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Oxidation Potentials of N-Modified Derivatives of the Analgesic Flupirtine Linked to Potassium K_V7 Channel Opening Activity But Not Hepatocyte Toxicity

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In memory of Professor Sidney D. Nelson

Openers of neuronal voltage-gated potassium channels (K_V) are of interest as therapeutic agents for treating pain (flupirtine) and epilepsy (retigabine). In an effort to better understand the mechanisms of action and toxicity of flupirtine, we synthesized nine novel analogues with varying redox behavior. Flupirtine can be oxidatively metabolized into azaquinone diimines; thus, the oxidation potentials of flupirtine and its analogues were measured by cyclic voltammetry. $K_V7.2/3$ (KCNQ2/3) opening activity was determined by an established assay with HEK293 cells overexpressing these channels. A link was found between the oxidation potentials of the compounds and their EC_{50} values for potassium channel opening activity. On the other hand, no correlation was observed between oxidation potentials and cytotoxicity in cultures of transgenic mouse hepatocytes (TAMH). These results support the idea that oxidative metabolites of flupirtine contribute to the mechanism of action, similar to what was recently proposed for acetaminophen (paracetamol), but not to hepatotoxicity.

Introduction

Flupirtine (**1**, Figure 1) is approved for use in a number of European countries as well as in Brazil and India as a centrally acting analgesic, offering an alternative to non-opioid, nonsteroidal anti-inflammatory drugs in patients that have contraindications to weak opioids or NSAIDs.[1] The mechanism of action of flupirtine has been attributed to the ability of the drug to open voltage-gated K^+ channels (K_V) in the central nervous system, which indirectly leads to antagonism of *N*-methyl-D-aspartate (NMDA) receptors.[2]

Depending on the primary factors that lead to channel opening and closing, ion channels are divided into ligand- or voltage-gated subfamilies, with or without a physiological ligand binding site.[3] Even in the latter case, modulation by small molecules is conceivable, and both subfamilies are druggable. K_V7 channels (also referred to as KCNQ after the corresponding genes) belong to the voltage-gated K^+ channel superfamily. The five members ($K_V7.1$–$K_V7.5$) are six-transmembrane channels based on four subunits and an appended transmembrane helix voltage-sensor domain.[4] The four subunits create a central ion-conducting pore with a constricted selectivity filter at the top, surrounded by the voltage-sensing domain (VSD). The VSD responds to membrane depolarization and initiates opening of the gate at the base of the pore through concerted movements of all subunits. In this way, the ion channel converts between the closed and open states.

$K_V7.2$ channels are expressed at high levels in the brain, often as functional heterotetramers with $K_V7.3$.[5,6] These heterotetrameric channels are also referred to as KCNQ2/3. A

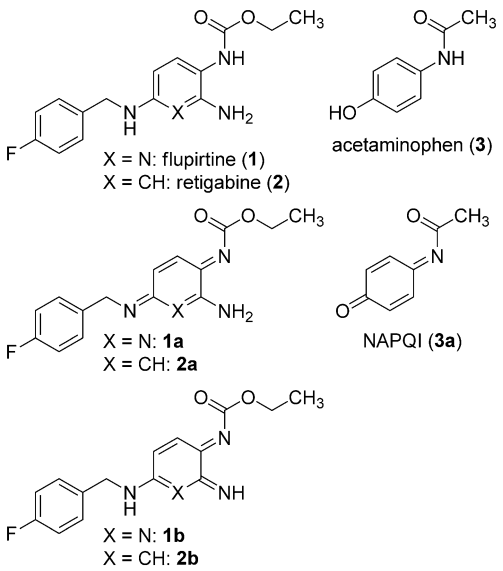

Figure 1. Flupirtine (**1**), retigabine (**2**), and their putative oxidation products (**1a**,**b** and **2a**,**b**, respectively) in comparison with acetaminophen (**3**) and its known metabolite *N*-acetyl-*p*-quinone imine (NAPQI, **3a**).

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number of positive and negative $K_V7.2/3$ channel modulators have been reported, and the discovery of compounds that affect these channels has attracted attention in the development of novel analgesics.[7–9] K_V7 openers, including flupirtine and retigabine (**2**, an antiepileptic drug), are also potential tools for understanding ion channel function, especially because considerable clinical data are available. However, it can be difficult to draw firm conclusions from these data because of poorly characterized polypharmacology.

Although rare, the most severe adverse effect of flupirtine is related to hepatocellular toxicity.[10] The incidence of flupirtine-related hepatotoxicity was estimated to be about eight in 100 000 patients based on a re-evaluation of 226 unselected, spontaneously reported cases in the German Health Authority (BfArM) database.[11] Nonetheless, the Drug Commission of the German Medical Association issued a drug safety warning in 2013 regarding the use of flupirtine in the management of chronic pain.[12] Thus, an understanding of the molecular pharmacology of the various channel subfamily members should be complemented by toxicology data.

Flupirtine's toxicity may be related to its metabolism, which has been shown to undergo both oxidative and conjugative reactions both in vitro[13] and in vivo.[14,15] Although the cytochrome P450 system does not appear to be involved in oxidation, peroxidases easily oxidize flupirtine to azaquinone diimine metabolites (**1a**,**b**).[13] These are conjugated with glutathione without the need for catalysis and after further metabolism they appear in the urine as mercapturic acids. Despite the striking molecular similarity between flupirtine and retigabine, hepatotoxicity has never been reported to be a problem with the latter. This goes to show that minute alterations in electronic features and/or reactivity might be important in terms of drug safety and a deeper understanding of underlying mechanisms. NAPQI (**3a**), the reactive quinone imine metabolite formed during oxidation of acetaminophen (**3**), has been implicated in both the activity[16] and toxicity[17,18] of this widely used analgesic. In analogy to **3a**, the azaquinone diimine metabolites of flupirtine **1a**,**b** may also be involved in both the mechanism of action as well as drug hepatotoxicity.

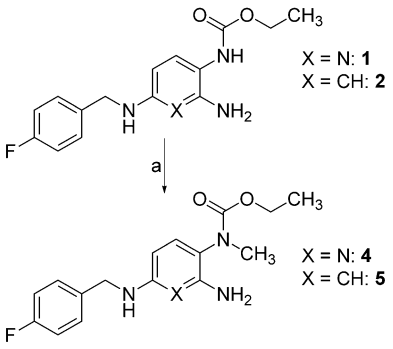
Herein we report the synthesis of a series of novel flupirtine analogues and their use in studying the activity at the proposed molecular target as well their potential to cause toxicity in liver cell culture models. These analogues closely resemble flupirtine, but possess substituents designed to interfere with azaquinone diimine formation by inductive electronic effects, replacement of removable hydrogen atoms, and steric bulk. Additionally, one derivative lacks an amino group necessary for the formation of *ortho*-azaquinone diimine. The altered redox properties associated with modified reactivity makes them ideal probes for these studies.

Results

Chemistry

Synthesis of carbamate methylated flupirtine/retigabine

Regarding the nitrogen-substituted pyridine ring of flupirtine (**1**) it can be assumed that the nitrogen atom incorporated into the carbamate group is weakly acidic, whereas the other two aromatic amines have weak basic properties. A prerequisite for the desired regioselective monoalkylation was therefore to exclusively improve the nucleophilicity of the appropriate nitrogen atom. Deprotonation of the carbamate group with subsequent addition of methyl iodide was applied (Scheme 1) by using established procedures[19] for the alkylation of carbamates. Alkyl bromides with longer alkyl chains led to better results than the corresponding iodides. However, in the case of methylation, methyl iodide was preferred over gaseous methyl bromide due to safety and handling concerns. Chromatographic analysis of the reaction products indicated that only minor changes in lipophilicity resulted from methylation while the photochemical stability was altered.

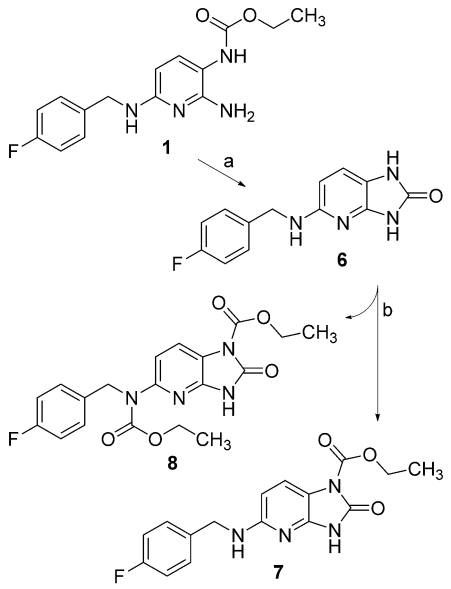


Scheme 1. Selective alkylation of the carbamate nitrogen atom. *Reagents and conditions*: a) NaH, CH_3I, THF, 0 °C, 30 min, then CH_3I, 0 °C→RT, overnight.

Synthesis of bicyclic flupirtine derivatives

Another possible way to modify the properties of the carbamate nitrogen atom is to form a bicyclic derivative. A bridge between nitrogen atoms on C2 and C3 of the flupirtine pyridine ring, forming a five-membered ring, would leave the backbone of the molecule unaltered, but would slightly change the angle of the C–N bonds on C2 and C3. Attempts to force ring closure by insertion of formic acid to obtain a methine bridge or cyanogen bromide to form a guanidine group were not successful. However, dry heating of **1** in an open vessel, following similar solvent-free procedures[20,21] led to **6** in good yield (Scheme 2).

Compound **6** is a known process impurity formed during large-scale synthesis of flupirtine and was recently identified and synthesized by Zhang et al.[22] Our straightforward preparation of **6** by dry heating, however, is advantageous over the



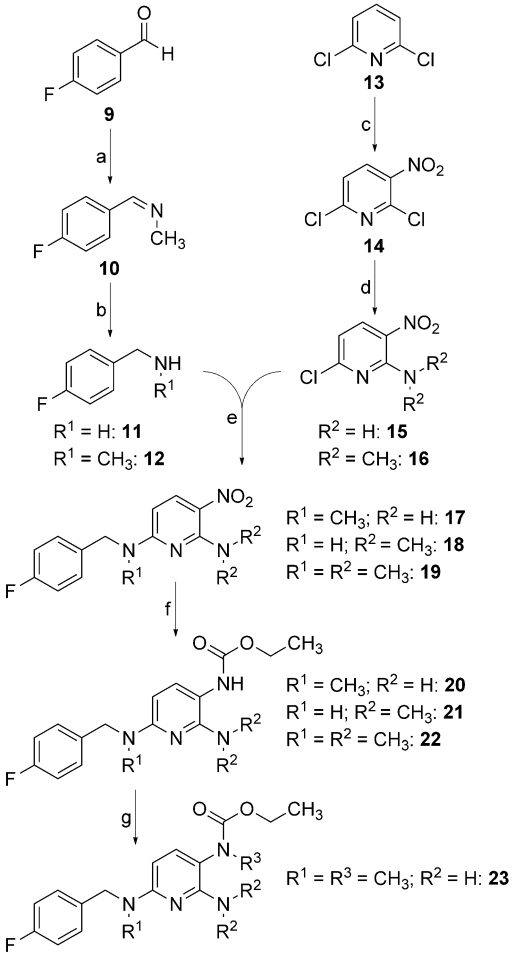
Scheme 2. Dry heating of flupirtine (**1**) followed by re-acylation. *Reagents and conditions*: a) Δ (200 °C), 30 min; b) ethyl pyridine-2-yl carbonate, DMF, MeCN, 75 °C, overnight.

method described by Zhang et al., who used 2,3-diamino-6-(4-fluorobenzylamino)pyridine, a compound extremely sensitive to oxidation, as an intermediate in a multistep sequence.

As a consequence of ring closure by dry heating, the ethyl carbamate group of **1** was lost. To improve the molecular similarity of the bicyclic derivative **6** and flupirtine, we envisioned reintroduction of such an ethyl carbamate substituent to **6**. This was achieved by use of an acylating carbonate with high electrophilicity brought about by low mesomeric stabilization due to incorporation of an electron-deficient aromatic ring. Thus, ethyl pyridin-2-yl carbonate was prepared as described[23] and used without further purification. The main product of the reaction of this electrophile with the cyclic urea **6** was the desired carbamate **7**, but a small quantity of a second compound, **8**, bearing two carbamate groups, was isolated. The formation of compound **8** was not expected, as Zhang et al. only found the regioisomer with N1 and N3 acylated among the process impurities.[22]

Synthesis of N-methylated derivatives following an established route for the synthesis of flupirtine

Direct alkylations of flupirtine in our hands always yielded N-methylated flupirtine derivatives with substitution at the carbamate nitrogen only. To obtain derivatives with a divergent N-alkylation pattern, multistep syntheses were required. In retrospect, there were no conformities between any side products of the synthesis of **4** detected by thin-layer chromatography (TLC) and any monoalkylated compounds described later on. This indicates that the alkylation of N^2 and N^6 cannot be accomplished by direct alkylation with standard procedures. We focused on a well-described route for the synthesis of flupirtine,[24–26] which allows the regioselective introduction of methyl groups in intermediary reaction steps (Scheme 3).



Scheme 3. Synthesis of flupirtine derivatives, introducing methyl groups at intermediary reaction steps (a, d, and g). *Reagents and conditions*: a) CH_3NH_2, MeOH, reflux, overnight; b) $NaBH_4$, MeOH, 0 °C→RT, overnight; c) conc. HNO_3, conc. H_2SO_4, reflux (~100–105 °C), overnight; d) (for **15**): aq. NH_3, 2-propanol, 35 °C, 3 d; (for **16**): $(CH_3)_2NH$, Et_3N, MeCN, 0 °C→RT, 30 min; e) (for **17**): **15**, **12**, 2-propanol, Et_3N, reflux, overnight; (for **18**, **19**): 4-fluorobenzylamine (**11** or **12**), **16**, Et_3N, MeCN, RT, overnight, then reflux, ≥24 h; f) (for **20**): **17**, Pd/C, H_2, EtOH, overnight, then Et_3N, ethyl chloroformate, 4 h; (for **21**, **22**): nitropyridine (**18** or **19**), Pd/C, H_2, EtOH, overnight, then Et_3N, ethyl chloroformate, 8 h; g) **20**, NaH, THF, 0 °C, 30 min, then CH_3I, 0 °C→RT, overnight.

Whereas 4-fluorobenzylamine (**11**) was commercially available, the N-methylated secondary amine **12** had to be prepared from 4-fluorobenzaldehyde (**9**) by reductive amination in a two-step synthesis.[27,28] The second component was the chlorinated pyridine ring **13**, which was nitrated by using hot nitrosulfuric acid to obtain **14**. In the next step, selective nucleophilic aromatic substitution was achieved; in this reaction, ammonia was far less reactive than dimethylamine, which was an advantage in matters of selectivity, and thus **15** was far more easily purified than **16**.

The subsequent transformation of compounds **15** and **16** in a second nucleophilic substitution with the 4-fluorobenzylamines **11** and **12** at elevated temperatures afforded **17**–**19**.

Again, the derivatives with a dimethylamino group proved to be more difficult to purify, as the resulting compounds (**18** and **19**) were not crystalline like **17**, but brown sticky oils. Whereas **18** has been reported previously in a patent[29] along with similar derivatives with various N^2 substituents,[30–32] **19** has not been reported until now.

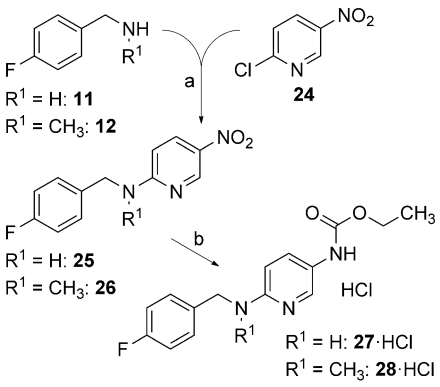
Subsequent hydrogenation of the nitro groups of **17**, **18**, and **19** was carried out without workup of the resulting amino intermediates because of their notorious sensitivity toward oxygen. Thus, ethyl chloroformate and triethylamine were added directly to the reaction mixture in the closed vessel and allowed to react for several hours.

Lastly, crystallization was enforced by the addition of a mixture of water and 2-propanol, as described for the maleate salt of flupirtine.[26] In the case of **20**, this could also be achieved, but even the free base was directly obtained in crystalline form. On the other hand, the crystalline salts of **21** and **22** could not be obtained in an analogous fashion, neither with nor without maleic acid. Thus, the crude products were subjected to liquid–liquid extraction by using dilute hydrochloric acid and dichloromethane, where the salts **21**·HCl and **22**·HCl fractioned into the organic phase. These salts could, in part, be obtained in solid form by lyophilization. Recrystallization was achieved by dissolution in ethyl acetate followed by the addition of *n*-hexane.

Compound **20** was alkylated with methyl iodide in an analogous manner to the preparation of **4** and **5** to obtain the dimethylated derivative **23**, which required an even more demanding workup und crystallized only slowly.

Synthesis of deaminoflupirtine derivatives

Starting from 2-chloro-5-nitropyridine (**24**), the flupirtine analogues **27** and **28** could be obtained in just three steps as their hydrochloride salts (Scheme 4). The synthetic route was analogous to that of **21** and **22**, yielding the solid intermediates **25** and **26**. Nucleophilic aromatic substitution again was driven by the electron-withdrawing effect of the nitro group and the electron deficiency of the pyridine ring.



Scheme 4. Synthesis of deaminoflupirtine derivatives. *Reagents and conditions*: a) Et_3N, MeCN, RT, overnight, then reflux, 24 h; b) Pd/C, H_2, EtOH, overnight, then Et_3N, ethyl chloroformate, 8 h.

Analytical investigations

Cyclic voltammetry studies

Electrochemistry can be used for mimicking some types of biological reactions, such as oxidative drug metabolism, including formation of reactive metabolites. Specifically, cyclic voltammetric determination of anodic peak potential is suited to indicate chemical properties in the context of oxidation; for example, the electrochemical properties of the widely used analgesic diclofenac were studied by cyclic voltammetry by Madsen et al.[33,34]

Flupirtine (**1**) undergoes facile autoxidation even when dissolved in aqueous solution at room temperature, as can be visualized directly by a change from a colorless solution to one with a blue–green hue within hours. Peroxidases are known to accelerate this reaction considerably, leading to reactive azaquinone diimines that can react further to form dimers and trimers of flupirtine.[13] Thus, we investigated the oxidation potentials of the new analogues by cyclic voltammetry and compared them with that of flupirtine (Figure 2).

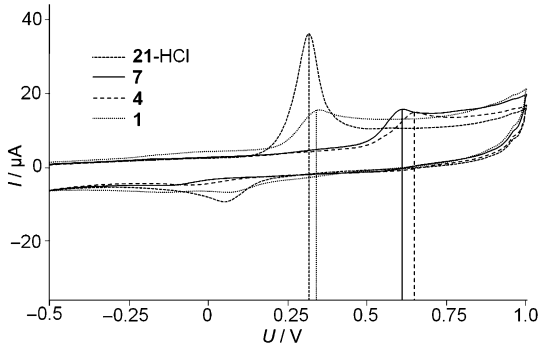


Figure 2. Cyclic voltammograms of selected compounds in 100 mM Tris buffer (pH 7.4); shown is the first cycle, swept through −1.0 to 1.0 V, displaying only −0.5 to 1.0 V.

Figure 2 shows representative cyclic voltammograms of flupirtine and three of the analogues, which are characterized by more or less well-defined anodic peaks between 0.25 and 0.75 V (Table 1). In contrast, the cathodic peaks were very weak or nonexistent, evidence of the irreversible nature of the redox process, and consistent with the high reactivity of the azaquinone diimine products, which deposit as a polymer film on the surface of the electrode. In fact, after each cyclic voltammetric run, the glassy carbon electrode had to be polished, or else the next cyclic voltammogram showed poorly formed peaks of decreased intensity.

Interestingly, the most intense anodic and cathodic peaks were found with the *N*,*N*-dimethyl-substituted analogue **21** (Figure 2); substitution of the primary aromatic amine likely prevents nucleophilic attack of the azaquinone diimine structure, lowering the propensity to form polymers. Nevertheless, substitution of either the primary or secondary aromatic amines of flupirtine with methyl groups (**21** and **20**, respectively) had little effect on the position of the anodic peak potentials ($E_{p,a}$) relative to flupirtine. On the other hand, substitution

Table 1. Comparisons of anodic peak potentials ($E_{p.a}$) of flupirtine (**1**) and derivatives, EC_{50} values toward $K_V7.2/3$ channels in HEK293 cells, and LD_{50} values in TAMH cells.

Compound	$E_{p.a}$ [mV][a]	EC_{50} [μM][b]	LD_{50} [μM][c]
20	294	1.9 ± 0.1	94 ± 34
retigabine (**2**)	299	1.9 ± 0.2[d]	174 ± 27
21·HCl	320	2.6 ± 0.4	119 ± 49
flupirtine (**1**)	350	3.6 ± 0.9[e]	87 ± 60[e]
22·HCl	385	3.5 ± 0.7	35 ± 17
5	591	> 100	205 ± 53
7	612	> 100	202 ± 57
27·HCl	642	> 100	204 ± 47
4	652	> 100	131 ± 67
28·HCl	653	> 100	69 ± 33
23	667	> 100	125 ± 61
acetaminophen (**3**)	602	ND[f]	> 1000
N-methylacetaminophen (**29**)	773	ND[f]	> 1000

[a] Determined with 1.0 mM compound in 100 mM Tris buffer (pH 7.4); values are the mean ± SD of 4–5 independent determinations. [b] Determined after 30 min exposure; values are the mean ± SD of 4–5 independent determinations. [c] Determined after 24 h exposure. [d] Ref. [36]. [e] Flupirtine maleate salt was used. [f] Not determined.

of the carbamate nitrogen (**4**, **5**, **7**, **23**) as well as lack of the amino group on C2 (**27**·HCl and **28**·HCl) caused a very notable shift in the anodic potential of about + 0.3 V.

Biology

In vitro tests on $K_V7.2/3$ channels

The nine synthesized flupirtine derivatives were tested in a FLIPR™-based assay for their ability to increase $K_V7.2/3$ potassium channel activity relative to flupirtine. Special HEK293 cells co-expressing the *KCNQ2* and *KCNQ3* genes were used in this assay, which was carried out by using a FluxOR potassium ion channel assay (Life Technologies).[35] In this assay, a specific thallium-sensitive dye is loaded into the HEK293 cells which is then cleaved by esterase activity to the active Tl-complexing agent, which forms fluorescent complexes with thallium ions that enter the cells selectively via the opened $K_V7.2/3$ potassium channel. The intensity of cellular fluorescence is an indication of the efficacy of ion channel opening.

Figure 3 shows the dose–response curves for the active compounds **1**, **20**, **21**, and **22** as $K_V7.2/3$ channel openers. Table 1 reports the average EC_{50} values. All flupirtine derivatives with a substituted carbamate nitrogen atom were ineffective at channel opening up to 100 μM. The two deaminoflupirtine derivatives **27**·HCl and **28**·HCl were not completely inactive at the measured concentrations, but estimates of their EC_{50} values were not possible, as they were > 100 μM, the highest concentration used (Figure 3). In contrast, derivatives that are methyl substituted at only N^2 and/or N^6 (**20**, **21**·HCl, and **22**·HCl) had EC_{50} values in the same low micromolar range as flupirtine. Interestingly, these are the same three flupirtine derivatives that show similar $E_{p.a}$ values to that of flupirtine, whereas the inactive derivatives have considerably greater $E_{p.a}$ values (Table 1).

In comparing the active and inactive compounds based on their anodic peak potentials by the Mann–Whitney U-test, a highly significant difference was found between the $E_{p.a}$ of the two groups of compounds (Figure 4a). This indicates that more easily oxidized flupirtine derivatives are more potent than those less easily oxidized. In fact, a slight trend toward greater potency of the active compounds can be observed with decreasing $E_{p.a}$ values (Table 1).

Cytotoxicity in TGF-α transgenic mouse hepatocytes

TGF-α transgenic mouse hepatocytes (TAMH cells) were used to estimate the hepatotoxicity of the compounds in cell culture. This cell line overexpresses TGF-α, but retains many of the characteristics of hepatocytes while replicating for months in culture.[37] Acute cytotoxicity was estimated by exposing confluent cell cultures to test compounds for 24 h and measuring cell viability by MTT assay. Compound concentrations that decreased cell viability by 50% relative to untreated controls were defined as LD_{50}. Figure 5 shows dose–response curves for three representative compounds in the TAMH cell line.

Table 1 summarizes the LD_{50} values for flupirtine and the various analogues, of which some show relatively high and others, like flupirtine, low $E_{p.a}$ values. It is clear that there is no correlation between $E_{p.a}$ and LD_{50} values. Moreover, Figure 4b shows that there is no significant difference between the LD_{50} values for the active and inactive compounds; i.e., $K_V7.2/3$ channel opening activity and hepatotoxicity are not linked.

Discussion

Earlier structure–activity relationship (SAR) studies with flupirtine were concerned mainly with the effect of the substitution pattern in the phenyl ring, as well as substitutions at the car-

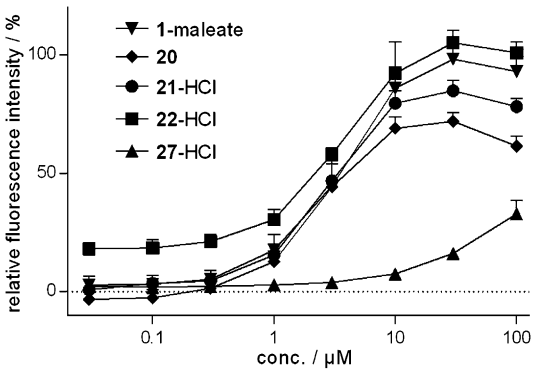


Figure 3. Dose–response curves of **20**, **21**·HCl, **22**·HCl, and **27**·HCl compared with that of flupirtine (**1**) in the $K_V7.2/3$ channel-opening assay. Relative fluorescence intensity was measured by setting stimulus buffer alone at 0%, and 100 μM flupirtine maleate at 100%; data are the average ± SD of four independent determinations.

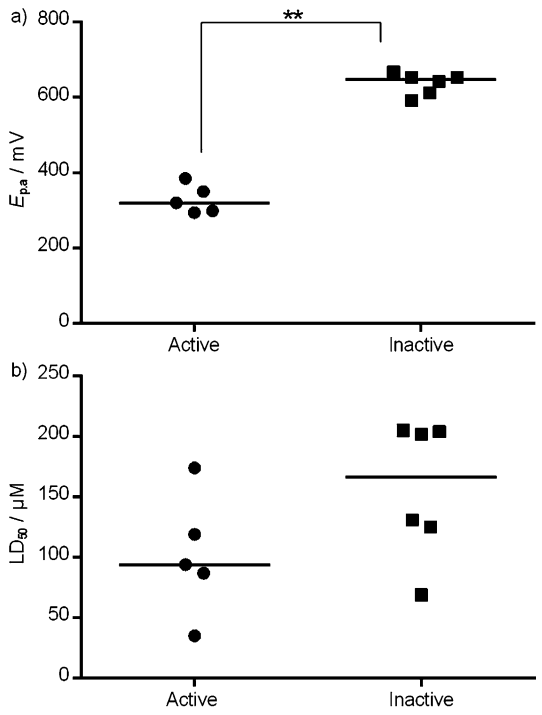


Figure 4. Statistical evaluation of the differences between active and inactive compounds with respect to a) $E_{p,a}$ and b) LD_{50}. Significance: $^{**}p < 0.01$ by two-tailed Mann–Whitney U-test (GraphPad Prism 6.0).

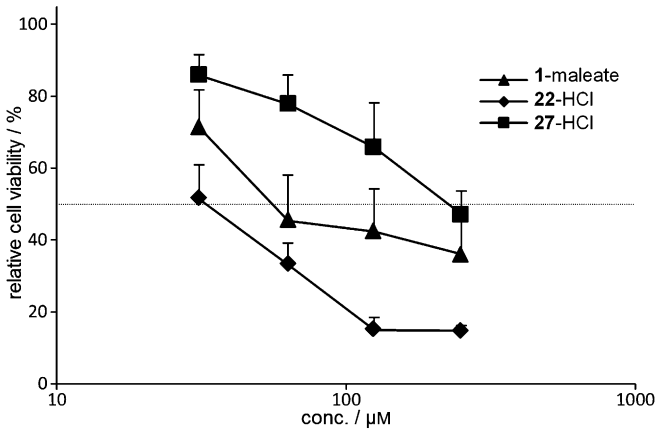


Figure 5. Dose-dependent cytotoxicity of flupirtine (**1**) and representative derivatives in TAMH cells after 24 h exposure, determined by the MTT method. Data are the average ± SD of four independent determinations.

bamate oxygen atom, on analgesic activity.[38] Until now, no reports have been published concerning the effects of N-alkylation either on activity or toxicity. In this study, all nitrogen atoms attached to the pyridine ring of flupirtine were selectively methylated, although only the carbamate nitrogen could be alkylated directly from flupirtine itself. All nine N-modified flupirtine derivatives were previously unreported, thus offering a unique opportunity to conduct SAR studies with respect to oxidation potential, $K_V7.2/3$ channel-opening activity, and hepatotoxicity of this widely used drug. Figure 6 summarizes the

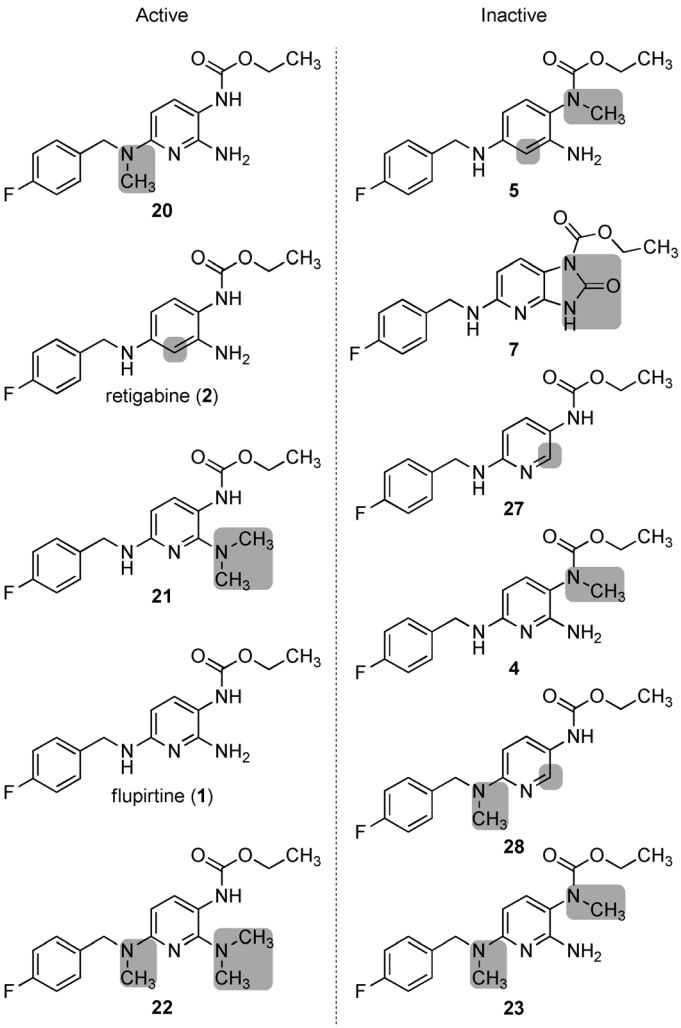


Figure 6. Structures of compounds active as $K_V7.2/3$ channel openers (left) compared with their inactive analogues (right). Shaded areas highlight structural differences from the lead compound flupirtine (**1**).

SAR of the 11 compounds investigated in this work with respect to their ability to open $K_V7.2/3$ channels.

The ease of oxidation, based on the cyclic voltammetric anodic peak potentials of the N-methylated analogues relative to flupirtine (**1**), can be rationalized based on their structures. For example, if the carbamate nitrogen atom is methylated (i.e., **4** and **23**), oxidation to an azaquinone diimine would place a permanent positive charge on that carbamate nitrogen directly adjacent to a partial positively charged carbonyl carbon, which is energetically unfavorable. On the other hand, methylation at N^2 and N^6 (i.e., **20**–**22**) can theoretically facilitate oxidation due to the electropositive nature of the two methyl groups on that nitrogen. Furthermore, the primary amino group of flupirtine would be expected to increase the electron density in the pyridine ring owing to resonance effects, which should also facilitate oxidation and explain the higher anodic peak potentials of the derivatives without this specific group (i.e., **27** and **28**).

SAR clearly show that substitution of the proton on the carbamate nitrogen atom with methyl group causes a total loss

of $K_V7.2/3$ channel-opening activity while dramatically increasing the oxidation potential by +300 mV. This observation is reminiscent of earlier studies with the analgesic acetaminophen, in which N-methylation increases the oxidation potential by +171 mV, resulting in a complete loss of analgesic activity.[39] This is consistent with recent evidence indicating that reactive quinone imine metabolite of acetaminophen, NAPQI (**3a**), is the active agent responsible for analgesic activity.[16]

An alternative explanation for the poor $K_V7.2/3$ channel-opening activity of the analogues with an N-methylated carbamate could be that the loss of the carbamate hydrogen results in the loss of a critical hydrogen bond within the binding site of the potassium channel. However, **27** and **28**, both of which belong to the group with higher oxidation potentials (Table 1), retain this hydrogen, but are still poor channel openers. Thus, oxidation potential appears more closely correlated with good channel-opening activity. The primary aromatic amino group of flupirtine also appears important for good channel-opening activity, as analogues **27** and **28**, which lack it, are notably less active. In contrast, methylation at either N^2 or N^6 resulted in analogues (i.e., **21** and **22**) with good channel-opening activity, but methylation also had little effect on their oxidation potentials (Table 1).

It is not yet known if these effects at the $K_V7.2/3$ channel translate directly to analgesic activity in animals. However, a number of other variables will affect in vivo activity that do not factor into an in vitro system, e.g., pharmacokinetics and metabolism. Thus, mechanism of action studies with a simpler cellular system would appear suitable for defining SAR.

There is growing interest in the regulation of potassium channels by the cellular redox environment.[40] Studies indicate that these heteromeric $K_V7.2/3$ channels are sensitive to oxidation, for example, through hydrogen peroxide mediated oxidation of cysteine residues, which affects current activity.[41] Once bound to the channel in the oxidized form (i.e., as the azaquinone diimine **1a** or **1b**), flupirtine might participate directly in such redox reactions, facilitating the opening of $K_V7.2/3$ channels. This could explain why easily oxidized analogues are better channel openers than those less easily oxidized. Understanding how the cellular redox environment influences the $K_V7.2/3$ channel-opening properties of flupirtine and derivatives will be at the center of future research.

For evaluation of the hepatotoxic potential of flupirtine and derivatives, an established in vitro model system based on cultured TAMH cells was used. TAMH cells show many of the same attributes as hepatocytes,[37] and have been used to study mechanisms of hepatotoxicity in drugs such as acetaminophen,[42] flutamide,[43] and lapatinib[44] (not shown).

In the case of flupirtine, a compound known to cause liver toxicity in a small group of patients, 24 h exposure led to a notable decrease in cell viability at concentrations below 100 μM. In fact, all the analogues tested showed roughly the same cytotoxic potency regardless of their oxidation potential (Table 1). In particular, **4** showed no significant decrease in cytotoxicity relative to flupirtine, even though methylation of the carbamate nitrogen atom led to an increase in oxidation potential by ~300 mV. This is in contrast to the case observed with acetaminophen (**3**), for which N-methylation led to an increase in $E_{p.a}$ of 171 mV and also protected against hepatotoxicity. Nelson and co-workers rationalized this by the failure of *N*-methylacetaminophen (**29**) to oxidatively form the hepatotoxic NAPQI.[39] In the case of flupirtine, however, hepatotoxicity would not appear to be directly linked to oxidative processes. The very different physicochemical properties of acetaminophen (hydrophilic, weak acid) relative to flupirtine (lipophilic, weak base) could result in differences as to how these compounds are taken up and compartmentalized by hepatocytes. Ongoing studies on the biochemical changes in hepatocytes treated with flupirtine and various analogues are aimed at gaining a better understanding of the mechanism of toxicity.

Conclusions

While the redox behavior appears to be linked to the pharmacological activity of flupirtine, as is also the case with acetaminophen, unlike acetaminophen, it does not appear to play a central role in hepatocyte toxicity. Thus, analgesic activity and hepatotoxicity would appear separable in this class of drugs. This knowledge should be useful for the rational design of new $K_V7.2/3$ channel openers.

Experimental Section

General

Reaction control by TLC was carried out with ALUGRAM SIL G/UV_{254} silica gel plates from Macherey–Nagel (4×8 cm, silica layer: 20 mm, substances plotted in 1 cm bends). Melting Points were measured with a Kofler hot-stage microscope (Boëtius PHMK 81/3035, VEB Wägetechnik Rapido) with 16-fold amplification. Compound purities were determined after HPLC by DAD chromatograms (100% method, 254 nm). NMR spectra were recorded with a Bruker Avance III instrument with Ultrashield 400 (25 °C, ppm scale) internal standard, resonance frequency, and solvent as indicated. HRMS data were obtained after HPLC with an LC-IT-TOF mass spectrometer (Shimadzu). IR spectra were recorded on a Nicolet IR200 FT-IR instrument (Thermo Electron Corporation) with diamond ATR accessory. For cyclic voltammetry, a 797 CA Computrace from Metrohm was used.

Chemistry

Ethyl [2-amino-6-(4-fluorobenzylamino)pyridine-3-yl]carbamate (flupirtine; 1): Five capsules of Trancopal Dolo (each containing 100 mg flupirtine maleate salt) were cut open, and the contents were added to a separation funnel containing H_2O (50 mL), EtOAc (50 mL) and Et_3N (1 mL). After shaking the mixture, the insoluble components were filtered off, and the organic layer of the filtrate was washed once with brine (50 mL), dried over Na_2SO_4 and concentrated in vacuo. Light-yellow solid, turning grey (which subsequently turned brown) upon exposure of the substance on the TLC plate to UV light for 15 min (315 mg, 87%); mp: 114–116 °C (from EtOAc); ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.20 (br s, 3H, CH_3), 4.03 (q, J = 6.8 Hz, 2H, O-CH_2), 4.35 (d, J = 6.0 Hz, 2H, C(α′)H_2), 5.19 (s, 2H, N^2H_2), 5.67 (t, J = 8.0 Hz, 1H, C(5)H), 6.52 (t, J = 6.0 Hz, 1H, N^6H), 7.01 (m, 1H, C(4)H), 7.10 (m, 2H, C(3′)H and C(5′)H), 7.34 (m, 2H, C(2′)H and C(6′)H), 8.17 ppm (br s, 1H, N^3H); ^{13}C NMR

(100 MHz, [D$_6$]DMSO): δ = 14.5, 43.5, 59.1, 106.5, 114.7, 128.9, 135.6, 137.2, 153.0, 155.0, 155.6, 159.7, 162.1 ppm; IR: $\tilde{\nu}$ = 3371, 3202, 2985, 2904, 2841, 1687, 1616, 1495, 1213 cm^{-1}.

Ethyl [2-amino-4-(4-fluorobenzylamino)phenyl]carbamate (retigabine; 2): One film-coated tablet of Trobalt (containing 300 mg retigabine) was pulverized in a mortar and diluted with Et_2O (25 mL) and brine (25 mL). After shaking the mixture, the insoluble components were filtered off, and the organic layer of the filtrate was washed once with brine (25 mL), dried over Na_2SO_4 and concentrated in vacuo. Light-yellow solid, evolving a pale-pink color when the substance on a TLC plate was exposed to UV light for 15 min (250 mg, 83%); mp: 136–138 °C (from Et_2O); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.19 (brs, 3H, CH_3), 4.02 (q, J = 7.2 Hz, 2H, O-CH_2), 4.16 (d, J = 6.0 Hz, 2H, C(α')H_2), 4.52 (s, 2H, N^2H_2), 5.82 (dd, J = 8.8 Hz, J = 2.4 Hz, 1H, C(3)H), 5.89 (t, J = 6.0 Hz, 1H, N^4H), 5.92 (d, J = 2.4 Hz, 1H, C(3)H), 6.79 (d, J = 8.0 Hz, 1H, C(6)H), 7.12 (m, 2H, C(3′)H and C(5′)H), 7.35 (m, 2H, C(2′)H and C(5′)H) 8.14 ppm (s, 1H, N^1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 14.7, 45.8, 59.8, 98.9, 101.6, 113.0, 114.8, 127.0, 128.8, 136.8, 143.4, 147.1, 155.1, 159.8, 162.2 ppm; IR: $\tilde{\nu}$ = 3421, 3375, 3268, 3187, 3037, 2984, 2918, 2850, 1697, 1507, 1214, 825 cm^{-1}.

General procedure A

Compound **1** or **2** (1 mmol) and NaH (1.5 mmol, 60 mg; 65% solution in *n*-hexane) were set under an argon atmosphere, stirred in THF (12 mL), cooled to 0 °C in the dark. After 30 min CH_3I (2 mmol, 124 μL) was added, then stirred overnight while the reaction was allowed to reach room temperature. TLC of the reaction progress showed almost no change in R_f values, but a change in color under UV light occurred after 15 min. The reaction was deemed complete when the spot was a consistent orange color without any hue of the original substance on the lower rim. Afterward, the solvent was removed under reduced pressure, the residue diluted with CH_2Cl_2 (50 mL) and then washed with brine (50 mL). The organic layer was dried over Na_2SO_4, concentrated in vacuo, and if necessary lyophilized to give a brown solid, which was washed with small amounts of Et_2O to give the desired product.

Ethyl [2-amino-6-(4-fluorobenzylamino)pyridine-3-yl](methyl)carbamate (4): Following general procedure A starting from **1** (304 mg) gave a light-brown solid (127 mg, 40%); purity: 100%; mp: 112–113 °C (from CH_2Cl_2); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.06 and 1.22 (brs and brs, 2H and 1H, O-CH_2-CH_3), 2.96 (s, 3H, N^3CH_3) 3.97 (brs, 2H, O-CH_2), 4.36 (m, 2H, C(α')H_2), 5.37 (s, 2H, N^2H_2), 5.65 (d, J = 8.4 Hz, 1H, C(5)H), 6.63 (t, J = 6.0 Hz, 1H, N^6H), 6.90 (d, J = 8.4 Hz, 1H, C(4)H), 7.12 (t, J = 9.0 Hz, 2H, C(3′)H and C(5′)H), 7.37 ppm (m, 2H, C(2′)H and C(6′)H), ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 14.7, 36.5, 43.6, 60.5, 95.3, 111.4, 114.8, 129.2, 137.0, 137.1, 154.1, 155.7, 156.5, 159.8 and 162.2 ppm; IR: $\tilde{\nu}$ = 3419, 3166, 1676, 1589, 1508, 1212 cm^{-1}; HRMS-ESI m/z $[M+H]^+$ calcd for $C_{16}H_{19}N_4O_2F$: 319.1565, found: 319.1550; Anal. calcd for $C_{16}H_{19}N_4O_2F$: C 60.4, H 6.0, N 17.6, found: C 60.25; H 5.7, N 17.4.

Ethyl [2-amino-4-(4-fluorobenzylamino)phenyl](methyl)carbamate (5): Following general procedure A starting from **2** (303 mg) gave a light-yellow solid (92 mg, 29%); purity: 100%; mp: 113–115 °C (from CH_2Cl_2); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.04 and 1.21 (brs and brs, 2H and 1H, O-CH_2-CH_3), 2.95 (s, 3H, N^1-CH_3), 3.95 (brs, 2H, O-CH_2), 4.15 (d, J = 5.2 Hz, 2H, C(α')H), 4.63 (s, 2H, N^2H_2), 5.79 (d, J = 8.4 Hz, 1H, C(5)H), 5.90 (s, 1H, C(3)H), 5.99 (t, J = 5.6 Hz, 1H, N^4H), 6.57 (d, J = 8.0 Hz, 1H, C(6)H), 7.13 (t, J = 8.6 Hz, 2H, C(3′)H and C(5′)H), 7.36 ppm (m, 2H, C(2′) and C(6′)H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 14.7, 36.8, 45.8, 60.4, 98.2, 101.7, 114.9, 118.0, 128.1, 128.9, 136.8, 144.6, 148.2, 155.9, 159.8, 162.2 ppm; IR: $\tilde{\nu}$ = 3428, 3381, 3340, 2981, 2856, 1686, 1507, 828 cm^{-1}; HRMS-ESI m/z $[M+H]^+$ calcd for $C_{17}H_{20}N_3O_2F$: 318.1612, found: 318.1603; Anal. calcd for $C_{17}H_{20}N_3O_2F$: C 64.3, H 6.35, N 13.2, found: C 64.0, H 6.0, N 13.1.

5-(4-Fluorobenzylamino)-1,3-dihydro-2*H*-imidazo[4,5-*b*]pyridine-2-one (6): Compound **1** (2 mmol, 608 mg) was heated at 200 °C for 30 min in an open vessel. After cooling, the crude product was washed with a small volume of MeOH and dried under reduced pressure. Light-brown solid (538 mg, 88%); purity: 100%; mp: 242–244 °C; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 4.37 (d, J = 6.0 Hz, 2H, C(α')H_2), 6.08 (d, J = 8.4 Hz, 1H, C(6)H), 6.61 (t, J = 6.0 Hz, 1H, N^5H), 6.98 (d, J = 8.0 Hz, 1H, C(7)H), 7.12 (m, 2H, C(3′)H and C(5′)H), 7.35 (m, 2H, C(2′)H and C(6′)H), 10.18 (s, 1H, N(1)H) and 10.79 (s, 1H, N(3)H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 44.1. 99.1, 113.7, 114.7, 117.7, 128.9, 136.9, 142.8, 153.8, 154.1, 159.7, 162.1 ppm; IR: $\tilde{\nu}$ = 3433, 3132, 2997, 2829, 1682, 1613, 1219 cm^{-1}; HRMS-ESI m/z $[M+H]^+$ calcd for $C_{13}H_{11}N_4OF$: 259.0990, found: 259.0985; Anal. calcd for $C_{13}H_{11}N_4OF$: C 60.5, H 4.3, N 21.7, found: C 60.1, H 4.1, N 21.5.

General procedure B

Compound **6** (1 mmol, 258 mg) and ethyl pyridine-2-yl carbonate (2 mmol; 333 mg) were suspended in a mixture of DMF (2.5 mL) and MeCN (2.5 mL) and stirred overnight in a capped round-bottom flask at 75 °C. The reaction was concentrated under reduced pressure and acidified with HCl (1 M) to pH ~ 1, filtered, and the residue was washed with H_2O and dried in vacuo. The obtained crude product was purified by column chromatography (*n*-hexane/EtOAc gradient) with silica gel.

Ethyl 5-(4-fluorobenzylamino)-2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridine-1-carboxylate (7): Following general procedure B yielded a light-beige solid (70 mg, 21%); purity: 100%; mp: 206–208 °C (from *n*-hexane/EtOAc); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.32 (t, J = 7.2 Hz, 3H, CH_3), 4.34 (q, J = 7.2 Hz, 2H, O-CH_2), 4.40 (d, J = 6.0 Hz, 2H, C(α')H_2), 6.19 (d, J = 8.8 Hz, 1H, C(6)H), 7.05 (t, J = 6.0 Hz, 1H, N^5H), 7.13 (m, 2H, C(3′)H and C(5′)H), 7.35 (m, 2H, C(2′)H and C(5′)H), 7.61 (d, J = 8.4 Hz, 1H, C(7)H), 11.48 ppm (s, 1H, N(3)H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 14.0, 43.7, 62.6, 99.9, 110.6, 114.8, 123.3, 128.9, 136.4, 141.8, 149.6, 149.8, 155.6, 159.8, 162.2 ppm; IR: $\tilde{\nu}$ = 3392, 3060, 2995, 2934, 2856, 2746, 1755, 1721, 1607, 1514 cm^{-1}; HRMS-ESI m/z $[M+H]^+$ calcd for $C_{16}H_{15}N_4O_3F$: 331.1201, found: 331.1186; Anal. calcd for $C_{16}H_{15}N_4O_3F$: C 58.2, H 4.6, N 17.0, found: C 58.3, H 4.5, N 16.8.

Ethyl 5-[(ethoxycarbonyl)(4-fluorobenzyl)amino]-2-oxo-2,3-dihydro-1*H*-imidazo[4,5-*b*]pyridine-1-carboxylate (8): Following general procedure B yielded a light-brown solid (7 mg, 2%); purity 100%; mp: 157–160 °C (from *n*-hexane/EtOAc); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.15 (t, J = 7.2 Hz, 3H, N^5-COO-CH_2-CH_3), 1.35 (t, J = 7.2 Hz, 3H, N(1)-COO-CH_2-CH_3), 4.13 (q, J = 7.2 Hz, 2H, N^5-COO-CH_2), 4.39 (q, J = 7.2 Hz, 2H, N(1)-COO-CH_2), 4.99 (s, 2H, C(α')H_2), 7.11 (m, 2H, C(3′)H and C(5′)H), 7.21 (d, J = 8.4 Hz, 1H, C(6)H), 7.30 (m, 2H, C(2′)H and C(6′)H), 7.88 (d, J = 8.4 Hz, 1H, C(7)H), 11.98 ppm (s, 1H, N(3)H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 14.0, 14.3, 49.7, 61.7, 63.2, 113.4, 115.0, 118.3, 122.2, 129.3, 134.4, 142.1, 149.5, 149.9, 154.5, 160.0, 162.4 ppm; IR: $\tilde{\nu}$ = 3219, 2972, 1763, 1731, 1708, 1613, 1509, 1217 cm^{-1}; HRMS-ESI m/z $[M+H]^+$ calcd for $C_{19}H_{19}N_4O_5F$: 403.1412, found: 403.1414.

***N*-Methyl-4-fluorbenzylamine (12)**: 4-Fluorobenzaldehyde (**9**; 10 mmol, 1056 μL) and methylamine (30 mmol, 3 mL, 40% solution

in MeOH) were diluted with MeOH (10 mL) and held at reflux overnight. The solution was cooled to 0 °C, and $NaBH_4$ (25 mmol, 1 g) was slowly added. The mixture was allowed to warm to room temperature and was stirred overnight. A small volume of H_2O was added to quench residual $NaBH_4$, then the mixture was concentrated under reduced pressure, the residue diluted with CH_2Cl_2 (20 mL) and washed with brine (2×20 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo to give the desired compound as a nearly colorless solution (1.2 g, 87%); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 2.01$ (br s, 1H, NH), 2.24 (s, 3H, CH_3), 3.60 (s, 2H, CH_2), 7.11 (m, 2H, C(3)H and C(5)H), 7.34 ppm (m, 2H, C(2)H and C(6)H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 35.4$, 54.2, 114.8, 129.6, 136.9, 159.7, 162.1 ppm; IR: $\tilde{\nu} = 2986$, 2944, 2856, 1603, 1508, 1219, 821 cm^{-1}.

***N*-Methyl-4-fluorbenzylidenamine (10)**: Intermediate of the synthesis of **12** was isolated by taking a sample of the solution after holding at reflux and concentrating it in vacuo, to give a colorless liquid; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 3.43$ (d, $J = 1.6$ Hz, 3H, CH_3), 7.27 (m, 2H, C(3)H and C(5)H), 7.78 (m, 2H, C(2)H and C(6)H), 8.34 ppm (d, $J = 1.6$ Hz, 1H, C(α)H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 47.4$, 115.6, 129.8, 132.8, 160.8, 162.2, 164.6 ppm; IR: $\tilde{\nu} = 2942$, 2845, 2773, 1650, 1602, 1507, 1228, 832 cm^{-1}.

2,6-Dichloro-3-nitropyridine (14): 2,6-Dichloropyridine (**13**; 30 mmol, 4.5 g), HNO_3 (35 mL, 65%) and conc. H_2SO_4 (90 mL) were held at reflux overnight at ~100–105 °C under an atmosphere of argon. The mixture was then cooled to 0 °C and poured into ice (~ 600 mL). The white precipitate was filtered off, rinsed several times with H_2O until neutral and dried for several days in a desiccator under reduced pressure. Colorless solid (3.3 g, 57%); mp: 56–59 °C (from H_2O); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 7.89$ (d, $J = 8.4$ Hz, 1H, C(5)H), 8.65 ppm (d, $J = 8.4$ Hz, 1H, C(4)H); ^{13}C NMR (100 MHz,): $\delta = 125.0$, 138.5, 141.5, 143.8, 151.9 ppm; IR: $\tilde{\nu} = 3059$, 1561, 1524, 1340 cm^{-1}.

2-Amino-6-chloro-3-nitropyridine (15): Compound **14** (20 mmol, 3.86 g) was dissolved in 2-propanol (200 mL) and aqueous NH_3 (10 mL, 25%), warmed in a stoppered round-bottom flask to 35 °C and stirred for 3 days, resulting in a yellow precipitate. The precipitate was filtered off, washed twice with H_2O (20 mL) and dried for several days under reduced pressure. Yellow solid (1.77 g, 51%); mp: 180–185 °C (from 2-propanol); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 6.77$ (d, $J = 8.8$ Hz, 1H, C(5)H), 8.26 (br s, 2H, NH_2), 8.39 ppm (d, $J = 8.8$ Hz, 1H, C(4)H); ^{13}C NMR (100 MHz, [D6]DMSO): $\delta = 112.0$, 126.1, 138.4, 153.5, 155.0 ppm; IR: $\tilde{\nu} = 3443$, 3279, 3157, 3098, 1632, 1556, 1496, 761 cm^{-1}.

6-Chloro-2-dimethylamino-3-nitropyridine (16): Compound **14** (5 mmol, 1 g) was dissolved in MeCN (20 mL) and Et_3N (7.5 mmol, 1 mL) and cooled to 0 °C. Then dimethylamine (6 mmol, 760 μL, 40% aqueous solution) was slowly added, the mixture stirred for 10 min and then allowed to warm to room temperature. After 30 min the mixture was reduced to dryness under a stream of air and the crude yellow crystalline product was purified by silica gel chromatography (solvent: toluene) to give a yellow solid (510 mg, 51%); mp: 70–72 °C (from toluene); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 2.97$ (s, 6H, N(CH_3)$_2$), 6.83 (d, $J = 8.4$ Hz, 1H, C(5)H) 8.25 ppm (d, $J = 8.4$ Hz, 1H, C(4)H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 39.9$, 111.4, 130.0, 138.9, 150.8, 151.7 ppm; IR: $\tilde{\nu} = 3114$, 3082, 2935, 2805, 1593, 1546, 1509 cm^{-1}.

2-Amino-6-[(4-fluorobenzyl)(methyl)amino]-3-nitropyridine (17): Compound **15** (10 mmol, 1.74 g) was suspended in 2-propanol (150 mL), **12** (15 mmol, 2 mL) and Et_3N (20 mmol, 2.8 mL) were added, and the mixture was held at reflux overnight. The resulting yellow solution was reduced to dryness under a stream of air, then the remaining crystals were washed twice with H_2O and once with a small amount of 2-propanol to give a yellow solid (2.58 g, 93%); purity 99%; mp: 127–128 °C (from 2-propanol); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 2.71$ (br s, 3H, CH_3), 4.85 (br s, 2H, C(α')H$_2$), 6.23 (d, 1H, C(5)H), 7.17 (m, 2H, C(3′)H and C(5′)H), 7.33 (m, 2H, C(2′)H and C(6′)H), 7.77 and 7.99 (br s and br s, 2H, NH_2), 8.08 ppm (d, 1H, C(4)H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 35.9$, 51.1, 98.7, 115.2, 118.0, 129.3, 133.6, 135.7, 154.4, 159.8, 160.1, 162.5 ppm; IR: $\tilde{\nu} =$ 3461, 3341, 1594, 1549, 1504, 1214; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{13}H_{13}N_4O_2F$: 277.1095, found: 277.1082; Anal. calcd for $C_{13}H_{13}N_4O_2F \cdot {}^1/_4 H_2O$: C 55.6, H 4.85, N 19.95, found: C 55.4, H 4.75, N 19.7.

General procedure C

Compound **16** (5 mmol, 1 g) was dissolved in MeCN (10 mL), Et_3N (14 mmol, 1.9 mL), and the appropriate 4-fluorobenzylamine (**11** or **12**; 6 mmol). The mixture was stirred overnight at room temperature, then more of the appropriate 4-fluorobenzylamine (6 mmol) was added, and the solution was held at reflux for 24 h (if necessary even longer). After cooling, the reaction mixture and HCl solution (2.5 mL, 1 M) were combined in a separation funnel and extracted with EtOAc (3×10 mL). The combined organic layers were washed with brine (25 mL), dried over Na_2SO_4, and concentrated to dryness under a stream of air.

2-Dimethylamino-6-(4-fluorobenzylamino)-3-nitropyridine (18): Following general procedure C starting from **11** (2×680 μL) yielded a brown oil (1.17 g, 81%); purity 98%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 2.90$ (s, 6H, $N^2(CH_3)_2$), 4.52 (d, $J = 5.6$ Hz, 2H, C(α')H$_2$), 5.99 (d, $J = 9.2$ Hz, 1H, C(5)H), 7.15 (m, 2H, C(3′)H and C(5′)H), 7.36 (m, 2H, C(2′)H and C(6′)H), 7.97 (d, $J = 8.8$ Hz, 1H, C(4)H), 8.28 ppm (br s, 1H, N^6H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta =$ 40.1, 43.4, 100.4, 114.9, 120.1, 129.2, 135.7, 136.6, 155.1, 157.8, 159.8, 162.3 ppm; IR: $\tilde{\nu} = 3344$, 3074, 2922, 2800, 1584), 1528, 1506, 1217 cm^{-1}; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{14}H_{15}N_4O_2F$: 291.1252, found: 291.1255.

2-Dimethylamino-6-[(4-fluorobenzyl)(methyl)amino]-3-nitropyridine (19): Following general procedure C starting from **12** (2× 800 μL) to yield a brown oil (1.38 g, 91%); purity 99%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 2.92$ (s, 6H, $N^2(CH_3)_2$), 3.12 (s, 3H, N^6CH_3), 4.83 (s, 2H, C(α')H$_2$), 6.21 (d, $J = 9.2$ Hz, 1H, C(5)H), 7.16 (m, 2H, C(3′)H and C(5′)H), 7.29 (m, 2H, C(2′)H and C(6′)H), 8.08 ppm (d, $J =$ 9.2 Hz, 1H, C(4)H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 36.3$, 40.2, 51.9, 97.3, 115.3, 120.9, 129.0, 134.0, 137.7, 154.1, 157.5, 160.1, 162.5 ppm; IR: $\tilde{\nu} = 3060$, 2927, 2800, 1571, 1503, 1218 cm^{-1}; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{15}H_{17}N_4O_2F$: 305.1408, found: 305.1405.

Ethyl {2-amino-6-[(4-fluorobenzyl)(methyl)amino]pyridine-3-yl}-carbamate (20): Compound **17** (1 mmol, 276 mg) and Pd/C (25 mg, 10% Pd) were carefully set under a hydrogen atmosphere, diluted with EtOH (10 mL), and stirred vigorously overnight. Then ethyl chloroformate (1.25 mmol, 119 μL) and Et_3N (1.5 mmol, 207 μL) were added and stirred for another 4 h. The suspension was filtered through a glass filter (por. 3) with a 1 cm layer of silica 60 H to remove all catalyst, and the filtrate was concentrated in vacuo. H_2O (1 mL) and 2-propanol (1 mL) were added to the oily residue and after storing overnight at 2–8 °C, a colorless precipitate formed, which was collected by filtration and dried under reduced pressure. Colorless solid (208 mg, 65%); purity 99%; mp: 114–116 °C (from H_2O/2-propanol); ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta =$ 1.21 (br s, 3H, O-CH_2-CH_3), 2.89 (s, 3H, N^6-CH_3), 4.05 (q, $J = 6.8$ Hz, 2H, O-CH_2), 4.69 (s, 2H, C(α')H$_2$), 5.33 (s, 2H, N^2H$_2$), 5.80 (d, $J =$

8.4 Hz, 1H, C(5)H), 7.11 (m, 3H, C(3′)H, C(5′)H and C(4)H), 7.25 (t, 2H, C(2′)H and C(6′)H), 8.25 ppm (brs, 1H, N^3H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 14.6, 35.7, 51.3, 60.0, 93.2, 107.2, 115.0, 129.1, 135.5, 135.7, 152.6, 155.1, 155.2, 159.9, 162.3 ppm; IR: $\tilde{\nu}$ = 3459, 3367, 2981, 2904, 1712, 1604, 1500, 1205 cm^{-1}); HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{16}H_{19}N_4O_2F$: 319.1565, found: 319.1551; Anal. calcd for $C_{16}H_{19}N_4O_2F$: C 60.4, H 6.0, N 17.6, found: C 60.5, H 6.0, N 17.4.

General procedure D

The appropriate nitropyridine derivative (2 mmol **18** or **19**) and Pd/C (150 mg, 10% Pd) were diluted with EtOH (15 mL) and stirred vigorously overnight under an atmosphere of hydrogen. Then ethyl chloroformate (2.5 mmol, 238 μL) and Et_3N (3 mmol, 414 μL) were added and stirred for another 8 h. The suspension was filtered through a glass filter (por. 3) with a 1 cm layer of silica 60 H to remove the catalyst, and the filtrate was concentrated in vacuo. The residue was diluted with CH_2Cl_2 (10 mL) and HCl solution (2 mL, 1 M) and in a separation funnel washed with brine (2× 5 mL). The combined organic layers were again concentrated in vacuo, and the residue was diluted with EtOAc (8 mL). Insoluble residues were removed by decanting the solution. *n*-Hexane (2 mL) was added to the solution, resulting in the precipitation of crystals, which were collected by filtration.

Ethyl [2-(dimethylamino)-6-(4-fluorobenzylamino)pyridine-3-yl]-carbamate hydrochloride (21·HCl): Following general procedure D starting from **18** (580 mg) yielded a red–brown solid (75 mg, 10%); purity 95%; mp: 155–158 °C (from *n*-hexane/EtOAc); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.20 (brs, 3H, O-CH$_2$-CH_3), 2.82 and 3.01 (m and brs, 1H and 5H, $N^2(CH_3)_2$), 4.07 (q, *J* = 6.8 Hz, 2H, O-CH$_2$), 4.46 (s, 2H, C(α′)H$_2$), 6.24 (brs, 1H, C(5)H), 7.12 (m, 3H, C(3′)H, C(5′)H and N^6H), 7.40 (m, 3H, C(2′)H, C(6′)H and C(4)H), 8.82 (brs, 1H, N^3H), 12.6 ppm (brs, 1H, HCl); ^{13}C NMR (100 MHz, CDCl$_3$): δ = 14.7, 42.4, 45.9, 62.2, 116.0, 128.8, 132.2, 152.3, 155.1, 161.3, 163.7 ppm; IR: $\tilde{\nu}$ = 3191, 3157, 3056, 2987, 2916, 2359, 1718, 1608, 1508, 1219 cm^{-1}; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{17}H_{21}N_4O_2F$: 333.1721, found: 333.1721; Anal. calcd for $C_{17}H_{22}N_4O_2FCl\cdot{}^1/_4$ EtOAc: C 55.3, H 6.2, N 14.3, found: C 55.2, H 5.8, N 14.4.

Ethyl {2-(dimethylamino)-6-[(4-fluorobenzyl)(methyl)amino]pyridine-3-yl}carbamate hydrochloride (22·HCl): Following general procedure D starting from **19** (608 mg) yielded a light colorless solid (200 mg, 26%); purity 92%; mp: 142–147 °C (from *n*-hexane/EtOAc); ^{1}H NMR (400 MHz): δ = 1.22 (brs, 3H, O-CH$_2$-CH_3), 2.96 (s, 6H, $N^2(CH_3)_2$), 3.07 (s, 3H, N^6CH_3), 4.08 (q, *J* = 7.2 Hz, 2H, O-CH$_2$); 4.76 (s, 2H, C(α′)H$_2$), 6.61 (brs, 1H, C(5)H), 7.13 (m, 2H, C(3′)H and C(5′)H), 7.29 (m, 2H, C(2′)H and C(6′)H), 7.48 (d, *J* = 5.6 Hz, 1H, C(4)H), 8.94 ppm (s, 1H, N^3H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 14.5, 36.4, 42.9, 52.0, 60.7, 112.9, 115.2, 129.0, 134.7, 139.2, 147.0, 155.0 160.0, 162.4 ppm; IR: $\tilde{\nu}$ = 3155, 3049, 2995, 2870, 1709, 1211, 811 cm^{-1}; HRMS-ESI *m/z* $[M+H]^+$ calcd for $[M+H]^+$ $C_{18}H_{24}N_4O_2F$: 347.1878, found: 347.1877; Anal. calcd for $C_{18}H_{25}N_4O_2FCl$: C 56.5, H 6.4, N 14.6, found: C 56.3, H 6.1, N 14.3.

Ethyl [2-(dimethylamino)-6-(4-fluorobenzylamino)pyridine-3-yl]-carbamate (21): Compound **21**·HCl (0.05 mmol, 20 mg) was added to a NaOH solution (2 mL, 2 M) and CH_2Cl_2 (2 mL) and vigorously extracted. The organic layer was separated, dried over Na_2SO_4, and concentrated in vacuo. Red–brown slurry (16 mg, 89%); purity 89%; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.17 (brs, 3H, O-CH$_2$-CH_3), 2.77 (s, 6H, $N^2(CH_3)_2$), 4.02 (q, *J* = 7.2 Hz, 2H, O-CH$_2$), 4.39 (d, *J* = 6.0 Hz, 2H, C(α′)H$_2$), 5.88 (d, *J* = 8.0 Hz, 1H, C(5)H), 6.82 (t, *J* = 6.0 Hz, 1H, N^6H), 7.01 (d, *J* = 7.6 Hz, 1H, C(4)H), 7.11 (m, 2H, C(3′)H and C(5′)H), 7.35 (m, 2H, C(2′)H and C(6′)H), 8.22 ppm (s, 1H, N^3H); ^{13}C NMR (100 MHz, [D6]DMSO): δ = 14.7, 40.3, 43.7, 59.8, 97.9, 109.8, 114.7, 128.9, 137.4, 138.8, 154.9, 155.3, 155.7, 159.6, 162.1 ppm; IR: $\tilde{\nu}$ = 3391, 2993, 2938, 2842, 1707, 1603, 1587, 1215, 821 cm^{-1}; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{17}H_{21}N_4O_2F$: 333.1721, found: 333.1718.

Ethyl {2-amino-6-[(4-fluorobenzyl)(methyl)amino]pyridine-3-yl}-(methyl)carbamate (23): Compound **20** (0.5 mmol, 159 mg) and NaH (0.75 mmol, 30 mg, 65% solution in *n*-hexane) in THF (8 mL) were stirred under an atmosphere of argon at 0 °C in the dark. After 30 min CH_3I (1 mmol, 62 μL) was added and stirred overnight while allowing the reaction to reach room temperature. The reaction mixture was concentrated under reduced pressure, and the residue was diluted with HCl solution (10 mL, 1 M) and washed with Et_2O (10 mL). The aqueous layer was then made alkaline with NaOH solution (10 mL) and extracted with Et_2O (2×10 mL). The combined organic layers were concentrated in vacuo, and the remaining colorless oil was stored at 2–8 °C until a precipitate formed (2–3 days). Colorless solid (40 mg, 24%); purity: 100%; mp: 73–76 °C (from Et_2O); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.07 and 1.23 (brs and brs, 2H and 1H, O-CH$_2$-CH_3), 2.91 (s, 3H, N^6-CH$_3$), 2.99 (s, 3H, N^3-CH$_3$), 3.98 (brs, 2H, O-CH$_2$), 4.71 (m, 2H, C(α′)H$_2$), 5.50 (s, 2H, N^2H_2), 5.76 (d, *J* = 8.4 Hz, 1H, C(5)H), 7.02 (d, *J* = 8.4 Hz, 1H, C(4)H), 7.12 (m, 2H, C(3′)H and C(5′)H) 7.28 ppm (m, 2H, C(2′)H and C(6′)H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 14.6, 35.7, 36.5, 51.2, 60.5, 93.2, 111.8, 114.9, 129.0, 135.4, 137.2, 153.7, 155.5, 156.1, 159.8, 162.2 ppm; IR: $\tilde{\nu}$ = 3443, 3346, 2983, 2847, 1675), 1603, 1217, 773 cm^{-1}; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{17}H_{21}N_4O_2F$: 333.1721, found: 333.1708; Anal. calcd for $C_{17}H_{21}N_4O_2F$: C 61.4, H 6.4, N 16.6, found: C 61.5, H 6.1, N 16.6.

2-(4-Fluorobenzylamino)-5-nitropyridine (25): 2-Chloro-5-nitropyridine (**24**; 5 mmol, 0.8 g) was diluted with MeCN (10 mL), Et_3N (15 mmol, 2.1 mL), and **11** (6 mmol, 680 μL), and the mixture was stirred overnight at room temperature. More of compound **11** (6 mmol) was then added, and the solution was held at reflux for 24 h. The reaction mixture was cooled to initiate crystallization, the suspension was diluted with HCl (12 mL, 1 M), and the precipitating light-brown crystals were filtered off, washed with H_2O and dried under reduced pressure. Light-yellow–brown solid (1.09 g, 88%); purity 97%; mp: 167–168 °C (from MeCN); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 4.61 (s, 2H, C(α′)H$_2$), 6.63 (m, 1H, C(3)H), 7.17 (m, 2H, C(3′)H and C(5′)H), 7.37 (m, 2H, C(2′)H and C(6′)H), 8.14 (dd, *J* = 9.2 Hz, *J* = 2.4 Hz, 1H, C(4)H), 8.59 (brs, 1H, NH) 8.92 ppm (d, *J* = 2.8 Hz, 1H, C(6)H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 43.4, 108.6, 115.1, 129.3, 131.8, 134.5, 135.0, 146.7, 160.0, 161.1, 162.4 ppm; IR: $\tilde{\nu}$ = 3220 (NH), 3097, 3048, 2991, 2935, 2881, 1600, 1498 cm^{-1}; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{12}H_{10}N_3O_2F$: 248.0830, found: 248.0837; Anal. calcd for $C_{12}H_{10}N_3O_2F$: C 58.3, H 4.1, N 17.0, found: C 57.95, H 4.0, N 16.8.

2-[(4-Fluorobenzyl)(methyl)amino]-5-nitropyridine (26): 2-Chloro-5-nitropyridine (**24**; 5 mmol, 0.8 g) was diluted with MeCN (10 mL), Et_3N (15 mmol, 2.1 mL), and **12** (6 mmol, 800 μL), and the mixture was stirred overnight at room temperature. More of **12** (6 mmol) was then added, and the solution was held at reflux for 24 h. The reaction mixture was cooled and diluted with HCl (12 mL, 1 M) and extracted with EtOAc (3×10 mL). The combined organic layers were washed with brine (25 mL), dried over Na_2SO_4 and concentrated in vacuo, slowly leading crystallization of the residue. Light-yellow solid (1.16 g, 89%); purity 99%; mp: 76–78 °C (from EtOAc); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 3.18 (s, 3H, CH$_3$), 4.93 (s, 2H, C(α′)H$_2$), 6.80 (d, *J* = 9.6 Hz, 1H, C(3)H), 7.17 (m, 2H, C(3′)H and C(5′)H), 7.29 (m, 2H, C(2′)H and C(6′)H), 8.24 (dd, *J* = 9.6 Hz, *J* = 2.8 Hz, 1H, C(4)H), 8.99 ppm (d, *J* = 2.8 Hz, 1H, C(6)H); ^{13}C NMR

(100 MHz, [D$_6$]DMSO): δ = 36.6, 52.0, 105.2, 115.3, 129.1, 132.8, 133.4, 134.4, 146.1, 160.2, 160.4, 162.6 ppm; IR: $\tilde{\nu}$ = 3088, 2934, 1519, 1219, 809 cm^{-1}; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{13}H_{12}N_3O_2F$: 262.0986, found: 262.0980; Anal. calcd for $C_{13}H_{12}N_3O_2F$: C 59.8, H 4.6, N 16.1, found: C 59.9, H 4.5, N 16.0.

Ethyl [2-(4-fluorobenzylamino)pyridine-5-yl]carbamate hydrochloride (27·HCl): Compound **25** (2 mmol, 494 mg) and Pd/C (150 mg, 10% Pd) were diluted with EtOH (15 mL) and stirred vigorously overnight under a hydrogen atmosphere. Then ethyl chloroformate (2.5 mmol, 238 μL) and Et_3N (3 mmol, 414 μL) were added to the reaction and stirred for another 8 h. The suspension was filtered through a glass filter (por. 3) with a 1 cm layer of silica 60 H to remove the catalyst, and the filtrate was concentrated in vacuo. The residue was diluted with CH_2Cl_2 (10 mL) and HCl (2 mL, 1 M), and the precipitated crystals were collected by filtration, washed with a small volume of H_2O and dried under reduced pressure. Colorless solid (215 mg, 33%); purity 100%; mp: 187–189 °C (from CH_2Cl_2); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.24 (t, *J* = 7.2 Hz, 3H, CH_3), 4.13 (q, *J* = 7.2 Hz, 2H, O-CH_2), 4.60 (br s, 2H, C(α')H_2), 7.10 (d, *J* = 9.6 Hz, 1H, C(3)H), 7.21 (m, 2H, C(3′)H and C(5′)H), 7.47 (m, 2H, C(2′)H and C(6′)H), 7.86 (dd, *J* = 2.4 Hz, *J* = 9.2 Hz, 1H, C(4)H), 8.10 (br s, 1H, C(6)H), 8.95 (br s, 1H, N^5H), 9.87 (s, 1H, N^2H), 13.96 ppm (br s, 1H, HCl); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 14.3, 44.4, 60.7, 113.7, 115.2, 123.6, 126.3, 129.8, 132.9, 136.0, 149.2, 153.5, 160.3, 162.7 ppm; IR: $\tilde{\nu}$ = 3305, 3167, 3091, 3046, 2989, 2920, 2739, 1692, 1216, 822 cm^{-1}; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{15}H_{16}N_3O_2F$: 290.1299, found: 290.1299; Anal. calcd for $C_{15}H_{17}N_3O_2FCl\cdot{}^1/_4\,H_2O$: C 54.55, H 5.3, N 12.7, found: C 54.5, H 5.0, N 12.7.

Ethyl {2-[(4-fluorobenzyl)(methyl)amino]pyridine-5-yl}carbamate hydrochloride (28·HCl): Following general procedure D starting from 523 mg **26** yielded a colorless solid (155 mg, 23%); purity: 98%; mp: 180–185 °C (from *n*-hexane/EtOAc); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.24 (t, *J* = 7.2 Hz, 3H, CH_3), 3.24 (s, 3H, N^2CH_3), 4.14 (q, *J* = 7.2 Hz, 2H, O-CH_2), 4.90 (s, 2H, C(α')H_2), 7.19 (m, 2H, C(3′)H and C(5′)H), 7.29 (d, *J* = 9.6 Hz, 2H, C(3)H), 7.36 (m, 2H, C(2′)H and C(6′)H), 7.97 (d, *J* = 9.2 Hz, 1H, C(4)H), 8.27 (s, 1H, C(6)H) and 9.97 ppm (s, 1H, N^5H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 14.4, 38.0, 53.4, 60.8, 112.2, 115.6, 125.4, 126.4, 129.2, 132.1, 135.8, 149.2, 153.6, 160.4, 162.8 ppm; IR: $\tilde{\nu}$ = 2984, 2776, 1716, 812 cm^{-1}; HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{16}H_{18}N_3O_2F$: 304.1456, found: 304.1442; Anal. calcd for $C_{16}H_{19}N_3O_2FCl\cdot{}^1/_2\,H_2O$: C 55.1, H 5.8, N 12.05, found: C 54.9, H 5.55, N 11.9.

***N*-(4-Hydroxyphenyl)-*N*-methylacetamide (29)**: This compound was synthesized as described by Fernando et al.[45] to yield a colorless solid (1.68 g; 70%); mp: 243–244 °C (from CH_2Cl_2/MeOH); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.71 (s, 3H, CO-CH_3), 3.07 (s, 3H, N-CH_3), 6.79 (d, *J* = 8.4 Hz, 2H, C(3′)H and C(5′)H), 7.10 (d, *J* = 8.4 Hz, 2H, C(2′)H and C(6′)H), 9.62 ppm (s, 1H, OH); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 21.9, 36.6, 115.8, 128.1, 135.7, 156.4, 169.3 ppm; IR: $\tilde{\nu}$ = 3098, 3016, 2907, 2804, 1622, 1587, 1239, 837 cm^{-1}.

Cyclic voltammetry studies

The voltammeter consisted of a glassy carbon working electrode, a platinum auxiliary electrode, and an Ag/AgCl reference electrode. Solutions of compound were prepared in 100 mM Tris buffer (pH 7.4) at a concentration of 1.0 mM, and were purged with N_2 for 3 min prior to analysis. Cycles were swept through −1.0 to 1.0 V (displaying only −0.5 to 1.0 V) at a sweep rate of 0.5 V s^{-1}. Between measurements, the working electrode required hand polishing, which was done by using a polishing cloth soaked in an aqueous suspension of aluminum oxide powder (particle size: 0.3 μm) for several minutes followed by sonication in deionized water.

K_V7.2/3-channel opening assay

KCNQ2/3-expressing HEK293 cells (cultivated with DMEM/F12, 10% FCS, 100 U mL^{-1} penicillin G sodium, 100 μg mL^{-1} streptomycin sulfate) were plated in 384-well plates (black wall, flat, clear-bottom microtiter plates, BD Biocoat poly-D-lysine multiwell cell culture plate) incubated overnight (37 °C) to achieve near confluent monolayer (10000–30000 cells per well). Treatment with loading buffer, assay buffer, and stimulus buffer was carried out according to the FluxOR Potassium Ion Channel Assay protocol (Life Technologies).[35] Cells were incubated with test compounds dissolved in assay buffer (concentrations: 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 μM) for 30 min at room temperature prior to stimulus buffer addition. Measurement of relative fluorescence intensity (stimulus buffer alone: 0%, 100 μM flupirtine maleate: 100%, excitation/emission wavelengths: 460–490/520–540 nm) was done by using a fluorescence imaging plate reader (FLIPR^{TETRA}).

Cytotoxicity assessment in TAMH cells

TGF-α transgenic mouse hepatocytes (TAMH) were obtained from Sidney D. Nelson (Department of Medicinal Chemistry, University of Washington, Seattle, USA). Cultivation conditions for this cell line and the MTT assay were described previously.[43] Briefly, cells between passages 31 and 40 at ~90% confluence were seeded into 96-well microtiter plates (Sarstedt) in 1:1 mixture of DMEM/F-12 culture medium containing ITS (5 μg mL^{-1} insulin, 5 μg mL^{-1} transferrin, 5 ng selenium), 0.1 μM dexamethasone, 10 mM nicotinamide, and 50 μg mL^{-1} gentamicin. After cells became confluent (usually 3–5 days following seeding), the medium was aspirated off and replaced with 200 μL of the same medium containing test compound and 1% DMSO. Typically, 4–8 serial dilutions of test compound were used for determination of the LD_{50} value. Untreated control rows were prepared with medium containing 1% DMSO alone. After 24 h exposure to drug, medium was aspirated off and replaced with 100 μL medium, as well as 20 μL per well of a 2.5 mg mL^{-1} solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in phosphate-buffered saline (PBS). Plates were returned to the incubator for 4 h. To each well was then added 100 μL 0.04 N HCl in 2-propanol. Plates were sonicated until all crystals had dissolved before the optical densities were measured on a plate reader (Sunrise, Tecan) at λ = 570 nm. LD_{50} values were calculated by linear regression of the *T/C* (%) versus log(concentration) curves.

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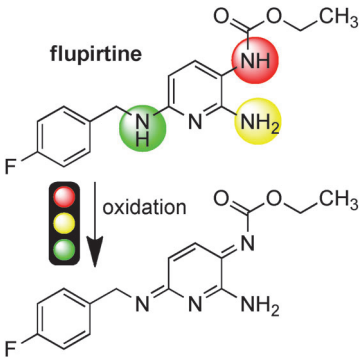
Oxidation–activation–toxicity? The connection between activity, toxicity, and oxidation potential of the pain killer flupirtine was investigated. N-Modified analogues of flupirtine displayed varying oxidation potentials that appeared to be linked to potassium channel opening activity but not to in vitro hepatotoxicity. These data suggest that oxidative metabolites of flupirtine contribute to the mechanism of action, but not to liver toxicity.



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Oxidation Potentials of N-Modified Derivatives of the Analgesic Flupirtine Linked to Potassium K_V7 Channel Opening Activity But Not Hepatocyte Toxicity