Synthesis, SAR, Crystal Structure, and Biological Evaluation of Benzoquinoliziniums as Activators of Wild-Type and Mutant Cystic Fibrosis Transmembrane Conductance Regulator Channels

Cécile Marivingt-Mounir,[†] Caroline Norez,[‡] Renaud Dérand,[‡] Laurence Bulteau-Pignoux,[‡] Dung Nguyen-Huy,[§] Bernard Viossat,[||] Georges Morgant,[§] Frédéric Becq,[‡] Jean-Michel Vierfond,[†] and Yvette Mettey*[,†]

Laboratoire de Chimie Organique, Faculté de Médecine et de Pharmacie, Université de Poitiers, 34 rue du Jardin des Plantes, BP 199, 86005 Poitiers Cedex, France, LBSC, CNRS, UMR 6558, Université de Poitiers, 86022 Poitiers Cedex, France, Laboratoire de Cristallochimie Bioinorganique, Faculté de Pharmacie, Université de Paris XI, 5 rue J. B. Clément, 92296 Châtenay-Malabry Cedex, France, and Laboratoire de Cristallographie et RMN Biologiques, Faculté de Pharmacie, Université de Paris V, 4 avenue de l'observatoire, 75270 Paris Cedex 06, France

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Chloride channels play important roles in homeostasis and regulate cell volume, transepithelial transport, and electrical excitability. Despite recent progress made in the genetic and molecular aspect of chloride channels, their pharmacology is still poorly understood. The cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-regulated epithelial chloride channel for which mutations cause cystic fibrosis. Here we have synthesized benzo[*c*]quinolizinium and benzo[*f*]indolo[2,3-*a*]quinolizinium salts (MPB) and performed a SAR to identify the structural basis for activation of the CFTR chloride channel. Synthesized compounds were evaluated on wild-type CFTR and on CFTR having the glycine-to-aspartic acid missense mutation at codon 551 (G551D-CFTR), using a robot and cell-based assay. The presence of an hydroxyl group at position 6 of the benzo[*c*]quinolizinium skeleton associated with a chlorine atom at position 10 or 7 and an alkyl chain at position 5 determined the highest activity. The most potent product is 5-butyl-7-chloro-6-hydroxybenzo[*c*]quinolizinium chloride (**8u**, MPB-104). **8u** is 100 times more potent than the parent compound **8a** (MPB-07).

Introduction

The CFTR gene encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a chloride channel that normally mediates Cl^- transepithelial transport in epithelia.[1,2,3] The channel activity of CFTR is controlled by two processes: (i) phosphorylation of serine and threonine residues within the regulatory R domain by cAMP-dependent protein kinases and (ii) the interaction of ATP molecules (binding and/or hydrolysis) at two distinct cytoplasmic domains, the nucleotide binding domains NBD1 and NBD2.[1,4,5]

Cystic fibrosis (CF) is the most common lethal autosomal recessive genetic disease in Caucasians and results from more than 1000 mutations of the CF gene (http://www.genet.sickkids.on.ca/cftr). These numerous mutations can be classified according to the fate of the final product.[6] Classes III and IV lead to proteins with an altered chloride channel activity; class V produces functional protein but with a reduced synthesis; and classes I and II mutations lead to defective CFTR folding, trafficking, and channel activity. The class III mutation glycine-to-aspartic acid at codon 551 (G551D) is found with a frequency of 2−5% in chromosomal analysis, depending on the population of origin. G551D is indeed one of the five most frequent CF mutations being always associated with a severe CF phenotype, pulmonary dysfunction, and pancreatic insufficiency.[7] The G551D mutation is located within the first nucleotide binding domain of CFTR.[7] Despite the fact that G551D mutated protein is fully glycosylated[6,8] and normally phosphorylated at the R domain by cAMP-dependent protein kinases,[9] its chloride channel activity cannot be stimulated pharmacologically by cAMP-elevating agents.[6,7,10] G551D mutation also confers a decreased nucleotide binding[11] and a reduced ATPase activity at NBD1.[12,13]

Searching for potent and specific small molecules able to modulate normal and mutated CFTR is crucial for both our understanding of the physiological role of CFTR in epithelial cell function and for the development of molecules of therapeutic interest to cure CF. In this regard, the pharmacology of G551D-CFTR chloride channel activity has not been clearly and systematically characterized and compared to that of wild-type CFTR channel. Recently, we have demonstrated that the benzo[*c*]quinolizinium derivatives MPB-07 (**8a**), MPB-27 (**8d**), and MPB-91 (**8t**) are activators of wild-type (wt) CFTR.[14,15] Among these, only MPB-91[15] but not MPB-07[15,16] was found capable to activate G551D-CFTR chloride channels. Moreover, some of these compounds exhibit a good inhibitory activity for protein kinase CKII.[17,18] In the present report, we have studied the structure−activity relationship of a series of benzo[*c*]-quinoliziniums and benzo[*f*]indolo[2,3-*a*]quinolizinium

* Corresponding author. Tel: 33 (0)5 49 45 43 96. Fax: 33 (0)5 49 45 43 98. E-mail: yvette.mettey@univ-poitiers.fr.
[†] Laboratoire de Chimie Organique, Université de Poitiers.
[‡] LBSC, CNRS, UMR 6558, Université de Poitiers.
[§] Laboratoire de Cristallochimie Bioinorganique, Université de Paris XI.
[||] Laboratoire de Cristallographie et RMN Biologiques, Université de Paris V.

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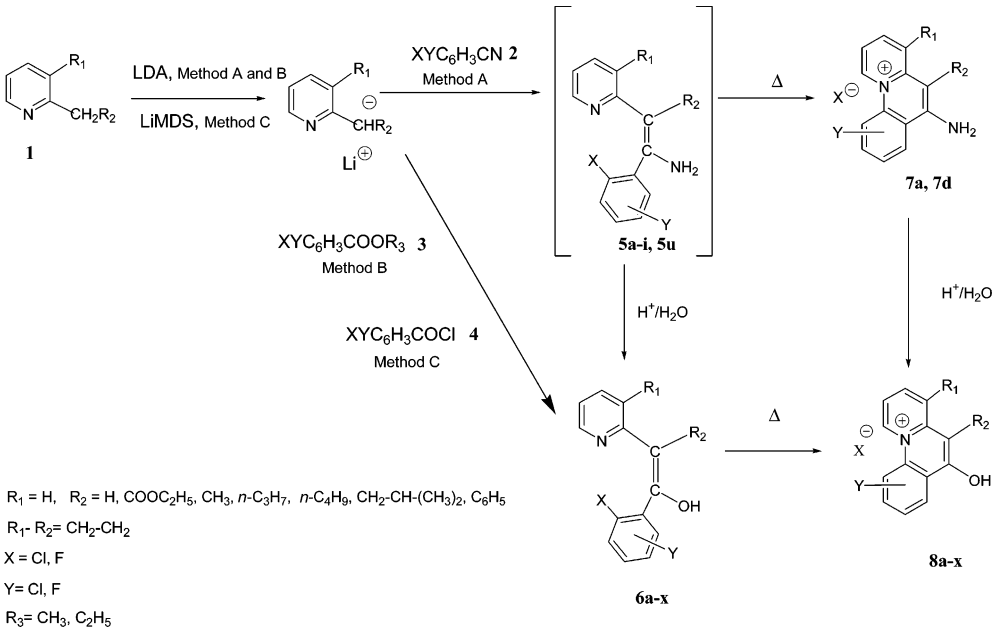
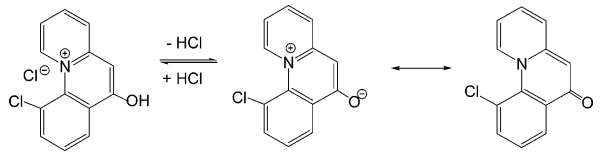
Scheme 1



Scheme 2

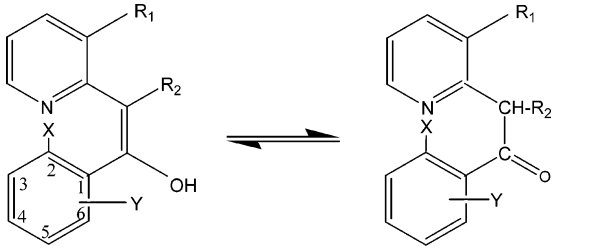


salts and generated many derivatives that have been tested on both wt- and G551D-CFTR chloride channel activity. A comparison of the dose−response was systematically done, which allows us to determine the most favorable chemical structure of MPB compounds for activation of both wild-type and mutant CFTR channels.

Chemistry

In a previous study[19] we reported a new synthesis of benzo[*c*]quinoliziniums from methyl pyridine and halogenobenzonitriles. This method (route A) and two others (routes B and C[20]) are outlined in Scheme 1. They were used to prepare the target compounds **8**. The 2-methylpyridine was metalated either with lithium diisopropylamide (LDA) (methods A and B) or lithium bis(trimethylsilyl)amide (LiHMDS) (method C), using dry tetrahydrofurane as solvent. The condensation with *o*-halogenobenzonitriles **2** (method A) gave nonisolated imines−enamines **5**, which led to keto−enols **6**. Compounds **6** could also be obtained using esters **3** (method B) or acid chlorides **4** (method C). Yields and formulas of the products **6a**−**x** are listed in Table 1. By heating, imines−enamines **5** and keto−enols **6** led to tricyclic derivatives benzo[*c*]quinoliziniums **7** and **8**, respectively. The cyclization occurred at about 200 °C for the 2-chloro derivatives or 160 °C for the fluoro ones. All benzo[*c*]-quinoliziniums **7** and **8** prepared appear in Table 2. As shown in Scheme 2, compounds **8** are quaternary ammoniums, in equilibrium between acidic enol form and keto base form **9**. This feature was sometimes used for purification of crude benzo[*c*]quinoliziniums.

Table 1. Structure, Yields, and Formulas of Compounds **6a**−**x**



	substituents						
compound	R_1	R_2	X	Y	method	% yield	formula
6a	H	H	Cl	3-Cl	A	23	$C_{13}H_9NOCl_2$
					B	64	
6b	H	H	Cl	4-Cl	A	45	$C_{13}H_9NOCl_2$
6c	H	H	Cl	5-Cl	A	33	$C_{13}H_9NOCl_2$
					B	33	
6d	H	H	Cl	6-Cl	A	33	$C_{13}H_9NOCl_2$
6e	H	H	F	3-F	A	29	$C_{13}H_9NOF_2$
6f	H	H	F	4-F	A	12	$C_{13}H_9NOF_2$
6g	H	H	F	5-F	A	60	$C_{13}H_9NOF_2$
6h	H	H	F	6-F	A	47	$C_{13}H_9NOF_2$
6i	H	H	F	6-Cl	A	50	$C_{13}H_9NOClF$
6j	H	COOEt	F	3-F	C	−	$C_{16}H_{13}NO_3F_2$
6k	H	COOEt	F	4-F	C	−	$C_{16}H_{13}NO_3F_2$
6l	H	COOEt	F	5-F	C	57	$C_{16}H_{13}NO_3F_2$
6m	H	COOEt	F	6-F	C	−	$C_{16}H_{13}NO_3F_2$
6n	H	COOEt	Cl	3-Cl	C	49	$C_{16}H_{13}NO_3Cl_2$
6o	H	COOEt	Cl	4-Cl	C	66	$C_{16}H_{13}NO_3Cl_2$
6p	H	COOEt	Cl	6-Cl	C	52	$C_{16}H_{13}NO_3Cl_2$
6q	H	C_6H_5	Cl	3-Cl	B	72	$C_{19}H_{13}NO_3Cl_2$
6r	H	CH_3	Cl	3-Cl	B	31	$C_{14}H_{11}NOCl_2$
6s	H	*n*-C_3H_7	Cl	3-Cl	B	52	$C_{16}H_{15}NOCl_2$
6t	H	*n*-C_4H_9	Cl	3-Cl	B	55	$C_{17}H_{17}NOCl_2$
6u	H	*n*-C_4H_9	F	6-Cl	A	20	$C_{17}H_{17}NOClF$
6v	H	*n*-C_5H_{11}	Cl	3-Cl	B	40	$C_{18}H_{19}NOCl_2$
6w	H	isobutyl	Cl	3-Cl	B	55	$C_{17}H_{17}NOCl_2$
6x	CH_2CH_2		F	6-F	B	−	$C_{15}H_{11}NOF_2$

For the synthesis of benzo[*f*]indolo[2,3-*a*]quinolizinium salts **12** (Scheme 3), the starting material was harmalane **10**. It was metalated with butyllithium and then reacted with *o*-dihalogenobenzonitriles **2** to obtain the imines−enamines **11**, which were heated at about 200 °C to give 6,7-dihydro-12*H*-benzo[*f*]indolo[2,3-*a*]-quinoliziniums **12** (Table 3).

Scheme 3

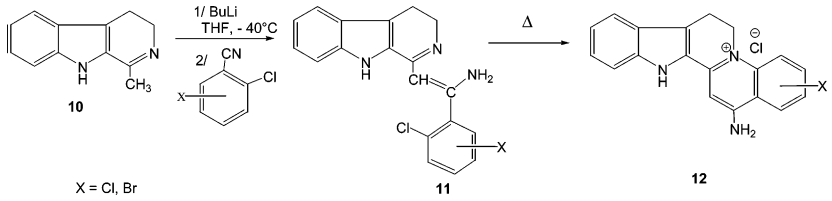
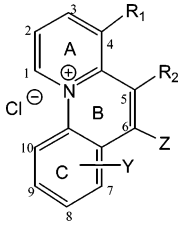


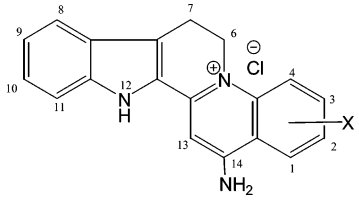
Table 2. Structure and Activation by Compounds **7** and **8** of Wild-Type CFTR



compound		substituents Z	R1	R2	Y	% activation[a]	*t*-test[b]	*n*
	Fsk (5 μM)					100 ± 7	***	20
7a	MPB-02	NH_2	H	H	10-Cl	<5	NS	4
7d	MPB-04	NH_2	H	H	7-Cl	<5	NS	4
8a	MPB-07	OH	H	H	10-Cl	24 ± 4	*	8
8b	MPB-08	OH	H	H	9-Cl	<5	NS	8
8c	MPB-30	OH	H	H	8-Cl	18 ± 4	NS	11
8d	MPB-27	OH	H	H	7-Cl	20 ± 6	*	4
8e	MPB-80	OH	H	H	10-F	<5	NS	4
8f	MPB-29	OH	H	H	9-F	16 ± 2	NS	4
8 g	MPB-79	OH	H	H	8-F	11 ± 1	NS	4
8h	MPB-78	OH	H	H	7-F	<5	NS	4
8j	MPB-77	OH	H	COOEt	10-F	37 ± 3	***	8
8k	MPB-86	OH	H	COOEt	9-F	6 ± 1	NS	4
8l	MPB-75	OH	H	COOEt	8-F	<5	NS	4
8m	MPB-73	OH	H	COOEt	7-F	8 ± 4	NS	4
8q	MPB-67	OH	H	C_6H_5	10-Cl	6 ± 4	NS	10
8r	MPB-94	OH	H	CH_3	10-Cl	19 ± 6	*	16
8s	MPB-96	OH	H	*n*-C_3H_7	10-Cl	32 ± 4	**	4
8t	MPB-91	OH	H	*n*-C_4H_9	10-Cl	45 ± 3	***	12
8u	MPB-104	OH	H	*n*-C_4H_9	7-Cl	33 ± 7	**	4
8v	MPB-97	OH	H	*n*-C_5H_{11}	10-Cl	38 ± 4	***	8
8w	MPB-95	OH	H	isobutyl	10-Cl	26 ± 4	*	4
8x	MPB-92	OH	CH_2CH_2		7-F	<5	NS	4

[a] Experiments were performed with the iodide efflux method. Data are given as mean percentage activation of CFTR-dependent efflux ± SEM for the indicated number (n) of experiments for each compound. [b] *p < 0.05, **p < 0.01, ***p < 0.001 (NS = not significant).

Table 3. Structure and Activation by Compounds **12** of Wild-Type CFTR



compound		X	% activation[a]	*t*-test[b]	*n*
	Fsk (5 μM)		100 ± 7	***	20
12a	MPB-54	4-Cl	46 ± 7	***	4
12b	MPB-51	3-Cl	ND		ND
12c	MPB-50	2-Cl	6 ± 4	NS	4
12d	MPB-49	1-Cl	30 ± 6	**	4
12y	MPB-52	3-Br	ND		ND

[a] Experiments were performed with the iodide efflux method. Data are given as mean percentage activation of CFTR-dependent efflux ± SEM for the indicated number (n) of experiments for each compound. [b] *p < 0.05, **p < 0.01, ***p < 0.001 (NS = not significant, ND = not determined).

Results

Following recrystallization in CH_3CN, X-ray diffraction analysis of **8a**, **8t**, and **13** (**13** = **8a** with Cl^- replaced by Br^-) confirmed their respective expected structure and provided proof that the compounds **8a** and **13** are isotypic and that they are solvated by water molecules, in contrast to **8t**.

Functional Analysis of the Effect of Benzo[*c*]-quinolizinium and Benzo[*f*]indolo[2,3-*a*]quinolizinium Salts on the Chloride Channel Activities of Wt- and G551D-CFTR. To test the synthesized compounds **7**, **8**, and **12**, we developed a cell-based primary screening assay using iodide efflux measurement. Chinese hamster ovary (CHO) cell lines stably expressing wt-CFTR[14] and G551D-CFTR[15] were used. Our assay allows the rapid detection of channel activity from cells cultured in 24-well plates. Comparative experiments were thus performed between cells treated with the test drug, resting cells, or cells stimulated with saturating or submaximal concentration of forskolin i.e., 5 and 1 μM, respectively. Forskolin is an agonist of the cAMP pathway used as a standard CFTR-activator.[2,5,14] These concentrations of forskolin were chosen because in preliminary experiments in which dose−response curves were constructed, we found an half-maximal effective concentration of 767 nM and maximal effect from 2 to 10 μM (not shown). Compounds producing a minimum of 20% of the activity elicited by 5 μM forskolin were further studied, and dose−response curves could be constructed to determine the half-maximal effective concentration, EC_{50}.

As a control experiment, Figure 1A illustrates the typical response of wt-CFTR to 5 μM forskolin (Fsk) compared to experiments in the absence of forskolin (noted control). The stimulation of the iodide efflux occurred within 2 min after drug addition (indicated by the arrow). Wt-CFTR cells were then exposed to 250 μM *o*f derivatives **7a**, **7d**, **8a**−**x**, **12a**−**d**, and **12y**, and the iodide efflux was quantified during a 10 min period. The results are shown in Tables 2 and 3. Of the series **7** and **8**, corresponding to benzo[*c*]quinoliziniums derivatives, seven have no effect, i.e., stimulation <5% (**7a**, **7d**, **8b**, **8e**, **8h**, **8l**, and **8x**). A second group of eight derivatives gave a stimulation inferior (**8c**, **8f**, **8g**, **8k**, **8m**, **8q**) or equal to (**8d**, **8r**) our 20% criterion. Finally, compounds **8a**, **8d**, **8j**, **8s**, **8t**, **8u**, **8v**, and **8w** stimulated wt-CFTR activity above 20% and were selected for further analysis. Although benzo[*f*]indolo[2,3-*a*]quinolizinium compounds **12a** and **12d** stimulated CFTR, they were not further studied because we observed a rapid cell toxicity (not shown).

Since CFTR is mainly regulated by a phosphorylation process,[2,5] we thought it would be important to study the effect of benzo[*c*]quinoliziniums derivatives on par-

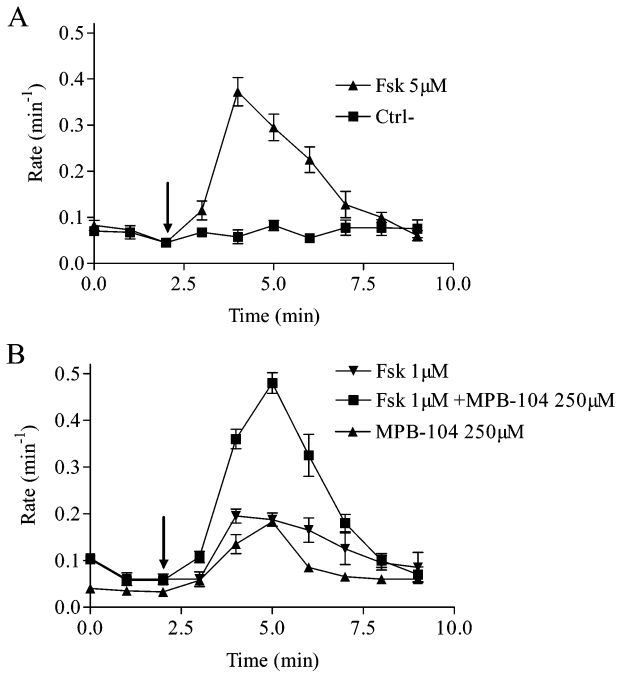


Figure 1. Stimulation of CFTR activity by forskolin and MPB-104 (**8u**). (A) ^{125}I efflux measured in CHO cells expressing wild-type CFTR in the presence of 5 μM Forskolin (Fsk). Control cells were not stimulated (noted ctrl). In this and subsequent figures, the agonist is present from its addition (indicated by an arrow) to the end of the experiment. Note that forskolin stimulated the efflux to a peak within 2 min after its addition. (B) ^{125}I efflux measured in CHO cells expressing wild-type CFTR in the presence of 1 μM Fsk, 250 μM MPB-104, or Fsk+MPB-104 at the indicated concentration. Note the dramatic potentiation when MPB-104 was added together with Fsk. Errors bars are SEM for $n = 4-6$ in A and B. Some error bars are smaller than the symbol.

tially phosphorylated CFTR. For that, wt-CFTR cells were simultaneously exposed to 1 μM Fsk and to different compounds **8** (the eight most potent in Table 2). Of these, **8u** (MPB 104) was the most promising. As shown in the Figure 1B, the stimulation of wt-CFTR by either **8u** or forskolin gave similar activation. However, when both drugs were added simultaneously, the stimulation of wt-CFTR was much more important, as shown Figure 1B. Thus although **8u** alone was able to stimulate wt-CFTR, it was much more potent when CFTR cells were prestimulated. Dose−response curves were then generated using increasing doses of **8u** with 1 μM forskolin. Results are shown in the Figure 2A. The EC_{50} was determined to 1.70 ± 0.15 μM for **8u** (Table 4).

The pharmacological characteristics of CFTR chloride channel having the glycine-to-aspartate mutation at codon 551 (G551D) were then studied. Several control experiments were first performed. G551D-CFTR channels expressed in CHO cells are not responsive to up to 10 μM Fsk, as previously reported.[15,21] This lack of responsiveness of G551D-CFTR to cAMP agonists constitutes a hallmark of the mutant and a major difference with wild-type CFTR. To determine the effect of compounds **8** on G551D-CFTR activity, cells were first exposed to 10 μM Fsk and then to these derivatives. In G551D-CFTR cells, in the absence of Fsk, the peak rate of ^{125}I efflux was 0.09 ± 0.01 min^{-1} (n = 14) and did not significantly differ from experiments in which 10 μM Fsk was added (0.10 ± 0.01 min^{-1}, n = 14, Figure 2B). We then studied the effect of compounds **8** on G551D-CFTR activity in the presence of 10 μM Fsk. As shown in the Figure 2B, when Fsk and **8u** were simultaneously present, a dramatic increase of the efflux was observed. The stimulation of G551D-CFTR activity by **8u** increased dose-dependently with an EC_{50} = 0.75 ± 0.15 μM (Figure 2B and Table 4). Interestingly, this value is similar to the EC_{50} calculated from wt-CFTR experiments (compare Figure 2A and 2B). A similar study was conducted with the different MPB derivatives listed Table 4. For each compound, the respective EC_{50} was determined for both wt-CFTR and G551D-CFTR channels. As can be seen from Table 4, compounds **8a** and **8d** were considered inactive on G551D-CFTR, because we could not determine the EC_{50}, which must be above 200 μM. In contrast, the remaining six compounds **8u, 8v**, **8t**, **8w**, **8s**, and **8j** were potent activators of both wt-CFTR and G551D-CFTR. It is also interesting to note that EC_{50} values for all compounds were similar, irrespective of the CFTR channel studied. The order of potency is **8j** < **8s** < **8w** ∼ **8t** < **8v** < **8u.** We have evaluated the cell toxicity of the compounds presented in Table 4 and found no cell toxicity at 50 μM (Figure 3A). However, at higher concentration (250 μM) only compounds **8v**, **8t**, and **8w** are toxic (Figure 3B). Interestingly, compound **8u**, being the most active on CFTR, is not toxic at 50 or 250 μM (Figure 3).

Table 4. Half-Maximal Effective Concentration (EC_{50}) Determined for the Eight Most Potent Benzo[*c*]quinolizinium Derivatives[a]

compound		EC_{50} (μM) wt	EC_{50} (μM) G551D
8u	MPB-104	1.70 ± 0.15	0.75 ± 0.15
8v	MPB-97	21.0 ± 1.5	20.0 ± 1.5
8t	MPB-91	23.5 ± 1.5	34.0 ± 2
8w	MPB-95	26.0 ± 1.5	32.5 ± 1.5
8s	MPB-96	54.2 ± 1.2	48.0 ± 1.3
8j	MPB-77	70.5 ± 1.5	95.0 ± 1.5
8a	MPB-07	141 ± 15	>200
8d	MPB-27	146 ± 14	>200

[a] Data are given as mean ± SEM for n = 6 for each drug tested for wt-CFTR and G551D-CFTR. For the effect of **8a** and **8d** on G551D-CFTR, the dose−response relationship was not completed and the EC_{50} was estimated to be above 200 μM, a concentration too high compared to the other congeners. Experiments were performed in the presence of 1 μM Fsk (wt-CFTR) or 10 μM Fsk (G551D-CFTR).

Discussion

Our knowledge of the pharmacological modulation of wild-type CFTR chloride channel activity is now on an exponential increase. In early studies phosphodiesterase inhibitors (e.g. IBMX) were the only molecules found capable of stimulating CFTR channel activity.[2,22] A great number of molecules have been now described as activators.[23] These compounds are phenylimidazothiazole,[10,24] milrinone,[25] flavonoids,[26,27] pyrazole derivatives,[27] benzimidazolones,[28] xanthines,[10,22,29−31] tetrahydrocarbazole, hydroxycoumarin, thiazolidine,[32] and benzo[*c*]quinoliziniums.[14,15,33] Most of these agents activate CFTR through cAMP-independent pathways,[14,15,28,34] but the precise mechanism of activation remains unknown. Some of these compounds have also been found to be effective on delF508 CFTR.[22,28,33] Genistein has been proposed as an activator of the

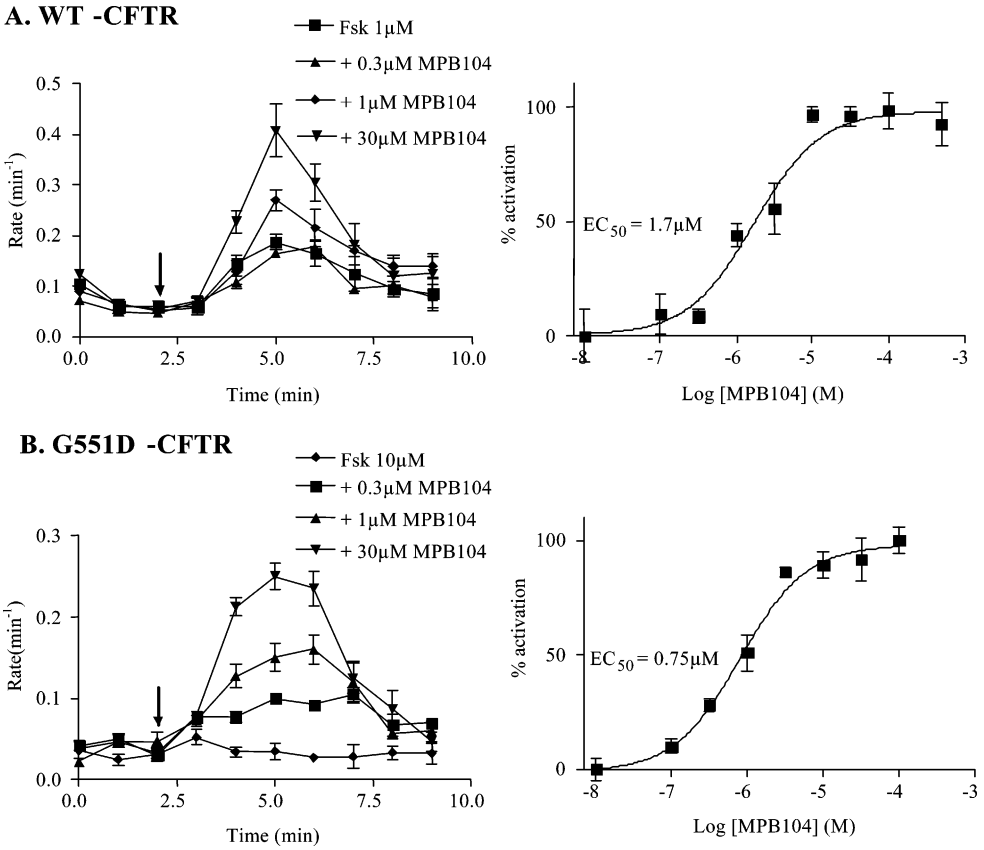


Figure 2. Effect of MPB-104 (8u) on wt-CFTR (A) and G551D-CFTR (B) activity. Example of experiments showing ^{125}I efflux in the presence of 1 μM Fsk (A) or 10 μM Fsk (B). On the right are presented the dose−response relationships for MPB-104 in the presence of 1 μM Fsk (wt-CFTR, A) or 10 μM Fsk (G551D-CFTR, B). The percentage of maximal effect is plotted as function of the concentration of MPB-104. The half-maximal effective concentration EC_{50} was 1.70 ± 0.15 μM and 0.75 ± 0.15 μM for wt-CFTR and G551D-CFTR, respectively. Errors bars are SEM for $n = 6$ for each concentration tested in A and B. Some error bars are smaller than the symbol.

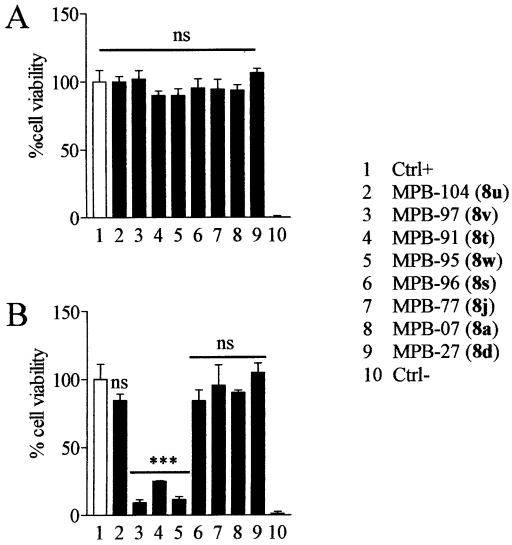


Figure 3. Evaluation of the cytotoxicity of the eight most potent benzo[*c*]quinolizinium derivatives. The toxicity of the compounds was evaluated as described in the Experimental Section. Results are presented as percent of cell viability for CHO cells treated 2 h with (A) 50 μM of each compound and (B) the maximal concentration used (250 μM) of MPB derivatives as indicated; $n = 4$ for each. Ns: no significant differences with respect experimental condition in which no drug was added (noted Ctrl+, Ctrl− indicated 0% cell viability), ***$p < 0.001$ using the *t*-test.

trafficking-competent G551D mutant after forskolin exposure.[21,35]

In our first report concerning the activity of benzo[*c*]-quinoliziniums on wt-CFTR chloride channel expressed in CHO cells,[14] we studied only four compounds. Chloro-6-hydroxybenzo[*c*]quinolizinium chlorides **8a** (MPB-07) and **8d** (MPB-27) were shown to be activators, whereas 6-amino derivatives **7a** (MPB-02) and **7d** (MPB-04) were inactive.[14] We also prepared benzo[*c*]quinolizinium chloride, without OH or NH_2, and 10-chloro-benzo[*c*]quinolizin-6-one (**9a**, MPB-70). These compounds have been tested and shown to be inactive on wt-CFTR. Since the OH group appeared to be essential for the activity, we prepared a series of 6-hydroxybenzo[*c*]quinoliziniums salts **8** listed in Table 2. These compounds were first tested with cells stably expressing wt-CFTR (Tables 2 and 3). An approach to study the structure−activity relationship was to examine the different sites of substitution. A halogen atom (Cl or F) was first introduced in different positions of cycle C. The most favorable positions for the chlorine atom were 10 (**8a**) and 7 (**8d**) (activation 24% and 20%, respectively). In contrast, 10- and 7-fluoro derivatives (**8e** and **8h**) were found to be ineffective, though other fluoro compounds (**8f** and **8g**) have moderate activity (16% and 11%). The introduction of an ester group COOEt in position 5 of 10-fluorocompound **8e** to give **8j** greatly increased the activity (37%), although the same substitution in 7-, 8-, and 9-fluoro derivatives produced inactive compounds **8k**, **8l**, and **8m**. Unfortunately, we failed to prepare 5-carbethoxy-10-chloro-6-hydroxybenzo[*c*]quinolizini-

um chloride, because thermal decomposition occurred by heating compound **6n** at 160 °C. The results obtained with **8j** encouraged us to substitute position 5. We thus synthesized new compounds having a chlorine atom in position 10 or 7 and an alkyl chain in position 5. We investigated the efficacy of the chain according to length, ramification, and rigidity. The presence of an alkyl chain at this position clearly increased the activity. The most potent compounds obtained had a *n*-butyl substituent in position 5 and a chlorine atom in position 10 (**8t**) or 7 (**8u**). Extension (**8v**), diminution (**8r**, **8s**), or ramification (**8w**) were unfavorable factors. Immobilization of the alkyl chain (substituent R_1 to R_2) in a rigid cycle joined to the ring A (**8x**) abolished the activity. We also prepared compound **8q** bearing a 5-phenyl ring, but the activity was reduced in comparison with compound **8a**, having no substituent in position 5.

It is interesting to note that between MPB-07 (**8a**) and MPB-27 (**8d**), the two MPB derivatives originally described,[14] and the novel MPB-104 (**8u**), we obtained a 100-fold better efficacy. MPB-104, being the most potent drug of the series, has the same efficacy on both wt- and G551D-CFTR. In addition, absence of cell toxicity was found with MPB-104 (**8u**) at 50 or 250 μM.

Among the benzo[*f*]indolo[2,3-*a*]quinoliziniums tested, only the 4-chloro derivative **12a** showed an interesting activity (activation factor 46%), but a rapid cell toxicity was observed.

Conclusion

Development of new compounds able to activate the CFTR is an important goal, because the channel is defective in CF and such compounds may lead to a pharmacotherapy of CF. In this report, we described the synthesis of new benzo[*c*]quinolizinium derivatives using three routes. These compounds were tested on wild-type CFTR and on G551D-CFTR, a class III CF mutation, using a robot and cell-based assay. Our study shows that the hydroxyl in position 6, the ammonium function, and a chlorine atom in position 7 or 10 are essential for the activation of the CFTR chloride channel. In addition, an alkyl chain in position 5 is also of crucial importance, since the most potent product is 5-butyl-7-chloro-6-hydroxybenzo[*c*]quinolizinium chloride (**8u**, MPB-104). Compound **8u** is indeed 100 times more potent than the parent compounds **8a** (MPB-07) and nontoxic. Stability,[36] mutagenicity, and preliminary pharmacokinetics are currently being studied.

Experimental Section

Chemistry. ^{1}H NMR spectra were recorded on a Varian EM 360A spectrometer (60 MHz). Chemical shifts (ppm) were reported relative to tetramethylsilane (TMS). ^{19}F NMR spectra were recorded on a Brucker ARX 200 spectrometer. Coupling constants (*J*) are reported in hertz (Hz), and s, d, t, q, m, and bs refer to singlet, doublet, triplet, quartet, multiplet, and broad singlet, respectively. Infrared spectra (IR) were recorded on an ATI Mattson genesis series FTIR. Mass spectra (MS) were taken on a VG 70-70F instrument operating in electrospray ionization (ESI) mode. High-resolution mass spectra were obtained by LSIMS positive ionization (FAB), at an accelerating voltage of 8 kV (ZABSpec TOF from Micromass). *m*-Nitrobenzylic alcohol was used as the sample matrix. Elemental analyses were indicated by the symbol of the elements, and the results were within ± 0.4% (for C, H, N) of the theorical values, unless otherwise noted; they were performed on a Perkin-Elmer elemental analyzer (2400). Melting points were measured on a Köfler apparatus and are uncorrected.

All experiments involving butyllithium (BuLi) were carried out in dried apparatus under an atmosphere of dry, oxygen-free nitrogen. Tetrahydrofuran (THF) was distilled from benzophenone−sodium. Diisopropylamine and heterocycles were distilled and stored over barium oxide. Butyllithium (1.6 M solution in hexane) was supplied by Acros and was assayed by titration against diphenylacetic acid. Alkylpyridines were prepared according to usual procedures. Benzo[*c*]quinolizinium chloride was synthesized from 2-methylpyridine and 2-chlorobenzaldehyde.[37] Matrex silica gel (60 Å, 20−45 μm), Merck alumina gel (90 Å , 63−200 μm), and Acros Florisil (60−100 mesh) were employed for column chromatographies.

Synthesis of Compounds 6a−x. Method A (Compounds 6a−i, 6u). Diisopropylamine (2.23 g, 22 mmol) in THF (20 mL) was cooled to 0 °C, and butyllithium (22 mmol) was added dropwise. After stirring for 30 min at 0 °C, the solution was cooled to −40 °C before addition of 2-methylpyridine (1.86 g, 20 mmol), or 2-pentylpyridine (2.98 g, 20 mmol) for compound **6u**, in THF (20 mL). The mixture was stirred for 15 min, and a solution of 20 mmol of dihalogenobenzonitrile in THF (30 mL) was added: 3.44 g of the dichloro derivatives **2a**−**d**, 2.78 g of the difluoro derivatives **2e**−**h**, or 3.11 g of 2-chloro-6-fluorobenzonitrile **2i** (for compounds **6i** and **6u**). The mixture was stirred at −40 °C for 30 min and 3 h at 20 °C and was hydrolyzed with water (20 mL). Then THF was evaporated under reduced pressure, and the pH was adjusted to 2 with sulfuric acid (30 mL of a 6 N solution). The mixture was heated for 3 h at 0 °C and then extracted with CH_2Cl_2 (50 mL). The organic layer was dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The crude product was chromatographed on silica gel eluting with CH_2Cl_2.

Method B (Compounds 6a, 6c, 6q−t, 6v−x). Some compounds **6** could be obtained from 2-substituted (or not) picolyllithium (20 mmol), prepared as above, with dihalogeno aromatic esters (10 mmol) in the place of nitriles: 2.05 g of methyl 2,3-dichlorobenzoate (compounds **6a, 6q−t, 6v-6w**), 2.19 g of ethyl 2,5-dichlorobenzoate (compound **6c**), and 1.72 g of methyl 2,6-difluorobenzoate (compound **6x**).

Method C (Compounds 6j−p). To a solution of butyllithium (25 mmol) at −5 °C was added 1,1,1,3,3,3-hexamethyldisilazane (4.03 g, 25 mmol) in THF (10 mL). The mixture was stirred for 30 min and a solution of ethyl 2-pyridylacetate (3.30 g, 20 mmol) in THF (25 mL) was added dropwise in 2 h. Then, difluorobenzoyl chloride **4j**−**m** (3.18 g, 18 mmol) or dichlorobenzoyl chloride **4n**−**p** (3.77 g, 18 mmol) in THF (10 mL) was added dropwise over 1 h. The mixture was stirred at −5 °C for 1 and for 2 h at room temperature. Water was added (20 mL), the organic layer was dried, and the solvent removed under reduced pressure.

1-(2,3-Dichlorophenyl)-1-hydroxy-2-(2-pyridyl)ethylene (6a): yellow powder; yield 1.22 g, 23% for method A, and 1.70 g, 64% for method B; mp 112 °C; ^{1}H NMR ($CDCl_3$) δ 15.3 (bs, 0.8H), 8.5 (d, J = 4 Hz, 0.2H), 8.2 (d, J = 4 Hz, 0.8H), 7.7−6.8 (m, 6H); 5.7 (s, 0.8H), 4.4 (s, 0.4H); IR (KBr) 3080, 1637 cm^{-1}. Anal. ($C_{13}H_9NOCl_2$) C, H, N.

1-(2,4-Dichlorophenyl)-1-hydroxy-2-(2-pyridyl)ethylene (6b): pale-yellow powder; yield 2.39 g, 45% for method A; ^{1}H NMR ($CDCl_3$) δ 15.3 (bs, 0.7H), 8.5 (d, J = 5 Hz, 0.3H), 8.2 (d, J = 5 Hz, 0.7H), 7.8−6.8 (m, 6H); 5.8 (s, 0.7H), 4.4 (s, 0.6H); IR (KBr) 3075, 3038, 2810, 2608, 1668, 1647 cm^{-1}. Anal. ($C_{13}H_9NOCl_2$, HCl) C, H, N.

1-(2,5-Dichlorophenyl)-1-hydroxy-2-(2-pyridyl)ethylene (6c): yellow crystal; yield 1.75 g, 33% for method A, and 0.88 g, 33% for method B; mp 176 °C; ^{1}H NMR ($CDCl_3$) δ 15.4 (bs, 0.9H), 8.6 (m, 0.1H), 8.2 (d, J = 5 Hz, 0.9H), 7.5−6.8 (m, 6H), 5.8 (s, 0.9H), 4.4 (s, 0.2H); IR (KBr) 3086, 1596 cm^{-1}. Anal. ($C_{13}H_9NOCl_2$) C, H, N.

1-(2,6-Dichlorophenyl)-1-hydroxy-2-(2-pyridyl)ethylene (6d): yellow powder; yield 1.75 g, 33% for method A; mp (HCl) 237 °C; ^{1}H NMR ($CDCl_3$) δ 8.4 (d, J = 5 Hz, 0.2H), 8.2 (d, J = 5 Hz, 0.8H), 7.5−6.7 (m, 6.8H), 5.4 (s, 0.8H), 4.4 (s,

0.4H); IR hydrochloride (KBr) 3064, 3019, 1649 cm^{-1}. Anal. ($C_{13}H_9NOCl_2 \cdot HCl$) C, H, N.

1-(2,3-Difluorophenyl)-1-hydroxy-2-(2-pyridyl)ethylene (6e): yellow powder; yield 1.35 g, 29% for method A; mp 65 °C; ^1H NMR ($CDCl_3$) δ 15.8 (bs, 0.8H), 8.8 (d, $J = 6$ Hz, 0.2H), 8.4 (d, $J = 6$ Hz, 0.8H), 8.0−7.7 (m, 2H), 7.5−7.0 (m, 4H), 6.4 (s, 0.8H), 5.5 (s, 0.4H); IR (KBr) 3440, 3080, 1624 cm^{-1}. Anal. ($C_{13}H_9NOF_2$) C, H, N.

1-(2,4-Difluorophenyl)-1-hydroxy-2-(2-pyridyl)ethylene (6f): yellow powder; yield 0.56 g, 12% for method A; mp (HCl) 177 °C (dec); ^1H NMR ($CDCl_3$) δ 13.2 (bs, 0.7H), 8.9 (d, $J = 7$ Hz, 0.3H), 8.6 (d, $J = 7$ Hz, 0.7H), 8.4−7.8 (m, 2H), 7.7−7.0 (m, 4H), 6.4 (s, 0.7H), 4.7 (s, 0.6H); IR (KBr) 3421, 3070, 1629 cm^{-1}. Anal. ($C_{13}H_9NOF_2 \cdot HCl$) C, H, N.

1-(2,5-Difluorophenyl)-1-hydroxy-2-(2-pyridyl)ethylene (6g): yellow powder; yield 2.80 g, 60% for method A; mp (HCl) 167 °C; ^1H NMR ($CDCl_3$) δ 16.2 (bs, 0.8H), 8.9 (d, $J = 7$ Hz, 0.2H), 8.6 (d, $J = 7$ Hz, 0.8H), 7.9 (t, $J = 7$ Hz 1H), 7.6−7.1 (m, 5H), 6.5 (s, 0.8H), 4.6 (s, 0.4H); IR (KBr) 3448, 3074, 2920, 1623 cm^{-1}. Anal. $C_{13}H_9NOF_2 \cdot HCl$) C, H, N.

1-(2,6-Difluorophenyl)-1-hydroxy-2-(2-pyridyl)ethylene (6h): yellow powder; yield 2.19 g, 47% for method A; mp 88 °C; ^1H NMR ($CDCl_3$) δ 16.0 (bs, 0.8H), 8.9 (d, $J = 6$ Hz, 0.2H), 8.6 (d, $J = 6$ Hz, 0.8H), 8.2−7.6 (m, 4H), 7.5−7.0 (m, 2H), 6.0 (s, 0.8H), 4.6 (s, 0.4H); IR (KBr) 3079, 1621 cm^{-1}. Anal. ($C_{13}H_9NOF_2$) C, H, N.

1-(2-Chloro-6-fluorophenyl)-1-hydroxy-2-(2-pyridyl)-ethylene (6i): yellow powder; yield 2.49 g, 50% for method A; mp (HCl) 190 °C (dec); ^1H NMR ($CDCl_3$) δ 16.9 (bs, 0.8H), 8.4 (m, 0.2H), 8.2 (d, $J = 5$ Hz, 0.8H), 7.6−6.8 (m, 6H), 5.5 (s, 0.8H), 4.3 (s, 0.4H); IR (KBr) 3084, 1632 cm^{-1}. Anal. ($C_{13}H_9NOClF \cdot HCl$) C, H, N.

Ethyl-3-oxo-3-(2,5-difluorophenyl)-2-(2-pyridyl)propanoate (6l): yellow powder; yield 3.13 g, 57% for method C; mp 97 °C (dec); ^1H NMR ($CDCl_3$) δ 17.4 (s, 1H), 8.4 (d, $J = 8$ Hz, 1H), 8.1−7.7 (m, 2H), 7.1−6.9 (m, 4H), 4.0 (q, $J = 8$ Hz, 2H), 0.9 (t, $J = 8$ Hz, 3H); IR (KBr) 3120, 3074, 3053, 2986, 2949, 2897, 1696 cm^{-1}. Anal. ($C_{16}H_{13}NO_3F_2$) C, H, N.

The intermediates **6j, 6k, 6m** were not isolated and led directly to **9j, 9k and 9m** when the reaction solvent was removed.

Ethyl-3-oxo-3-(2,3-dichlorophenyl)-2-(2-pyridyl)propanoate (6n): white powder; yield 2.98 g, 49% for method C; mp 135 °C; ^1H NMR (DMSO-d_6) δ 8.4 (d, $J = 8$ Hz, 1H), 8.0−6.9 (m, 7H), 3.8 (q, $J = 7$ Hz, 2H), 0.8 (t, $J = 7$ Hz, 3H); IR (KBr) 3457, 3100, 2976, 2899, 1663 cm^{-1}. Anal. ($C_{16}H_{13}NO_3Cl_2$) C, H, N.

Ethyl-3-oxo-3-(2,4-dichlorophenyl)-2-(2-pyridyl)propanoate (6o): yellow powder; yield 4.02 g, 66% for method C; mp 120 °C; ^1H NMR (DMSO-d_6) δ 17.8 (s, 1H), 8.3 (d, $J = 9$ Hz, 1H), 7.9−6.8 (m, 6H), 3.8 (q, $J = 8$ Hz, 2H), 0.8 (t, $J = 8$ Hz, 3H); IR (KBr) 3500, 3049, 2979, 2897, 1661 cm^{-1}. Anal. ($C_{16}H_{13}NO_3Cl_2$) C, H, N.

Ethyl-3-oxo-3-(2,6-dichlorophenyl)-2-(2-pyridyl)propanoate (6p): white powder; yield 4.99 g, 82% for method C; mp 136 °C; Anal. ($C_{16}H_{13}NO_3Cl_2$) C, H, N; ^1H NMR (DMSO-d_6) δ 17.1 (s, 1H), 8.5 (d, $J = 6$ Hz, 1H), 8.3−8.0 (m, 2H), 7.4 (bs, 4H), 3.8 (q, $J = 7$ Hz, 2H), 0.8 (t, $J = 7$ Hz, 3H); IR (KBr) 3641, 3074, 2978, 1650 cm^{-1}.

1-(2,3-Dichlorophenyl)-1-hydroxy-2-phenyl-2-(2-pyridyl)ethylene (6q): yellow powder; yield 2.46 g, 72% for method B; mp 130 °C (dec); ^1H NMR ($CDCl_3$) δ 16.5 (s, 1H), 8.1 (d, $J = 5$ Hz, 1H), 7.4−6.5 (m, 11H); IR (KBr) 3060, 2960, 1623, 1572, 1541, 1386, 1310, 740 cm^{-1}. Anal. ($C_{19}H_{13}NOCl_2$) C, H, N;

1-(2,3-Dichlorophenyl)-1-hydroxy-2-methyl-2-(2-pyridyl)-ethylene (6r): yellow powder; yield 0.87 g, 31% for method B; mp 60 °C (dec); ^1H NMR ($CDCl_3$) δ 15.8 (s, 1H), 8.1 (d, $J = 5$ Hz, 1H), 7.7−6.8 (m, 6H), 1.8 (s, 3H); IR (KBr) 3407, 3081, 2935, 1706 cm^{-1}. Anal. ($C_{14}H_{11}NOCl_2$) C, H, N.

1-(2,3-Dichlorophenyl)-1-hydroxy-2-propyl-2-(2-pyridyl)-ethylene (6s): yellow powder; yield 1.60 g, 52% for method B; ^1H NMR ($CDCl_3$) δ 8.4 and 8.2, (2d, $J = 6$ Hz, 0.8H and 0.2H), 7.7−6.9 (m, 6.2H), 4.5 (t, $J = 7$ Hz, 0.8H), 2.0 (m, 2H), 1.5−1.2 (m, 2H), 0.9 (t, $J = 7$ Hz, 3H); IR (KBr) 2965, 2924, 2867, 1701 cm^{-1}. Anal. ($C_{16}H_{15}NOCl_2$) C, H, N.

1-(2,3-Dichlorophenyl)-1-hydroxy-2-butyl-2-(2-pyridyl) ethylene (6t): yellow powder; yield 1.77 g, 55% for method B; ^1H NMR ($CDCl_3$) δ 8.5 and 8.3, (2d, $J = 6$ Hz, 0.8H and 0.2H), 7.8−6.9 (m, 6.2H), 4.6 (t, $J = 6$ Hz, 0.8H), 2.1 (m, 2H), 1.6−1.1 (m, 4H), 0.9 (t, $J = 6$ Hz, 3H); IR (KBr) 3085, 2955, 2930, 2869 1707 cm^{-1}. Anal. ($C_{17}H_{17}NOCl_2$) C, H, N.

1-(6-Chloro-2-fluorophenyl)-1-hydroxy-2-butyl-2-(2-pyridyl)ethylene (6u): yellow powder; yield 1.22 g, 20% for method A; ^1H NMR ($CDCl_3$) δ 8.5 and 8.3, (2d, $J = 4$ Hz, 0.5H each), 7.5 (t, $J = 7$ Hz, 1H), 7.4−6.9 (m, 5.5H), 4.4 (t, $J = 6$ Hz, 0.5H), 2.1 (m, 2H), 1.5−1.1 (m, 4H), 1.0−0.6 (t, $J = 6$ Hz, 3H); IR (KBr) 2955, 2932, 2870, 1713 cm^{-1}. Anal. ($C_{17}H_{17}$-NOClF) C, H, N.

1-(2,3-Dichlorophenyl)-1-hydroxy-2-pentyl-2-(2-pyridyl)-ethylene (6v): colorless liquid; yield 1.34 g, 40% for method B; ^1H NMR ($CDCl_3$) δ 8.5 and 8.3, (2d, $J = 4$ Hz, 0.7H and 0.3H), 7.7−6.8 (m, 6.3H), 4.5 (t, $J = 6$ Hz, 0.7H), 2.1 (t, $J = 6$ Hz, 2H), 1.5−1.1 (m, 6H), 0.8 (t, $J = 6$ Hz, 3H); IR (KBr) 2955, 2929, 2870, 1709 cm^{-1}. Anal. ($C_{18}H_{19}NOCl_2$) C, H, N.

1-(2,3-Dichlorophenyl)-1-hydroxy-2-isobutyl-2-(2-pyridyl)ethylene (6w): yellow powder; yield 1.77 g, 55% for method B; ^1H NMR ($CDCl_3$) δ 8.5 and 8.3, (2d, $J = 6$ Hz, 0.7H and 0.3H), 7.7−6.9 (m, 6.3H), 4.6 (t, $J = 6$ Hz, 0.7H), 2.2−1.8 (m, 3H), 0.9−0.8 (d, $J = 6$ Hz, 6H); IR (KBr) 2957, 1702 cm^{-1}. Anal. ($C_{17}H_{17}NOCl_2$) C, H, N.

The intermediate 6x was not isolated because of its light sensitivity and rapid degradation on silica gel.

Synthesis of Compounds 7a (MPB-02) and 7d (MPB-04). Lithium diisopropylamide (22 mmol) was prepared as above at 0 °C and cooled to −40 °C before addition of 2-methylpyridine (1.86 g, 20 mmol) in THF (20 mL). After 30 min, 2,3- or 2,6-dichlorobenzonitrile **2a** or **2d** (3.44 g, 20 mmol) in THF (20 mL) was added, and the solution was stirred for 30 min at − 40 °C and a further 3 h at 20 °C and then hydrolyzed with water. The organic layer was dried over Na_2SO_4 and concentrated under vacuum. The crude product was warmed to 200 °C for 1 h and then dissolved in boiling ethanol, and ethyl acetate was added until no precipitation occurred. After filtration the product was crystallized from ethanol.

6-Amino-10-chloro-benzo[*c*]quinolizinium chloride (7a, MPB-02): yellow powder; yield 1.10 g, 20%; mp 260 °C (dec); ^1H NMR (DMSO-d_6) δ 9.2 (d, $J = 7$ Hz, 1H, 1-H), 9.0−7.5 (m, 6H), 7.4−6.5 (m, 3H); IR (KBr) 3430, 3281, 3115 cm^{-1}. Anal. ($C_{13}H_{10}N_2Cl_2 \cdot 0.5H_2O$) C, H, N.

6-Amino-7-chloro-benzo[*c*]quinolizinium chloride (7d, MPB-04): yellow powder; yield 2.53 g, 42%); mp 260 °C (dec); ^1H NMR (DMSO-d_6) δ 10.1 (d, $J = 7$ Hz, 1H, 1-H), 9.4 (m, 1H), 8.6−8.2 (m, 5H), 8.1−7.5 (m, 3H); IR (KBr) 3417, 3176 cm^{-1}. Anal. ($C_{13}H_{10}N_2Cl_2 \cdot 2H_2O$) C, H, N.

Preparation of Compounds 8a−h, 8j−m, and 8q−x. Pure products **6** (0.50 g) were warmed for 30 min at 165−170 °C for the 2-fluoro derivatives **6e−i, 6l**, and **6u**; at 185−190 °C for the 2-chloro derivatives **6a−d**; and 195−200 °C for the 2-chloro derivatives **6q−w**. Crude fluorides **8** were washed with acetone, dissolved in CH_2Cl_2, and converted into the hydrochloride to give **8e−h, 8d** (from **6i**), **8l**, and **8u.** Crude chlorides **8a−d** were washed with acetone and recrystallized from ethanol or dimethylformamide, and **8q−w** were purified by Florisil gel column chromatography (CH_2Cl_2) giving the cyclic ketones **9q−w**. As noted above, the compounds **6j**, **6k**, and **6m** were cyclized into **9j**, **9k**, and **9m** when the reaction solvent was removed, and crude **6x** was warmed at 150 °C during 5 min to give **9x**. Compounds **9** were converted into the hydrochloride in diethyl ether.

10-Chloro-6-hydroxybenzo[*c*]quinolizinium chloride (8a, MPB-07): pale-yellow powder; yield 0.21 g, 42%; mp 196 °C (dec); ^1H NMR (DMSO-d_6) δ 10.0 (d, $J = 7$ Hz, 1H, 1-H), 8.9−7.6 (m, 7H), 7.5 (bs, 1H, OH); IR (KBr) 3143, 3029, 2920, 2416 cm^{-1}; FABMS calcd (C^+, $C_{13}H_9NO^{35}Cl$) 230.03727, found 230.0377. Anal. ($C_{13}H_9NOCl_2$) C, H, N;

9-Chloro-6-hydroxybenzo[*c*]quinolizinium chloride (8b, MPB-08): yellow powder; yield 0.36 g, 72%; ^{1}H NMR (DMSO-d_6) δ 9.8 (d, J = 8 Hz, 1H, 1-H), 9.0 (s, 1H, 10-H), 8.2−7.6 (m, 6H), 7.4 (bs, 1H, OH); IR (KBr) 3410, 3102, 3056, 2700, 2356 cm^{-1}; FABMS calcd (C^+, $C_{13}H_9NO^{35}Cl$) 230.03727, found 230.0371. Anal. ($C_{13}H_9NOCl_2\cdot 0.25H_2O$) C, H, N.

8-Chloro-6-hydroxybenzo[*c*]quinolizinium chloride (8c, MPB-30): brown powder; yield 0.34 g, 68%; mp 210 °C (dec); ^{1}H NMR (DMSO-d_6) δ 9.8 (d, J = 7 Hz, 1H, 1-H), 8.9 (d, J = 10 Hz, 1H), 8.2−7.9 (m, 6H), 7.4 (bs, 1H, OH); IR (KBr) 3383, 3200, 3090, 2455, 1903 cm^{-1}; FABMS calcd (C^+, $C_{13}H_9NO^{35}Cl$) 230.03727, found 230.0371. Anal. ($C_{13}H_9NOCl_2\cdot 0.5H_2O$) C, H, N.

7-Chloro-6-hydroxybenzo[*c*]quinolizinium chloride (8d, MPB-27): yellow powder; yield 0.22 g, 45%; mp 210 °C (dec); ^{1}H NMR (DMSO-d_6) δ 9.8 (d, J = 7 Hz, 1H, 1-H), 8.9 (m, 1H), 8.2−7.1 (m, 7H); IR (KBr) 3097, 3045, 2396 cm^{-1}; FABMS calcd (C^+, $C_{13}H_9NO^{35}Cl$) 230.03727, found 230.0372. Anal. ($C_{13}H_9NOCl_2$) C, H, N.

10-Fluoro-6-hydroxybenzo[*c*]quinolizinium chloride (8e, MPB-80): yellow powder; yield 0.21 g, 43%; mp 186 °C; ^{1}H NMR (CF_3COOD) δ 10.0 (m, 1H, 1-H), 8.6 (m, 1H), 8.4−7.7 (m, 6H), 7.6 (s, 1H, 5-H); ^{19}F NMR (DMSO-d_6) δ −113.4 (ddd, $J_{F/9-H}$ = 16 Hz, $J_{F/1-H}$ = 8.1 Hz and $J_{F/8-H}$ = 4.7 Hz, 1F); IR (KBr) 3164, 2467, 1885 cm^{-1}; FABMS calcd (C^+, $C_{13}H_9NOF$) 214.06682, found 214.0664. Anal. ($C_{13}H_9NOClF\cdot H_2O$) C, H, N.

9-Fluoro-6-hydroxybenzo[*c*]quinolizinium chloride (8f, MPB-29): beige powder; yield 0.32 g, 65%; ^{1}H NMR (CF_3COOD) δ 9.4 (d, J = 7 Hz, 1H, 1-H), 8.9−8.6 (m, 1H), 8.5−8.1 (m, 3H), 8.0−7.6 (m, 3H), 7.5 (s, 1H, 5-H); ^{19}F NMR (DMSO-d_6) δ −103.4 (td, $J_{F/7-H}$ = $J_{F/8-H}$ = 7 Hz and $J_{F/10-H}$ = 11.6 Hz, 1F); IR (KBr) 3263, 3065, 2550, 2000 cm^{-1}; FABMS calcd (C^+, $C_{13}H_9NOF$) 214.06682, found 214.0666. Anal. ($C_{13}H_9NOClF\cdot 0.5H_2O$) C, H, N.

8-Fluoro-6-hydroxybenzo[*c*]quinolizinium chloride (8g, MPB-79): yellow powder; yield 0.30 g, 60%; mp 250 °C; ^{1}H NMR (DMSO-d_6) δ 10.0 (d, J = 7 Hz, 1H, 1-H), 9.4−9.0 (m, 1H), 8.4−8.2 (d, 2H), 8.0 (s, 1H), 7.9−7.6 (m, 3H), 7.2 (bs, OH + H_2O); ^{19}F NMR (DMSO-d_6) δ −109.9 (td, $J_{F/7-H}$ = $J_{F/9-H}$ = 8.2 Hz and $J_{F/10-H}$ = 4.2 Hz, 1F); IR (KBr) 3274, 3102, 3062, 2362 cm^{-1}; FABMS calcd (C^+, $C_{13}H_9NOF$) 214.06682, found 214.0671. Anal. ($C_{13}H_9NOClF$) C, H, N.

7-Fluoro-6-hydroxybenzo[*c*]quinolizinium chloride (8h, MPB-78): beige powder; yield 0.22 g, 45%; ^{1}H NMR (DMSO-d_6) δ 9.9 (d, J = 9 Hz, 1H, 1-H), 8.9 (d, J = 9 Hz, 1H), 8.3−8.1 (m, 2H), 8.0−7.6 (m, 5H); ^{19}F NMR (DMSO-d_6) δ −107.9 (dd, $J_{F/8-H}$ = 11.3 Hz and $J_{F/9-H}$ = 5.6 Hz, 1F); IR (KBr) 3247, 3107, 3056, 2679, 2401 cm^{-1}; FABMS calcd (C^+, $C_{13}H_9NOF$) 214.06682, found 214.0669. Anal. ($C_{13}H_9NOClF$) C, H, N.

5-Carbethoxy-10-fluoro-6-hydroxybenzo[*c*]quinolizinium chloride (8j, MPB-77): beige powder; yield reported from 2,6-difluorobenzoyl chloride (18 mmol), 0.43 g, 7%; mp 134 °C; ^{1}H NMR (DMSO-d_6) δ 8.9 (bd, 1H, 1-H), 8.2−6.9 (m, 6H), 6.3 (s, 1H, OH), 4.3 (q, J = 7 Hz, 2H), 1.3 (t, J = 7 Hz, 3H); ^{19}F NMR (DMSO-d_6) δ −116.7 (td, $J_{F/8-H}$ = $J_{F/1-H}$ = 5.0 Hz and $J_{F/9-H}$ = 15 Hz, 1F); IR (KBr) 3420, 3171, 3080, 2973, 1718 cm^{-1}; FABMS calcd (C^+, $C_{16}H_{13}NO_3F$) 286.08795, found 286.0878. Anal. ($C_{16}H_{13}NO_3ClF\cdot H_2O$) C, H, N.

5-Carbethoxy-9-fluoro-6-hydroxybenzo[*c*]quinolizinium chloride (8k, MPB-86): yellow powder; yield reported from 2,5-difluorobenzoyle chloride (18 mmol), 2.02 g, 35%; mp 154 °C; ^{1}H NMR (DMSO-d_6) δ 9.2 (d, J = 8 Hz, 1H, 1-H), 8.7−7.1 (m, 6H), 5.7 (s, 1H, OH), 4.3 (q, J = 7 Hz, 2H), 1.3 (t, J = 7 Hz, 3H); ^{19}F NMR (DMSO-d_6) δ −105.7, (td, $J_{F/7-H}$ = $J_{F/8-H}$ = 8 Hz and $J_{F/10-H}$ = 12 Hz, 1F); IR (KBr) 3454, 3104, 3047, 3016, 2960, 1727 cm^{-1}; FABMS calcd (C^+, $C_{16}H_{13}NO_3F$) 286.08795, found 286.0877. Anal. ($C_{16}H_{13}NO_3ClF$) C, H, N.

5-Carbethoxy-8-fluoro-6-hydroxybenzo[*c*]quinolizinium chloride (8l, MPB-75): yellow powder; yield 0.09 g, 18%; mp 168 °C; ^{1}H NMR (DMSO-d_6) δ 9.4 (d, J = 9 Hz, 1H, 1-H), 8.9−8.7 (m, 1H), 8.2−7.0 (m, 5H), 6.0 (s, 1H, OH), 4.3 (q, J = 8 Hz, 2H), 1.3 (t, J = 8 Hz, 3H); IR (KBr) 3500, 3157, 3049, 2960, 1670 cm^{-1}. Anal. ($C_{16}H_{13}NO_3ClF$) C, H, N.

5-Carbethoxy-7-fluoro-6-hydroxybenzo[*c*]quinolizinium chloride (8m, MPB-73): beige powder; yield reported from 2,6-difluorobenzoyle chloride (18 mmol), 0.52 g, 9%; mp 173 °C; ^{1}H NMR (DMSO-d_6) δ 9.0 (d, J = 8 Hz, 1H, 1-H), 8.4−6.8 (m, 7H), 4.2 (q, J = 7 Hz, 2H), 1.3 (t, J = 7 Hz, 3H); ^{19}F NMR (DMSO-d_6) δ −112.3 (dd, $J_{F/8-H}$ = 12.0 Hz and $J_{F/9-H}$ = 5.6 Hz, 1F); IR (KBr) 3420, 3160, 3101, 3046, 2984, 1720 cm^{-1}; FABMS calcd (C^+, $C_{16}H_{13}NO_3F$) 286.08795, found 286.0878. Anal. ($C_{16}H_{13}NO_3ClF\cdot 0.5H_2O$) C, H, N.

10-Chloro-6-hydroxy-5-phenylbenzo[*c*]quinolizinium chloride (8q, MPB-67): yellow powder; yield 0.05 g, 9%; mp 187 °C (dec); ^{1}H NMR (DMSO-d_6) δ 9.5 (d, J = 7 Hz, 1H, 1-H), 8.8 (d, J = 6 Hz, 1H), 8.5−7.2 (m, 10H), 4.8 (s, 1H, OH); IR (KBr) 3445, 3062, 3021 cm^{-1}; FABMS calcd (C^+, $C_{19}H_{13}NO^{35}Cl$) 306.06857, found 306.0685. Anal. ($C_{19}H_{13}NOCl_2$) C, H, N.

10-Chloro-6-hydroxy-5-methylbenzo[*c*]quinolizinium chloride (8r, MPB-94): beige powder; yield 0.02 g, 4%; mp 160 °C; ^{1}H NMR ($CDCl_3$) δ 9.8 (d, J = 7 Hz, 1H, 1-H), 9.4−8.6 (m, 2H), 8.2−7.2 (m, 5H), 2.8 (s, 3H); IR (KBr) 3256, 3142 cm^{-1}; FABMS calcd (C^+, $C_{14}H_{11}NO^{35}Cl$) 244.05292, found 244.0525. Anal. ($C_{14}H_{11}NOCl_2\cdot 2H_2O$) C, H, N.

10-Chloro-6-hydroxy-5-propylbenzo[*c*]quinolizinium chloride (8s, MPB-96): cream powder; yield 0.11 g, 23%; mp 187 °C (dec); ^{1}H NMR (DMSO-d_6) δ 9.4 (d, J = 7 Hz, 1H, 1-H), 8.5 (d, J = 7 Hz, 1H), 8.2−7.2 (m, 6H), 3.0 (t, J = 6 Hz, 2H), 1.8−1.2 (m, 2H), 1.0 (t, J = 6 Hz, 3H); IR (KBr) 2963, 2915, 2870, 2464 cm^{-1}; FABMS calcd (C^+, $C_{16}H_{15}NO^{35}Cl$) 272.08422, found 272.0839. Anal. ($C_{16}H_{15}NOCl_2$) C, H, N.

5-Butyl-10-chloro-6-hydroxybenzo[*c*]quinolizinium chloride (8t, MPB-91): cream powder; yield 0.3 g, 60%; mp 186 °C (dec); ^{1}H NMR (DMSO-d_6) δ 9.3 (d, J = 7 Hz, 1H, 1-H), 8.5 (d, J = 7 Hz, 1H), 8.1−6.9 (m, 6H), 2.9 (t, J = 4 Hz, 2H), 1.5−1.3 (m, 4H), 0.9 (t, J = 5 Hz, 3H); IR (KBr) 2951, 2923, 2895, 2863, 2450 cm^{-1}; MS (ESI) 286 (M^+ − HCl); FABMS calcd (C^+, $C_{17}H_{17}NO^{35}Cl$) 286.09987, found 286.0999. Anal. ($C_{17}H_{17}NOCl_2$) C, H, N.

5-Butyl-7-chloro-6-hydroxybenzo[*c*]quinolizinium chloride (8u, MPB-104): yellow powder; yield 0.3 g, 60%; mp 190 °C; ^{1}H NMR (DMSO-d_6) δ 9.4 (d, J = 7 Hz, 1H, 1-H), 8.7 (m, 1H), 8.0−7.7 (m, 5H), 7.4−7.1 (m, 1H), 2.9 (t, J = 5 Hz, 2H), 1.7−1.3 (m, 4H), 1.0 (t, J = 5 Hz, 3H); IR (KBr) 3100, 3060, 2951, 2925, 2868, 2853 cm^{-1}; FABMS calcd (C^+, $C_{17}H_{17}NO^{35}Cl$) 286.09987, found 286.0999. Anal. ($C_{17}H_{17}NOCl_2\cdot 0.2H_2O$) C, H, N.

10-Chloro-6-hydroxy-5-pentylbenzo[*c*]quinolizinium chloride (8v, MPB-97): cream powder; yield 0.04 g, 8%; mp 177 °C (dec); ^{1}H NMR (DMSO-d_6) δ 9.6 (d, J = 7 Hz, 1H, 1-H), 8.6 (d, J = 7 Hz, 1H), 8.3−7.1 (m, 6H), 3.3 (t, J = 4 Hz, 2H), 1.6−1.2 (m, 6H), 1.0 (t, J = 5 Hz, 3H); IR (KBr) 2958, 2925, 2850 cm^{-1}; FABMS calcd (C^+, $C_{18}H_{19}NO^{35}Cl$) 300.11552, found 300.1155. Anal. ($C_{18}H_{19}NOCl_2$) C, H, N.

10-Chloro-6-hydroxy-5-isobutylbenzo[*c*]quinolizinium chloride (8w, MPB-95): cream powder; yield 0.28 g, 55%; mp 185 °C (dec); ^{1}H NMR (DMSO-d_6) δ 9.3 (d, J = 7 Hz, 1H, 1-H), 8.6 (d, J = 7 Hz, 1H), 8.2−7.2 (m, 6H), 3.0 (d, J = 6 Hz, 2H), 2.2−1.3 (m, 1H), 1.0 (d, J = 6 Hz, 6H); IR (KBr) 3083, 3042, 2958, 2863, 2550 cm^{-1}; FABMS calcd (C^+, $C_{17}H_{17}NO^{35}Cl$) 286.09987, found 286.0997. Anal. ($C_{17}H_{17}NOCl_2$) C, H, N.

7-Fluoro-6-hydoxybenzo[*c*]cyclopentano[*i,j*]quinolizinium chloride (8x, MPB-92): beige powder; yield reported from methyl 2,6-difluorobenzoate (10 mmol), 0.05 g, 2%; mp 245 °C; ^{1}H NMR (CF_3COOD) δ 11.3 (bs, 1H, OH), 9.3 (d, J = 6 Hz, 1H, 1-H), 8.6 (d, J = 8 Hz, 1H), 8.2−7.4 (m, 4H), 3.6 (s, 4H); IR (KBr) 3418, 3100, 3055, 2927, 2367 cm^{-1}; FABMS calcd (C^+, $C_{15}H_{11}NOF$) 240.08247, found 240.0824. Anal. ($C_{15}H_{11}NOClF\cdot 0.25H_2O$) C, H, N.

Synthesis of Compounds 9. Heating compounds **6j**−**m**, **6q**−**r**, and **6x** led to compounds **9**. When chromatographed on an Alumine or Florisil column, compounds **8a** and **8s**−**w** were converted into **9**. All products **9** could also be purified by column chromatography on silica gel (elution with CH_2Cl_2).

10-Chlorobenzo[*c*]quinolizin-6-one (9a, MPB-70): yellow powder; yield 80%; mp 144 °C; ^{1}H NMR ($CDCl_3$) δ 8.9 and 8.5 (2d, J = 7 Hz, 1H each), 7.8−7.4 (m, 2H), 7.2−7.0 (m, 2H,), 6.6−6.3 (m, 2H); IR (KBr) 3126, 3046, 1680 cm^{-1}. Anal. ($C_{13}H_8NOCl$) C, H, N.

Ethyl 10-fluoro-6-oxo-6*H*-benzo[*c*]quinolizine-5-carboxylate (9j): yellow powder; yield 0.77 g, 15%; mp 138 °C; ^{1}H NMR ($CDCl_3$) δ 9.2 (t, J = 6 Hz, 1H, 1-H), 8.8 (bs, 1H), 8.1−7.4 (m, 4H), 7.0 (t, J = 6 Hz, 1H), 4.6 (q, J = 7 Hz, 2H), 1.5 (t, J = 7 Hz, 3H); IR (KBr) 3380, 3160, 3120, 2982, 2940, 1715 cm^{-1}; MS *m*/*z* 285, 240, 213, 185, 158. Anal. ($C_{16}H_{12}NO_3F$) C, H, N.

Ethyl 9-fluoro-6-oxo-6*H*-benzo[*c*]quinolizine-5-carboxylate (9k): yellow powder; yield 2.87 g, 56%; mp 160 °C; ^{1}H NMR ($CDCl_3$) δ 8.6 (d, J = 6 Hz, 1H, 1-H), 8.4 (d, J = 8 Hz, 1H, 4-H), 7.8−7.2 (m, 4H), 6.7 (t, J = 6 Hz, 1H), 4.5 (q, J = 7 Hz, 2H), 1.5 (t, J = 7 Hz, 3H); IR (KBr) 3502, 3380, 3160, 3090, 2953, 1693 cm^{-1}. Anal. ($C_{16}H_{12}NO_3F$) C, H, N.

Ethyl 8-fluoro-6-oxo-6*H*-benzo[*c*]quinolizine-5-carboxylate (9l): yellow powder; yield 3.39 g, 66%; mp 154 °C; ^{1}H NMR ($CDCl_3$) δ 8.5 (d, J = 9 Hz, 1H, 1-H), 8.4−8.1 (m, 2H), 7.8−7.2 (m, 3H), 6.9 (t, J = 9 Hz, 1H), 4.5 (q, J = 7 Hz, 2H), 1.5 (t, J = 7 Hz, 3H); IR (KBr) 3447, 3180, 3160, 2960, 1727, 1716 cm^{-1}. Anal. ($C_{16}H_{12}NO_3F$) C, H, N.

Ethyl 7-fluoro-6-oxo-6*H*-benzo[*c*]quinolizine-5-carboxylate (9m): yellow powder; yield 1.69 g, 33%; mp 133 °C; ^{1}H NMR ($CDCl_3$) δ 8.8 (d, J = 8 Hz, 1H, 1-H), 8.1−7.0 (m, 6H), 4.6 (q, J = 7 Hz, 2H), 1.5 (t, J = 7 Hz, 3H); IR (KBr) 3537, 3180, 2980, 1713 cm^{-1}. Anal. ($C_{16}H_{12}NO_3F \cdot 0.25H_2O$) C, H, N.

10-Chloro-5-phenylbenzo[*c*]quinolizin-6-one (9q): yellow powder; yield 0.22 g, 36%; mp 209 °C; ^{1}H NMR ($CDCl_3$) δ 8.4 (t, J = 6 Hz, 2H), 7.7−6.8 (m, 9H), 6.3 (m, 1H); IR (KBr) 3433, 3120, 3080, 1656 cm^{-1}. Anal. ($C_{19}H_{12}NOCl \cdot 0.25H_2O$) C, H, N.

5-Methyl-10-chlorobenzo[*c*]quinolizin-6-one (9r): yellow powder; yield 0.02 g, 5%; mp 149 °C; ^{1}H NMR ($CDCl_3$) δ 8.3 (t, J = 6 Hz, 2H), 7.6−6.3 (m, 5H), 2.2 (s, 3H); IR (KBr) 3060, 2929, 2857, 1638 cm^{-1}. Anal. ($C_{14}H_{10}NOCl \cdot 0.25H_2O$) C, H, N.

5-Butyl-7-chlorobenzo[*c*]quinolizin-6-one (9u): yellow powder; yield 60%; mp 141 °C; ^{1}H NMR ($CDCl_3$) δ 8.2 (d, J = 7 Hz, 1H, 1-H), 7.8−7.6 (m, 1H), 7.5−7.0(m, 4H), 6.6−6.3 (m, 1H), 2.9−2.5 (m, 2H), 1.7−1.2 (m, 4H), 1.1−0.8 (m, 3H); IR (KBr) 2952, 2924, 2868, 2852, 1644 cm^{-1}. Anal. ($C_{17}H_{16}NOCl$) C, H, N.

7-Fluorobenzo[*c*]cyclopentano[*i*,*j*]quinolizine-6-one (9x): yellow powder; yield 0.57 g, 12%; mp 258 °C; ^{1}H NMR ($CDCl_3$) δ 7.9 (d, J = 6 Hz, 1H, 1-H), 7.6−6.2 (m, 5H), 4.0 (s, 2H), 3.1 (s, 2H); IR (KBr) 3468, 3113, 2928, 1601, cm^{-1}; MS *m*/*z* 239, 238, 210, 95. Anal. ($C_{15}H_{10}NOF \cdot 0.25H_2O$) C, H, N.

Synthesis of Compounds 11a−d, 11y. Harmalane **10** was prepared from *N*-acetyltryptamine by a Bischler−Napieralsky procedure. A solution of harmalane (1.84 g, 10 mmol) in THF (40 mL) was cooled at −40 °C and BuLi (20 mmol) was added dropwise. Dichlorobenzonitriles (1.72 g, 10 mmol) **2a−d** or 4-bromo-2-chlorobenzonitrile (2.16 g, 10 mmol) (for synthesis of compound **11y**) was added. Instable compounds **11a,b** could not be isolated and were directly cyclized, but compounds **11c**, **11d**, and **11y** were caracterized.

1-Amino-1-(2,5-dichlorophenyl)-2-[1-(3,4-dihydropyrido-[3,4-*b*]indolyl)]ethylene (11c): orange powder; yield 1.49 g, 42%; mp 128 °C (dec); ^{1}H NMR ($CDCl_3$) δ 7.8 (bs, 3H, NH + NH_2), 7.7−7.0 (m, 7H), 5.3 (s, 1H), 3.6 (t, J = 6 Hz, 2H), 2.9 (t, J = 6 Hz, 2H); IR (KBr) 3429, 3270, 3055, 2928, 2835 cm^{-1}.

1-Amino-1-(2,6-dichlorophenyl)-2-[1-(3,4-dihydropyrido-[3,4-*b*]indolyl)]ethylene (11d): orange powder; yield 2.06 g, 58%; mp 228 °C; ^{1}H NMR ($CDCl_3$) δ 8.4 (bs, 3H, NH + NH_2), 7.7−7.0 (m, 7H), 5.0 (s, 1H), 3.7 (t, J = 6 Hz, 2H), 3.0 (t, J = 6 Hz, 2H); IR (KBr) 3428, 3255, 3134, 2944, 2846 cm^{-1}. Anal. ($C_{19}H_{15}N_3Cl_2$) C, H, N.

1-Amino-1-(4-bromo-2-chlorophenyl)-2-[1-(3,4-dihydropyrido[3,4-*b*]indolyl)]ethylene (11y): yellow powder; yield 1.20 g, 30%; ^{1}H NMR ($CDCl_3$) δ 8.1 (bs, 3H, NH + NH_2), 7.7−7.0 (m, 7H), 5.1 (s, 1H), 3.8 (t, J = 6 Hz, 2H), 2.9 (t, J = 6 Hz, 2H); IR (KBr) 3435, 3287, 3054, 2932, 2834 cm^{-1}. Anal. ($C_{19}H_{15}N_3BrCl$) C, H, N.

Synthesis of Compounds 12a−d, 12y. A small amount of previously obtained imine (0.50 g, 1.4 mmol of compounds **11a−d** or 1.25 mmol of compound **11y**) was warmed to 200 °C for 10 min. The crude product was washed with propanone and recrystallized from ethanol.

14-Amino-6,7-dihydro-12*H*-4-chlorobenzo[*f*]indolo[2,3-*a*]quinolizinium chloride (12a, MPB-54): brown powder; yield 0.03 g, 6%; mp >260 °C; IR (KBr) 3170 cm^{-1}; MS *m*/*z* 320 (M^+ − HCl), 305, 284, 271, 150; FABMS calcd (C^+, $C_{19}H_{15}N_3{}^{35}Cl$) 320.09545, found 320.0959. Anal. ($C_{19}H_{15}N_3Cl_2 \cdot H_2O$) C, H, N.

14-Amino-6,7-dihydro-12*H*-3-chlorobenzo[*f*]indolo[2,3-*a*]quinolizinium chloride (12b, MPB-51): brown powder; yield 0.09 g, 18%; mp 254 °C (dec); IR (KBr) 3139 cm^{-1}; MS *m*/*z* 320 (M^+ − HCl), 305, 267, 249, 187. Anal. ($C_{19}H_{15}N_3Cl_2$) C, H, N.

14-Amino-6,7-dihydro-12*H*-2-chlorobenzo[*f*]indolo[2,3-*a*]quinolizinium chloride (12c, MPB-50): brown powder; yield 0.04 g, 7%; mp >260 °C; IR (KBr) 3127 cm^{-1}; MS *m*/*z* 320 (M^+ − HCl), 305, 284, 150; FABMS calcd (C^+, $C_{19}H_{15}N_3{}^{35}Cl$) 320.09545, found 320.0951. Anal. ($C_{19}H_{15}N_3Cl_2 \cdot H_2O$) C, H, N.

14-Amino-6,7-dihydro-12*H*-1-chlorobenzo[*f*]indolo[2,3-*a*]quinolizinium chloride (12d, MPB-49): brown powder; yield 0.09 g, 17%; mp >260 °C; ^{1}H NMR (CF_3COOD) δ 7.9−6.4 (m, 11H), 4.5 (bt, 2H), 3.0 (bt, 2H); IR (KBr) 3458, 3371, 3073 cm^{-1}; FABMS calcd (C^+, $C_{19}H_{15}N_3{}^{35}Cl$) 320.09545, found 320.0965. Anal. ($C_{19}H_{15}N_3Cl_2 \cdot 0.5H_2O$) C, H, N.

14-Amino-6,7-dihydro-12*H*-3-bromobenzo[*f*]indolo[2,3-*a*]quinolizinium chloride (12y, MPB-52): brown powder; yield 0.06 g, 12%; mp >260 °C; IR (KBr) 3427, 3161 cm^{-1}; MS *m*/*z* 364 (M^+ − HCl), 324, 286; FABMS calcd (C^+, $C_{19}H_{15}N_3{}^{79}Br$) 364.04493, found 364.0449. Anal. ($C_{19}H_{15}N_3BrCl \cdot 2H_2O$) C, H, N.

Synthesis of 10-Chloro-6-hydroxybenzo[*c*]quinolizinium Bromide 13. Compound **9a** (0.20 g, 0.87 mmol) was dissolved in ethanol (2 mL), and hydrobromidic acid was added dropwise with stirring. Propanone (20 mL) and diethyl ether (20 mL) were then added. The mixture was filtered to give a pale yellow powder: yield (0.26 g, 91%); mp 221 °C (dec); ^{1}H NMR (DMSO-d_6) δ 9.9 (d, J = 7 Hz, 1H, 1-H), 8.5−7.6 (m, 7H), 7.3 (s, 1H, 5-H); IR (KBr) 3258, 3047, 2518 cm^{-1}. Anal. ($C_{13}H_9NOBrCl \cdot H_2O$) C, H, N.

Crystallographic Studies of 8a, 8t, and 13. Suitable crystals were obtained from the compounds produced by our syntheses after recrystallization in CH_3CN. Unit-cell parameters were refined by a least-squares fitting procedure using 25 reflections. An $\omega-2\theta$ scan was used for data collection. The structure was solved by the program package Crystals 2000 (compounds **8a** and **13**) or Crystals 2001 (compound **8t**).[38] Atomic scattering factors were taken from Cromer and Mann and *International Tables for X-ray Crystallography*.[39] All nonhydrogen atoms were found on the E-map[40] and refined anisotropically.[41] Hydrogen atom positions were placed geometrically after each cycle and not refined. The figures were generated by Cameron[42] as implemented in Crystals 2000/2001 package.

Cell Culture. Chinese hamster ovary (CHO) cells stably transfected with pNUT vector alone (pNUT CHO) or containing wild-type CFTR (CFTR(+) CHO) or G551D (G551D CHO) mutation were provided by J. R. Riordan and X.-B. Chang, Scottsdale, AZ.[2,9] Cells cultured at 37 °C in 5% CO_2 were maintained in MEM containing 7% fetal bovine serum, 0.5% antibiotics (50 IU/mL penicillin and 50 μg/mL streptomycin) and 100 or 20 μM methotrexate for CFTR(+) and G551D or pNUT CHO cells, respectively. For detailed procedures see elsewhere.[15,30]

Biological Evaluation of CFTR Chloride Channel Activity. CFTR chloride channel activity was assayed by measuring the rate of iodide (^{125}I) efflux from transfected CHO cells as previously described.[15,30] All experiments were performed with a robotic liquid handling system (Perkin-Elmer Life Sciences, Courtaboeuf, France). Cells were cultured in 24-well plates in order to perform parallel experiments and comparison analysis. At the beginning of each experiment, cells were washed with efflux buffer containing (in mM): 137 NaCl, 5.36 KCl, 0.8 $MgCl_2$, 5.5 glucose, and 10 HEPES, pH 7.4. Cells were then incubated in efflux buffer containing 1 μM KI and

1 μCi of ^{125}I Na /mL (NEN, Boston, MA) for 30 min at 37 °C to permit the ^{125}I to reach equilibrium. Cells were then washed with efflux medium to remove extracellular ^{125}I. The loss of intracellular ^{125}I was determined by removing the medium with efflux buffer every 1 min for up to 11 min. The first four aliquots were used to establish a stable baseline in efflux buffer alone. A medium containing the appropriate drug was used for the remaining aliquots. Residual radioactivity was extracted with 0.1 N NaOH and determined using a Packard Cobra II γ-counter (Perkin-Elmer Life Sciences, Courtaboeuf, France). The fraction of initial intracellular ^{125}I lost during each time point was determined and time-dependent rates of ^{125}I efflux were calculated from $\ln(^{125}I_{t_1}/^{125}I_{t_2})/(t_1 - t_2)$ where $^{125}I_t$ is the intracellular ^{125}I at time t, and t_1 and t_2 are successive time points.[43] Curves were constructed by plotting rate of ^{125}I versus time. In Tables 2 and 3, the data are given as mean percentage activation of CFTR-dependent efflux ± SEM for the indicated number (n) of experiments for each compound. The activation is the percentage corresponding to the maximal peak rate value measured for a given compound assuming the stimulation of efflux being maximal with 5 μM forskolin and equal to 100%. Relative rates (R) were determined and correspond to R_{peak}/R_{basal}. All comparisons are based on maximal values for the time-dependent rates excluding the points used to establish the baseline.[15,30]

Cytotoxicity Assay. The cytotoxicity of CFTR modulators was assessed by measuring cellular deshydrogenase activity using the water-soluble tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], which is converted to purple formazan. For the determination of growth rate and cytotoxicity assay, cells were grown to confluency in 96-well plates at 37 °C. After incubation with the test compound for 3−7 days, 10 μM of 5 mg/mL MTT was added (final concentration 0.5 mg/mL) to each well. After 4 h incubation, culture medium was removed and 100 μL of dimethyl sulfoxide (DMSO) added to solubilize the blue formazan product. The optical density at 595 nm was quantified with a microplate photometer (Packard Biosciences) and compared to the control value without drug (100% cell viability). A second control corresponds to 0% cell viability (air exposed). Cell viability was measured using the trypan blue exclusion as an indicator of cell death. Results are means ± SEM for $n = 4$ separate determinations.

Statistics. Results are expressed as means (SEM of n observations). To compare sets of data, we used either an analysis of variance (ANOVA) or Student's t test. Differences were considered statistically significant when $p < 0.05$. All statistical tests were performed using GraphPad Prism version 3.0 for Windows (Graphpad Software).

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Supporting Information Available: X-ray analysis of **8a**, **8t**, and **13**, including tables of atomic coordinates, thermal parameters, bond distances and angles, and figures concerning crystal structures. This material is available free of charge via the Internet at http://pubs.acs.org.

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