ABSTRACT
The cystic fibrosis transmembrane conductance regulator (CFTR) represents the main Cl⁻ channel in the apical membrane of epithelial cells for cAMP-dependent Cl⁻ secretion. Here we report on the synthesis and screening of a small library of nontoxic α-aminoazaheterocycle-methylglyoxal adducts, inhibitors of wild-type (WT) CFTR and G551D-, G1349D-, and F508del-CFTR Cl⁻ channels. In whole-cell patch-clamp experiments of Chinese hamster ovary (CHO) cells expressing WT-CFTR, we recorded rapid and reversible inhibition of forskolin-activated CFTR currents in the presence of the adducts 5a and 8a,b at 10 pM concentrations. Using iodide efflux experiments, we compared compound-dependent inhibition of CFTR with glibenclamide (IC₅₀ = 14.7 μM), 3-[(3-trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone (CFTRinh-172) (IC₅₀ = 1.2 μM), and α-aminoazaheterocycle-methylglyoxal adducts and identified compounds 5a (IC₅₀ = 71 pm), 8a,b (IC₅₀ = 2.5 nM), and 7a,b (IC₅₀ = 3.4 nM) as the most potent inhibitors of WT-CFTR channels. Similar ranges of inhibition were also found when these compounds were evaluated on CHO channels with the cystic fibrosis mutations F508del (in temperature-corrected human airway epithelial F508del/F508del CF15 cells)-, G551D-, and G1349D-CFTR (expressed in CHO and COS-7 cells). No effect of compound 5a was detected on the volume-regulated or calcium-regulated iodide efflux. Picomolar inhibition of WT-CFTR with adduct 5a was also found using a 6-methoxy-N-(3-sulfopropyl)-quinolinium fluorescent probe applied to the human tracheobronchial epithelial cell line 16HBE14o. Finally, we found comparable inhibition by 5a or by CFTRinh-172 of forskolin-dependent short-circuit currents in mouse colon. To the best of our knowledge, these new nontoxic α-aminoazaheterocycle-methylglyoxal adducts represent the most potent compounds reported to inhibit CFTR chloride channels.

Identifying potent and specific small molecules able to modulate Cl⁻ channels is crucial for an understanding of their physiological role in cellular functions and also for the development of molecules of therapeutic interest for human diseases caused by mutations of these proteins (cystic fibrosis, myotonia, Barter’s syndrome, Dent’s disease, and osteopetrosis) or alternatively due to loss of regulation of their channel activity (secretory diarrheas and cholera) (Nilius and Droogmans, 2003). Among the Cl⁻ channels expressed by mammalian cells, the CFTR protein is a CAMP-regulated Cl⁻ channel, which normally mediates Cl⁻ transepithelial transport in epithelia and is encoded by the CFTR gene and mutated in the genetic disease cystic fibrosis (CF) (Riordan et
2-Amino-3-hydroxypripridine adducts 3a,b (75.25, respectively). 77 mg (0.30 mmol, 73% yield) obtained from 46 mg (0.41 mmol) of 2-amino-3-hydroxypripridine in 0.5 ml of 40% aqueous solution of methylglyoxal (2.6 mmol) and 4.5 ml of water after 4 h at 50°C. m.p.: 142°C (decomposition). 1H NMR (200 MHz, D2O, pH 7 phosphate buffer): 7.32 (1H, m, CH); 6.64 (2H, m, CH); 4.36/3.99 (1H, s, CH); 1.61/1.58 (3H, bs, CH3); 1.49 (3H, s, CH3). 13C NMR (50 MHz, D2O, pH 7 phosphate buffer): 177.9 (COO); 147.7 (Cpy); 171.7 (CH2); 116.7 (CH2); 113.9/113.7 (CH2); 87.6/89.2 (Cpy); 73.1 (Cpy); 70.3 (CH); 61.2 (CO, 3H, 28 (CH3)); 22.4 (CH3). MS (DCI NH3/subssobutane): m/z 255 ([M+H]+). Analysis calculated for C19H15N5O10: 0.1% H2O: C, 49.52; H, 6.71. Found: C, 49.50; H, 6.47. UV absorption (H2O): λmax = 278 nm (9000).

1-Aminoisoquinoline 8a,b (60-40, respectively). The mixture of adducts was precipitated from the reaction mixture. After filtration, it was washed with water and 2.065 g (7.2 mmol, 82% yield) of diethyl ether obtained from 1.00 g (8.8 mmol) of 1-aminoisoquinoline in 11 ml of 40% aqueous solution of methylglyoxal (70 mmol) and 11 ml of water after 14 h at 50°C. m.p.: 179°C (decomposition). 1H NMR (200 MHz, D2O, pH 7 phosphate buffer): 8.32/8.28 (1H, d, CHAr); 7.9–7.5 (4H; m; CHAr); 7.13/7.09 (1H, d, CHAr); 4.44/4.11 (1H, s, CH); 1.71 (3H, s, CH3); 1.63 (3H, s, CH3). 13C NMR (50 MHz, D2O, pH 7 phosphate buffer): 177.1/175.7 (COO); 151.5/148.5 (Cpy); 137.3/135.3 (Cpy); 134.3 (CH); 129.0 (CH); 127.6 (CH); 124.4 (CH); 123.9 (CH); 117.5 (Cpy); 113.1 (CH); 87.8 (Cpy); 70.3 (CH); 63.1 (Cpy); 25/125.0 (CH2); 22.4 (CH3). MS (DCI NH3/subssobutane): m/z 289 ([M+H]+). Analysis calculated for C19H15N5O10: 0.1% H2O: C, 61.53; H, 5.68; N, 9.57. Found: C, 61.50; H, 5.73; N, 9.52. m.p.: 179°C (decomposition). 1H NMR (200 MHz, D2O, pH 7 phosphate buffer): 8.32/8.28 (1H, d, CHAr); 7.9–7.5 (4H; m; CHAr); 7.13/7.09 (1H, d, CHAr); 4.44/4.11 (1H, s, CH); 1.71 (3H, s, CH3); 1.63 (3H, s, CH3). 13C NMR (50 MHz, D2O, pH 7 phosphate buffer): 177.1/175.7 (COO); 151.5/151.5 (Cpy); 137.3/135.3 (Cpy); 134.3 (CH); 129.0 (CH); 127.6 (CH); 124.4 (CH); 123.9 (CH); 117.5 (Cpy); 113.1 (CH); 87.8 (Cpy); 70.3 (CH); 63.1 (Cpy); 25/125.0 (CH2); 22.4 (CH3). MS (DCI NH3/subssobutane): m/z 289 ([M+H]+). Analysis calculated for C19H15N5O10: 0.1% H2O: C, 61.53; H, 5.68; N, 9.57. Found: C, 61.50; H, 5.73; N, 9.52. UV absorption (H2O): λmax = 335 (5400) and 245 nm (12,000).

Other Chemicals. The CFTR chloride channel activator MBP-91 was prepared as described previously (Marvingt-Mounir et al., 2004). CFTRinh-172 (Ma et al., 2002) and forskolin were purchased from Calbiochem (VWR International, Fontenay/bois, France) and LC Laboratories (a division of PKC Pharmaceuticals, Inc., Woburn, MA), respectively. All other chemicals were from Sigma-Aldrich. All methylglyoxal adducts were dissolved in H2O. Forskolin, genistein, benzoquinolizinium, glibenclamide, and CFTRinh-172 were dissolved in dimethyl sulfoxide (DMSO) (final concentration = 0.1%). Currents were not altered by DMSO alone.

Cell Culture. All cell lines were grown under standard culture conditions, as follows. CHO cells stably transfected with pNUT vector alone (mock-COH) or containing wild-type CFTR (WT-CFTR-COH) or the mutant G551D-CFTR were provided by J. R. Rioran.
and X. B. Chang (Scottsdale, AZ) (Tabcharani et al., 1991; Beecq et al., 1994, 1999). They were maintained at 37°C in 5% CO₂ in a minimal essential medium-Glutamax containing 7% fetal bovine serum (FBS), 50 IU/ml penicillin and 50 μg/ml streptomycin, and methotrexate for cell selection (WT-CFTR-CHO: 100 μM, G535D-CF: 20 μM) (Tabcharani et al., 1991; Beecq et al., 1994). The G1349D mutation was created by site-directed mutagenesis as described previously (Melin et al., 2004) and transiently expressed in COS-7 cells, 12 to 24 h after seeding, using cationic lipids jetPEI; Qiagen Inc., Carlsbad, CA) with 1 μg/ml of plasmid. Media were refreshed 24 h post-transfection. COS-7 cells were cultured at 37°C in 5% CO₂ as described previously (Melin et al., 2004) and used 72 h after transfection for iodide efflux experiments. The human pulmonary epithelial cell line Calu-3 (American Type Culture Collection, Manassas, VA) (Shen et al., 1994) was maintained at 37°C in 5% CO₂ in Dulbecco’s modified Eagle’s medium-Ham’s F-12 (1:1) nutritive mix supplemented by 10% FBS and 100 μg/ml streptomycin, 5 μg/ml insulin, 5.5 μM epinephrine, 180 μM adenine, 1.64 mM epidermal growth factor, 2 mM 3,5,3’-triiodo-L-thyronine sodium salt and 1.1 μM hydrocortisone. To record F508del-CFTR activity, JME/CF15 cells were exposed for 24 h at 27°C to allow functional membrane expression of F508del proteins. F508del-CFTR activity, JME/CF15 cells were exposed for 24 h at 27°C to allow functional membrane expression of F508del proteins (Denning et al., 1992; Norez et al., 2006). The human tracheobronchial epithelial cell line, 16HBE14o− (Jefferson et al., 1991; Melin et al., 2004) and transiently expressed in COS-7 cells, was obtained from the generous gift of Professor D. C. Gruenert (California Pacific Medical Center Research Institute, University of California, San Francisco, CA). Cells were grown in Eagle’s minimal essential medium with Earle’s salt supplemented with 10% FBS, l-glutamine, and penicillin-streptomycin at 37°C under 5% CO₂. The cell culture surface was coated with LHC basal medium (Biosource International, Camarillo, CA) supplemented with 10% bovine serum albumin, collagen (Vitrogen 100; Cohesion Technologies, Palo Alto, CA) and human fibronectin. All culture media and antibiotics were from Gibco BRL (now known as Invitrogen, Cergy-Pontoise, France). FBS was from PerbioScience (Brebieres, France). Hormones and growth factors were from Sigma-Aldrich.

Whole-Cell Patch-Clamp Recordings. Whole-cell patch clamp experiments were performed on WT-CFTR-CHO cells at room temperature. Ionic currents were measured in the broken-patch, whole-cell configuration of the patch-clamp method using an EPC-7 amplifier (List Electronic, Darmstadt, Germany). The holding potential was −40 mV in all whole-cell experiments. Current/voltage (I-V) relationships were built by clamping the membrane potential to −40 mV and by pulses from −100 mV to +100 mV in 20 mV increments. Pipettes were prepared by pulling borosilicate glass capillary tubes (GCL150-TF10; Clark Electromedical Inc., Reading, UK) using a two-step vertical puller (Narishige, Tokyo, Japan). They were connected to the head stage of the patch-clamp amplifier through an Ag-AgCl pellet (pipette resistance of 3–5 MΩ). Pipette capacitance was electronically compensated for in the cell-attached mode. Membrane capacitance and series resistances were measured in the whole-cell mode by fitting capacitance currents, obtained in response to hyperpolarization of 10 mV, with a first-order exponential and by integrating the surface of the capacitance current. Voltage-clamp signals, allowing the membrane potential to be held at different values, were recorded via a microcomputer equipped with an analog/digital-analog/analogue conversion board (Digidata 1200 interface; Molecular Devices, Sunnyvale, CA). Results were analyzed with pCLAMP software (version 9.2; Axon Instruments). The external solution contained 113 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 3 mM MgATP (ex temporary), and 10 mM TES (titrated with CsOH to pH 7.2). The osmolarity was 285 ± 5 mOsmol. The pipette solution was always hypotonic (with respect to the bath solution) to prevent cell swelling and activation of the volume-sensitive chloride channels. Time course experiments were performed by clamping the membrane potential from −40 mV to +40 mV. The calculated chemical equilibrium potential for chloride (ECl−) is −42 mV. All currents traces shown are single traces.

Iodide Efflux. Screening of small molecules and concentration-response curves were determined by measuring the rate of iodide (125I) efflux with a high-capacity robotic system (MultiProbe II EXT; PerkinElmer Life and Analytical Sciences, Courtabeuf, France) adapted to the determination of iodide efflux as described previously (Marvingt-Mounir et al., 2004; Norez et al., 2004). Our protocol of screening is as follows. CHO, COS-7, Calu-3, and CF15 cells were cultured in multiwell plates and incubated at 37°C in Krebs’ solution containing 1 μM KI and 1 μCi of Na125I/ml (PerkinElmer Life and Analytical Sciences, Boston, MA) during 30 min (CHO and COS-7 cells) or 1 h (Calu-3 and CF15 cells) to permit the 125I to reach equilibrium. The first three aliquots were used to establish a stable baseline in cold Krebs’ buffer (from t₀ to t₂). A medium containing the appropriate drug was then used for the remaining aliquots from t₂ to tₙ. Residual radioactivity was extracted at the end of the experiment with a mixture of 0.1 N NaOH and 0.1% SDS and determined using a gamma counter (Cobra II; PerkinElmer Life and Analytical Sciences). The fraction of initial intracellular 125I lost during each time point was determined, and time-dependent rates (k = peak rate, per minute) of 125I efflux were calculated from the following equation: k = ln125I(t₂)/125I(t₀/t₂) t (t₂ − t₀), where 125I is the intracellular 125I at time t, and t₁ and t₂ are successive time points (Norez et al., 2004). Relative rates were calculated as kpeak − kbasal (per minute), i.e., the maximal value for the time-dependent rate (kpeak, per minute) excluding the third point used to establish the baseline (kbasal, per minute). Concentration-dependent activation curves were constructed as a percentage of maximal activation (set at 100%) transformed from the calculated relative rates. CFTR-dependent iodide efflux was stimulated either by forskolin (WT-CFTR) or by a cocktail containing forskolin with genistein (F508del- and G535D-CFTR) or with the benz[c]quinolizinium derivative MPB-91 (G1349D-CFTR). Other details have been described elsewhere (Marvingt-Mounir et al., 2004; Melin et al., 2004).

SPQ Fluorescence Assay. In 16HBE14o− cells, CFTR activity was assessed using the halide-sensitive fluorescent probe SPQ (Molecular Probes, Leiden, The Netherlands). Cells were loaded with intracellular SPQ dye by incubation in Ca²⁺-free hypotonic (50% dilution) medium containing 10 mM SPQ for 15 min at 37°C. Coverslips were mounted on the stage of an inverted microscope (Nikon, Diaphot; Nikon, Tokyo, Japan) equipped for fluorescence and illuminated at 360 nm. The emitted light was collected at 456 ± 33 nm by a high-resolution image intensifier coupled to a video camera (Extended ISIS camera system; Photonic Science, Roberts-bridge, UK) connected to a digital image processing board controlled by FLUO software (Imstar S.A., Paris, France). Cells were maintained at 37°C and continuously superfused with an extracellular solution containing 145 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES, and 5 mM glucose, pH 7.4. A microperfusion system allowed local application and rapid change of the different experimental media. Iodide (I⁻)− and nitrate (NO₃−)-containing media were identical to the extracellular solution except that I⁻ and NO₃− replaced Cl⁻ as the dominant extracellular anions. All extracellular media also contained 10 μM bumetanide to inhibit the Cl⁻/Na⁺/K⁺ cotransporter. Single-cell fluorescence intensity was measured from digital image processing and displayed against time. Fluorescence intensity was standardized according to the equation ΔF = (Fmax − Frest) × 100, where Fmax is the relative fluorescence and Frest is the fluorescence intensity measured in presence of I⁻. The membrane permeability of the halide was determined as the rate of SPQ dequenching upon per-
fusion with nitrates. At least three successive data points were collected immediately after the NO$_3^-$-containing medium application and then fitted using linear regression analysis. The slope of the straight line reflecting the membrane permeability to halides (noted as p in Fig. 7 and measured per minute) was used as an index of CFTR activity (Leblais et al., 1999).

**Short-Circuit Current Measurements.** Experiments were carried out on the colon epithelium of wild-type (Cftr$^{-/-}$) mice (B6 129-CFTRtm1Unc) obtained from Centre National de la Recherche Scientifique-Center de Distribution, de Typage et Archivage Animal (Orléans, France). Animals were killed by cervical dislocation, a procedure approved by the local animal ethics committee of the University of Poitiers. One animal per experiment was used (n is the number of animals). The ascending colon was removed and prepared as described previously (Noel et al., 2006). The epithelium was mounted in a vertical Ussing chamber (Easymount, 0.166 cm$^2$ surface area; Physiologic Instruments, San Diego, CA). Luminal and serosal sides were bathed at 37°C with a nutrient buffer containing 120 mM NaCl, 1.2 mM CaCl$_2$, 1.2 mM MgCl$_2$, 0.8 mM K$_2$HPO$_4$, 3.3 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, and 10 mM D-glucose (pH 7.4). They were connected to the preamplifier head stage of an amplifier (Physiologic Instruments). Data were collected with the Acquire and Analyze package software (Physiologic Instruments). All experiments were carried out in the presence of amiloride (500 µM) in apical solution to prevent sodium transport. Other details have been described previously (Noel et al., 2006).

**Cytotoxicity Assay.** The cytotoxicity of CFTR inhibitors was assessed by measuring the cellular dehydrogenase activity using the water-soluble tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is converted to purple formazan. For the determination of growth rate and the cytotoxicity assay, CHO cells were grown to confluence in 96-well plates at 37°C. After incubation with the test compound for 2 to 24 h, 0.5 µg/ml MTT was added to each well. After a 4-h incubation, culture medium was removed, and 100 µl of DMSO was added to solubilize the blue formazan product. The optical density at 595 nM was quantified with a microplate photometer (Packard BioScience, Meriden, CT) and compared with the control value without drug (100% cell viability, noted as ctrl). Results are means ± S.E.M. for n = 4 separate determinations.

**Statistics.** All statistical tests were performed using GraphPad Prism (version 4.0 for Windows; GraphPad Software Inc., San Diego, CA). All results are expressed as means ± S.E.M. of n observations. Sets of data were compared with either an analysis of variance or

Fig. 1. Reaction between methylglyoxal 1 and an aromatic α-aminoazaheterocycle. a and b indicate the major and minor isomers, respectively.

Fig. 2. Structure of the methylglyoxal adducts, glibenclamide and CFTRinh-172. Adducts 2 to 4, 7, and 8; one enantiomer present in the obtained racemic mixture a or b is drawn; adducts 5 and 6: one diastereomer present in the obtained 50:50 mixture of two diastereomers a or b is drawn.
Student's $t$ test. Differences were considered statistically significant from the control when $P < 0.05$. Dose-response curves were compared using a post-test after two-way analysis of variance applying the Bonferroni correction. The half-maximal effective concentration for inhibition ($IC_{50}$) was calculated after nonlinear regression of the data.

**Results**

Synthesis of Novel $\alpha$-Aminoazaheterocycle-Methylglyoxal Adducts. Previously, we had reported a new reaction between methylglyoxal and an aromatic $\alpha$-aminoazaheterocycle such as 2-aminopyridine or adenine, leading in water to heterocycles of a new class (Routaboul et al., 2002). Condensation in water of two molecules of methylglyoxal on the heterocycle results in the formation in good yields of a new six-membered ring carrying a carboxylate function, two hydroxyl and two methyl groups (Fig. 1). Four isomers were formed and isolated in the form of two diastereomeric mixtures of enantiomers, the major enantiomers $a$ and the minor ones $b$. The enantiomers $2a$ and their isomers $2b$ obtained from 2-aminopyridine and the corresponding adenine adducts $4a$ have been characterized by X-ray crystallography (Fig. 2) (Routaboul et al., 2002). After the reaction and removal of methylglyoxal in excess, the isomers $a$ and $b$ could be detected by NMR spectrometry in a ratio of 60:40. Then, these very polar derivatives were isolated after indifferent purification by chromatography on silica gel and C18 reverse phase. The reaction has been observed with different adenine derivatives, with the adenine nucleosides and with poly(A) and the cytosine base. From 2'-deoxyadenosine or adenosine, because of the chirality of the sugar in the starting nucleosides, the 50:50 mixture of two diastereomers $a$ and the corresponding 50:50 mixture of diastereomers $b$ were obtained (adducts 5 and 6 from 2'-deoxyadenosine and adenosine, respectively, Fig. 2). Both diastereomers $a$ and $b$ could be detected by NMR spectrometry but could not be separated (Routaboul et al., 2002). To modulate the lipophily of the adducts, new methylglyoxal adducts $3a,b$, $7a,b$, and $8a,b$ were prepared. The adducts $3a,b$, which were more hydrophilic than adducts $2a,b$, were obtained from 2-amino-3-hydroxypyridine in a 73% yield (75:25 ratio, respectively). The adducts $7a,b$ (60:40 ratio) were synthesized in a 74% yield from 1-propylcytosine prepared in three steps from cytosine: 1) protection of the 4-amino group by acetylation with acetic anhydride in pyridine; 2) alkylation with 1-bromopropane in the presence of potassium carbonate; and 3) deprotection by treatment with methanolic ammonia (58% overall yield). The adducts $8a,b$ (60:40 ratio) were prepared from 1-aminoisoquinoline in 82% yield. During heating of the aminoazaheterocycle with methylglyoxal in water, the adducts $8a,b$ precipitated as a result of their low solubility in water.

Evaluation of the Methylglyoxal Adducts on the Activity of CFTR Chloride Channels. We have developed a simple and robust robotic high throughput screening assay to detect small molecules on Cl$^-$ channel activity (activator or inhibitor). The assay has been adapted for the radiotracer flux

![Fig. 3. Screening of $\alpha$-aminoazaheterocycle-methylglyoxal adducts. A. effect on iodide efflux in WT-CFTR-CHO cells. All compounds were tested at 1 $\mu$M ($n = 4$) in presence of 1 $\mu$M Fsk. The corresponding effect was normalized to that of 1 $\mu$M Fsk (100%). Inset, cytotoxicity expressed as a percentage of cell viability of WT-CFTR-CHO cells incubated with adducts $5a$ with variable times of incubation and concentrations as indicated below each bar. B, typical iodide efflux experiments showing concentration-response time-dependent curves for adducts $5a$ and $8a,b$ in WT-CFTR-CHO cells in the presence of 1 $\mu$M Fsk ($n = 4$ for each data point). C, concentration-response relationships for adducts $3a,b$, $4a,b$, $5a$, $7a,b$, and $8a,b$ and $\text{CFTR}_{\text{inhib}}$ on iodide efflux in WT-CFTR-CHO cells ($n = 4$ for each concentration). All data are means ± S.E.M. **, $P < 0.01$; ***, $P < 0.001$. ns, non significant.](https://jpet.aspetjournals.org/doi/abs/10.1124/jpet.1027.101207)
method and applied to various epithelial and nonepithelial cells
(Marivating-Mounir et al., 2004; Melin et al., 2004; Norez et al.,
2004). The present study was undertaken on the following
cells: CHO cells overexpressing WT-CFTR or mutated G551D-CFTR
and COS-7 cells expressing G1349D-CFTR (both G551D and
G1349D are class III CF mutations); two human airway epithelial
cells, Calu-3 and 16HBE14o–, expressing endogenous WT-
CFTR; and the human airway epithelial cells JME/CF15, endo-
genously expressing F508del-CFTR (class II CF mutation). A
first round of screening of α-aminoazaheterocycle-methyl-
glyoxal adducts was performed at a 1 μM concentration. For
WT-CFTR-expressing cells, we used the adenylate cyclase acti-
(27°C. Experiments were performed in the presence of 1
vator forskolin (Fsk, 1 μM) to stimulate CFTR channel activity.
The effects of the previously described adducts (Routaboul et
al., 2002) were first evaluated from the mixtures of adducts
5a and 6a by NMR Spectrometry) -deoxyadenosine and adenosine ad-
ducts was performed at a 1 μM concentration. For
WT-CFTR-expressing cells, we used the adenylate cyclase acti-
visor forskolin (Fsk, 1 μM) to stimulate CFTR channel activity.
The effects of the previously described adducts (Routaboul et
al., 2002) were first evaluated from the mixtures of adducts a
and b for 2-aminopyridine and adenine derivatives 2 and 4,
respectively, and from 2'-deoxyadenosine and adenosine ad-
ducts 5a and 6a, respectively (Fig. 3A). Significant inhibition
of forskolin-activated CFTR-dependent efflux was observed for
the 3, 4, 5, 7, and 8 adducts. However, the 6a,b adenosine and
2-aminopyridine adducts did not inhibit CFTR channels (60:40
mixture 2a,b, respectively, and also, separately, adducts 2a and
2b as shown in Fig. 3A). Before extensive analysis of the inhibi-
tion of CFTR by these new methylglyoxal adducts, the cyto-
toxicity of CFTR inhibitors has been assessed by measuring
Cellular dehydrogenase activity using the water-soluble tetra-
zolium salt MTT (see Materials and Methods). Importantly,
no cytotoxicity was detected with any of the α-aminoazahetero-
cycle-methylglyoxal adducts after a 2- or 24-h incubation of
CHO cells. An example is provided Fig. 3A (inset) for compound
5a evaluated on CHO cells incubated with the compound for 2
or 24 h at different concentrations. Therefore, these experi-
ments identified novel nontoxic inhibitors of CFTR-dependent
iodide efflux.

New α-Aminoazaheterocycle-Methylglyoxal Adducts Have Picomolar and Nanomolar Efficacy as CFTR Inhibitors. The inhibition of forskolin-dependent iodide efflux by α-aminoazaheterocycle-methylglyoxal adducts was con-

<table>
<thead>
<tr>
<th>Compounds (Ratio a,b Determined by NMR Spectrometry)</th>
<th>Endogenous CFTR</th>
<th>Heterologous Expression of CFTR</th>
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<tr>
<td></td>
<td>WT-CFTR</td>
<td>F508del-CFTR</td>
</tr>
<tr>
<td>Glibenclamide</td>
<td>11.7 ± 1.1 μM</td>
<td>11.8 ± 1.1 μM</td>
</tr>
<tr>
<td>CFTRinh-172</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>5a</td>
<td>93.3 ± 1.3 μM</td>
<td>67.3 ± 1.3 μM</td>
</tr>
<tr>
<td>5a,b (60:40)</td>
<td>15.7 ± 1.1 nM</td>
<td>8.7 ± 1.2 nM</td>
</tr>
<tr>
<td>7a,b (60:40)</td>
<td>2.3 ± 1.5 nM</td>
<td>6.3 ± 2.1 nM</td>
</tr>
<tr>
<td>3a,b (75:25)</td>
<td>11.2 ± 1.6 μM</td>
<td>7.0 ± 1.4 μM</td>
</tr>
<tr>
<td>4a,b (60:40)</td>
<td>11.9 ± 1.7 μM</td>
<td>6.0 ± 3.3 μM</td>
</tr>
</tbody>
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N.D., complete dose-response curve was not determined, but the inhibition for each cell type with 10 μM concentrations of inhibitors was confirmed.

![Fig. 4](https://via.placeholder.com/150)

**Fig. 4.** Inhibition of CFTR chloride channels by 5a in whole-cell patch-clamp experiments. A, representative original chloride currents recorded in CHO cells expressing WT-CFTR, in the presence of 1 μM Fsk (top traces), in the presence of 10 μM Fsk (middle traces), and Fsk + 10 μM 5a (bottom traces). B, corresponding current density-voltage relationships for CFTR currents before (basal), after application of Fsk, and after application of 5a (n = 7), C, representative time course of WT-CFTR currents (between −40 and +40 mV) showing the kinetics of inhibition by 10 μM 5a and the reversibility of inhibition after a 20-min washout. The wash period is indicated.
concentration-dependent as shown, for example, for adducts 5a and 8a,b (Fig. 3B). Thus, the IC50 was determined for the adducts 3a,b, 4a,b, 5a, 7a,b, and 8a,b (Fig. 3C) and compared with those for glibenclamide and CFTRinh-172, both used as reference inhibitors (see Fig. 2 for chemical structures). With iodide efflux experiments performed on WT-CFTR-CHO cells, we measured low micromolar IC50 values of 4.4 ± 1.3 and 5.7 ± 1.3 μM for 3a,b and 4a,b adducts, respectively, and much lower values of 71.0 ± 1.2 nM, 2.5 ± 1.7 nM for 5a, 7a,b, and 8a,b adducts, respectively (Fig. 3C; Table 1). For glibenclamide, we determined an IC50 of 14.7 ± 1.3 μM (Table 1) and for CFTRinh-172 an IC50 of 1.2 ± 0.9 μM (Fig. 3C; Table 1). Thus, the main product of the reaction between 2-deoxyadenosine and methylglyoxal 5a is extremely efficient in inhibiting WT-CFTR with picomolar affinity. The adducts 7a,b and 8a,b are CFTR inhibitors at low nanomolar concentrations, and 3a,b and 4a,b are less potent with a low micromolar range.

**Effects of Adducts 5a and 8a,b on Whole-Cell CFTR Chloride Currents.** Whole-cell patch-clamp experiments were performed on WT-CFTR-CHO cells to directly evaluate the efficacy of adducts 5a and 8a,b. WT-CFTR chloride currents were stimulated after bath application of forskolin (Fig. 4). After stable activation (Fig. 4A, middle trace) of the whole-cell current, 5a (10 pM) was added to the bath. A very rapid and complete inhibition of both inward and outward CFTR chloride currents was recorded (Fig. 4, A, right traces, and B). Moreover, the inhibition by 5a was reversible and reproducible as shown by the time-dependent plot of the whole-cell chloride current measured between −40 mV and +40 mV (Fig. 4C). The current amplitude (picoamperes) was measured between +40 mV and −40 mV and plotted as function of time and normalized by cell capacitance (picoamperes/picofarads) to allow comparison between cell densities (picoamperes/picofarads) as described for a previous study (Melin et
Effects of α-Aminoazaheterocycle-Methylglyoxal Adducts, CFTR Inhibitors, on Human Airway Epithelial Cells. The effects of α-aminoazaheterocycle-methylglyoxal adducts on endogenous CFTR chloride channel activity were also determined in several human airway epithelial cells. Chloride channel activity of endogenous human CFTR was measured by iodide efflux experiments for WT-CFTR (Calu-3 cells) and F508del-CFTR (JME/CF15 cells). Fsk at 10 μM was used to stimulate WT-CFTR (Bulteau et al., 2000), and a mixture of 10 μM Fsk plus 30 μM genistein was used to stimulate F508del-CFTR (Norez et al., 2006). As shown in Table 1, adducts 3a,b, 4a,b, 5a, 7a,b, and 8a,b inhibited endogenous WT- and F508del-CFTR with similar affinities. With the potent adducts 5a, IC_{50} values were 67.6 ± 1.3 μM for F508del-CFTR (Fig. 8A) and 93.3 ± 1.3 μM for WT-CFTR (Table 1) determined with iodide efflux experiments. Another functional assay was used to evaluate 5a potency. In 16HBE14o− cells expressing endogenous WT-CFTR, the CFTR Cl− activity was measured by microcytofluorimetry using the halide-sensitive fluorescent probe SPQ (Mohammad-Panah et al., 1998; Leblais et al., 1999). Cells responded with an increase (4–5-fold) in SPQ fluorescence in response to cAMP stimulation by 10 μM Fsk (Fig. 7). The simultaneous application of compound 5a reduced this effect in a concentration-dependent manner. Fluorescence intensity in the presence of 10 μM Fsk plus 100 μM 5a revealed 63 ± 9% inhibition (Fig. 7A). The Fsk effect was also inhibited in a similar manner by 10 μM CFTRinh-172 (66 ± 7%) or 100 μM glibenclamide (73 ± 7%) (Fig. 7B).

Methylglyoxal Adducts Inhibit the Activity of Mutated CFTR Chloride Channels. Responses to various adducts were also studied on CFTR chloride channels affected by class III CF mutations, G551D- and G1349D-CFTR. These mutations altered the pharmacological properties of the channels (Illek et al., 1999; Melin et al., 2004, 2005). G551D-CFTR activity, expressed in CHO cells, was analyzed in response to α-aminoazaheterocycle-methylglyoxal adducts. Iodide efflux was stimulated by a mixture of 10 μM Fsk plus 30 μM genistein (Melin et al., 2004). Glibenclamide inhibited G551D-CFTR with an IC_{50} of 7.9 ± 1.6 μM, similar to that for WT-CFTR (Table 1). It is interesting to note that to inhibit G551D-CFTR (whereas glibenclamide is equally potent com-
pared with WT-CFTR; see Table 1), higher IC\textsubscript{50} values than that for WT-CFTR were found with adducts 5a (Fig. 8B; Table 1), 3a,b, 4a,b, 7a,b, and 8a,b (Table 1). For G1349D-CFTR, iodide efflux was stimulated by a mixture of 10 \mu M Fsk plus CFTR activator MPB-91 (250 \mu M) because genistein failed to stimulate G1349D-CFTR as reported earlier (Melin et al., 2004). The IC\textsubscript{50} for glibenclamide inhibition of G1349D-CFTR (9 \pm 1.4 \mu M; Table 1) was in this case similar to that of WT-CFTR. In contrast with G551D, adducts 3a,b, 4a,b, 5a (IC\textsubscript{50} of 72.3 \pm 1.1 \mu M), 7a,b, and 8a,b inhibited G1349D-CFTR with affinities similar to that of WT-CFTR (Table 1). Thus, G551D- and G1349D-CFTR channels are both inhibited (although with different potencies) by the \alpha-aminooazaheterocycle-methylglyoxal adducts.

**Effect of \alpha-Aminooazaheterocycle-Methylglyoxal Adducts on Volume- and Calcium-Activated Chloride Channels.** To begin to define a selectivity of these compounds, we evaluated the action of adducts 5a on two non-CFTR chloride transport pathways: the calcium- and volume-regulated Cl\textsuperscript{−} channels. In WT-CFTR-CHO and human airway epithelial Calu-3 cells, the calcium ionophore A23187 was used to stimulate the activity of calcium-dependent Cl\textsuperscript{−} channels, and a hypo-osmotic challenge was used to stimulate the activity of volume-regulated Cl\textsuperscript{−} channels. Both maneuvers stimulated a significant iodide efflux resistant to adducts 5a (Fig. 9), indicating a relatively selective inhibition of CFTR by 5a.

**Inhibition of Cl\textsuperscript{−} Secretion in the Proximal Colon of CFTR Wild-Type Mice.** Finally, the efficacy of adducts 5a and CFTR\textsubscript{inh}-172 was tested ex vivo. To that end, we compared the effects of adducts 5a, CFTR\textsubscript{inh}-172, and glibenclamide on transepithelial ion transport in the mouse colon under the short-circuit (I\textsubscript{sc}) condition. In short-circuited colon of Cfr\textsuperscript{+/+} mice the application of 500 \mu M amiloride inhibited the resting Na\textsuperscript{+} current set by the activity of the amiloride-sensitive epithelial sodium channel channels (not shown) (Noel et al., 2006). Figure 10A shows a control experiment in which the serosal application of 1 \mu M Fsk stimulates CFTR-dependent Cl\textsuperscript{−} secretion (\Delta I\textsubscript{sc} = 5 \pm 0.5 \mu A/cm\textsuperscript{2}, n = 7) as described previously (Noel et al., 2006). In contrast, with mouse colon preincubated during 10 min with 100 \mu M CFTR\textsubscript{inh}-172 (Fig. 10B) or 100 \mu M adducts 5a (Fig. 10C) the serosal application of 1 \mu M Fsk had only a very modest effect on I\textsubscript{sc} (CFTR\textsubscript{inh}-172: \Delta I\textsubscript{sc} = 0.43 \pm 0.14 \mu A/cm\textsuperscript{2}, n = 4; adducts 5a: \Delta I\textsubscript{sc} = 0.5 \pm 0.16 \mu A/cm\textsuperscript{2}, n = 4) (Fig. 10D). These results provide electrophysiological evidence for comparable CFTR-dependent inhibition of chloride secretion in mouse colon by 100 \mu M adducts 5a and 100 \mu M CFTR\textsubscript{inh}-172.

**Stereochemistry in the Inhibitory Activities of \alpha-Aminooazaheterocycle-Methylglyoxal Adducts.** Finally, to examine the role of stereochemistry in the inhibitory activities observed, IC\textsubscript{50} values of the major adducts a were compared with IC\textsubscript{50} values of the minor adducts b in WT-CFTR-CHO cells for the most active derivatives. The most potent inhibitor 5a, obtained from 2′-deoxyadenosine, was significantly more efficient than the corresponding minor adducts 5b (***, P < 0.001), but IC\textsubscript{50} values are both in picomolar range, 71 and 194 \mu M, respectively (Table 2). Differences between the effects of adducts a and b in a mixture and separately for compounds 3, 4, 7, and 8 were not significant (Table 2). Thus, the stereochemistry relative to positions of carboxylic and hydroxyl functions in the new ring formed after condensation of two molecules of methylglyoxal on the starting heterocycle affects the inhibitory effects of CFTR activity only weakly.

**Discussion**

In this report, a new chemical class of CFTR channel inhibitors was discovered; among these are new, highly potent, water-soluble, nontoxic molecules suitable for studying CFTR with iodide efflux, microcytofluorimetry, and whole-cell patch-clamp techniques in epithelial and nonepithelial cells and for CFTR-dependent transepithelial current analysis with a Ussing chamber in mouse colon. To our knowledge, the 2′-deoxyadenosine derivative 5a represents the most potent inhibitor of CFTR channels with picomolar affinity in WT-, F508del- and G1349D-CFTR-expressing cells and nano-
molar affinity in G551D-CFTR cells. Compounds 7a,b and 8a,b also showed inhibitory effects with IC_{50} values in the nanomolar range. Whole-cell patch-clamp experiments confirmed rapid and reversible inhibition with adducts 5a and 8a,b of CFTR chloride current stimulated by Fsk in CHO cells. We found no inhibition of volume- and calcium-activated chloride channels with 5a. Importantly, transepithelial chloride secretion in mice colon can be inhibited by adducts 5a in a manner comparable to that with the thiazolidinone CFTRinh-172 but with a very marked difference in the concentration range (adducts 5a being 10^6 less concentrated) and the advantage of a water-soluble compound.

**Progress in the Development of CFTR Inhibitors.** Recent classes of CFTR inhibitors have been identified by a systematic high throughput screening assay. The thiazolidinone CFTRinh-172 (Ma et al., 2002) and the glycine hydrazide GlyH-101 (Muanprasat et al., 2004) are lead compounds reversibly inhibiting CFTR (K_i 0.3 and 5 \mu M, respectively). CFTRinh-172 has no effect on various ionic channels, including calcium-activated and volume-regulated chloride channels, K_ATP channels, and the multidrug resistance ABC transporter (Ma et al., 2002) but has reduced potency in intact tissues (K_i 5 \mu M) (Ma et al., 2002; Wang et al., 2004). CFTRinh-172 has poor water solubility (~20 \mu M) (Ma et al., 2002; Taddei et al., 2004). This agent is now commercially available and is generally used at 10 \mu M concentrations to achieve complete CFTR inhibition. This agent has also been used to study the CFTR dependence of the nasal potential difference in mice and pigs (Salinas et al., 2004). With freshly isolated microperfused human sweat ducts endogenously expressing a high level of CFTR, CFTRinh-172 at a maximal dose, limited by its aqueous solubility of 5 \mu M, partially blocked CFTR, with better efficacy from the cytosolic side (Wang et al., 2004). In this particular tissue, the inhibition was relatively slow and poorly reversible, and sodium conductance appeared to be affected as well (Wang et al., 2004). GlyH-101 (Muanprasat et al., 2004) and more recent highly polar derivatives (Sonawane et al., 2006) have better water solubility. GlyH-101 causes a voltage-dependent block of whole-cell CFTR chloride currents, and single-channel analysis in the presence of the drug suggests that GlyH-101 is an open-channel blocker of CFTR, occluding an extracellular vestibule (Muanprasat et al., 2004). However, at 50 \mu M, GlyH-101 also inhibits calcium-activated chloride channels (Muanprasat et al., 2004). Two new classes of CFTR inhibitors have been very recently reported (Muanprasat et al., 2007): a substituted thiazole derivative named INH 1 and a sulfonamide compound named INH 2. Both compounds inhibit CFTR with K_i values of 15 to 25 \mu M, depending of the model used. However, 100 \mu M INH 1 (but not INH 2) also inhibits calcium-activated chloride secretion in T84 human colonic epithelial cells. In vivo studies in mice showed that INH 1 (3 mg/kg) reduced 40% of cholera toxin-induced intestinal fluid secretion, whereas INH 2 had no effect (Muanprasat et al., 2007). Finally, inhibitors of multidrug resistance-associated proteins, among them sulfipyrazone, probenecid, and benz bromarone, inhibit CFTR activity with low affinity, i.e., K_i values of 191, 1244, and 11.5 \mu M, respectively (Diena et al., 2007). This latter observation is nevertheless interesting because it confirms pharmacological similarities within members of the family of ABC transporters. Indeed, earlier findings identified the first relatively good, although not selective, inhibitor of CFTR within the sulfonylureas (glibenclamide) acting on the ABC protein SUR, forming with the K_ATP channel, the K_ATP channel (Sheppard and Welsh, 1992).

**CFTR Inhibitors in Human Diseases.** A number of human renal and gastrointestinal diseases may benefit from the development of new potent and selective CFTR inhibitors. The recent literature illustrates this exciting
new area. CFTRinh-172 has been found to be effective in reducing cholera toxin-induced intestinal fluid secretion in mice (Ma et al., 2002). In mice, a single i.p. injection of CFTRinh-172 (at 250 μg/kg) almost completely reduced cholera toxin-induced fluid secretion in the small intestine over 6 h. A further in vivo pharmacology study explored pharmacokinetic parameters in rodents (Sonawane et al., 2005). No toxicity of the compound was reported after high-dose administration in mice over the first 6 weeks of life (Sonawane et al., 2005). An important recent development based on CFTR inhibitors is the finding that treating cells with CFTRinh-172 creates a CF model with the CF-related lung inflammatory response leading to the hypothesis that CFTR has an important role as a modulator of airway inflammation (Perez et al., 2007). Furthermore, in a rat model for duodenal ulcer formation induced by cys-

![Figure 10](https://example.com/image.png)

**Fig. 10.** Effect of adducts 5a, CFTRinh-172, and glibenclamide on transepithelial ion transport in the mouse colon under the short-circuit condition. A, time-dependent short-circuit (Isc) recording on Cfr+/− mouse colon. The tracing starts after the application of 500 μM amiloride to inhibit the resting Na+ current set by the activity of epithelial sodium channel channels. The serosal application of 1 μM Fsk stimulates CFTR-dependent Cl− secretion (n = 7). B and C, time-dependent Isc recordings as in A, but forskolin was now added after a 10-min incubation with the CFTR inhibitor CFTRinh-172 (100 μM, n = 4) (B) or adducts 5a (100 μM, n = 4) (C). At the end of the each experiment 500 μM glibenclamide was added to block all residual CFTR-dependent Isc. D, corresponding histograms showing means ± S.E.M. of ΔIsc. ***P < 0.001.

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<tr>
<td>IC50 determined for the different isomers of the most potent methylglyoxal adducts prepared</td>
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<tr>
<td>Data are from iodide efflux experiments and are given as mean ± S.E.M. for n = 4 for each drug tested. All dose-response experiments were performed with CHO cells overexpressing WT-CFTR in presence of 1 μM Fsk.</td>
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<th>Isomers</th>
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<tr>
<td>a</td>
<td>b</td>
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<tr>
<td>Isomer 3</td>
<td>5.7 ± 1.3 μM (60:40)</td>
</tr>
<tr>
<td>Isomer 4</td>
<td>1.8 ± 1.2 μM</td>
</tr>
<tr>
<td>Isomer 5</td>
<td>7.1 ± 2 μM</td>
</tr>
<tr>
<td>Isomer 6</td>
<td>2.2 ± 1.2 μM (60:40)</td>
</tr>
<tr>
<td>Isomer 7</td>
<td>3.0 ± 1.2 μM (60:40)</td>
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N.D., not determined.  
* Isomers 8b could not be obtained without traces of isomers a.
teamine treatment, i.p. injection of CFTR<sub>nh</sub>-172 (at 1 mg/kg) 1 h before cysteamine treatment reduced duodenal ulceration (Akiba et al., 2005). Finally, CFTR inhibitors, such as CFTR<sub>nh</sub>-172, glibenclamide, and 5-nitro-2-(3-phenylpropylamino)-benzoic acid, by inhibiting transepithelial ion transport, retard renal cyst growth using MDCK cells as a model (Li et al., 2004), suggesting that such molecules could contribute to the pharmacological treatment of autosomal-dominant polycystic kidney disease.

**Advantage of α-Aminoazaheterocycle-Methylglyoxyal Adducts to Inhibit CFTR.** In our first report on reaction of methylglyoxal with α-aminoazaheterocycle leading to a new class of compounds, X-ray studies of crystals 2a, 2b, and 4a revealed the zwitterionic character of the products (Routaboul et al., 2002). The presence of an amino acid-like function confers to the products interesting water solubility. The water solubility of compound 5a is higher than 55 mM, and the less soluble inhibitors in water are compounds 8a,b, showing a maximum solubility around 8 mM. The resulting zwitterionic character of the inhibitors in water under neutral conditions also could be a major element in the mechanism of the inhibitory effect. The opposite charges present in the inhibitors could lead to their strong binding to charged amino acid residues in the CFTR protein and produce a major switch in the protein conformation controlling the Cl<sup>-</sup> channel activity. Although the mechanism of inhibition of CFTR by adducts 5a and 8a,b has not yet been studied, these compounds already have some advantages over existing inhibitors. First, these agents are water-soluble and nontoxic; second, they are effective at extremely low concentrations; third, the inhibition in the whole-cell patch-clamp experiments of CFTR activity is very rapid, reversible, and reproduducible, and fourth, these agents are able to inhibit cAMP-dependent chloride secretion in an intact tissue, the mouse colon, at picomolar concentrations. To our knowledge pico-molar efficacy is not common in the pharmacology area of ionic channels for a small molecule, except for some channel-specific toxins (Mouhat et al., 2004; Terlau and Olivera, 2004). Therefore, α-aminoazaheterocycle-methylglyoxyal adducts could be useful tools for studying CFTR structure and functions and epithelial pharmacology. Future development of these compounds may potentially provide new therapeutic anti diarrheal, anti cholERIC, anti ulceration, and anti-autooso-

dominant polycystic kidney disease agents.

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