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Structure-Activity Relationship of 1,4-Dihydropyridines as Potentiators of the Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channel[S]

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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, such as ΔPhe508, give rise to a protein with both a defective maturation and altered channel gating. Mutations belonging to class III, such as G551D and G1349D, cause only a gating defect. We have previously identified antihypertensive 1,4-dihydropyridines (DHPs), a class of drugs that block voltage-dependent Ca^{2+} channels, as effective potentiators of CFTR gating, able to correct the defective activity of CFTR mutants (*Mol Pharmacol* **68:**1736–1746, 2005). However, optimization of potency for CFTR versus Ca^{2+} channels is required to design selective compounds for CFTR pharmacotherapy. In the present study, we have established DHP structure-activity relationship 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 strong effect as potentiators on the ΔPhe508, 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 of 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).

So far, more than 1400 mutations have been described, distributed along the entire amino acidic 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 seems particularly appropriate for mutations belonging to classes 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 (ΔPhe508) that is present in at least one allele in approximately 50 to 90% of patients with CF (Bobadilla et al., 2002). The ΔPhe508 mutant pre-

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ABBREVIATIONS: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; NBD, nucleotide-binding domain; DHP, 1,4-dihydropyridine; VDCC, voltage-dependent Ca^{2+} channel; FRT, Fischer rat thyroid; YFP, yellow fluorescent protein; BayK-8644, 1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-[trifluoromethyl]phenyl)-3-pyridine carboxylic acid methyl ester.

sents 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 ΔPhe508 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, such as G1349D, are much rarer. However, the total number of missense mutations causing a gating defect may be present in a significant fraction of all patients with CF.

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 ΔPhe508- and G551D-CFTR channel gating (Hwang et al., 1997; Illek et al., 1999; Zegarra-Moran et al., 2002). In addition, high-throughput screening has allowed the identification of new classes of CFTR potentiators with improved potency (Yang et al., 2003; Pedemonte et al., 2005a). Some potentiators, such as tetrahydrobenzothiophenes and sulfonamides, are active only on the ΔPhe508 mutant. Conversely, phenylglycines, another class of compounds identified by high-throughput screening, are effective also on G551D and G1349D channels. In a recent screening of a set of compounds that included drugs and natural compounds, we identified antihypertensive 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 ΔPhe508- and G551D-CFTR. DHPs act as antihypertensive drugs by blocking L-type voltage-dependent Ca^{2+} channels (VDCCs), thereby causing the relaxation of arterial smooth muscle cells (Harrold, 2002).

We have shown previously that activation of mutant CFTR by DHPs occurs through a mechanism that does not involve the inhibition of Ca^{2+} channels, 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 versus 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 relationships 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 (ΔPhe508, 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.

Materials and Methods

Cell Culture. Fischer rat thyroid (FRT) cells were stably transfected with ΔPhe508, G551D, or G1349D-CFTR, and the halide-sensitive yellow fluorescent protein mutant YFP-H148Q/I152L (Galietta et al., 2001a). The cells were cultured on plastic in Coon's modified Ham's F-12 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For fluorescence assays of CFTR activity, cells were plated (100,000 cells/well) on clear-bottomed 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 Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For fluorescence assays of L-VDCC activity, cells were plated (100,000 cells/well) on clear-bottomed 96-well microplates. After 24 h, 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 h.

Compounds. A set of 333 felodipine analogs was purchased from ChemDiv. The analogs were selected by 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 h (G551D and G1349D) or 48 h (ΔPhe508) after plating on microplates. Before the assay, ΔPhe508-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 Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$) 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 of 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: $dF/dt = (dF/dt)_{max} / [1 + (K_a / [compound])^{n_H}]$.

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 h, cells were washed with phosphate-buffered saline 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 at 485 nM; emission at 520 nM). Each assay consisted of 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 s. 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 to 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 KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM Na-HEPES, pH 7.3, and 10 mM glucose. For the apical side, this solution was modified by replacing half the NaCl with sodium gluconate and increasing $CaCl_2$ 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. ΔPhe508 cells were previously incubated at 27°C for 24 h 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

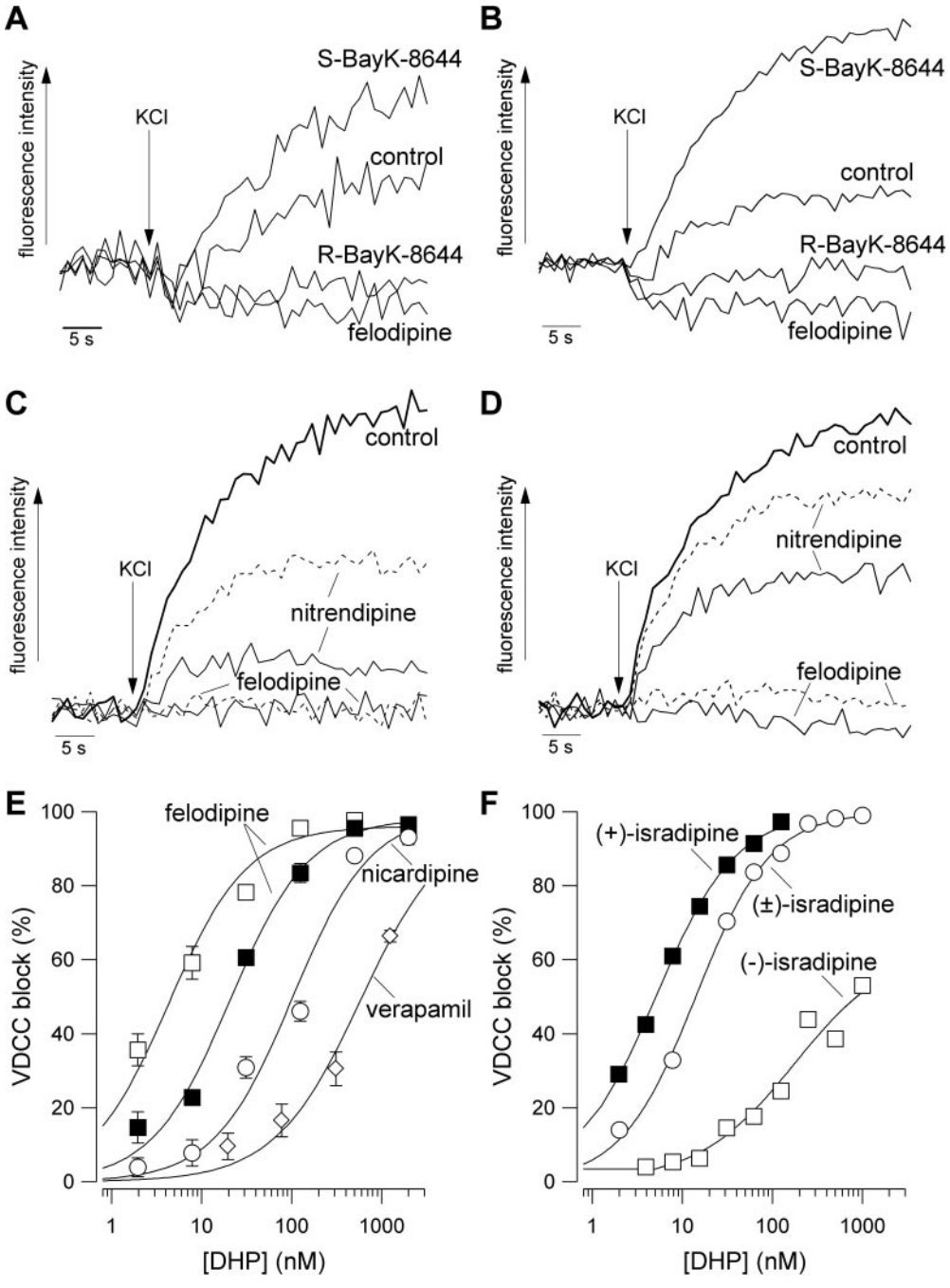


Fig. 1. Assay for the determination of VDCC activity in H9C2 cells. A and B, representative fluorescent traces showing the response to K^+ addition in the absence (saline) or in the presence of indicated DHPs (500 nM). Cells were untreated (A) or treated (B) for 24 h with 10 nM retinoic acid. Note the block of VDCC activity by felodipine and (*R*)-(+)-BayK-8644 and potentiation of KCl effect by (*S*)-(−)-BayK-8644. C and D, representative fluorescence traces showing the block of VDCC by nitrendipine and felodipine (dashed line, 200 nM; solid line, 1 μM). 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 ± S.E.M. of five experiments.

groups, 3) substitutions of the pyridine ring with other cyclic scaffolds, and 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 (Galietta 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, that endogenously express 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 that 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). Long-term treatment for 24 h 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. Figure 1, C and D, shows representative traces recorded with test compounds in the presence of 50 or 100 nM (*S*)-(−)-BayK-8644, respectively. The high-affinity drug felodipine applied at 0.25 and 1 μM fully blocked Ca^{2+} channels at both concentrations of the VDCC potentiator. The less potent compound nitrendipine instead caused a partial reduction of the Ca^{2+} influx. Figure 1E shows dose-response relationships established for felodipine in the presence of 50 or 100 nM (*S*)-(−)-BayK-8644. 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 described previously (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 FRT cells coexpressing the halide-sensitive mutant H148Q/I152L of the fluorescent protein YFP and the CFTR mutants ΔPhe508, G551D, and G1349D. Cells expressing ΔPhe508-CFTR were incubated at 27°C for 24 h 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 μM) 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 undergoing long-term treatment with retinoic acid and using the fluorescence assay described

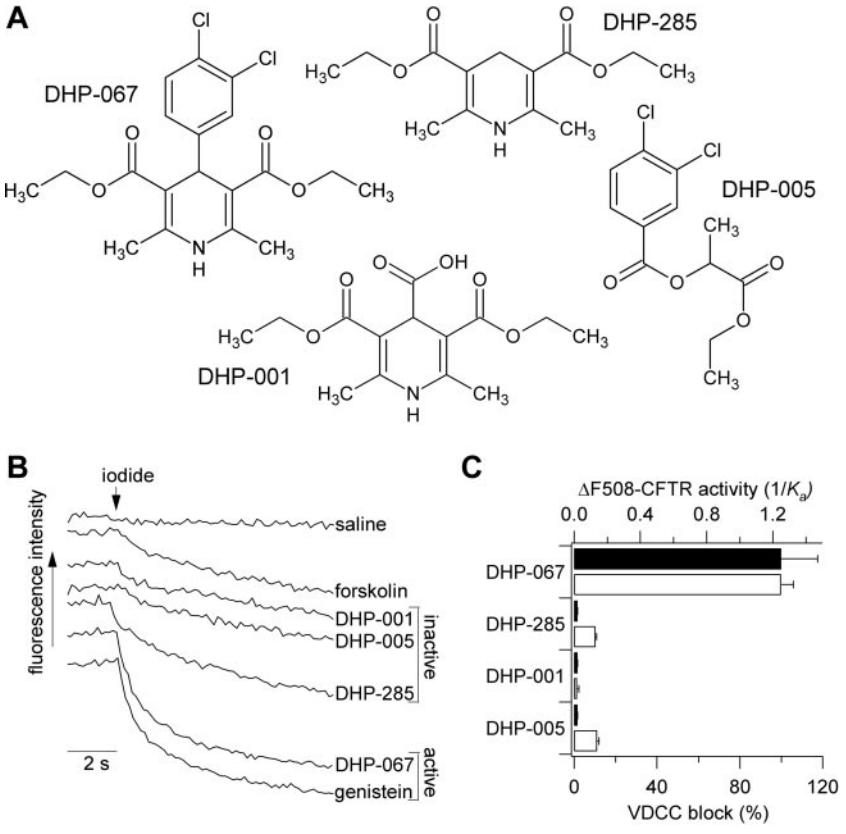


Fig. 2. DHP structural requirements for CFTR activation and VDCC block: DHP core structure. A, chemical structure of tested compounds. B, representative fluorescence traces showing the response to I^- addition in FRT cells expressing G1349D-CFTR under resting conditions (saline) or upon stimulation with forskolin alone (20 μM) or in the presence of test compounds. C, bar graph showing DHP analog activity on ΔPhe508-CFTR (filled bars) and on VDCC (empty bars). The activity on ΔPhe508-CFTR is expressed as the reciprocal of the K_a value obtained from dose-response relationships of fluorescence experiments (mean ± S.D., $n = 3$). Activity on VDCC is reported as the percentage block obtained at a single concentration (1 μM) on H9C2 cells (mean ± S.D., $n = 3$).

above [with 50 nM (*S*)-(−)-BayK-8644]. Compounds were tested at 1 μM, a concentration at which all antihypertensive DHPs cause complete blockade of Ca^{2+} channels. For the most interesting compounds, a dose-response curve was generated by testing concentrations in the range of 1 nM to 30 μM.

Selected sets of compounds are shown in Figs. 2 to 7 and Tables 1 to 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 with others having substitutions and modifications at a specific position. In general, conclusions arising from the examples shown in Figs. 2 to 7 and Tables 1 to 3 are supported also by data obtained from other DHPs present in the library. Data for all compounds are reported in the Supplemental 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) were necessary for CFTR potentiation as well as for VDCC block (Fig. 2). Indeed, compounds such as DHP-001 and DHP-285, which lack the 4-phenyl group, were 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, did not provide activity. The second major point is that modification of the pyridine ring heavily affected the ability of compounds to activate CFTR (Fig. 3). Substitution of the pyridine ring with a pyrimidine ring (DHP-035) completely abolished activity on both CFTR and VDCC. Other modifications of this part of the molecule, such as 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), resulted in the inability of the compounds to stimulate CFTR but maintained part of the VDCC inhibitor activity. Substitutions of the hydrogen bound to the nitrogen at position

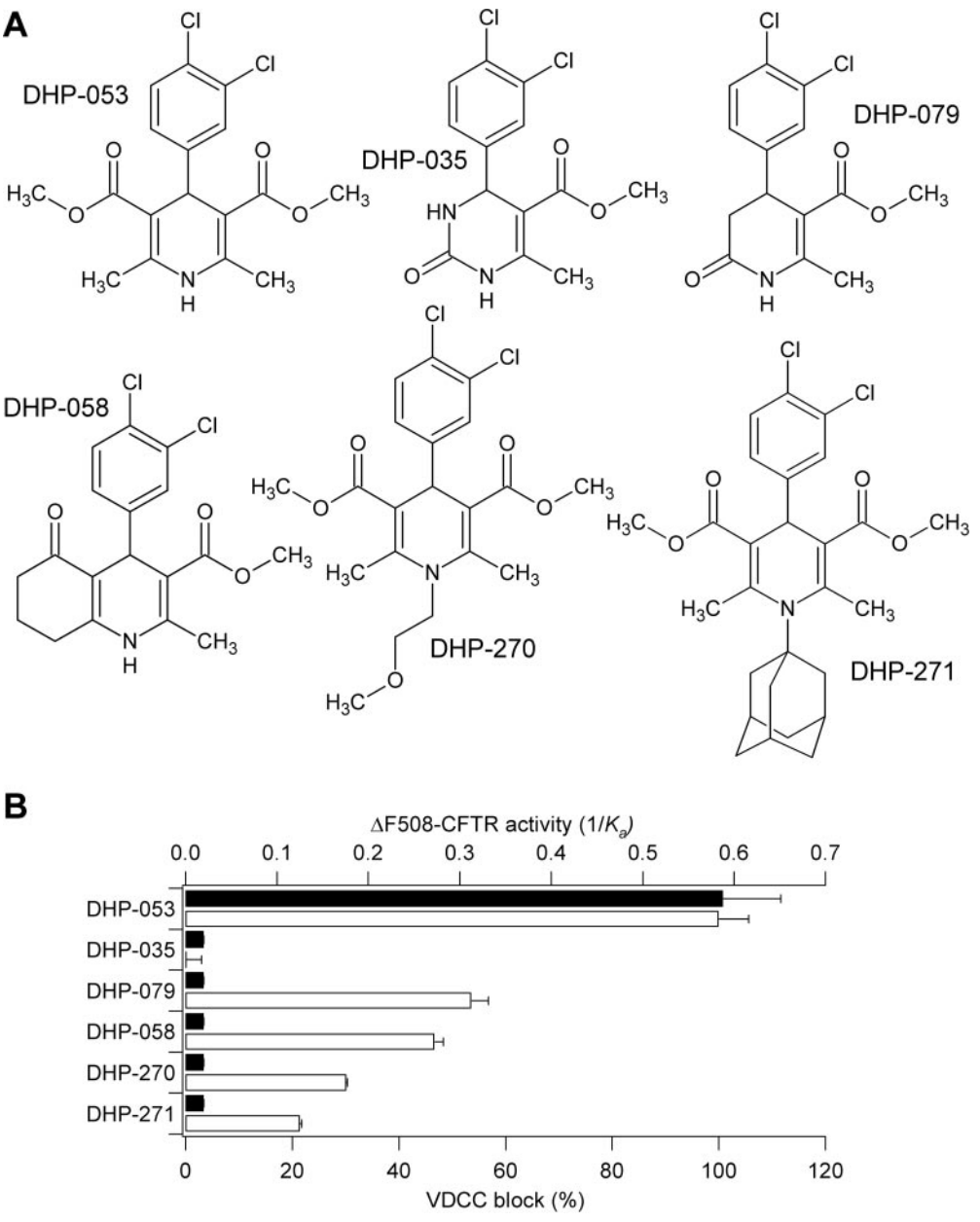


Fig. 3. DHP structural requirements for CFTR activation and VDCC block: modification of the pyridine ring. A, chemical structure of tested compounds. B, bar graph showing DHP analogs activity on ΔPhe508-CFTR (filled bars) and on VDCC (empty bars). Data reported as described in the legend to Fig. 2C.

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 were 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 preserved the activity. Among the active compounds, we found methyl, ethyl, isopropyl, benzyl, propenyl, and methoxyethyl esters (Fig. 5A, Table 2). It is noteworthy that potency data for CFTR activation demonstrated a ranking order for the size of the alkyl groups, which need to be not too small but not too bulky either. Accordingly, the ethyls were better than the methyl groups, and the esterification with propenyls caused 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 such as benzyls caused 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 modifications.

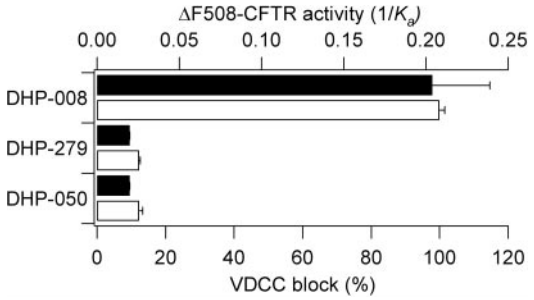


Fig. 4. Determination of DHP structural requirements for CFTR activation and VDCC block: substitutions at the pyridine ring. Bar graph showing DHP analogs activity on ΔPhe508-CFTR (filled bars) and on VDCC (empty bars). Data reported as described in the legend to Fig. 2C. Chemical structures of tested compounds are shown in Table 1.

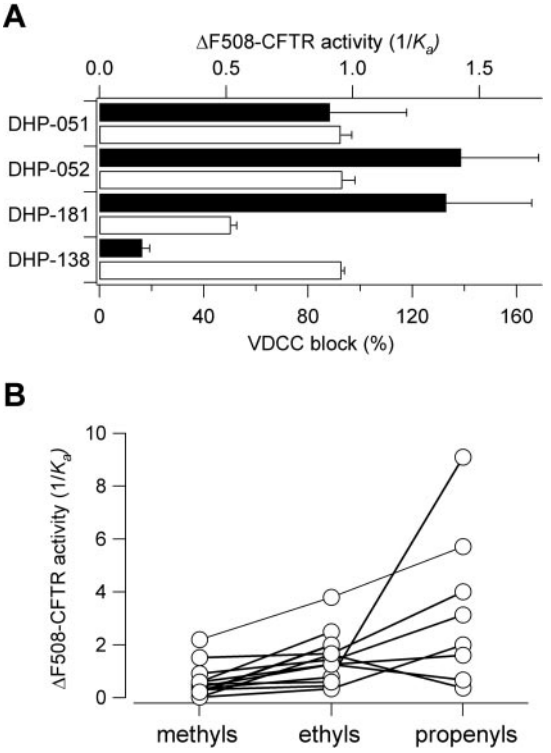


Fig. 5. Determination of DHP structural requirements for CFTR activation and VDCC block: alkyl esters at the carboxyl groups of the pyridine ring. A, bar graph showing DHP analog activity on ΔPhe508-CFTR (filled bars) and on VDCC (empty bars). Data reported as described in the legend to Fig. 2C. Chemical structures of tested compounds are shown in Table 2. B, role of alkyls esterified to carboxyl groups of DHPs on ΔPhe508-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.

It is noteworthy that the phenyl ring of DHPs was a very adaptable site. Indeed, activity of DHPs was maintained despite the presence of no 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). We found one very strict constriction: the presence of a hydroxyl at position 4 (R_3) of the phenyl ring severely impaired the ability to potentiate CFTR (see DHP-004, Table 3 and Fig. 6). Hydroxyls were also deleterious for CFTR activity in other positions, such as position 3 (see, for example, Supplemental Materials, DHP-013, DHP-014, and DHP-263). Multiple halogen substitutions were well tolerated at every position of the phenyl ring. Particularly interesting also was the presence of alkyl groups at position 4. In fact, substitutions at this position (*para*-substitutions) with a methyl, an isopropyl or a *tert*-butyl preserved 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, because this kind of modification caused a marked decrease of the inhibition of VDCC (Table 3, Fig. 6). More extensive changes in the chemical structure involving the phenyl ring did not influence dramatically the ability of the compounds to potentiate CFTR (Fig. 7). For example, substi-

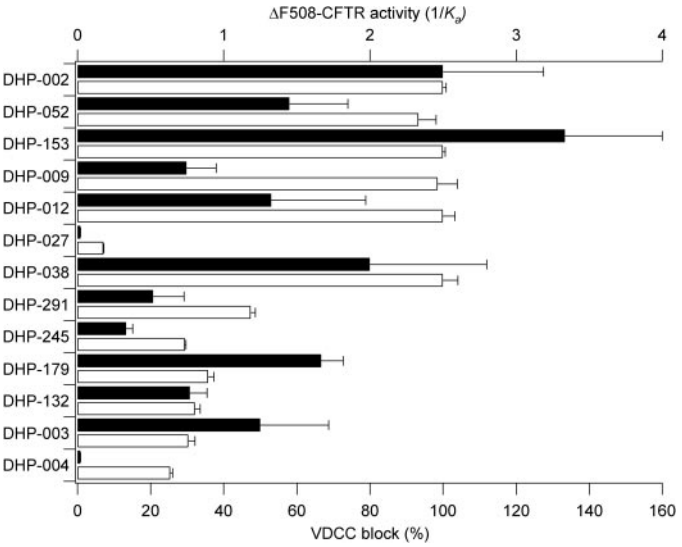


Fig. 6. Determination of DHP structural requirements for CFTR activation and VDCC block: substitutions at the phenyl ring. Bar graph showing DHP analog activity on ΔPhe508-CFTR (filled bars) and on VDCC (empty bars). Data reported as described in the legend to Fig. 2C. Chemical structures of tested compounds are shown in Table 3.

tution of the phenyl ring with a benzodioxole group (DHP-110) did not change the activity of the compound as a CFTR potentiator, compared with the phenyl ring without any substitution, but significantly decreased its ability to inhibit Ca^{2+} channels. Furthermore, the presence of a styryl group (DHP-134) instead of the phenyl ring also preserved activity on CFTR. However, when the phenyl ring was 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 of the sensitivity to DHP analogs of the three CFTR mutants is shown in Fig. 8. We found consistently that G551D was the most refractory to potentiation; the concentrations needed to stimulate this mutant were always 10 to 20 times higher than those active on ΔPhe508 (Fig. 8A, see also Supplemental Data). Conversely, the G1349D mutant displayed a sensitivity between that of ΔPhe508 and G551D (Fig. 8, B and C). The comparison between the potency data for ΔPhe508 activation and the extent of VDCC inhibition at 1 μM demonstrated the existence of a subset of compounds that could 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

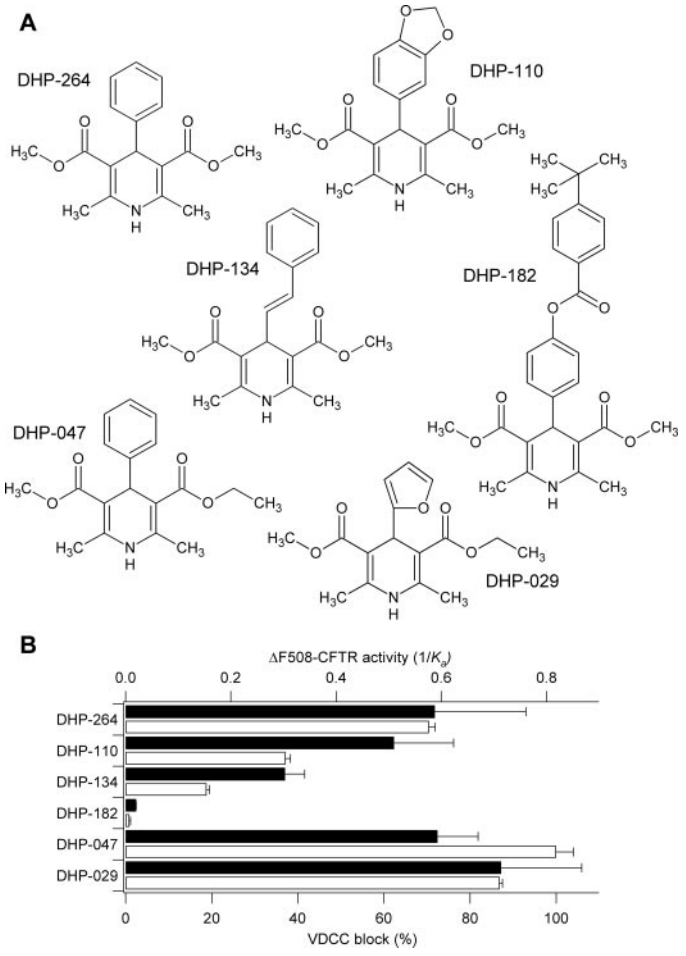
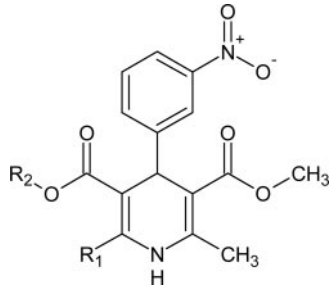


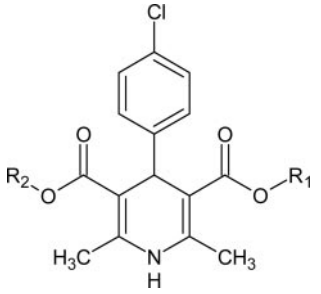
Fig. 7. Determination of DHP structural requirements for CFTR activation and VDCC block: modification of the phenyl ring. A, chemical structure of tested compounds. B, bar graph showing DHP analog activity on ΔPhe508-CFTR (filled bars) and on VDCC (empty bars). Data reported as described in Fig. 2C.

TABLE 1
DHPs with different substitutions at the pyridine ring



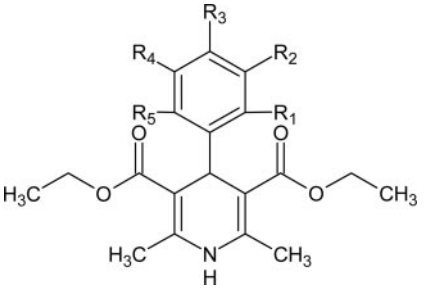
	R_1	R_2
DHP-008	CH_3	CH_3
DHP-279	CH_2COOCH_3	CH_3
DHP-050	CH_3	H

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_2CH_3	CH_2CH_3
DHP-181	$CH(CH_3)_2$	$CH(CH_3)_2$
DHP-138	$CH_2C_6H_6$	$CH_2C_6H_6$

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

shown in Fig. 9B, these compounds showed a dramatic decrease in activity on Ca^{2+} channels. In particular, the K_i for three of these compounds was in the range 1 to 2 μM, a decrease in potency of more than 250-fold with respect to felodipine (K_i = 4 nM). On the other hand, the same DHPs maintained a good activity on CFTR channels with an apparent K_a for the ΔPhe508 and G1349D mutants in the range 100 to 700 nM (Fig. 9, C and E). In agreement with all previous observations, activity on G551D required higher concentrations, with K_a values of 1 to 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 with genistein, the selected DHPs showed a marked ability to elicit CFTR activity at significantly lower concentrations. DHP-194 was the most potent compound, with apparent K_a values of approximately 0.11 and 1.2 μM for ΔPhe508- CFTR and G551D-CFTR, respectively, in agreement with the values derived from the fluorescence assay (0.11 and 2.0 μM). In addition, other compounds showed, in short-circuit current recordings, a potency comparable with that calculated from fluorescence experiments. For example, DHP-229 displayed K_a values of 0.32 and 2.6 μM for ΔPhe508 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 ΔPhe508 (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 ΔPhe508-CFTR cells was comparable with that to genistein, whereas in G551D-CFTR cells, the activity elicited by DHPs was at least 2-fold larger than that evoked by genistein, as already described for antihypertensive 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 ΔPhe508 mutant, once the trafficking defect is corrected, at least partially, by pharmacological chaperones, such as bisamimethylbitiazoles and quinazolinones (Pedemonte et al., 2005c; Van Goor et al., 2006).

In a previous study, we described the effect of antihypertensive 1,4-dihydropyridines as potentiators of ΔPhe508 and G551D-CFTR (Pedemonte et al., 2005b). This activity was not mediated by block of Ca^{2+} channels but probably by

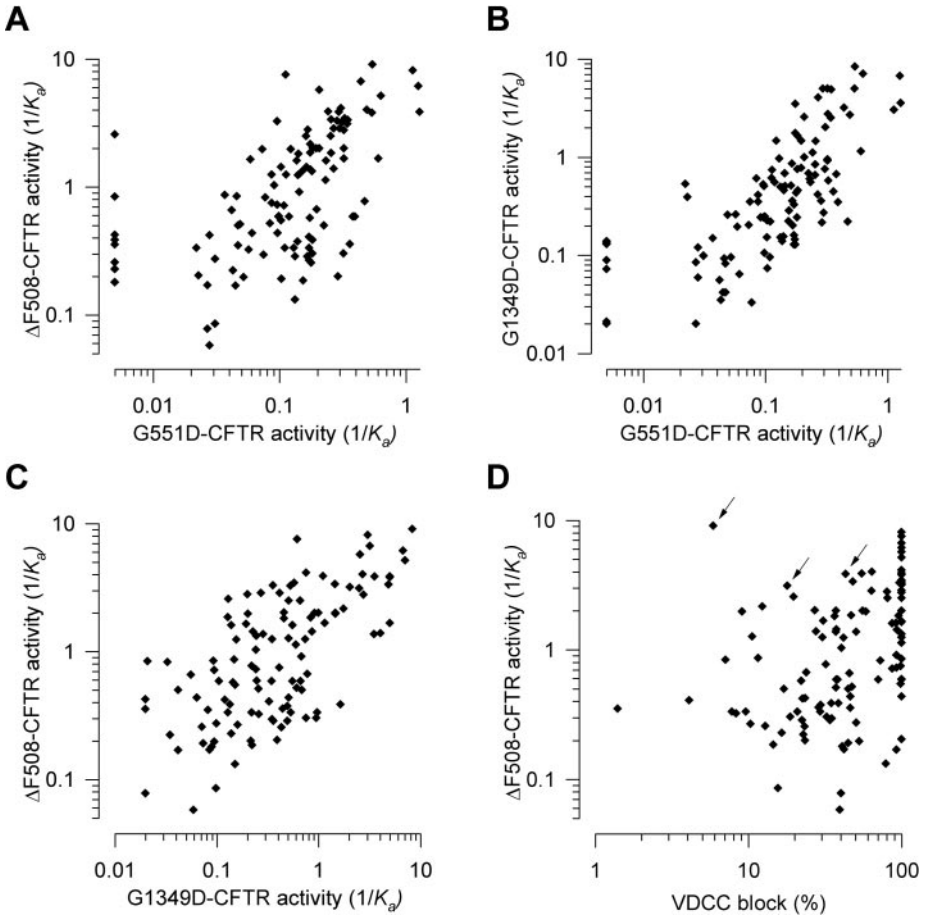


Fig. 8. Correlation between DHP analog activity on CFTR and VDCC. Each symbol represents the activity of a single DHP on mutant CFTR versus another CFTR mutant (A–C) or versus VDCC (D). Activities are reported as $1/K_a$ (CFTR) or percentage of VDCC block. Note that the concentrations needed to stimulate the G551D mutant are always 10 to 20 times higher than those effective on ΔPhe508 or G1349D. Arrows outline compounds having good activity on CFTR mutant and negligible effect on VDCC.

direct interaction with CFTR itself. However, activation of CFTR occurred at concentrations well above those effective on Ca^{2+} 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, such 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 VDCCs. This assay, based on the Ca^{2+}-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 YFPs to measure activity of compounds on ΔPhe508, 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 ΔPhe508 and G551D mutants. This finding indicates that DHPs represent a class of CFTR activators that, similarly to phenylglycines and differently from sulfonamides and tetrahydrobenzothiophenes (Yang et al., 2003; Pedemonte et al., 2005a), are effective on a wide panel of CFTR mutations that cause channel gating defect. However, the potency of DHPs changed in relation with the type of mutation. ΔPhe508 and G1349D were activated at nanomolar concentrations by the most potent compounds. In contrast, G551D required 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 Gly551 is located near a common binding site for potentiators and that mutation to aspartate dramatically alters this site (Moran and Zegarra-Moran, 2005; Moran et al., 2005; Zegarra-Moran et al., 2007). Our results also established the structural criteria required by DHPs for CFTR activation. For example, the esterification of the carboxyl groups with ethyls or propenyls, instead of methyls, increased potency on CFTR. In addition, substitutions at the various positions of the phenyl ring preserved activity, the only exception being the hydroxyls.

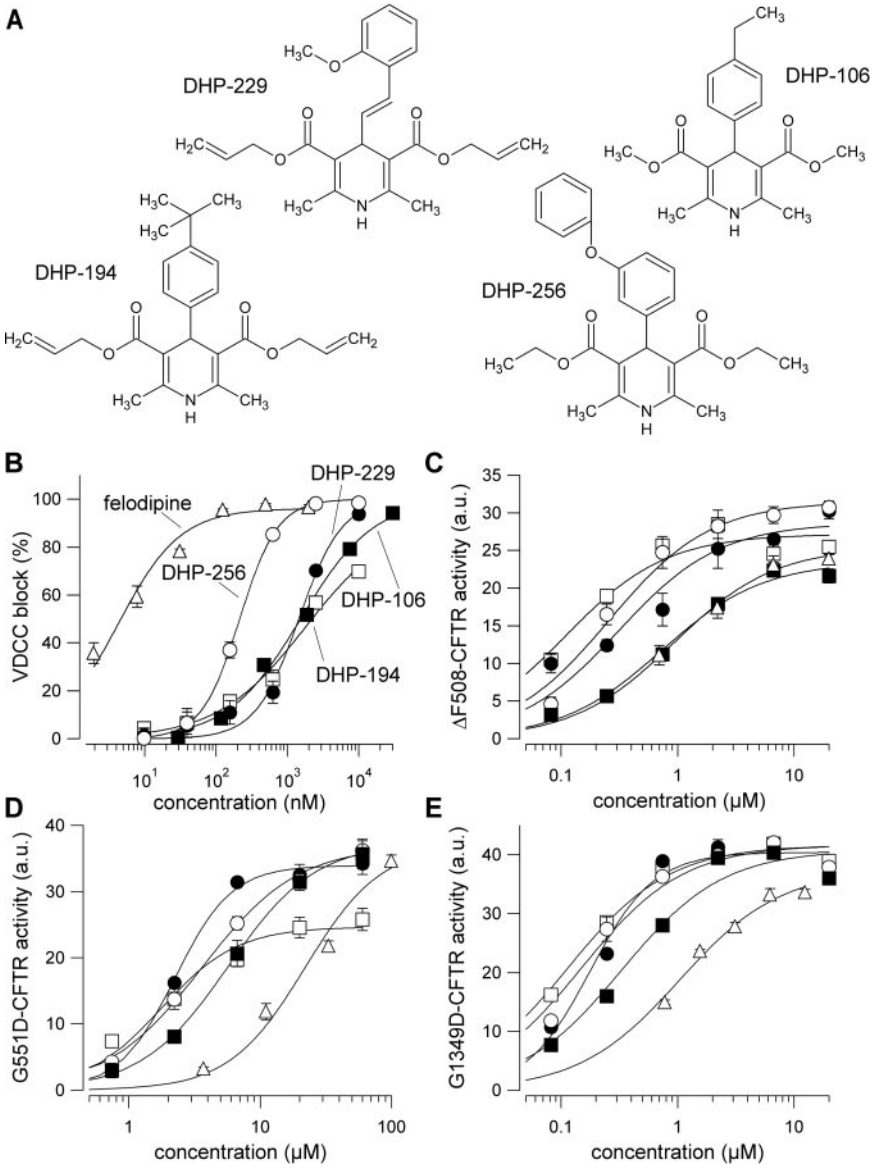


Fig. 9. Validation of selected DHP analogs. A, chemical structure of compounds. B–E, dose-response relationships obtained for indicated compounds on VDCC (B), ΔPhe508-CFTR (C), G551D-CFTR (D), and G1349D-CFTR (E). Felodipine dose-responses are shown for comparison. Data are the mean ± S.E.M. of five experiments.

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 but preserve 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 with 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 antihypertensive 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 ΔPhe508.

Many of the active DHPs identified in our screening were characterized by a phenyl ring at position 4 of the pyrimidine ring, in which substitutions with hydrophobic groups seemed to increase potency. Such 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 seemed to favor the activity of the molecules, although excessive bulkiness represented 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 conclusion, the best DHPs discovered in our study are highly selective for the CFTR channel with potencies comparable with or even better than those of previously described potentiators (Illek et al., 1999; Yang et al., 2003; Pedemonte et al., 2005a,b; 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.

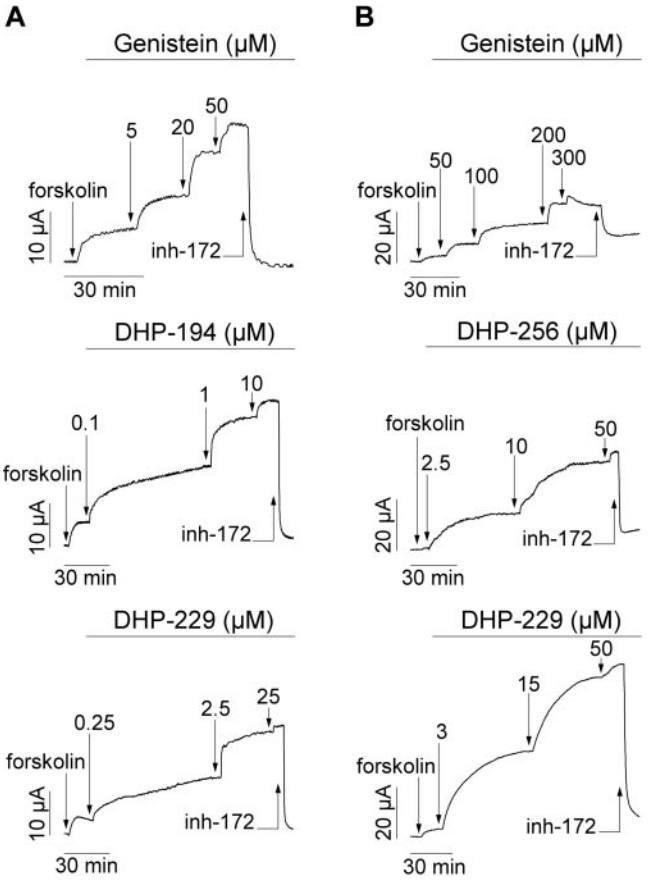


Fig. 10. Activation of CFTR Cl^- currents by DHPs. Graphs report transepithelial Cl^- currents in FRT cells stimulated with forskolin (20 μM) followed by genistein or DHPs at the indicated concentrations. At the end of the experiments, CFTR currents were blocked with $CFTR_{inh}$-172 (10 μM). A, data from FRT cells expressing ΔPhe508-CFTR. Cells were preincubated 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.

Acknowledgments

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