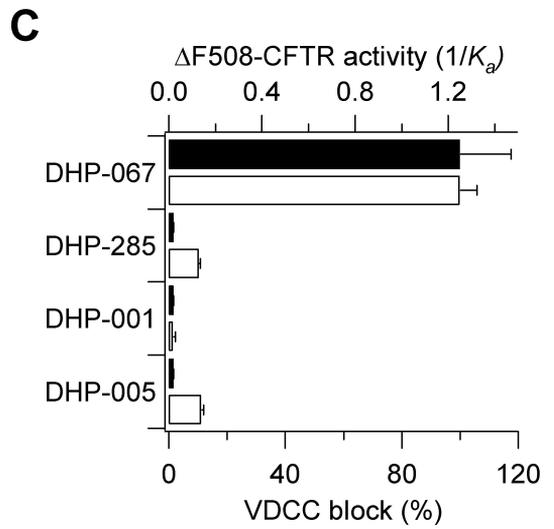
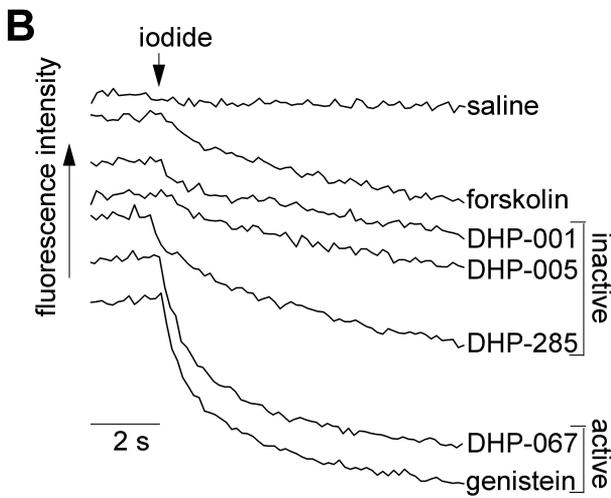
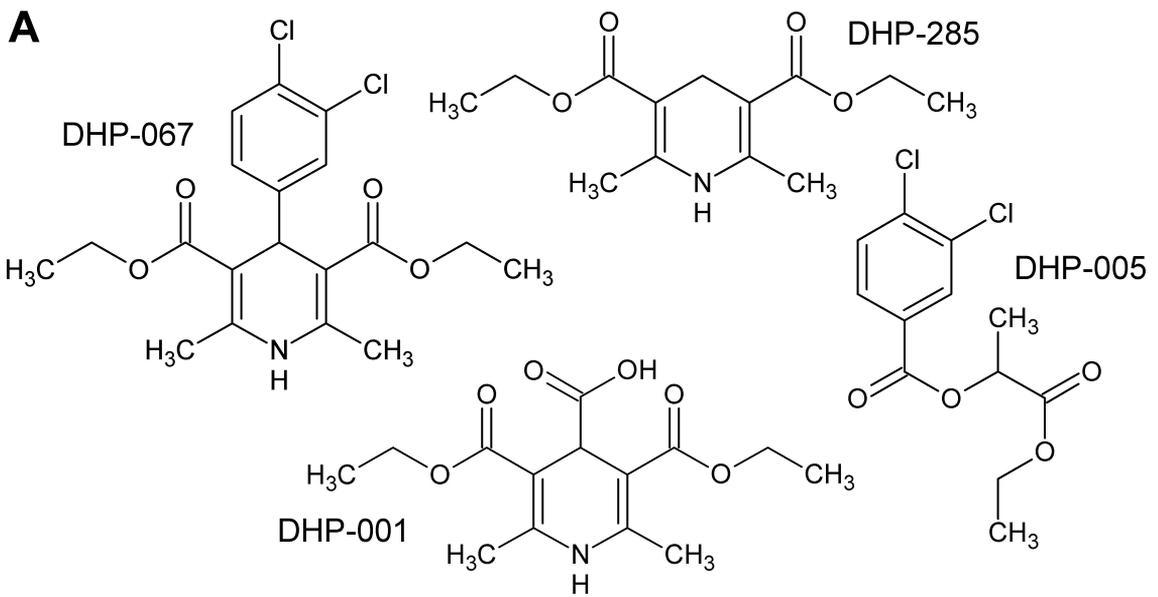


Figure 2

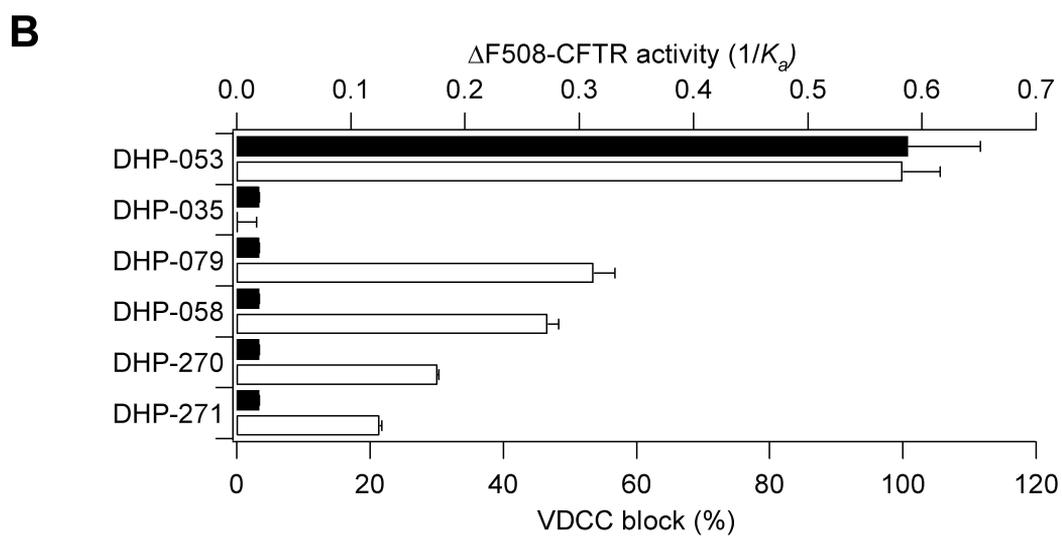
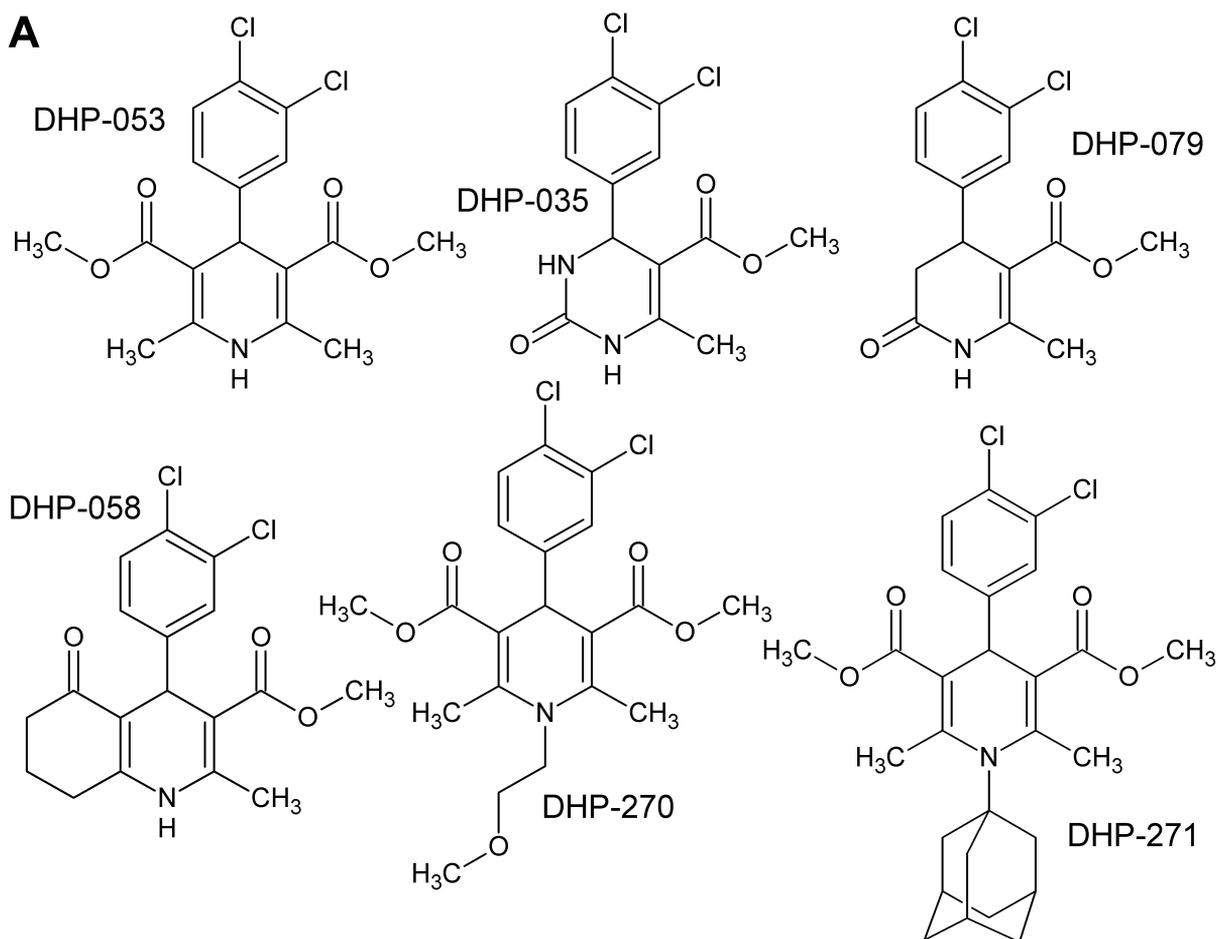


Figure 3

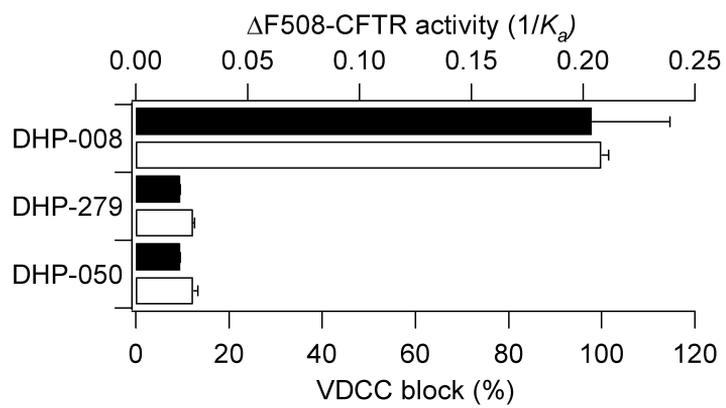


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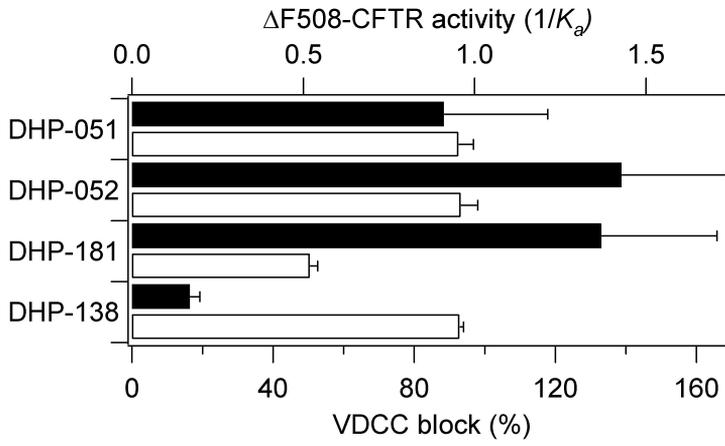
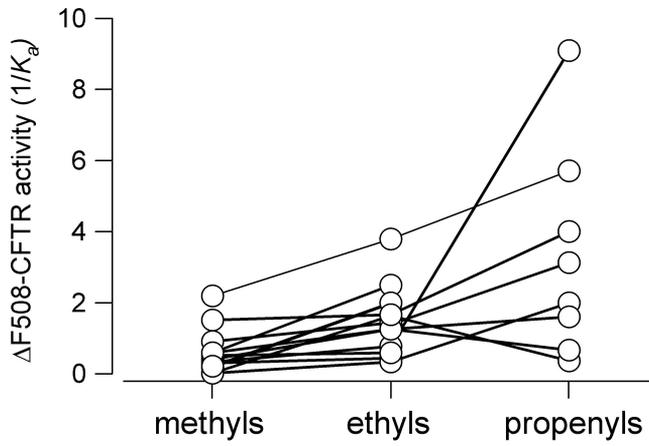
A**B**

Figure 5

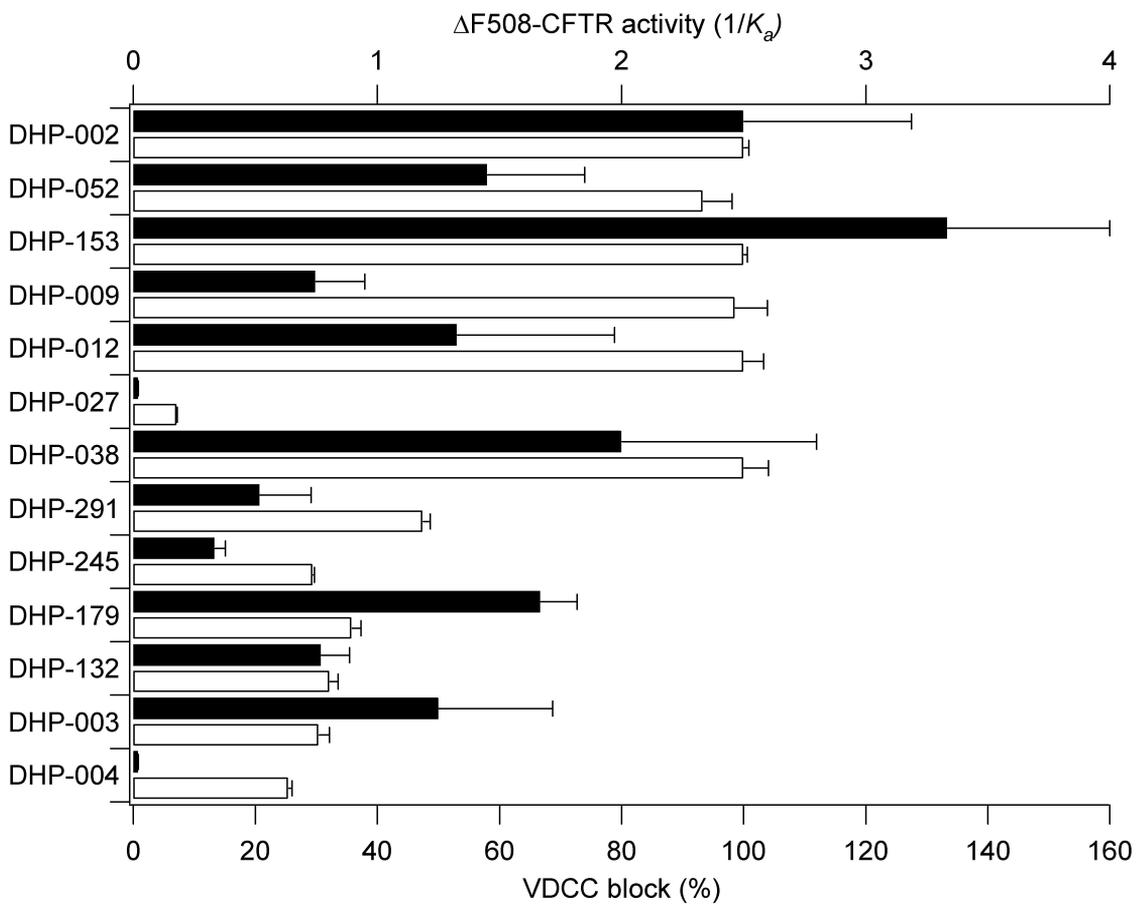


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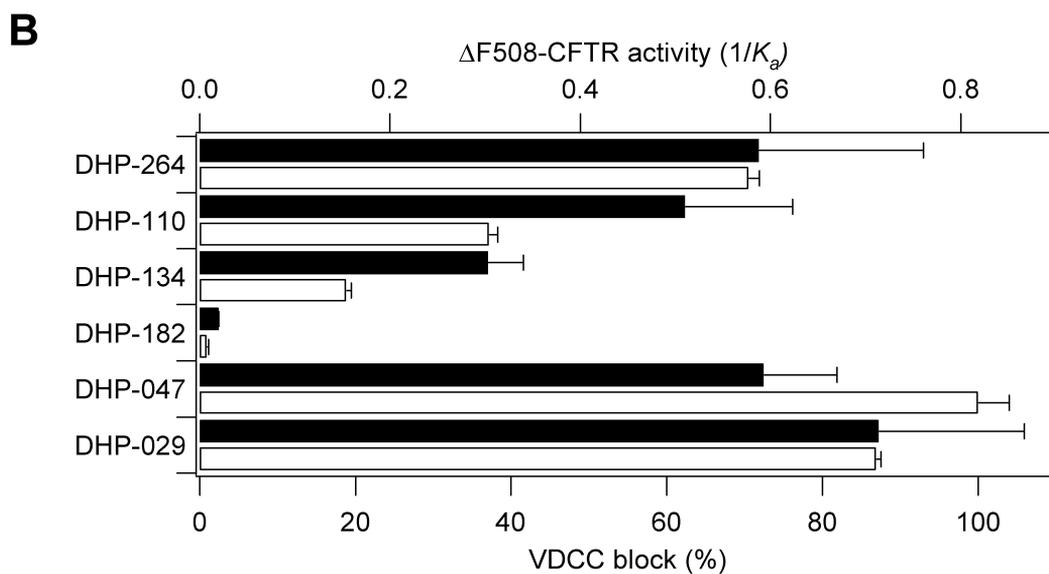
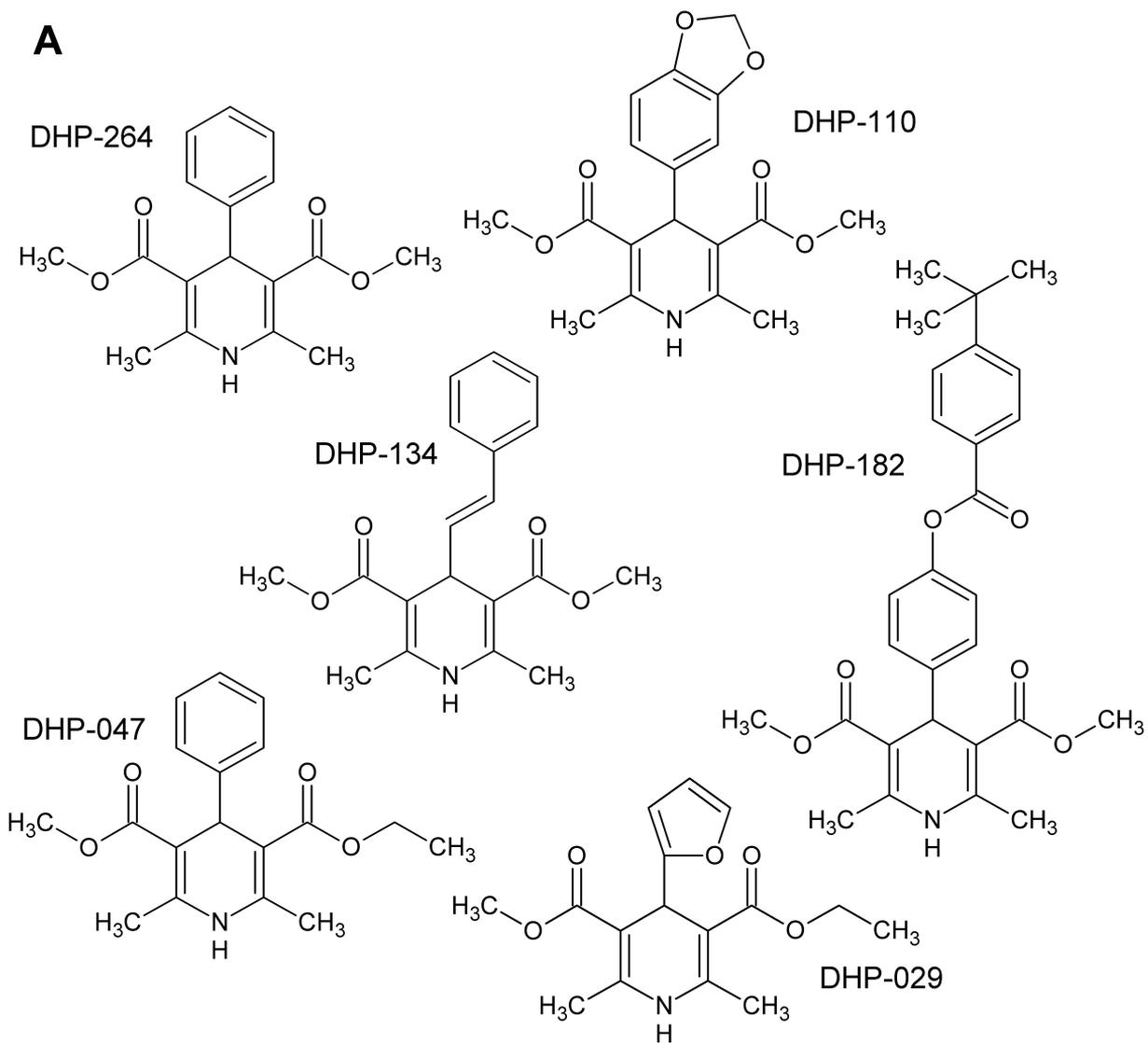


Figure 7

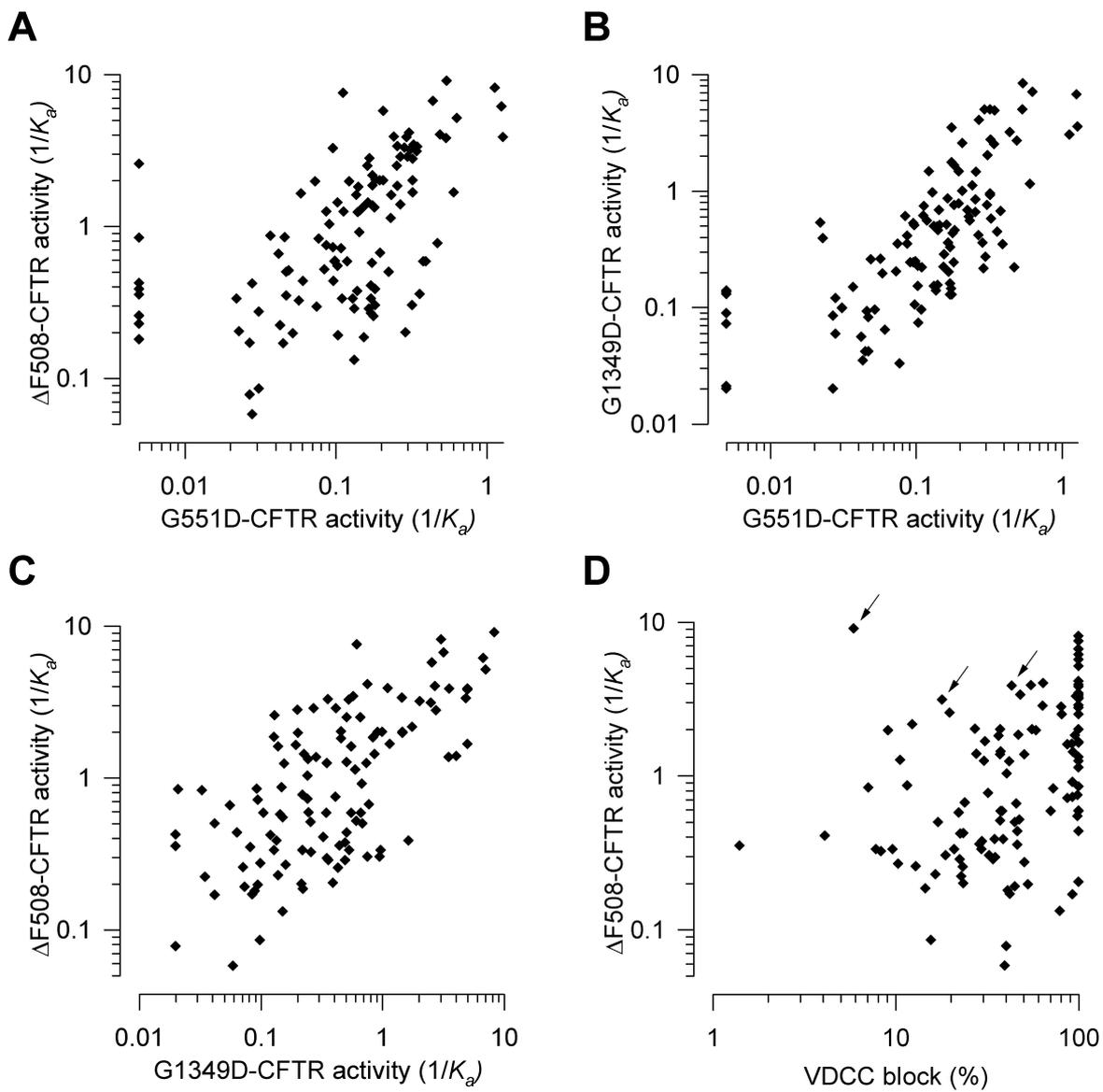


Figure 8

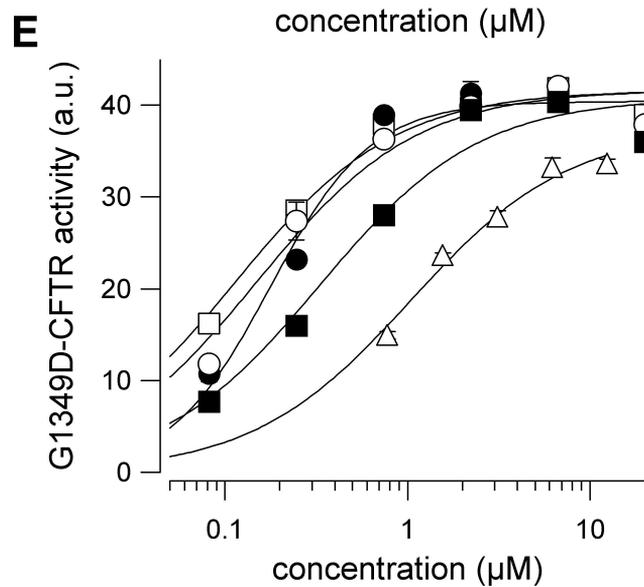
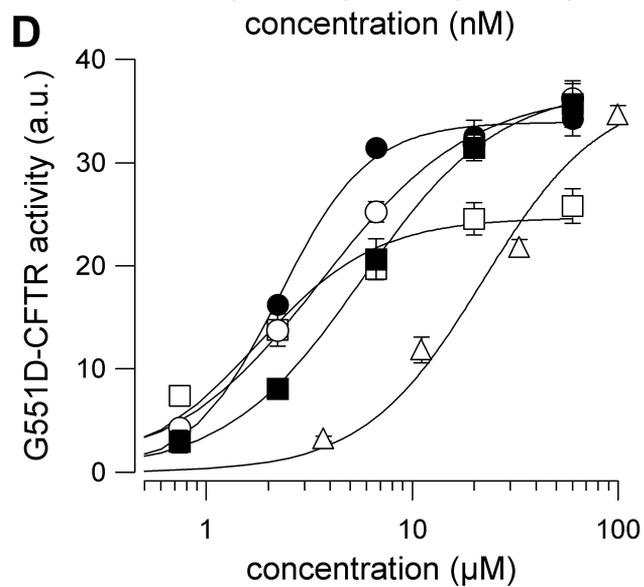
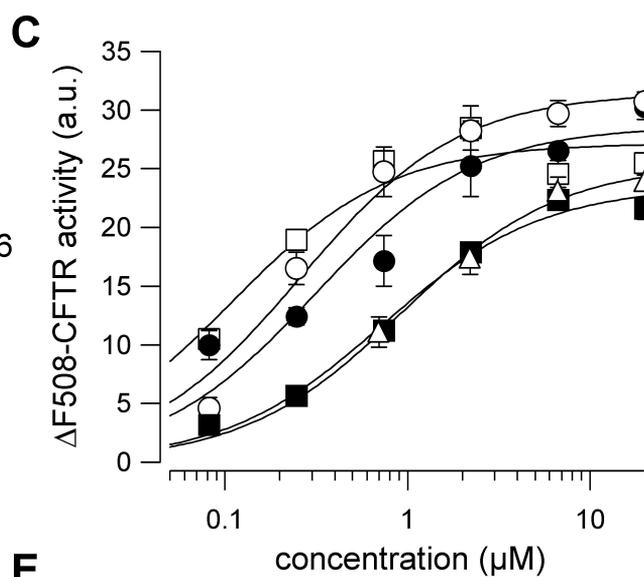
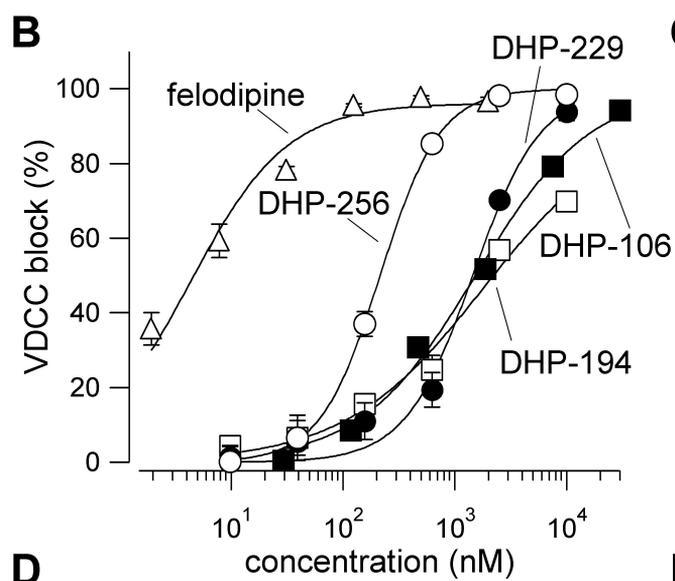
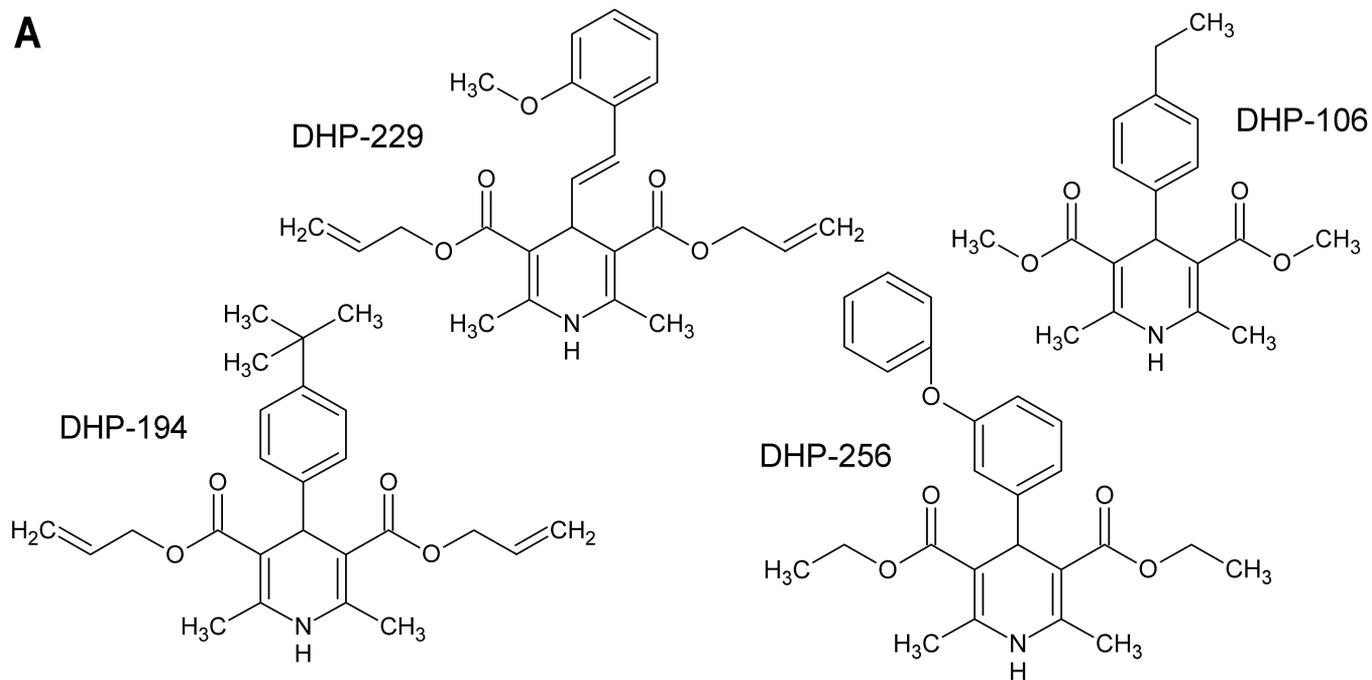


Figure 9

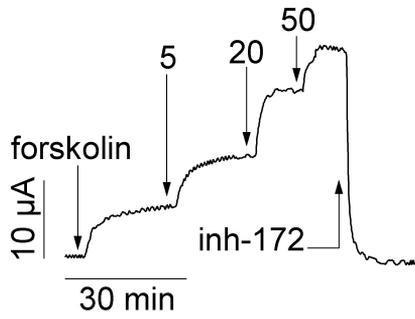
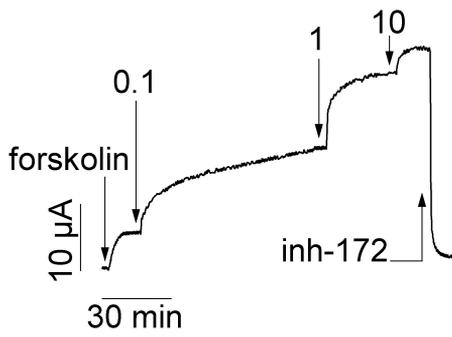
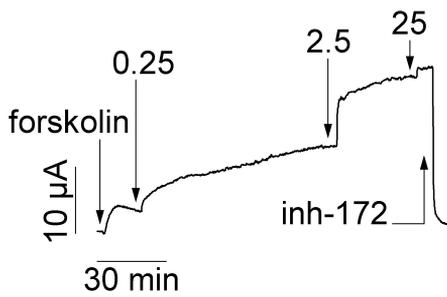
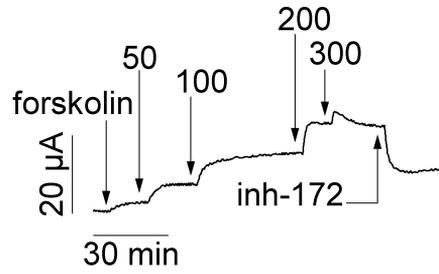
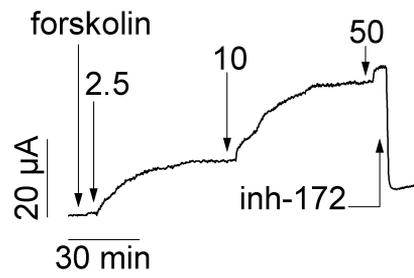
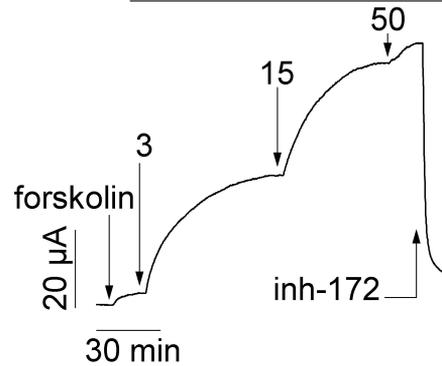
AGenistein (μM)DHP-194 (μM)DHP-229 (μM)**B**Genistein (μM)DHP-256 (μM)DHP-229 (μM)

Figure 10