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Strategies To Reduce hERG K^+ Channel Blockade. Exploring Heteroaromaticity and Rigidity in Novel Pyridine Analogues of Dofetilide

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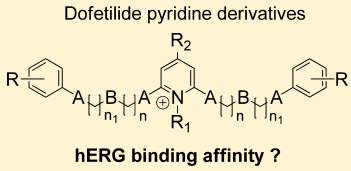
Supporting Information

ABSTRACT: Drug-induced blockade of the human ether-a-go-go-related gene K^+ channel (hERG) represents one of the major antitarget concerns in pharmaceutical industry. SAR studies of this ion channel have shed light on the structural requirements for hERG interaction but most importantly may reveal drug design principles to reduce hERG affinity. In the present study, a novel library of neutral and positively charged heteroaromatic derivatives of the class III antiarrhythmic agent dofetilide was synthesized and assessed for hERG affinity in radioligand binding and manual patch clamp assays. Structural modifications of the pyridine moiety, side chain, and peripheral aromatic moieties were evaluated, thereby revealing approaches for reducing hERG binding affinity. In particular, we found that the extra rigidity imposed close to the positively charged pyridine moiety can be very efficient in decreasing hERG affinity.



INTRODUCTION

Among the vast family of ion channels are the voltage-gated potassium channels (Kv), including the human ether-a-go-go-related gene (hERG) K^+ channel. Class III antiarrhythmic drugs interact with this channel. As a result, the QT interval of the cardiac cycle is prolonged, as observed in the electrocardiogram. However, nonantiarrhythmic drugs and their metabolites may also interact with the hERG channel, thereby increasing the risk of ventricular arrhythmia and fibrillation and sometimes culminating in Torsades de pointes and sudden death.[1,2] Drugs such as astemizole, cisapride, sertindole, and terfenadine have been withdrawn from most markets because of these unwanted cardiac side effects. Currently, hERG safety testing is an indispensable check in the drug discovery process and is required by the FDA.[3]

In the present study we synthesized many derivatives of dofetilide (Figure 1), a potent class III antiarrhythmic agent. We reasoned that such a repertoire of congeneric ligands could provide chemical alerts for hERG potassium channel toxicity.

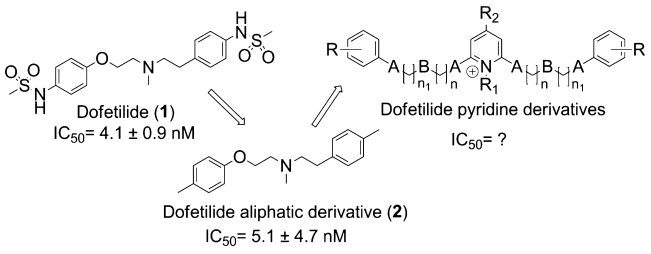


Figure 1. Schematic representation of the rationale behind heteroaromatic compound design. A: triple bonds; B: oxygen; *n*: chain length; R: CH_3, H, OMe, Cl, phenyl; R_1: CH_3; R_2: H or NH_2.

We have taken that approach previously and were successful in defining criteria for what a molecule should *not* have in order to display low or negligible affinity for the hERG channel.[4,5] In this particular case, we focused on the linker between the two aromatic moieties of dofetilide. Flexibility and a basic aliphatic amine in the center of the linker have been suggested as prominent characteristics in the hERG pharmacophore,[6] which we took as the starting point in the current study.

However, there are a number of hERG blockers which do not contain a positively charged nitrogen center,[7] and this apparent contradiction was explored by substituting the aliphatic amine with an aromatic pyridine moiety. The linker's flexibility was challenged by introducing rigidity in that part of the molecule. This synthetic approach yielded over 70 final products that were tested in a competition binding assay with [^{3}H]astemizole as the radioligand. A few of these were further evaluated in a patch clamp assay. From these data we derived chemical alerts and "do's and don'ts" for reduced hERG affinity.

RESULTS AND DISCUSSION

Chemistry. Several pyridine derivatives of dofetilide were synthesized as outlined in Schemes 1−3, with the aim of studying their ability to interact with the hERG channel and ultimately finding new chemical modifications that decrease hERG affinity. As depicted in Figure 1, and as previously shown by our group,[4] the *p*-methylphenyl derivative of dofetilide (**2**) displays about the same hERG binding affinity as dofetilide itself (**1**). Therefore, pyridine analogues of compound **2** were initially designed.

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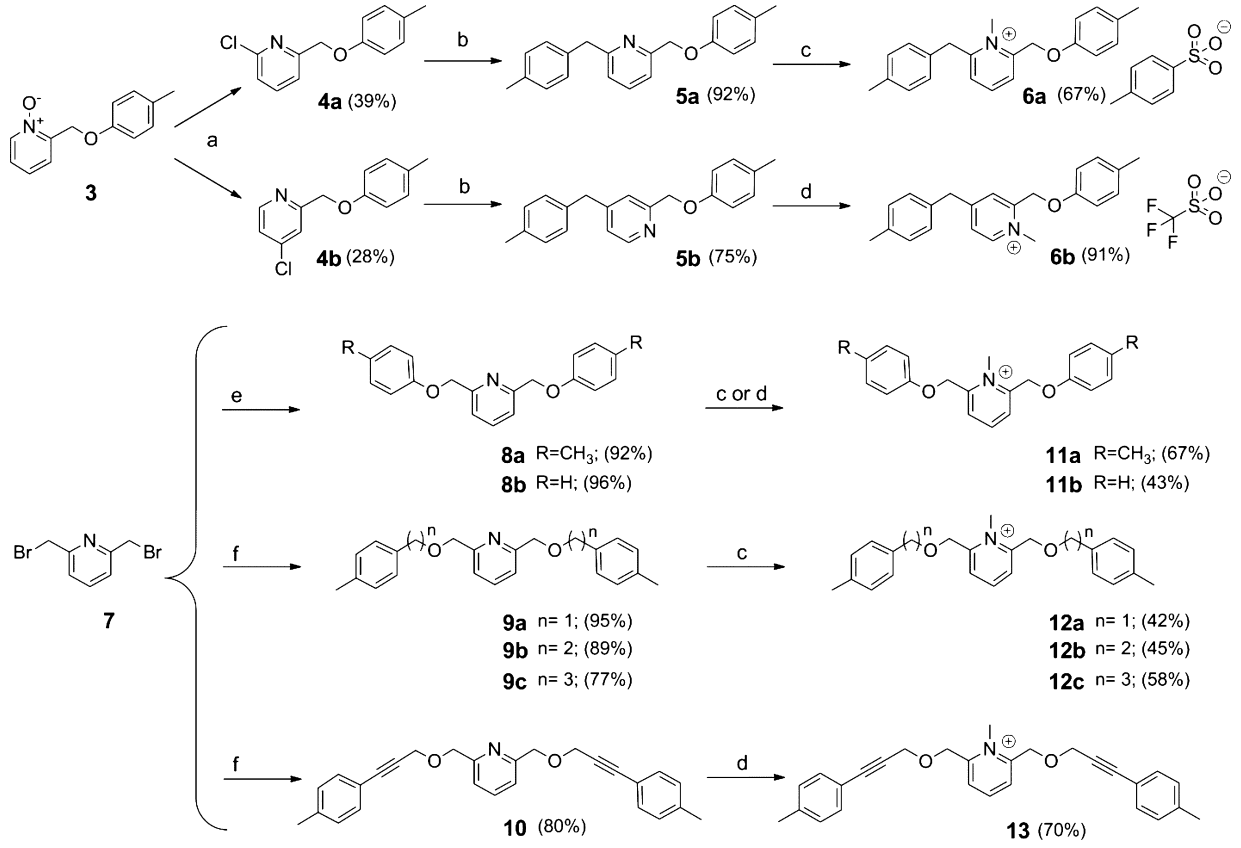
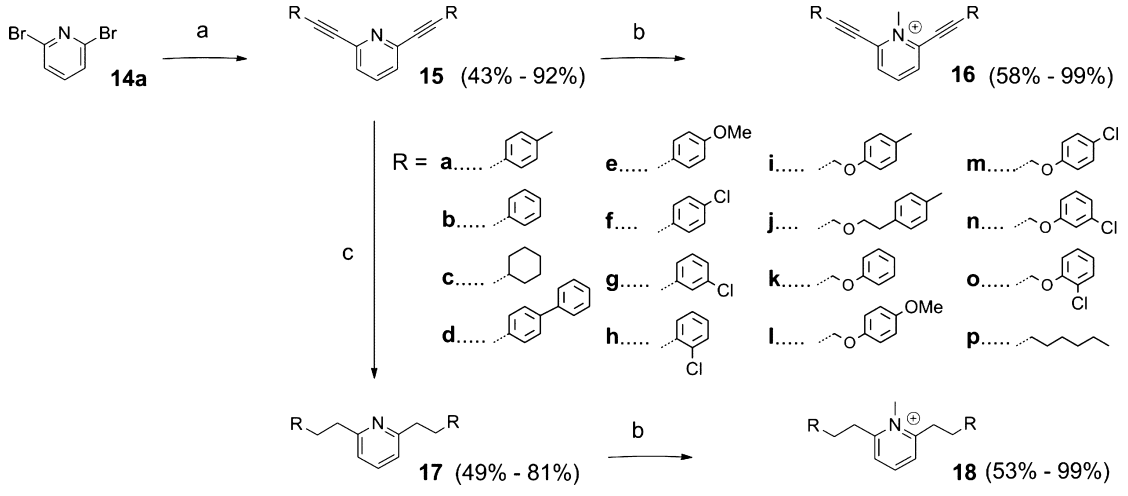
Scheme 1. Synthesis of Asymmetric and Symmetric 4-Methylphenyl Pyridine Derivatives of Dofetilide[a]



[a](a) $POCl_3$, reflux, overnight; (b) 4-methylbenzylzinc, $Pd(PPh_3)_4$, THF, N_2, 60 °C, overnight; (c) methyl *p*-toluenesulfonate, CH_3CN, reflux, overnight; (d) methyl trifluoromethanesulfonate, CH_2Cl_2, 0 °C → room temp, overnight; (e) *p*-cresol or phenol, K_2CO_3, CH_3CN, reflux, N_2, overnight; (f) (4-methylphenyl) alcohol, NaH, THF, 0 °C → reflux, N_2, overnight. For simplification purposes, positively charged pyridines **11a**, **11b**, and **13** are represented without the trifluoromethane sulfonate counterion, and **12a–c** without the *p*-toluenesulfonate counterion.

Scheme 2. Synthesis of Pyridines Bearing Side Chains with Different Degrees of Rigidity and Different Peripheral Aromatic Substituents[a]



[a](a) Alkyne, $PdCl_2(PPh_3)_2$, CuI, Et_3N, or Et_3N/CH_3CN, room temp or reflux; (b) methyl trifluoromethanesulfonate, CH_2Cl_2, 0 °C → room temp, overnight; (c) H_2, Pd/C, THF/MeOH, room temp. Positively charged pyridines are represented without the trifluoromethane sulfonate counterion.

Compounds **5a** and **5b** were first designed (Scheme 1), with the aim to understand the biological consequences of changing the pK_a of the basic amino compound **2** by introducing a pyridine moiety. The synthetic route was started by preparing the *N*-oxide **3**[8] (see Supporting Information for full synthetic details of all compounds). Chlorination of compound **3** in $POCl_3$ at reflux temperature afforded the ortho-chlorinated product **4a** and the para-chlorinated product **4b** in moderate yields (39% and 28%, respectively). Negishi cross-coupling of the pyridine chlorides **4a** and **4b** with 4-methylbenzylzinc,

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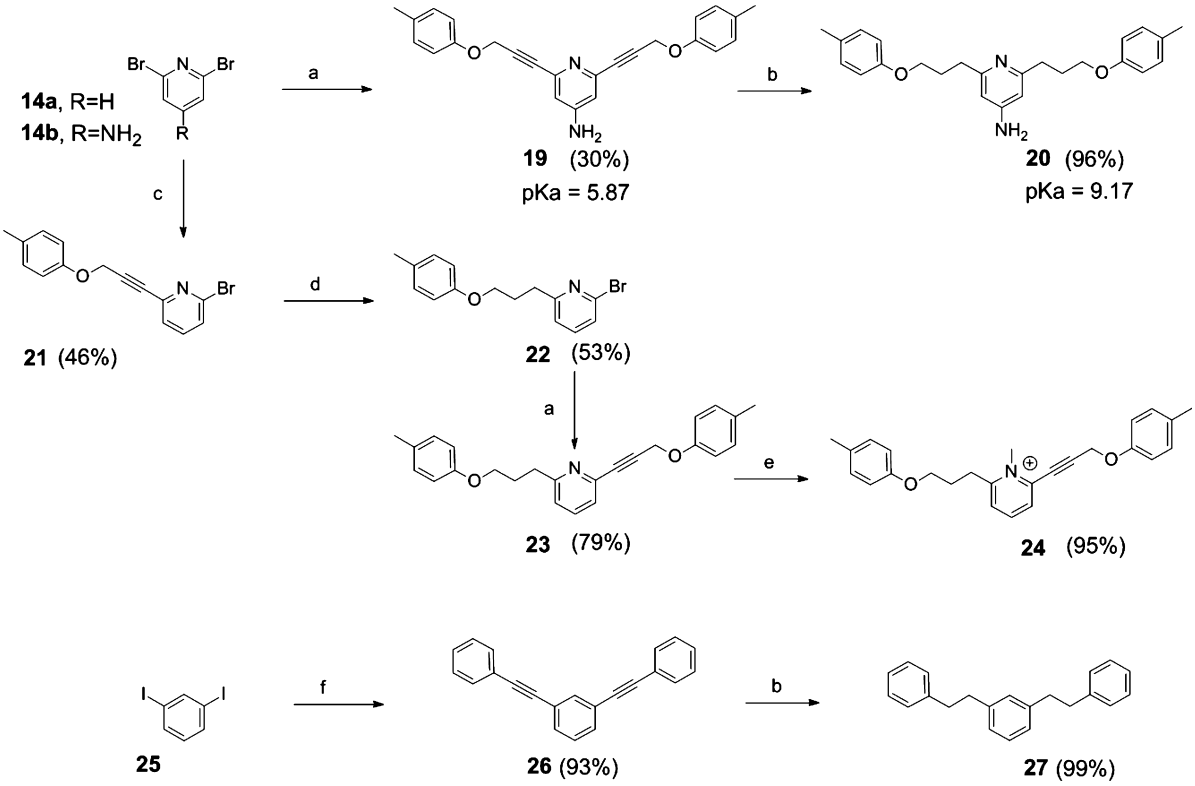
Scheme 3. Synthesis of *p*-Aminopyridines, Asymmetric Substituted Pyridines, and Aromatic Derivatives of 2,6-Diphenethylpyridine 17b[a]



[a](a) 1-Methyl-4-(prop-2-yn-1-yloxy)benzene, $PdCl_2(PPh_3)_2$, CuI, Et_3N/CH_3CN, reflux; (b) H_2, Pd/C, THF/MeOH, room temp; (c) 1-methyl-4-(prop-2-yn-1-yloxy)benzene, $PdCl_2(PPh_3)_2$, CuI, Et_3N, room temp; (d) H_2, PtO_2, EtOH, Et_3N, room temp; (e) methyl trifluoromethanesulfonate, CH_2Cl_2, 0 °C → room temp; (f) 4-ethynylbenzene, $PdCl_2(PPh_3)_2$, CuI, Et_3N, room temp. Positively charged pyridines are represented without the counterion for simplification purposes. pK_a values of pyridines **19** and **20** were estimated using Marvin software.[9]

catalyzed by $Pd(PPh_3)_4$, gave the desired ortho- and para-substituted 4-methylbenzylpyridines **5a** and **5b** in good yields (92% and 75%, respectively).

Compounds **6a** and **6b** were prepared by methylation of the pyridines **5a** and **5b**, in order to study the effect of the positive charge on the dofetilide pyridine derivatives. Methylation was achieved using two different methylating agents, specifically methyl *p*-toluenesulfonate or methyl trifluoromethanesulfonate.

A new set of dofetilide derivatives with symmetric, neutral, and positively charged pyridines (**8**–**13**), bearing extended side chains, was prepared by alkylation of the 2,6-dibromomethyl pyridine (**7**) with the corresponding alcohol using basic conditions, and subsequent methylation of the pyridine using one of the two methods described above. Compounds **8a** and **8b** were prepared very efficiently (yields >90%) by reaction of the corresponding aromatic alcohol using an excess of K_2CO_3 in CH_3CN at reflux temperature. Compounds **9a**–**c** and **10** were obtained by deprotonation of the corresponding aliphatic alcohol with NaH at 0 °C for 30 min and subsequent overnight stirring in the presence of the dibromide **7** at reflux temperature (THF), resulting in a very good yield (>75%).

The next step in our synthetic plan (Scheme 2) was performed to evaluate the influence of rigidity and aromatic modification on hERG affinity, for positively charged methylated pyridines. The rigid and neutral pyridines **15a**–**p** were synthesized in good yields through cross-coupling reactions of 2,6-dibromopyridine **14a** with the corresponding alkyne, using Sonogashira conditions (catalytic amounts of $PdCl_2(PPh_3)_2$ and CuI and an excess of Et_3N) at reflux (CH_3CN) or room temperature. The flexible and neutral pyridines **17a**,**b**,**i**–**p** were obtained in moderate to good yields by hydrogenation of the rigid pyridines **15a**,**b**,**i**–**p**. The rigid or flexible positively charged pyridines **16a**–**p** or **18a**,**b**,**i**–**p**, respectively, were prepared by methylation of the corresponding neutral pyridines using methyl trifluoromethanesulfonate, yielding moderate to high yields.

Next we explored how asymmetric rigidification and basicity of the pyridine moiety affect hERG interactions (Scheme 3). The *p*-aminopyridine compounds **19** and **20** were obtained starting from Sonogashira coupling of the commercially available dibromo-*p*-aminopyridine **14b**, which gave the acidic compound **19** in low yield, followed by efficient hydrogenation allowing access to the basic pyridine **20** ($pK_a \sim 9.17$, estimated using Marvin software),[9] in high yield. The positively charged hybrid pyridine **24**, bearing both a flexible and a rigid side chain, was prepared by hydrogenation of compound **21** using PtO_2 under basic conditions, subsequent Sonogashira coupling with 1-methyl-4-(prop-2-yn-1-yloxy)benzene yielding compound **23**, and finally methylation of the latter compound using methyl trifluoromethanesulfonate. Compound **27**, lacking the nitrogen on the central aromatic moiety, was prepared by Sonogashira coupling of 1,3-diiodobenzene (**25**) with ethynyl benzene and subsequent hydrogenation of the resulting compound **26**, as shown in Scheme 3.

Biology. *Radioligand Binding Experiments.* In the present study, 74 aromatic derivatives of dofetilide were evaluated in

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vitro for the ability to displace radiolabeled [^{3}H]astemizole from hERG K^+ channels stably expressed on the plasma membranes of HEK_{293} cells. In our biological approach, compounds were initially screened at 10 μM for [^{3}H]astemizole displacement in membranes of HEK_{293} cells stably expressing the hERG K^+ channel. Those capable of displacing more than 50% of the [^{3}H]astemizole at that concentration were then selected for generating concentration–effect curves (using at least 12 dilutions), and IC_{50} values were determined.

As shown in Figure 2, the concentration–effect curves of dofetilide (**1**) and the pyridine derivatives (**12c** and **18m**)

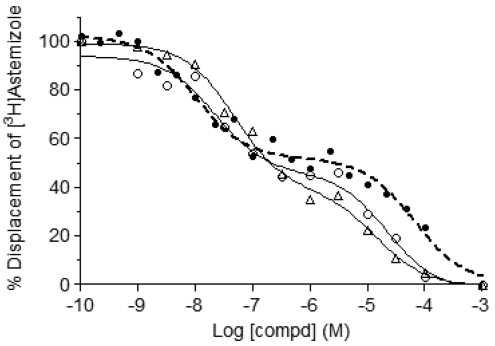


Figure 2. Representative displacement curves of specific [^{3}H]-astemizole binding to HEK_{293} cell membranes stably expressing the hERG K^+ channel by dofetilide (**1**, ●, dashed bold line)[4] and two positively charged pyridine analogues: compound **12c** (Δ, solid line) and compound **18m** (○, solid line). Curves are best described by a two-site model, representing a high- and low-affinity binding site.

reveal two affinity sites when displacing [^{3}H]astemizole with increasing concentrations of the unlabeled ("cold") ligand. This biphasic behavior in [^{3}H]astemizole binding studies is a more general phenomenon, although not observed with all compounds tested. It has been previously described by our group,[4,5] not only for dofetilide[4] but also for the experimental drug E-4031.[5] Both studies showed that the IC_{50} value for the high-affinity site is highly correlated with the IC_{50} value determined by whole-cell voltage clamp measurements. Thus, in the present study, the reported IC_{50} values represent the high-affinity site found for compounds with biphasic behavior (Tables 1–4).

For a few selected compounds, we eventually performed manual patch clamp experiments using the same HEK cells and compared the potencies in that assay with the affinities determined in the radioligand binding experiments (Table 5).

Structure–Affinity Relationships. Our biological study started by focusing on pyridine analogues of the *p*-methylphenyl derivative of dofetilide **2** (Table 1: symmetric and asymmetric analogues). Later we moved on to derivatives containing different substituents in the peripheral aromatic groups (Table 2: short-chained analogues, and Table 3: extended side-chained pyridine derivatives). Table 4 outlines hERG affinities of short-chained pyridines bearing phenyl substituents in order to discuss hERG activity of neutral pyridine **17b**. Finally, in Table 5 a comparison between the hERG affinity found for selected compounds by the radioligand binding assay on cell membranes and the manual patch clamp assay on intact cells is presented.

Effect of Central Pyridine Moiety. Out of the 72 heteroaromatic molecules tested, 36 compounds contained a central neutral pyridine moiety, and with the exceptions of compounds **17b** (Scheme 2, $IC_{50} \sim 1.6$ μM) and **17m** ($IC_{50} \sim 9.9$ μM), none of the other neutral pyridines evaluated in this study displaced [^{3}H]astemizole binding by more than 50% at 10 μM ($IC_{50} > 10$ μM).

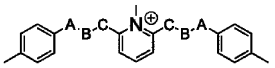
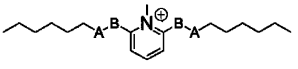
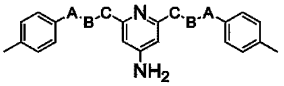
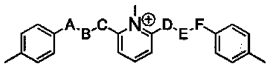
This observation addressed one of our fundamental questions regarding the possibility that compounds containing a central and neutral pyridine moiety could be effective hERG blockers. Indeed, it is known that a central basic amine is not always a requirement for hERG blockade,[7] and a recent study suggests a perpendicular binding mode for hERG blockers.[10] This new model is supported by the 4-fold symmetry of the tetrameric channel, containing four flexible Tyr 652 residues, in combination with the polarity of the selectivity filter. Presumably, the affinity of pyridine analogues of dofetilide could result from $\pi-\pi$ stacking interactions between the peripheral phenyl rings of the blocker and Tyr 652 of the channel, in combination with hydrogen bonds between the pyridine central moiety and the OH groups of Thr 623 and Ser 624. However, our biological results reveal that substituting a basic nitrogen with a pyridine central moiety can be regarded as a safe strategy to avoid potent hERG binding. The reduction in hERG affinity may result from the drastic decrease in pK_a and/or from the increased rigidity and bulkiness imposed by this modification. To better understand the reason for such a drastic decrease, positively charged pyridines were synthesized.

Positively Charged Pyridines. Positively charged pyridine compounds have been recently synthesized with a wide range of biological effects.[11−14] Nonetheless, scarce information is available in the literature regarding the ability of such pyridines to interact with the hERG channel.

Changing the pK_a, bulkiness, and rigidity of the potent hERG blocker, by introducing a pyridine moiety between the *p*-methylphenyl groups of dofetilide derivative **2** (Figure 1), results in a large decrease in the hERG binding affinity ($IC_{50} > 10$ μM) as demonstrated by compounds **5a** and **5b** (Scheme 1, 9% ± 10 and −3% ± 5 [^{3}H]astemizole displacement, respectively, at 10 μM). Additionally, the position of the nitrogen in the pyridine moiety does not affect the biological outcome for these neutral pyridines. However, when positively charged, distinct biological behavior was noticed. While the 2,6-disubstituted pyridine **6a** did not interact with the hERG channel (Table 1, asymmetric pyridines, $IC_{50} > 10$ μM), the 2,4-disubstituted pyridine **6b** bound effectively to the hERG channel ($IC_{50} = 346 \pm 125$ nM, data not shown in the table). These results demonstrate that introducing rigidity can affect hERG affinity, yet the biological outcome is highly dependent on the position of the positively charged nitrogen in the aromatic moiety. Most probably, this difference in hERG activity observed for the two analogues **6a** and **6b** resulted from the combination of the extra rigidity imposed by the pyridine moiety with the extra hindrance imposed on the positive charge by the two relatively short side-chained ortho substituents. These experiments demonstrate the importance of rigidity on hERG affinity when a central positive charge is present in the blocker. Additionally, the position of the positive charge on the pyridine can influence activity, and ortho-disubstituted methylated pyridines might be a solution to overcome the hERG affinity.

Having established that a positively charged pyridine analogue of dofetilide can effectively be accommodated by the hERG channel, we discuss below the parameters affecting hERG affinity of derivatives containing a central methylated pyridine moiety.

Table 1. hERG Channel Affinities of Symmetric and Asymmetric Pyridines[a]

		Chemical Modification						Displacement at 10 μM (%) or IC_{50} ± SEM (nM)
Symmetric Pyridines		**A**		**B**		**C**		
11a	Side Chain Lenght	--		O		CH_2		53 ± 12 nM
12a		CH_2		O		CH_2		107 ± 28 nM
12b		$(CH_2)_2$		O		CH_2		135 ± 32 nM
12c		$(CH_2)_3$		O		CH_2		24 ± 12 nM
13		$C{\equiv}CCH_2$		O		CH_2		40 ± 4 nM
16a	Side Chain Rigidity	--			C≡C			285 ± 47 nM
16i		OCH_2			C≡C			30 ± 2 %
16j		$(CH_2)_2OCH_2$			C≡C			14426 ± 952 nM
18a		--		CH_2		CH_2		24 ± 1 nM
18i		OCH_2		CH_2		CH_2		86 ± 31 nM
18j		$(CH_2)_2OCH_2$		CH_2		CH_2		371 ± 60 nM
Aliphatic Pyridines		**A**		**B**				
16p			C≡C					1992 ± 701 nM
18p		CH_2		CH_2				15 ± 5 nM
p-Amino Pyridines		**A**		**B**		**C**		
19		OCH_2			C≡C			-53 ± 13 %
20		OCH_2		CH_2		CH_2		170 ± 64 nM
Asymmetric Pyridines		**A**	**B**	**C**	**D**	**E**	**F**	
6a		--	--	CH_2	CH_2	O	--	21 ± 7 %
24		OCH_2	CH_2	CH_2	C≡C		CH_2O	2908 ± 940 nM

[a]High-affinity IC_{50} ± SEM or displacement (%) at 10 μM (n = 3) of [^{3}H]astemizole binding to membranes of HEK_{293} cells stably expressing the hERG K^+ channel. Positively charged pyridines are represented without the counterion.

Side-Chain Length (Table 1, symmetric pyridines). If flexibility is a limiting parameter for hERG interaction, then increasing the side-chain length of the nonactive positively charged pyridine **6a** results in a more flexible molecule which can hypothetically be more prone to hERG interaction. Indeed, the biological results of extended positively charged pyridines (**11a**, **12a**−**c**) revealed that increasing flexibility can enhance hERG binding. The positively charged pyridines, **11a**, **12a**−**c**, were all active in the low nanomolar range (24 to 135 nM). Interestingly, side-chain length is not directly proportional to hERG affinity. Remarkably, compound **12c**, with three carbons between the oxygen and aromatic group, displayed the most potent hERG affinity (24 nM, and is only about 6-fold less active than dofetilide, **1**).

Side-Chain Rigidity (Table 1). Several hERG blockers described in the literature contain flexible linkers joining the different fragments of the molecule. This flexibility translates into hERG affinity, allowing the molecule to assume optimal position on the hERG binding site. Increasing side-chain rigidity may, therefore, decrease hERG affinity.

To test the influence of rigidity in hERG affinity, triple bonds were introduced at different positions of the side chain. Introducing a triple bond close to the peripheral aromatic groups, such as in the symmetric pyridine **13**, only slightly affected hERG binding affinity (1.7-fold loss, compared to compound **12c**). In contrast, when the triple bond was introduced close to the pyridine moiety, a considerable decrease in hERG affinity was observed (compare **16a**, **16i**, and **16j** with **18a**, **18i**, and **18j**, respectively). The decrease in hERG affinity is related to the length of the side chain, where introducing triple bonds was less effective in reducing hERG affinity of shorter side-chained compounds (two carbon side chains, 12-fold decrease, compare compound **16a** with **18a**). Quite remarkably, introducing triple bonds close to the

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pyridine moiety in four-bonded side chains led to a strong decrease in hERG affinity (>116-fold, compare compound **16i** with **18i**). Increasing further flexibility by adding two more carbons to the side chain allowed some recovery of hERG affinity (~44-fold decrease, compare compound **16j** with **18j**).

Generally, the maximum free plasma drug concentration allowed for clinical use of a hERG blocker is at least 30-fold less than its IC_{50},[15−18] as this is considered the safety margin of a hERG blocker drug. We therefore considered a 30-fold decrease in hERG affinity as our threshold for desirability. The introduction of triple bonds close to the pyridine moiety is therefore a very efficient strategy to reduce hERG affinity for compounds possessing side chains with more than two bonds.

Having established that introducing triple bonds in each side chain can completely remove hERG affinity on molecules of a particular size, our attention was driven toward the biological relevance of a triple bond in only one of the side chains, as in the asymmetric pyridine **24**. This modification was still very effective in hampering hERG affinity, as it displayed a more than 30-fold decrease in hERG affinity (IC_{50} ~ 2.9 μM), when compared to the saturated side-chained analogue **18i** (IC_{50} ~ 86 nM).

Several strategies to circumvent hERG blockade have been reported in the literature using pharmacologically active compounds that target different receptors.[19] Based on the classical model for hERG binding,[20−22] modulation of pK_a recurs frequently as a satisfactory approach to reduce hERG affinity.[19] However, disruption of the nitrogen's basicity can also lead to decreased binding affinity for the desired target. This feature is particularly relevant for compounds where cation−π interactions are important for binding, such as aminergic GPCR ligands.[23] Here, we found a novel chemical modification, comprising a methylated pyridine conjugated with triple bonds, which allows substantial reduction of hERG affinity without interfering with the positive charge of the molecule.

Presence of Oxygens in the Side Chain (Table 1, symmetric pyridines). Comparing the hERG affinity of compound **18a** with the oxy analogue compound **11a** reveals that the presence of oxygens in the side chain plays a role in hERG binding. Specifically, approximately a 2-fold decrease in hERG activity is observed by introducing oxygen atoms in the side chains. Additionally, if we compare the activity of the long side-chained pyridine **12b** with analogue **18i**, a decrease in binding affinity was noticed when oxygens were moved toward the positively charged pyridine moiety, indicating that the location of the oxygen in the side chain also influences hERG activity.

Furthermore, by comparing the hERG activity of compounds **11a**, **16a**, and **18a**, rigidification of the side chains of short-chained pyridines results in a 5-fold increase in efficiency toward reducing hERG affinity compared with the introduction of oxygens.

Peripheral Aromaticity (Table 1, aliphatic pyridines). The literature indicates that π−π stacking interactions between the blocker and the hERG binding pocket are important for potent hERG affinity.[10,20−22] To evaluate the importance of peripheral aromaticity, compounds **16p** and **18p**, aliphatic analogues of compounds **16i** and **18i**, were evaluated for hERG affinity. Interestingly, positively charged pyridines bearing aliphatic side chains were found to be about 5-fold more efficient in binding the hERG channel than the corresponding aromatic derivatives. Remarkably, compound **18p** displayed one of the highest affinities for hERG observed in this study (same hERG affinity as compound **18m**, Table 3, discussed later). These experiments show that aromaticity is not essential for potent binding affinity, suggesting that van der Waals interactions can be as effective as π−π stacking interactions. In fact, these results are consistent with the evidence for a lipophilic binding site in the hERG cavity.[24] van der Waals interactions with the side chain of Phe656 were better correlated with the potency of hERG blockage than π−π stacking interactions for several structurally diverse drugs. Moreover, our results are also in agreement with the large body of literature showing a relationship between lipophilicity and potent hERG affinity.[19] Additionally, comparing the hERG affinity of compounds **16p** and **18p** reveals once more the ability to reduce hERG affinity by triple bonds close to the positively charged pyridine (hERG affinity decreases >116-fold for aromatic side chains and about 133-fold for aliphatic side chains).

p-Amino Substituent on the Pyridine Moiety (Table 1, p-aminopyridines). Quaternary pyridine compounds, because of their permanently positive charge, are pharmacokinetically problematic, particularly when it comes to drug absorption. Alternatively, *p*-aminopyridine compounds can be used. Resonance stabilization of the protonated nitrogen on the pyridine moiety renders *p*-aminopyridines basic and thus positively charged at physiological pH. Because the *p*-aminopyridine moiety is also present in its nonionized form, it can cross membranes[25,26] and thus potentially induce hERG blockade. Indeed, *p*-aminopyridine itself is known to block voltage-activated K^+ channels,[25,26] and quite recently, *p*-aminopyridine-containing compounds designed to target the neuropeptide Y Y1 receptor[27] or the δ-opioid receptor[28] were found to display potent hERG affinity.

By comparing the hERG affinity of the basic *p*-aminopyridine derivative **20** (estimated pK_a ~ 9.2) with the quaternary pyridine **18i**, we found that the *p*-amino substituent is 2-fold less active. This difference in hERG affinity may result from the increased hydrophilicity and/or bulkiness introduced on the para-position of the pyridine moiety.

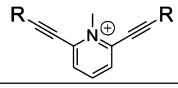
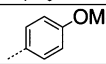
Introduction of triple bonds close to the *p*-aminopyridine moiety increases rigidity and substantially decreases the pK_a (pK_a < 7.4), resulting in predictable loss of hERG affinity, as confirmed by compound **19** (IC_{50}> 10 μM).

Taking into consideration the knowledge gained with the asymmetric pyridine **24**, where introduction of a single triple bond reduced hERG activity (>30-fold), one can speculate that introducing a triple bond in one of the side chains of compound **20** would also reduce hERG affinity efficiently, without interfering massively with the positive charge of the *p*-aminopyridine moiety. The estimated pK_a value of such a compound is 7.9 using Marvin software. Hypothetically, when designing a molecule for which a positive charge is crucial for desired activity, introducing a *p*-aminopyridine moiety conjugated with a single triple bond might allow a transiently positively charged compound to reach the inside of the cell, without displaying considerable hERG affinity or bioavailability problems.

Peripheral Aromatic Substituents. Introducing triple bonds close to the positive charge of pyridines leads to a very different hERG outcome, which varies according to the length of the side chain. Consequently, different chemical modifications on the peripheral aromatic groups were introduced to study the influence on hERG activity of the resulting molecules.

Short-Chain and Rigid Pyridines (Table 2). The short-chained and rigid pyridine **16a**, with 4-methylphenyl substituents, has a remarkably high affinity for the hERG channel (IC_{50} ~ 285 nM). Removing the *p*-methyl substituent leads to a 2-fold decrease in activity (compare compound **16a** with **16b**). Most probably, this loss in hERG activity is due to the resulting decrease in lipophilicity.

Table 2. hERG Channel Affinities of Rigid and Short-Chained Pyridines Bearing Different Substituents[a,b]

		IC_{50} ± SEM (nM)[c]
R[d]		
	**16a**	285 ± 47 nM
	**16b**	554 ± 129 nM
	**16c**	4340 ± 1264 nM
	**16d**	294 ± 141 nM
	**16e**	377 ± 73 nM
	**16f**	1159 ± 368 nM
	**16g**	4019 ± 937 nM
	**16h**	3901 ± 476 nM

[a]Positively charged pyridines are represented without the counterion. [b]The corresponding rigid (**15a–h**) and flexible (**17a,b**) neutral pyridines are not shown due to their negligible affinity for the hERG channel at 10 μM, with the exception of compound **17b** (see Table 4). [c]High-affinity IC_{50} ± SEM of [^{3}H]astemizole binding to membranes of HEK_{293} cells stably expressing the hERG K^+ channel; [d]Synthesis is schematically presented in Scheme 2.

We have shown that peripheral aromaticity is not required for potent hERG affinity of long side-chained and positively charged pyridines (see IC_{50} of aliphatic pyridine **18p**, Table 1). The loss of hERG affinity of the aliphatic derivative **16c** (8-fold decrease when compared to the aromatic analogue **16b**) shows that, in contrast to long-chained derivatives, the peripheral aromaticity of short-chained and rigid pyridines is crucial for hERG affinity. The role of aromaticity on the hERG affinity of short-chained and rigid pyridines is stronger than the lipophilicity gained by substituting a phenyl ring with a cyclohexyl group. Conversely, additional aromaticity introduced by a biphenyl substituent, as in compound **16d**, only slightly improved hERG affinity (compare compounds **16b** and **16d**). The fact that biphenyl compound **16d** is as active as the 4-methylphenyl analogue **16a** suggests a negative effect from the bulkiness of the second aromatic moiety.

The effect of electron-donating and -withdrawing groups on hERG affinity was studied by preparing aromatic analogues containing *p*-methoxy and *p*-chloro substituents (compounds **16e** and **16f**, respectively). Interestingly, both compounds were less active than the *p*-methyl analogue **16a**. Notably, the *p*-chloro analogue **16f** was about 4-fold less active than the *p*-methyl analogue **16a**. This decrease in hERG activity may result from the electron-withdrawing properties of the chloro substituent, destabilizing the required $\pi-\pi$ stacking interactions. Alternatively, the less impressive decrease in hERG activity observed for the *p*-methoxy analogue **16e** can be explained by a decrease in lipophilicity and/or increased bulkiness of this substituent when compared to the *p*-methyl group.

Additionally, introducing *m*- or *o*-chloro substituents on the peripheral phenyl moieties causes further loss of hERG activity (compounds **16g** and **16h**, about 14-fold decrease when compared to compound **16a**). Compounds **16g** and **16h** display hERG activity similar to that of the cyclohexyl derivative **16c**, which confirms the role of $\pi-\pi$ stacking destabilization mediated by the chloro substituent on the phenyl moieties. These observations also highlight a positive effect of the *p*-chloro substituent which counteracts its $\pi-\pi$ stacking destabilization features.

Long-Chained Pyridines (Table 3). Contrary to the rigid and short-chained compound **16a** with 4-methylphenyl substituents, the long-chained analogue **16i** was unable to target the hERG channel (IC_{50}> 10 μM). To explore how triple bonds in long-chained and positively charged pyridines impede interactions with hERG, other rigid analogues containing different aromatic substituents, such as phenyl **16k**, *p*-methoxy **16l**, and *p*-chloro **16m** analogues and their corresponding flexible derivatives, were evaluated. Results showed that rigid and extended pyridines, in contrast with shorter pyridines (Table 2), were unable to significantly interact with the hERG channel (IC_{50} > 10 μM). Once again, introducing triple bonds close to the positively charged pyridine proved to be very efficient in decreasing hERG affinity (>30-fold decrease), as depicted by the chemical index parameter presented in Table 3. Indeed, rigidity can decrease hERG activity from 67-fold (for phenyl analogues) to 2173-fold (*p*-chloro analogues), clearly indicating how effective this chemical transformation can be in reducing hERG affinity.

Considering hERG affinity of the flexible and positively charged pyridines, the *p*-chloro derivative **18m** was the most potent ligand (6-fold more potent than the *p*-methyl analogue **18i**). In fact, it is the strongest hERG blocker found in this study, with an IC_{50} value comparable to the extended and aliphatic side-chained **18p** (Table 1). In contrast with the short and rigid analogues discussed above, where introduction of a *p*-chloro substituent disrupted hERG activity, the introduction of a *p*-chloro substituent on long-chained and flexible positively charged pyridines enhances hERG binding. As discussed previously, the electron-withdrawing properties of the chloro substituent destabilize $\pi-\pi$ stacking interactions and thus van der Waals forces may be the prevalent interactions driving the potent hERG affinity observed for long-chained compounds.

The 11-fold decrease in hERG activity of the *p*-methoxy analogue **18l**, when compared to the *p*-chloro derivative **18m**, may result from increased bulkiness and decreased lipophilicity of the methoxy substituent.

The *m*- (**16n** and **18n**) and *o*-choride analogues (**16o** and **18o**) were also evaluated for hERG affinity. While the rigid analogues (**16n** and **16o**) were not active, as expected, the activity of the flexible derivatives proved to be dependent on the position of the chloro substituent. The hERG affinity was decreased when the chloro atom moved away from the para-position, being 5-fold less active on the ortho-position (compound **18o**) when compared to the *p*-chloro analogue **18m**. Interestingly, the same trend was noticed for short-

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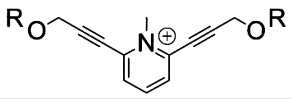
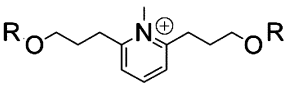
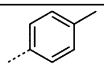
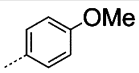
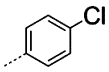
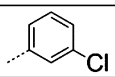
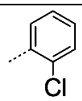
Table 3. hERG Channel Affinities of Positively Charged Pyridines Bearing Extended Side Chains with Different Aromatic Substituents[a,b]

Displacement at 10 μM (%) or IC_{50} ± SEM (nM)[c]					
R[d]					CI[e]
	**16i**	30 ± 2 %	**18i**	86 ± 31 nM	>116
	**16k**	14014 ± 3794 nM	**18k**	208 ± 77 nM	67
	**16l**	19 ± 4 %	**18l**	158 ± 28 nM	>63
	**16m**	31077 ± 5149 nM	**18m**	14 ± 4 nM	2173
	**16n**	15 ± 13 %	**18n**	~ 60 nM[f]	>200
	**16o**	16 ± 6 %	**18o**	74 ± 20 nM	>135

[a]Positively charged pyridines are represented without the counterion. [b]The corresponding neutral pyridines are not shown due to their nonaffinity for the hERG channel at 10 μM; with the exception of compound **17m** (IC_{50}= 9857 ± 239 nM). [c]High-affinity IC_{50} ± SEM or displacement (%) at 10 μM (n = 3) of [^{3}H]astemizole binding to membranes of HEK_{293} cells stably expressing the hERG K^+ channel. [d]Synthesis is schematically presented in Scheme 2. [e]Chemical index = IC_{50} of triple bond compound/IC_{50} of single bond compound. [f]Compound **18n** was insufficiently pure (<80%).

chained and rigid derivatives (**16f** and **16h**, Table 2), whereas moving the chloro substituent from the para- to the ortho-position led to an approximately 3-fold decrease in activity.

Interestingly, and corroborating how chloro substituents on the phenyl moieties of extended pyridines impact hERG affinity, we found that out of the 12 neutral derivatives of the compounds outlined in Table 3, only the *p*-chloro and flexible side-chained analogue **17m** (Scheme 2) displayed some hERG affinity (IC_{50} ~ 9.9 μM). Quite remarkably, this neutral and flexible pyridine **17m** is 3-fold more active on hERG than the positively charged and rigid analogue **16m**. This example shows that a neutral pyridine can be more active than a positively charged pyridine and reveals how effective introducing triple bonds can be in reducing hERG activity, because the resulting positively charged pyridine is considerably less active than the neutral analogue.

hERG Affinity of Neutral Pyridines (Table 4). In this study we found a neutral and flexible pyridine capable of inducing significant hERG blockade (Table 4, compound **17b**, IC_{50} ~ 1.6 μM). Interestingly, adding a 4-methyl substituent to the phenyl groups, as in compound **17a** (Scheme 2), hampered hERG affinity (IC_{50} > 10 μM, data not shown). This exceptional neutral pyridine **17b** displays only 3.5-fold less activity than the positively charged analogue **18b** or 2-fold less activity than the positively charged oxy analogue **11b**. As far as we know, this is the first report of a neutral compound with a central pyridine moiety capable of displaying a considerable binding affinity toward the hERG channel. Generally, this study shows that replacing a basic nitrogen with a pyridine moiety results in reduced hERG affinity. However, the significative hERG activity of **17b** reminds us that every general rule has an exception. This result should keep medicinal chemists alert about the introduction of pyridine moieties in look-a-like compounds.

The loss of activity (more than 6-fold) observed for compound **27**, the aromatic analogue of **17b**, reveals the nitrogen requirement for hERG interactions. Introducing triple bonds in the side chain of both heteroaromatic and aromatic rigid compounds **15b** and **26**, respectively, resulted in nondisplacement of [^{3}H]astemizole from the hERG channel at 10 μM. Introducing oxygens in the side chain, such as in the oxy analogue **8b**, also proved to be as capable of reducing the hERG affinity as rigidification (IC_{50}> 10 μM). In this respect, the introduction of oxygens in the side chains was much more effective in neutral pyridines than in positively charged pyridine derivatives. Although hERG affinity of neutral and positively charged pyridines appear to be ruled by different interactions, the same chemical modifications are effective for avoiding hERG activity in positively charged and neutral pyridines.

Patch Clamp Studies (Table 5). Previous work performed by our group showed that quaternary and tertiary amine compounds can be accommodated by the hERG binding pocket equally well.[5] Results obtained with the radioligand binding assay and the patch clamp assay were found to be quite comparable,[4,5] with the exception of quaternary amine derivatives.[5] In this regard, a considerable decrease in hERG potency (up to 75-fold) was observed for permanently positively charged compounds, as these compounds have difficulty in passing through the cell membrane. Generally, hERG blockers access the binding site inside the cell,[29] so the cell membrane becomes a barrier for hERG activity. This feature can be explored to abrogate off-target hERG binding of pharmacologically active compounds. In fact, chemical modifications that limit membrane permeability, such as the

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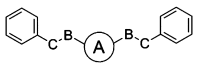
Table 4. hERG Channel Affinities of Short-Chained Compounds Bearing Phenyl Substituents[a,b]

	Chemical Modification			Displacement at 10 μM (%) or IC_{50} ± SEM (nM)
Compound	A	B	C	
8b		CH_2	O	15 ± 5 %
11b		CH_2	O	767 ± 237 nM
15b		C≡C		-11 ± 3 %
17b		CH_2	CH_2	**1608 ± 402 nM**
18b		CH_2	CH_2	466 ± 142 nM
26		C≡C		3 ± 3 %
27		CH_2	CH_2	8 ± 9 %

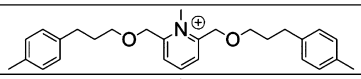
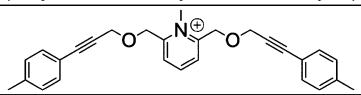
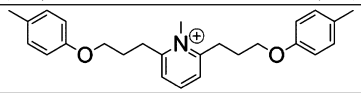
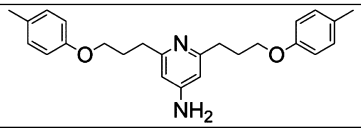
[a]High-affinity IC_{50} ± SEM or displacement (%) at 10 μM (n = 3) of [^{3}H]astemizole binding to membranes of HEK_{293} cells stably expressing the hERG K^+ channel. [b]Positively charged pyridines are represented without the counterion.

formation of zwitterions, have already been explored as a strategy to mitigate hERG liability.[19] Our current results, using a radiolabeled binding assay, reveal that flexible positively charged pyridines can effectively fit in the hERG channel. To investigate if permanently positively charged pyridine derivatives can be a strategy to overcome hERG liability, we selected three such compounds (**12c**, **13**, and **18i**) and a *p*-aminopyridine derivative (**20**) for manual patch clamp studies on intact cells (Table 5). Manual patch clamping is considered the most accurate, albeit laborious, functional method to unravel whether a particular compound interacts with the hERG channel.[30]

Using the radioligand binding assay, out of the four compounds selected, the *p*-aminopyridine derivative **20** was the least potent on the hERG channel. Conversely, in the patch clamp experiments, compound **20** was the most active (IC_{50} = 146 nM). In fact, this compound showed comparable hERG affinities in both methods. These results support our hypothesis that a transiently positive charge allows compound **20** to cross membranes easily. On the other hand, all the permanently positive charged pyridines (compounds **12c**, **13**, and **18i**) showed significant loss of hERG activity (>5-fold) in the patch clamp assay when compared to the respective radioligand binding experiments. Specifically, the flexible pyridines **12c** and **18i** are more than 8-fold less active on hERG when compared to the radioligand binding experiments, while rigidification of the side chain distant from the positive charge, such as in pyridine **13**, enhances hERG affinity, suggesting that it may facilitate membrane crossing.

In summary, radioligand binding experiments showed that permanently positively charged pyridines interact very efficiently with the hERG channel (low nanomolar range). Patch clamp results, however, which better reflect the consequences of in vivo hERG liability, reveal that these compounds can be up to 11-fold less active when compared to the values obtained with the radioligand binding assay. Previously, we had set a threshold of 30-fold decrease in activity for a chemical modification to be considered desirable. By comparing the hERG activity obtained with radioligand binding and patch clamp assays, we found that although a positively charged pyridine can suppress affinity, it is not enough to be considered a safe strategy by itself. Conversely, considering that quaternary amine compounds showed up to 75-fold decreased hERG potency with the patch clamp assay[5] while quaternary pyridines displayed only up to 11-fold impairment suggests that positively charged pyridines are less pharmacokinetically problematic (about 7-fold more cell membrane permeable) than positively charged amines.

Table 5. Comparison of hERG Affinity of Selected Compounds As Determined by the Radioligand Binding Assay on Cell Membranes and the Manual Patch Clamp Assay on Intact Cells[a]

Compound	Structure	IC_{50},RBA (nM)[b]	IC_{50},PCA (nM)[c]	AI[d]
12c		24 ± 12	194	8.1
13		40 ± 4	214	5.4
18i		86 ± 31	962	11.2
20		170 ± 64	146	0.86

[a]Positively charged pyridines are represented without the counterion. Compounds with *p*-toluene sulfonate counterion: **12c**. Compounds with trifluoromethane sulfonate counterion: **13** and **18i**. [b]IC_{50} ± SEM determined by radioligand binding assay (RBA): displacement of [^{3}H]astemizole in membranes of HEK_{293} cells stably overexpressing the hERG channel. [c]IC_{50} values, determined by patch clamp assay (PCA, n = 1). [d]Assay index = IC_{50} of PCA/IC_{50} of RBA.

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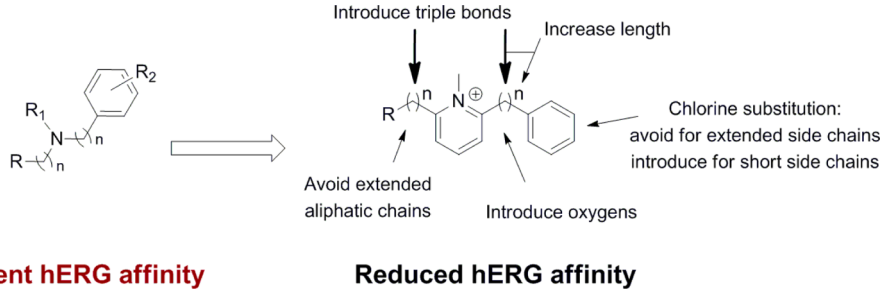

Figure 3. Graphical depiction of the proposed strategies to reduce the hERG K^+ affinity based on SAR found with the radioligand binding assay. Generally, introducing a central pyridine moiety reduces hERG affinity. When a positive charge is critical for the desired activity, methylation or *p*-amino substitution of the pyridine moiety renders the molecule positive. Permanently positively charged pyridines showed substantial loss of hERG activity (up to 11-fold) when evaluated with the patch clamp assay, in contrast to *p*-aminopyridines. Illustrated strategies for reduced hERG K^+ affinity were found on positively charged pyridines. Introducing rigidity and oxygens in the side chain of a neutral pyridine also reduced hERG affinity.

CONCLUSIONS

The pyridine moiety is found in a wide variety of biologically active molecules,[31−33] and, hence, in this study 72 aromatic analogues of dofetilide containing a central pyridine moiety were synthesized and evaluated for hERG affinity in a radioligand binding assay using [^{3}H]astemizole. Sixty nine of these pyridine analogues of dofetilide are novel chemical entities. This large collection of novel compounds allowed us to demonstrate that substituting a basic nitrogen on an effective hERG blocker with a pyridine moiety can be regarded as a reliable measure to reduce potent hERG affinity. However, such chemical modifications can also disrupt the interaction with the desired pharmacological target, particularly for compounds in which a positive charge determines the desired receptor interaction. In this regard, we found that the potent hERG activity of the basic dofetilide derivative **2** was efficiently suppressed by introducing a positively charged pyridine, as confirmed by compound **6a**. However, we also demonstrate that longer side-chained positively charged pyridines (permanently: methylated pyridines, or transiently: *p*-aminopyridines) can effectively bind to the hERG channel. Actually, in this study we identified four positively charged pyridine compounds (**12c**, **18a**, **18p**, and **18m**) with IC_{50} values ranging from 14 to 24 nM, as determined in radioligand binding assays, which are almost equivalent to the most potent hERG blockers known to date.[34]

In patch clamp assays, permanently positively charged pyridines were less effective (up to 11-fold), indicating this chemical modification as a strategy to reduce in vivo hERG affinity. Alone, though, such modification may not be enough to be considered a reliable approach. Thus, we searched for other chemical modifications that can decrease the hERG affinity of lengthy positively charged pyridines. We found that rigidification and oxygenation of both side chains can affect hERG binding affinity. In particular, the introduction of triple bonds and oxygens close to the positive charge lead to a stronger decrease in hERG affinity than distant modifications. Rigidification proved to be much more effective in decreasing hERG activity than oxygenation, in particular for more extended pyridine analogues. Successful mitigation of hERG activity by rigidification evidenced in this study is consistent with a recent report on the synthesis of histone deacetylase inhibitors.[35]

Upon closer inspection we identified two classes of positively charged pyridines, those with a short side chain in which hERG interaction is dependent on peripheral aromaticity, and those with longer side chains, which are less dependent on aromaticity for interacting with the hERG channel. Rigidification of short side-chained pyridines was less effective in reducing hERG affinity than in extended side-chained pyridines. In contrast, introduction of the electron-withdrawing chloro substituent on the aromatic groups of short side-chained pyridines was very efficient in mitigating hERG activity while for more extended pyridines the presence of the chloro substituent in peripheral aromatic moieties promoted stronger hERG affinity. Nonetheless, both classes showed decreased hERG affinity when the chlorine substituent was moved away from the para-position.

Rigidifying a molecule reduces its conformational flexibility and is often regarded in medicinal chemistry as an approach to increase affinity and selectivity.[36] Here we show that rigidification of an aliphatic amine by introduction of a neutral pyridine moiety promotes loss of hERG affinity. If positive charge is essential for primary activity, the pyridine moiety can be methylated without inducing hERG affinity for very short compounds. For lengthy side-chained compounds, introducing a positively charged pyridine moiety requires extra rigidity (imposed by adjacent triple bonds) for reduction of hERG interactions. Rigidification of only one of the side chains resulted in a lowering of hERG affinity, and the conjugation of this chemical modification with a *p*-aminopyridine moiety may lead to a transiently charged compound with reduced hERG affinity, which is able to cross cell membranes and thus be less prone to bioavailability problems. Finally, the chemical features found to reduce the affinity of potent positively charged pyridine hERG blockers could also be applied to reduce hERG affinity of neutral pyridines.

We believe these findings might have wide applications in the development of new drugs where a low off-target hERG profile is desired, and Figure 3 presents a schematic overview of the major strategies to modulate hERG affinity based on SAR found with the radioligand binding assay.

EXPERIMENTAL SECTION

Chemistry. *General Methods.* All commercially available chemicals were used without purification. Reactions were monitored by TLC using silica gel 60 F_{254} aluminum sheets. Reaction yields were determined by compound isolation through flash column chromatography using silica gel 60 (230−400 mesh ASTM). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV400 spectrometer (^{1}H NMR, 400 MHz; ^{13}C NMR, 100 MHz) at ambient temperature and analyzed with MestReNova software. Sample solutions were prepared in $CDCl_3$, MeOD, or mixtures of both deuterated solvents. Chemical shifts (δ)

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are given in ppm and coupling constants (J) in hertz. Trimethylsilane was used as internal standard for calibrating chemical shift for ^{1}H, ^{13}C NMR spectroscopy. HRMS was recorded on a Thermo Finnigan LTQ Orbitrap mass spectrometer. HPLC analysis, carried out on a C18 reversed-phase column (125 × 4.6 mm, particle size 5 μm, pore size 180 Å) at 25 °C coupled with a UV detector at 254 nm, was used to determine the purity of final compounds. Final compounds were dissolved in mixtures of CH_3CN/H_2O and eluted from the column at a flow rate of 0.6 mL min^{-1}, with a two-component system of CH_3CN/H_2O. Purity was confirmed to be ≥95% for all compounds, with the exception of compounds **10** (91%), **16i** (93%), and **23** (94%). Compounds **17n** and **18n** could not be purified to a desired standard (purity <80%) and the biological data obtained from these compounds was only used qualitatively (see Table 4). With the exception of compounds **8a**,[37] **15b**,[38] **17b**,[39] **26**,[38] and **27**,[40] all compounds synthesized and evaluated for hERG activity were considered novel chemical entities using CrossFire Beilstein Database. pK_a values of pyridines **19** and **20** were estimated using Marvin software (http://www.chemaxon.com/marvin/sketch/index.jsp).[9]

General Procedures. General Procedure for the Negishi Coupling Reaction of Chloropyridines. A 0.5 M solution of 4-methylbenzylzinc chloride in THF (20.7 mL, 10.356 mmol) was added dropwise to a solution of 6-chloro-2-((*p*-tolyloxy)methyl)pyridine (**4a**, 605 mg, 2.589 mmol) in dry THF (50 mL) at 60 °C and under inert atmosphere. The mixture was stirred overnight, and the reaction was stopped by evaporation under vacuum. FCC (petroleum ether/ethyl acetate, 60:1 to 20:1) afforded the desired product 2-(4-methylbenzyl)-6-((*p*-tolyloxy)methyl)pyridine (**5a**, 720 mg, 92%). Product was further purified by hydrochloride salt formation using ethanolic HCl and recrystallized from a mixture of EtOH and Et_2O. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 (t, J = 7.8 Hz, 1H), 7.93 (d, J = 7.8 Hz, 1H), 7.44 (d, J = 7.8 Hz, 1H), 7.31 (d, J = 7.8 Hz, 2H), 7.17 (d, J = 7.8 Hz, 2H), 7.11 (d, J = 7.8 Hz, 2H), 6.95 (d, J = 8.1 Hz, 2H), 5.78 (s, 2H), 4.67 (s, 2H), 2.34 (s, 3H), 2.30 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 157.10, 154.74, 153.25, 145.03, 137.61, 131.58 (2C), 130.18 (2C), 129.93 (2C), 129.29 (2C), 125.15, 121.93, 114.57 (2C), 64.62, 38.10, 21.02, 20.42. HRMS (ESI) m/z calcd for $C_{21}H_{21}NOH^+$: 304.16959, found: 304.16926. HPLC purity: 100.0% (t_R: 15.70 min).

General Procedure for the Methylation of Pyridines Using Methyl p-Toluenesulfonate. Methyl *p*-toluenesulfonate (906 μL, 6 mmol) was added to a solution of 2-(4-methylbenzyl)-6-((*p*-tolyloxy)methyl)-pyridine (**5a**, 303.4 mg, 1 mmol) in acetonitrile (15 mL) under inert atmosphere and the resulting mixture stirred at reflux temperature during overnight. The reaction was stopped by evaporation under vacuum and crystallization (petroleum ether/ethyl acetate) gave the desired product 1-methyl-2-(4-methylbenzyl)-6-((*p*-tolyloxy)methyl)-pyridin-1-ium 4-methylbenzenesulfonate (**6a**, 333 mg, 67%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10 (t, J = 7.8 Hz, 1H), 7.89 (d, J = 7.8 Hz, 1H), 7.57 (d, J = 7.8 Hz, 2H), 7.37 (d, J = 7.8 Hz, 1H), 7.11 (d, J = 7.8 Hz, 2H), 7.12−6.88 (m, 6H), 6.89 (d, J = 7.8 Hz, 2H), 5.39 (s, 2H), 4.42 (s, 2H), 4.23 (s, 3H), 2.32 (s, 3H), 2.25 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.21, 154.74, 153.97, 144.06, 143.42, 139.00, 137.52, 131.54, 130.39 (2C), 130.13 (2C), 129.96, 129.35 (2C), 128.44 (2C), 127.92, 125.70 (2C), 125.40, 114.76 (2C), 66.28, 40.43, 39.30, 21.12, 21.01, 20.39. HRMS (ESI) m/z calcd for $C_{22}H_{24}NO^+$: 318.18524, found: 318.18488. HPLC purity: 95.6% (t_R: 13.59 min).

General Procedure for the Methylation of Pyridines Using Methyl Trifluoromethanesulfonate. Methyl trifluoromethanesulfonate (50 μL, 0.456 mmol) was added to a solution of 4-(4-methylbenzyl)-2-((*p*-tolyloxy)methyl)pyridine (**5b**, 50 mg, 0.165 mmol) in dry DCM (5 mL) at 0 °C and under inert atmosphere. The mixture was stirred overnight, and the reaction was stopped by pouring the mixture into silica. FCC (dichloromethane/methanol, 60:1 to 30:1) afforded the desired 1-methyl-4-(4-methylbenzyl)-2-((*p*-tolyloxy)methyl)pyridin-1-ium trifluoromethanesulfonate product (**6b**, 70.2 mg, 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.76 (d, J = 6.4 Hz, 1H), 7.82 (d, J = 2.3 Hz, 1H), 7.59 (dd, J = 6.4, 2.3 Hz, 1H), 7.12 (d, J = 7.8 Hz, 2H), 7.08(d, J = 8.3 Hz, 2H), 7.02 (d, J = 7.8 Hz, 2H), 6.87 (d, J = 8.3 Hz, 2H), 5.29 (s, 2H), 4.29 (s, 3H), 4.12 (s, 2H), 2.31 (s, 3H), 2.27 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.08, 154.68, 152.24, 146.80, 137.39, 132.50, 132.01, 130.27 (2C), 129.92 (2C), 129.09 (2C), 127.33, 126.77, 114.70 (2C), 65.38, 44.82, 41.03, 20.99, 20.44. HRMS (ESI) m/z calcd for $C_{33}H_{21}N^+$: 318.18518, found: 318.18524. HPLC purity: 99.6% (t_R: 13.66 min).

General Procedure for Alkylation with Aromatic Alcohols. To a solution of *p*-cresol (1714 mg, 15.85 mmol) in acetonitrile (113 mL) under inert atmosphere were added sequentially K_2CO_3 (4172 mg, 30.19 mmol) and 2,6-bis(bromomethyl)pyridine (**7**, 2000 mg, 7.55 mmol) at room temperature. The resulting mixture was then stirred at reflux temperature overnight, and the reaction was stopped by filtration and evaporation under vacuum. FCC (petroleum ether/ethyl acetate, 20:1 to 10:1) afforded the desired 2,6-bis((*p*-tolyloxy)methyl)-pyridine (**8a**, 2210 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (t, J = 7.8 Hz, 1H), 7.43 (d, J = 7.8 Hz, 2H), 7.08 (d, J = 8.4 Hz, 4H), 6.88 (d, J = 8.3 Hz, 4H), 5.18 (s, 4H), 2.28 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.99 (2C), 156.22 (2C), 137.52, 130.34 (2C), 129.94 (4C), 119.98 (2C), 114.58 (4C), 70.57 (2C), 20.45 (2C). HRMS (ESI) m/z calcd for $C_{21}H_{21}NO_2H^+$: 320.16451, found: 320.16452. HPLC purity: 98.6% (t_R: 15. 80 min).

General Procedure for Alkylation with Aliphatic Alcohols. *p*-Tolylmethanol (338.6 mg, 2.77 mmol) was added to a suspension of NaH (60%, 147.8 mg, 3,69 mmol) in dry THF (30 mL) at 0 °C under inert atmosphere and the resulting mixture stirred for 30 min. Next, a solution of 2,6-bis(bromomethyl)pyridine (**7**, 350 mg, 1.32 mmol) in THF (30 mL) was added dropwise at 0 °C and then refluxed and stirred overnight. The reaction was stopped by filtration through Celite and evaporation under vacuum. The resulting crude product was purified by FCC (petroleum ether/ethyl acetate, 10:1 to 4:1) and afforded the pure 2,6-bis(((4-methylbenzyl)oxy)methyl)pyridine (**9a**, 435.6 mg, 95%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (t, J = 7.7 Hz, 1H), 7.37 (d, J = 7.7 Hz, 2H), 7.26 (d, J = 7.9 Hz, 4H), 7.14 (d, J = 7.9 Hz, 4H), 4.64 (s, 4H), 4.58 (s, 4H), 2.32 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 157.84 (2C), 137.25, 137.06 (2C), 134.78 (2C), 128.95 (4C), 127.80 (4C), 119.76 (2C), 72.75 (2C), 72.65 (2C), 21.04 (2C). HRMS (ESI) m/z calcd for $C_{23}H_{25}NO_2H^+$: 348.19581, found: 348.19578. HPLC purity: 98.6% (t_R: 15. 59 min).Method A

General Procedure for the Sonogashira Coupling of Bromopyridines $PdCl_2(PPh_3)_2$ (148 mg, 0.21 mmol) and CuI (120.6 mg, 0.63 mmol) were added to a solution of 2,6-dibromopyridine (1000 mg, 4.22 mmol) in Et_3N (10 mL) under N_2, and the resulting mixture was stirred for 30 min. Then 4-ethynyltoluene (1177 mg, 10.13 mmol) was added dropwise and the reaction mixture stirred overnight at room temperature. The reaction was stopped by addition of silica gel and evaporated under vacuum. FCC (petroleum ether/ethyl acetate, 40:1 to 1:1) afforded the desired 2,6-bis(3-(*p*-tolyloxy)prop-1-yn-1-yl)-pyridine product (**15a**, 1200 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (t, J = 7.6 Hz, 1H), 7.49 (d, J = 7.2 Hz, 4H), 7.43 (d, J = 7.6 Hz, 2H), 7.16 (d, J = 7.2 Hz, 4H), 2.37 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.91 (2C), 139.30 (2C), 136.28, 131.97 (4C), 129.12 (4C), 125.92 (2C), 119.03 (2C), 89.87 (2C), 87.82 (2C), 21.54 (2C). HRMS (ESI) m/z calcd for $C_{23}H_{17}NH^+$: 308.14338; found: 308.14322. HPLC purity: 97.7% (t_R: 17.64 min).Method B

$PdCl_2(PPh_3)_2$ (140.4 mg, 0.2 mmol), CuI (114.3 mg, 0.6 mmol), ethynylbenzene (2451 mg, 24 mmol), and Et_3N (3036 mg, 30 mmol) were added to a solution of 2,6-dibromopyridine (2369 mg, 10 mmol) in CH_3CN (20 mL) under N_2. The reaction mixture was stirred overnight at reflux temperature. The reaction was stopped by addition of silica gel and evaporated under vacuum. FCC (petroleum ether/ethyl acetate, 20:1 to 3:1) afforded the desired 2,6-bis(phenylethynyl)-pyridine product (**15b**, 2486 mg, 89%).^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66−7.60 (m, 5H), 7.45 (d, J = 7.7 Hz, 2H), 7.36 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 143.68 (2C), 136.36, 132.00 (4C), 129.01 (2C), 128.30 (4C), 126.14 (2C), 121.99 (2C), 89.56 (2C), 88.22 (2C). HRMS (ESI) m/z calcd for $C_{21}H_{13}NH^+$: 280.11208, found: 280.11188. HPLC purity: 100.0% (t_R: 15.44 min).

General Procedure for Reduction of Pyridine Alkynes. To a solution of 2,6-bis(3-(*p*-tolyloxy)prop-1-yn-1-yl)pyridine (**15a**, 400 mg, 1.3 mmol) in a mixture of THF (12 mL) and MeOH (12 mL) was added 10% Pd on carbon (80 mg), and the atmosphere was changed to H_2 (2.5 bar). The resulting mixture was stirred in a Parr

hydrogenation apparatus overnight at room temperature, followed by filtration over Celite and evaporation under vacuum. FCC (petroleum ether/ethyl acetate, 20:1) afforded the desired 2,6-bis(4-methylphenethyl)pyridine product (**17a**, 221.4 mg, 54%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 (t, J= 7.7 Hz, 1H), 7.09−7.04 (m, 8H), 6.86 (d, J = 7.7 Hz, 2H), 3.07 (ddd, J = 9.1, 6.1, 2.1 Hz, 4H), 3.00 (ddd, J = 9.1, 6.1, 2.1 Hz, 4H), 2.29 (s, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 160.70 (2C), 138.48 (2C), 136.38, 135.12 (2C), 128.87 (4C), 128.29 (4C), 120.19 (2C), 40.07 (2C), 35.64 (2C), 20.93 (2C). HRMS (ESI) m/z calcd for $C_{23}H_{25}NH^+$: 316.20598, found: 316.20585. HPLC purity: 99.6% (t_R: 13.99 min).

Biology. *Radioligand Binding Experiments.* Radioligand binding studies were performed as recently described by our group.[4,5]

Patch Clamp Experiments. These experiments are detailed in the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

Structural characterization of starting materials, intermediates, and final products synthesized and details of the patch-clamp assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AI, assay index; approx, approximately; CI, chemical index; FCC, flash column chromatography; GPCR, G-protein-coupled receptors; hERG, human ether-a-go-go-related gene; IC_{50}, concentration of unlabeled ligand which displaces 50% of [^{3}H]astemizole binding to membranes of HEK_{293} cells stably expressing the hERG K^+ channel; PCA, patch clamp assay; RBA, radioligand binding assay; SAR, structure−activity relationships; TdP, Torsades de pointes; temp, temperature

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