The most common mutation (F508del) causing cystic fibrosis (CF) results in misfolding of the CF transmembrane conductance regulator (CFTR), leading to its degradation via the proteasome pathway. To study the mechanism of action of several pharmacological chaperones benzol[c]quinolizinium (MPB), we analyzed their effects on two CF mutations; F508del-CFTR and G622D-CFTR. The replacement of Gly622 by an aspartic acid (G622D) alters the trafficking and activity of the protein. G622D, similar to F508del, was functionally rescued by the glucosidase inhibitor miglustat but, unlike F508del, could not be rescued by MPB. A structure-activity relationship for F508del functional correction revealed the following profile: MPB-104-91-07-80 > 05 > 89 > 9-hydroxyphenanthrene = phenanthrene. Immunoprecipitation experiments on human airway epithelial F508del/F508del CF15 cells showed that MPB did not prevent the interaction of F508del-CFTR with heat shock protein (HSP)70, HSP90, or calnexin. Functional rescue of F508del-CFTR by MPB and miglustat was abolished by brefeldin A (BFA) but potentiated by thapsigargin (TG) and geldanamycin. The proteasome inhibitor MG132 potentiated the effect of miglustat but only modestly affected that of MPB. It is noteworthy that MPB inhibited proteasome activity in F508del-CFTR-expressing cells but did not directly affect the activity of purified 20S proteasome. With the mutant G622D-CFTR, MPB did not inhibit proteasome activity, as in mock-transfected cells. Inhibition of cellular degradation machinery by MPB is not only CFTR-dependent, but it also follows similar structure-activity relationship as demonstrated by functional correction. We conclude that G622D is a partial trafficking-deficient mutant with dysfunctional chloride channel activity, and that Gly622 is part of the putative site for interaction of MPB with CFTR, protecting the channel from proteasome-mediated degradation.

The retention of a misfolded protein in the endoplasmic reticulum (ER) is linked to the activity of the cell quality control system (Ellgaard and Helenius, 2003). This retention generally causes the absence of the client protein at its target site, preventing its physiological function and leading to abnormal cell homeostasis and functions. Indeed, defective protein trafficking has been recognized as an important mechanism for a growing number of inherited human diseases (Aridor and Hannan, 2000, 2002). However, in many cases, the trafficking-defective mutant protein retains residual function if it can be rescued to its final destination (Dalemans et al., 1991; Burrows et al., 2000). These trafficking-defective proteins can be classified as class 2 mutations. For example, most patients suffering from cystic fibrosis (CF) have the F508del mutation (Fig. 1A, top), a class 2 mutation (Welsh and Smith, 1993), on at least one CFTR allele, generating misfolding and retention in the endoplasmic reticulum, followed by substantial degradation of the mutated protein via the ubiquitin-26S proteasome pathway (Cheng et al., 1990; Kartner et al., 1992). However, when the trafficking defect is corrected by incubation at low temperature, for example corrected by incubation at low temperature, for example
Denning et al., 1992), CFTR, it displays an altered gating (Dalemans et al., 1991). Several other less frequent CF mutations such as G551D or G1349D are classified as class 3 because the corresponding proteins, although correctly located at the plasma membrane, have a dysfunctional regulation (see for a recent review, MacDonald et al., 2007). The G622D-CFTR mutant, in which the pathophysiology is unclear, has been provisionally classified as a class 3 missense mutation after its identification in CF patients (http://www.genet.sickkids.on.ca/cftr) (Vankeerberghen et al., 1998). Gly622 is located within the N terminus of the R domain (Fig. 1A, bottom). The G622D mutant forms a cAMP-regulated chloride channel with significantly lower Po than the wild-type channels (Vankeerberghen et al., 1998).

In previous studies, we identified benzo[c]quinolizinium (MPB) derivatives (several chemical structures are shown in Fig. 1B together with two phenanthrene derivatives) as activators of wild-type CFTR, and G551D, G1349D, and F508del mutants (Becq et al., 1999; Dormer et al., 2001a; Marivingt-Mounir et al., 2004; Melin et al., 2004). However, in most cases, the mechanism of pharmacological rescue is not clearly understood. In particular, numerous efforts aimed at developing small molecule pharmacotherapy for CF focused on identifying compounds that can either stabilize the tertiary structure of the F508del-CFTR protein or modify the interactions of the mutant protein with ER chaperones. By preventing these interactions, the newly synthesized, misfolded but functional F508del-CFTR protein might escape recognition by mechanisms responsible for its retention and ultimate degradation (Powell and Zeitlin, 2002). A limited number of molecules have been shown to restore partial function in F508del-CFTR mutant cells, including 4-phenylbutyrate (4-PBA; Buphenyl) (Rubenstein et al., 1997; Rubenstein and Zeitlin, 2000), curcumin (Egan et al., 2002, 2004; Norez et al., 2006a), CFTRcor-325 (Wang et al., 2006), and the α1,2-glucosidase inhibitor miglustat (Norez et al., 2006b).

In the present work, we performed a comparative analysis of the effect of several chemically diverse MPB and phenanthrene derivatives on two CFTR mutants, F508del and G622D. We identified the Gly622 amino acid as part of the putative site interfering with the correcting effect of MPB and showed that the rescue of F508del-CFTR by MPB is due to prevention of proteasomal degradation.
Materials and Methods

Cells. For this study, we used the human nasal airway epithelial cell line JME/CF15, derived from a CF patient homozygous for the F508del mutation (Jefferson et al., 1990), and COS-7 cells stably transfected with green fluorescent protein (GFP)-tagged CFTR vectors containing diisothiocyanate donoracceptor (Di-4-ANEPPS-672/380) and wild-type CFTR (F508del, or G622D-CFTR). The F508del and G622D mutations were created and introduced into pS65T/enhanced GFP-C1/wt-CFTR constructs as described previously (Melin et al., 2004). The culture conditions are as described previously (Jefferson et al., 1990; Melin et al., 2004; Norez et al., 2006a).

Immunoprecipitation and Western Blot Analysis. For lysates, total protein was quantified using the Bradford (Bradford, 1976) protein assay reagent (Bio-Rad S.A., Marnes-la-Coquette, France), and 50 μg of protein was loaded onto an SDS-polyacrylamide gel electrophoresis apparatus. For immunoprecipitation, CF15 cell lysates were incubated with monoclonal anti-CFTR antibody (2 μg, IgG2a clone 24-1; R&D Systems, Minneapolis, MN). Immunoblots were probed with monoclonal mouse anti-GFP antibody (2 μg/ml; Sigma-Aldrich, St. Louis, MO), polyclonal rabbit anti-calnexin (CNX) antibody (2 μg/ml, SPA-860; Assay Designs, Ann Arbor, MI), polyclonal rabbit anti-heat shock protein (HSP70) antibody (1:80,000, SPA-757; Assay Designs), or polyclonal rabbit anti-HSP90 antibody (1:250, SPA-771; Assay Designs). The protein levels were quantified by densitometry and expressed as a percentage of control, which was replaced by either wild-type or F508del-CFTR (Norez et al., 2006a).

Immunofluorescence Study. CF15 cells were incubated with monoclonal anti-human CFTR antibody (1:100, IgG1 clone 24-1; R&D Systems) overnight at 4°C. Cells were then incubated with the fluoProbes 488 (1:400; fluoProbes, Interchim, France) secondary antibody. Nuclei were stained in red with TO-PRO-3 iodide (Invitrogen, Carlsbad, CA) for 15 min at room temperature (1:1000 in Tris-buffered saline). Fluorescence was examined with a spectral confocal station FV 1000 installed on an inverted microscope IX-81 (Olympus, Tokyo, Japan). For more details, see Norez et al., 2006a.

Functional Analysis of CFTR Channel Activity. The wt, F508del-, and G622D-CFTR C1 channel activities were assayed by measuring the rate of iodide (125I-) efflux from CF15 cells and COS-7 cells as described previously (Melin et al., 2004; Norez et al., 2006a). Time-dependent rates of 125I efflux were calculated from the following: ln([125I]t1/[125I]t2) = k (t1-t2), where k = 1/2 t1 is the intracellular 125I at time t1, and t1 and t2, successive time points. Curves were constructed by plotting rates of 125I versus time. All comparisons were based on maximal values for the time-dependent rates (k), excluding the points used to establish the baseline (k = basal, min^-1) (for other details, see Norez et al., 2006a).

Proteasome Activity Assay. Proteasome enzymatic activity was measured as described by Canu et al. (2000) and per the manufacturer’s protocol (20S Proteasome Activity Assay Kit; Millipore Bioscience Research Reagents, Temecula, CA). In brief, this assay is based on the detection of the fluorophore 7-aminomethylcoumarin (AMC) after cleavage from the labeled substrate LLVY-AMC by the proteasome machinery. The free AMC fluorescence was quantified using a 380/460 nm filter set. The intracellular proteasome activity was detected in cells after 2 h of treatment with several correctors, whereas testing the inhibition of purified protease enzyme by these correctors was performed after incubation of the 20S proteasome-positive control for 30 min according to the assay instructions.

Statistics. Results are expressed as the mean ± S.E.M. of n observations. Sets of data were compared with a Student’s t test. Differences were considered statistically significant when p < 0.05. Significant differences were **, p < 0.05, ***, p < 0.01, and ****, p < 0.001. All statistical tests were performed using Prism 4.0 software for Windows (GraphPad Software Inc., San Diego, CA).

Chemicals. The following MPB derivatives have been prepared in our laboratory as described previously (Marvingt-Mounir et al., 2004; MPB-07, MPB-80, MPB-91, MPB-104, MPB-89, and MPB-05. All other chemicals were obtained from Sigma-Aldrich, with the exception of miglustat (obtained from Toronto Research Chemicals, Toronto, ON, Canada), forskolin (Fsk), and genistein (Gst) (both obtained from PKC Pharmaceuticals, Woburn, MA). TS-TM calix[4]arene was provided by Dr. R. Bridges (University of Chicago, Chicago, IL).

Results

MPB Compounds Rescue F508del-CFTR but Not G622D-CFTR. The plasmid construction (see Materials and Methods) allowed stable expression in COS-7 cells of GFP-tagged F508del-CFTR and GFP-tagged G622D-CFTR. We evaluated the function of CFTR mutants by a rapid, robust, and robotic cell-based assay using the iodide efflux technique (Marvingt-Mounir et al., 2004; Norez et al., 2004). In this assay, a rapid change in the rate of iodide efflux on stimulation was indicative of the presence of active CFTR at the plasma membrane. The transport activity of CFTR was stimulated by a cocktail of Fsk (10 μM) and Gst (30 μM) (Norez et al., 2006a). Figure 2A shows an example of CFTR-dependent iodide efflux in F508del-, G622D-, and wt-CFTR-expressing COS-7 cells. As expected, in F508del-CFTR-expressing cells, Fsk + Gst could not stimulate iodide efflux due to trafficking deficiency (Fig. 2A, black circles), as in mock-transfected cells (data not shown). For G622D-CFTR-expressing cells (Fig. 2A, black triangles), Fsk + Gst stimulated the iodide efflux (kpeak - kbasal = 0.046 ± 0.020 min^-1) to a level ~3-fold lower than that of wt-CFTR cells (Fig. 2A, black squares; kpeak - kbasal = 0.155 ± 0.009 min^-1). All of the effluxes stimulated by Fsk + Gst were inhibited by the CFTR inhibitor CFTRinh-172 (Ma et al., 2002) (data not shown). These results suggest that, although functional (in agreement with Vankeerberghen et al., 1998), G622D-expressing COS-7 cells have a reduced transport activity compared with wt-CFTR cells. This difference in activity can be explained by a dysfunctional activity, as reported previously (Vankeerberghen et al., 1998). An alternative explanation would be that the trafficking of G622D is abnormal. To test this hypothesis, we explored the effect of several trafficking correctors; MPB derivatives (Dormer et al., 2001a,b) and miglustat (Norez et al., 2006b). All cells maintained at 37°C were incubated for 2 h with culture medium supplemented with 100 μM of the given chemical compound. After the incubation period, CFTR-dependent effluxes were stimulated by the Fsk + Gst cocktail. Figure 2B presents typical iodide efflux responses, showing that in G622D-CFTR cells incubated with miglustat (black triangles), Fsk + Gst increased ~2-fold) iodide efflux compared to untreated cells (Fig. 2B, open squares; p < 0.001). However, with G622D-CFTR cells incubated with MPB-104 (Fig. 2B, black circles), the Fsk + Gst-stimulated iodide efflux was not different from control. Normalized iodide effluxes for numerous experiments are presented in Fig. 2C. Therefore, these results show a partial trafficking defect of G622D-CFTR that can be reversed by miglustat as with the F508del mutant. We also observed that miglustat by itself is not a direct activator of CFTR, because replacement of Fsk + Gst by miglustat failed to stimulate CFTR-dependent efflux (data not shown). Altogether, our observations indicate successful functional rescue of F508del-CFTR by MPB-91 and MPB-07 and also by MPB-104 and MPB-80 to a similar level than cells corrected by miglustat (Fig. 2C, open bars). It is surprising to note that the incubation of cells with any of the MPB correctors did not rescue G622D-CFTR (Fig. 2C, gray bars).
If G622D is a partial trafficking-deficient mutant, as our functional data suggest, then we should observe less band C forms of the mutant compared to wt-CFTR, indicating a reduced pool of mature proteins. Thus, we performed biochemical assays to compare the G622D-CFTR protein expression in the presence or absence of these compounds. Anti-GFP immunoblotting from COS-7 cells stably expressing GFP-tagged wt-CFTR was used as a control to reveal the two characteristic bands of the CFTR protein (Fig. 2D, lane 1). Band C corresponds to the mature, fully glycosylated CFTR that underwent Golgi complex, and band B corresponds to the immature, core-glycosylated CFTR in the ER (Cheng et al., 1990). Anti-GFP immunoblotting in COS-7 cells expressing G622D-CFTR (Fig. 2D, lane 2) shows a reduced amount of band C form but a much higher quantity of band B form for G622D versus wt proteins, confirming the partial trafficking defect of G622D. In COS-7 cells expressing G622D-CFTR incubated for 2 h with 100 μM miglustat, anti-GFP immunoblotting revealed an increased band C intensity (Fig. 2D, lane 3). Increasing the band C level by the CFTR corrector miglustat is in good agreement with our functional results. However, and contrary to F508del-CFTR rescued by MPB derivatives (Dormer et al., 2001a), the abnormal processing of G622D-CFTR cannot be rescued by MPB. Again, this is in good agreement with our functional data.

**Effect of MPB Correctors on the Endogenous F508del-CFTR Trafficking.** Because heterologous expression of F508del-CFTR can disturb the ER quality control (ERQC), we extended our study to evaluate the mechanism of action of MPB correctors on endogenous F508del-CFTR in the human airway epithelial CF15 cells. We first localized F508del-CFTR in CF15 cells before and after treatment with MPB. We found clear restriction of F508del proteins around the nucleus in untreated CF15 cells (Fig. 3A, a–d). After 2 h of treatment at 37°C with 100 μM MPB-91 (Fig. 3A, e–h) or MPB-104 (Fig. 3A, i–l), the cellular distribution of F508del-CFTR changed, with a new location at the plasma membrane and throughout the cells. The right panel (Fig. 3A, d, h, and l) is a merge of fluorescent images (Fig. 3A, b, f, and j) with the corresponding light micrograph (Fig. 3A, c, g, and k) showing the plasma membrane and cytosol location of F508del-CFTR proteins after MPB treatment in comparison to untreated cells.

We also conducted experiments using iodide efflux assay, as in COS-7 cells. Results presented in Fig. 3B show functional rescue with MPB-104, -91, -07, and -80 to the same level compared to cells corrected by miglustat. It is noteworthy that these results are similar to those obtained with COS-7 cells expressing GFP-F508del proteins (Fig. 2C). A partial rescue was also found with MPB-89 and -05 (~30% and ~50% of the miglustat correction, respectively) (see chemical structures in Fig. 1B). No rescue was obtained with either phenanthrene or 9-hydroxyphenanthrene (Fig. 3B), two MPB-related compounds lacking the quaternary ammonium (Fig. 1B). Thus, the minimal chemical structure for trafficking correction corresponds to the MPB-89 compound. We measured increasing potency with the hydroxyl-substituted derivative MPB-104 (~50% of the miglustat correction, respectively) (see chemical structures in Fig. 1B).
stimulated by Fsk + Gst was inhibited by diphenylamide-2-carboxylic acid (DPC), CFTRinh-172, and glibenclamide, but not by calixarene or DIDS as expected for CFTR (Norez et al., 2006a). Similar results were obtained after correction by MPB-07, MPB-80, and MPB-91 (data not shown). We then addressed several questions regarding the correcting effect of MPB. First, could an increase of the duration of incubation with the drug improve the iodide efflux response? It is interestingly to note that, as shown Fig. 3D, the response increased as a function of the duration of treatment to a maximum of 0.170 ± 0.005 min⁻¹ obtained after 4 h of MPB-104 treatment. This response then declined, although there was still detectable rescue after 6 h (Fig. 3D) but not after 12 h (data not shown). In a separate series of experiments, we incubated CF15 cells for 2 h at 37°C

Fig. 3. Effect of MPB correctors in CF15 cells. A, confocal imaging showing the localization of F508del-CFTR in CF15 cells in untreated cells (a–d) or after 2-h incubation with 100 μM MPB-91 (e–h) or 100 μM MPB-104 (i–l). d, h, and l are a merge of b, f, and j, respectively, with their corresponding light micrograph c, g, and k. Scale bars, 10 μm. F508del-CFTR is stained green, and nucleus is stained red. B, bar graph showing Fsk + Gst-induced iodide efflux response in untreated CF15 cells (denoted 37°C), in CF15 cells treated by miglustat or by MPB compounds (100 μM, 2 h). C, effect of chloride channel inhibitors on iodide efflux stimulated by Fsk + Gst from MPB-104-corrected cells (MPB-104, 100 μM, 2 h) with calixarene (100 nM), CFTRinh-172 (10 μM), DIDS (500 μM), DPC (500 μM), or glibenclamide (100 μM). Basal corresponds to unstimulated MPB-104-corrected cells. Data are expressed as a percentage of CFTR maximal activity. D, time dependence of MPB-104 correction. CF15 cells incubated for different times with MPB-104 (100 μM) and stimulated by Fsk + Gst. E, persistence of correction. CF15 cells were treated for 2 h with MPB-104, and F508del-CFTR activity was assayed at different times after washout. B, the number of experiments is noted on each bar graph. C and D, n = 4 for each condition. ***, p < 0.001, **, p < 0.01, and *, p < 0.05, ns, nonsignificant difference.
with MPB-104 and then washed out the drug and analyzed the Fsk + Gst iodide efflux response every 2 h for 12 h. Figure 3E shows that rescue of F508del-CFTR function was maximal during the first 2 h after compound washout, and then it progressively declined between 4 and 12 h. Taken together, these data demonstrated reversible rescue of endogenous F508del-CFTR function with MPB in a time-dependent manner. These results also show that MPB is able to rescue endogenous F508del-CFTR with the following order of potency: MPB-104 = -91 = -07 = -80 > -05 > -89, whereas phenanthrene and 9-hydroxyphenanthrene are not active.

**Interactions between F508del-CFTR and Molecular Chaperones in MPB-Corrected CF15 Cells.** To study a potential effect of MPB on molecular chaperones, we performed coimmunoprecipitation experiments using CF15 cells. We first immunoprecipitated F508del-CFTR complexes using polyclonal anti-CFTR antibody followed by anti-calnexin Western blotting (Fig. 4A). Two conditions were used as positive controls: CF15 lysate (Fig. 4A, lane 1) and untreated CF15 cells immunoprecipitation (Fig. 4A, lane 3). Nonimmune mouse IgG (Fig. 4A, lane 2) was used as a negative control. In untreated CF15 cells (Fig. 4A, lane 3), we detected the calnexin band complexed with F508del-CFTR. This interaction was not affected when cells were incubated at 27°C (Fig. 4A, lane 4) but was prevented by the corrector miglustat (Fig. 4A, lane 5). Immunoblotting with CF15 cells corrected either by MPB-104, -91, -80, or -07 detected a similar intensity band (Fig. 4A, lanes 6–9) of calnexin as in the untreated CF15 cells (Fig. 4A, lane 3). Densitometry analysis of several independent experiments confirmed these results (Fig. 4, right panels). In a similar method, we evaluated the effect of MPB derivatives on the interaction between F508del-CFTR and the molecular chaperones CNX, HSP70, and HSP90. Histograms show an absence of effect of MPB-104, -91, -80, or -07 on the F508del-CFTR/HSP70 (Fig. 4B) or F508del-CFTR/HSP90 complexes (Fig. 4C). To verify that variations in the CFTR expression level did not affect the amount of chaperone that was pulled down, we compared the mRNA quantity of CFTR by the quantitative reverse transcription-polymerase chain reaction technique. Whatever the MPB treatment, we observed no variation of CFTR mRNA (data not shown). Taken together, these results show that the rescue of F508del-CFTR by MPB correctors cannot be explained by perturbation of the physical interaction between the mutant channel and the molecular chaperones CNX, HSP70, or HSP90.

To further study the rescue pathway of F508del-CFTR in MPB-treated cells, we investigated the effect of MPB-104 in competition with several inhibitors of the biosynthetic pathway and of the ER quality machinery (see the schematic drawing in Fig. 5) using the iodide efflux technique. We observed inhibition of the effect of MPB-104 and miglustat by BFA, a vesicular ER/Golgi-intermediate compartment traffic inhibitor (denoted BFA in Fig. 5A). This result indicates that F508del-CFTR proteins follow a conventional trafficking pathway when rescued by MPB-104 and miglustat. The sarcoendoplasmic reticulum calcium ATPase pump inhibitor TG is a partial corrector of F508del-CFTR (Fig. 5B, hatched bar), as shown previously (Egan et al., 2002; Norez et al., 2006a), and is known to alter the F508del-CFTR/calnexin interaction (Norez et al., 2006a). In the presence of TG, we observed a significant inhibition of the effect of MPB-104 and miglustat by BFA, indicating that F508del-CFTR proteins follow a conventional trafficking pathway when rescued by MPB-104 and miglustat.
very significant (p < 0.001) potentiation of the F508del-CFTR rescue by MPB-104 (Fig. 5B, black bars) and a significant potentiation (p < 0.05) of the F508del-CFTR rescue by miglustat (Fig. 5B, open bars). The HSP90 inhibitor geldanamycin has been shown to stabilize F508del-CFTR by preventing the interaction between the chaperone and the mutant protein (Fuller and Cuthbert, 2000). We showed that MPB compounds do not inhibit the interaction between HSP90 and F508del-CFTR in CF15 cells (Fig. 4C). We measured the iodide efflux in CF15 cells incubated with MPB-104 and geldanamycin (Fig. 5C, black bars) and observed a significant potentiation (p < 0.001) of the F5k + Gst response. When cells were treated with miglustat and geldanamycin (Fig. 5C, white bars), the Fsk + Gst response was also potentiated compared to cells treated only with miglustat, but the level of potentiation for miglustat/geldanamycin was smaller (p < 0.05) than for MPB-104/geldanamycin. Moreover, we observed only a small increase (p < 0.05) of the Fsk + Gst response after treating cells with MPB-104 in the presence of MG132, a proteasome inhibitor (Fig. 5D, black bars). When cells were treated with miglustat and MG132, the Fsk + Gst response was also potentiated compared to cells treated only with miglustat (Fig. 5D, white bars), but the level of potentiation was increased (p < 0.01) for miglustat/MG132 compared to MPB-104/MG132. MG132 by itself was not able to rescue F508del-CFTR function (Fig. 5D, hatched bar). Similar results were obtained with other MPB compounds (data not shown). These results suggest that the mechanism of correction of MPB and miglustat are different.

**Effect of MPB Correctors on the Degradation Machinery.** In a previous study, Stratford et al. (2003) suggested that MPB-07 and MPB-91 are inhibitors of the first cytoplasmic domain degradation of F508del-CFTR using an in vitro biochemical assay. To determine whether this mechanism of action takes place in CF15 cells, we evaluated the effect of correctors on the proteasome activity of CF15 cells. The proteasome activity of CF15 cells corrected by MPB-104, -07, -91, or -80 was significantly reduced compared to uncorrected and miglustat-corrected cells (Fig. 6A). In CF15 cells, we observed approximately 30% inhibition after MPB treatment in comparison to MG132 and lactacystin, two known proteasome inhibitors (91 and 79% inhibition, respectively). On the contrary, we did not detect inhibition of the purified 20S proteasome activity by any of the MPB correctors (Fig. 6B), whereas lactacystin and MG132 caused 97 and 91% inhibition, respectively. Miglustat has no effect on the proteasome activity in CF15 cells (Fig. 6A) or on the purified 20S proteasome activity (Fig. 6B). The inhibition of the cellular proteasome by MPB correctors was not due to a toxic effect because no cellular toxicity was observed (data not shown) for any of the MPB compounds [toxicity evaluated on CF15 cells with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test as described previously (Norez et al., 2006a)].
The results presented above show that after treatment of CF cells by MPB correctors, the cell proteasome activity was reduced. Because this effect could be attributed to a nonspecific action of MPB, we also measured the proteasome activity in mock COS-7 cells (Fig. 7A) or COS-7 cells stably expressing F508del-CFTR (Fig. 7B) or G622D-CFTR (Fig. 7C). In mock-transfected COS-7 cells, the overall proteasome activity was fully inhibited by MG132 and lactacystin. On the contrary, with MPB derivatives, phenanthrene or the vehicle (dimethyl sulfoxide), inhibition could not be detected (Fig. 7A). In sharp contrast, in COS-7 cells expressing F508del-CFTR, we obtained \(-50\%\) inhibition of the proteasome activity by MPB treatment (100 \(\mu\)M, 2 h; Fig. 7B). Interestingly, we did not observe inhibition by phenanthrene, a non-F508del-CFTR corrector, and noted only 25% of proteasome inhibition in the presence of MPB-89, a partial F508del-CFTR corrector (see Fig. 3B). Therefore, these experiments revealed that inhibition of the cellular degradation machinery by MPB correctors is not only CFTR-dependent, but it also follows the same structure-activity relationship as demonstrated for functional correction, i.e., MPB-104-91-07-80 \(>\) 89 \(>\) phenanthrene. Finally, because we showed that MPB correctors are not effective on G622D-CFTR, we asked whether this mutation could also affect the MPB-mediated inhibition of the degradation machinery. To study this theory, the proteasome activity was also measured in COS-7 cells expressing G622D proteins. Our results show that neither MPB derivatives nor phenanthrene were able to inhibit the cell proteasome activity (Fig. 7C). These results are undistinguishable from those obtained in mock COS-7 cells (Fig. 7A). Altogether, these results demonstrate that MPB correctors inhibit the cell proteasome activity in a CFTR-dependent manner and suggest that the mutation G622D prevents this inhibition.

**Discussion**

The present experiments demonstrate the following: 1) MPB derivatives are pharmacological chaperones of F508del-CFTR but not of G622D-CFTR mutants; 2) MPB corrects the F508del-CFTR-trafficking defect via a specific structure-activity relationship; 3) MPB does not prevent the interaction of F508del-CFTR with the ER resident calnexin or cytosolic HSP70 and HSP90 molecular chaperones; 4) MPB correctors inhibit the proteasome activity only in CFTR-expressing cells and have no effect on the activity of purified 20S proteasome; and 5) the mutant G622D-CFTR prevents rescue of CFTR by MPB but also abolishes the inhibition of the proteasome by MPB derivatives. Taken as a whole, we propose a mechanism for correction of misprocessed proteins by the pharmacological chaperones MPB interacting with CFTR on the amino acid Gly622 to protect the channel from proteasome-mediated degradation.
Structural Determinants of MPB Compounds. In a previous study analyzing the effect of CFTR channel activators, we reported a structure-activity relationship of MPB compounds (Marivingt-Mounir et al., 2004), and we demonstrated that some of these derivatives were able to stimulate the channel activity of wt-, G551D-, G1349D-, G551D/G1349D-, and F508del-CFTR (Becq et al., 1999; Dormer et al., 2001a; Marivingt-Mounir et al., 2004; Melin et al., 2004). We determined that presence of the hydroxyl group associated with a chlorine atom, and that an alkyl chain in the MPB skeleton was more effective at activating CFTR (Marivingt-Mounir et al., 2004). For example, the product MPB-104 seems to be 100 times more potent than the parental compound MPB-07. In the present study, we analyzed the structure-activity relationship of several MPB and phenanthrene derivatives for their ability to rescue the abnormal trafficking of mutant CFTR proteins. The present results indicate that F508del-CFTR-dependent iodide efflux was rescued by MPB (in CF15 and COS-7 cells) to the same level as cells corrected by miglustat. In addition, a modest rescue was found with MPB-89 and MPB-05 (30–50% of the miglustat correction). No rescue of mutant CFTR was found with phenanthrene and 9-hydroxyphenanthrene, arguing that the quaternary ammonium is important for F508del-CFTR rescue. Thus, we hypothesize that the minimal conformation to partially rescue F508del trafficking corresponds to the structure of MPB-89, i.e., to the MPB skeleton (see structure in Fig. 1B). To increase its efficacy on F508del-CFTR rescue, further chemical substituents (Fig. 8A) are required, such as the 6-hydroxy (MPB-05, -07, -80, -91, and -104). In contrast, adding an alkyl chain (as in MPB-91 and MPB-104) has no apparent effect on the rescue efficacy (for example, MPB-80 and MPB-91 have similar efficacy), whereas this greatly increases the activation efficacy of MPB-91 or MPB-104 as an activator of CFTR-chloride channels (Marivingt-Mounir et al., 2004).

Although several CFTR potentiators and/or activators have been reported in the literature, MPB compounds seem to be distinguished by their ability to activate several forms of CFTR (Becq et al., 1999; Dormer et al., 2001a; Marivingt-Mounir et al., 2004; Melin et al., 2004) and to rescue the defective trafficking of endogenous F508del-CFTR in human cells.

Fig. 8. Structural basis for specificity and schematic drawing illustrating the proposed mechanism of action of correctors. A, summary of the structural basis for specificity and potency of MPB derivatives as correctors and activators of CFTR. A to C indicate the three cycles of the molecules and the carbon atoms (numbers 1–10) starting from the ammonium. The quaternary ammonium associated with chloride ion is required both for activation and correction. X and Y substitutions by halogen atoms (positions 7 or 10, X = Cl or F) or by hydroxyl (position 6, Y = OH) are important determinants as indicated. However, the nature of the chemical group R1 at position 5 is not important for correction, whereas it improves the potency for channel activation as reported elsewhere (Marivingt-Mounir et al., 2004). B and C, drawings illustrating the proposed mechanism of action of miglustat (B) and MPB (C) to rescue F508del-CFTR. Miglustat inhibits the interaction of the ER resident chaperone calnexin with F508del-CFTR leading to plasma membrane location of the mutant protein via a BFA-sensitive pathway. MPB, through a direct interaction with F508del-CFTR in the cytosol on (at least in part) the Gly622, prevents recognition and degradation of F508del-CFTR by the cell proteasome and also leads to plasma membrane location of the mutant protein via a BFA-sensitive pathway.
airway epithelial cells (this study; Dormer et al., 2001a,b). Because a significant number of human diseases involve defective traffic of misfolded glycoproteins, albeit partially functional (Aridor and Hannan, 2000, 2002; Ulloa-Aguirre et al., 2004), the use of new MPB congeners tailored to rescue cell class 2 CF mutation might prove to be beneficial for the management of CF and other diseases.

**Mechanism of F508del-CFTR Rescue by MPB Correctors.** Some of the pharmacological chaperones described so far, including miglustat (Fig. 5B), alter the recognition of the mutant protein by calnexin (Robert et al., 2005; Gong et al., 2006; Norez et al., 2006a,b). In all of these cases, the complex pharmacological chaperone/traffic-deficient protein seemed to be stabilized in an intermediate state in its folding path that more closely resembled the native state of the wild-type protein (Bernier et al., 2004). Thus, until now, preventing the calnexin interaction with the mutant protein in the ER has been regarded as a major mechanism of rescue. In this study, we show dramatic action of MPB on the release of F508del-CFTR from the ER by a calnexin-independent effect. Interaction between HSP90 and F508del-CFTR was unaffected by MPB, arguing against an effect of MPB derivatives on ERQC but more likely on endoplasmic reticulum-associated degradation (ERAD) machinery. Furthermore, Stratford et al. (2003) provide evidence that MPB-07 and MPB-91 protect a proteolytic cleavage site by direct binding to the first cytoplasmic domain of F508del-CFTR. MPB derivatives probably affect the trafficking of F508del-CFTR by interfering with CFTR architecture from the cytosol. Thus, we hypothesize that within the CFTR structure, occupation of a drug binding site by MPB may stabilize the conformation of F508del-CFTR protein, allowing the MPB/F508del-CFTR complex to escape the proteasome-degradation system reaching the plasma membrane (Fig. 8C).

ERAD has important consequences for protein folding, transport, and degradation (Meusser et al., 2005), and it is a central element of the secretory pathway. A direct inhibition of the proteasome with lactacystin and MG132 did not promote F508del-CFTR to the cell surface (Jensen et al., 1995; Ward et al., 1995). We also found that MG132 was unable to rescue F508del-CFTR in CF15 cells after incubation and iodide efflux assay. It is interesting to note that deoxyxypquin, which competitively inhibits peptide binding to HSP70, leads to a restoration of CFTR function in cells expressing F508del-CFTR (Jiang et al., 1998). Moreover, Rubenstein and Zeitzlin (2000) demonstrated that decreased heat shock cognate 70 (HSC70) expression after 4-PBA treatment leads to improved F508del-CFTR trafficking. A recent study reported that a proteasome inhibition with bortezomib, which induces HSP70 and down-regulates valosin-containing protein expression, partially rescued mature F508del-CFTR in IB3-1 cells and human CF cells (Vij et al., 2006). These results are consistent with our data, demonstrating that MPB correctors prevent the recognition of F508del-CFTR by the proteasome machinery and allow restoration of F508del-CFTR to the cell surface via a BFA-sensitive pathway (Fig. 8C). Therefore, this study and others (Jiang et al., 1998; Rubenstein and Zeitzlin, 2000; Vij et al., 2006) provide evidence that ERAD is a potential target to rescue defective F508del-CFTR trafficking.

**Acknowledgments.** We thank Nathalie Bizard for cell culture, Fabrice Antigny for reverse transcription-polymerase chain experiments, and Elise Mok for critical reading of the manuscript.

**References**


Bernier RL, McNeilly CM, Morrisey ER, Pereira MM, Doull LJ, Beq F, Mettey Y, Vierfont JM, and McPherson MA (2001b) Localization of wild-type and DeltaF508-CFTR in IB3-1 cells and human CF cells (Vij et al., 2006). These results are consistent with our data, demonstrating that MPB-07 and MPB-91 protect a proteolytic cleavage site by direct binding to the first cytoplasmic domain of F508del-CFTR. MPB derivatives probably affect the trafficking of F508del-CFTR by interfering with CFTR architecture from the cytosol. Thus, we hypothesize that within the CFTR structure, occupation of a drug binding site by MPB may stabilize the conformation of F508del-CFTR protein, allowing the MPB/F508del-CFTR complex to escape the proteasome-degradation system reaching the plasma membrane (Fig. 8C). Therefore, this study and others (Jiang et al., 1998; Rubenstein and Zeitzlin, 2000; Vij et al., 2006) provide evidence that ERAD is a potential target to rescue defective F508del-CFTR trafficking.

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