Molecular Dissection of Lubeluzole Use–Dependent Block of Voltage-Gated Sodium Channels Discloses New Therapeutic Potentials

Jean-François Desaphy, Roberta Carbonara, Teresa Costanza, Giovanni Lentini, Maria Maddalena Cavalluzzi, Claudio Bruno, Carlo Franchini, and Diana Conte Camerino

Section of Pharmacology (J.-F.D., R.C., T.C., D.C.C.), and Section of Medicinal Chemistry (G.L., M.M.C., C.B., C.F.), Department of Pharmacy, University of Bari-Aldo Moro, Bari, Italy

Received June 25, 2012; accepted November 21, 2012

ABSTRACT

Lubeluzole, which acts on various targets in vitro, including voltage-gated sodium channels, was initially proposed as a neuroprotectant. The lubeluzole structure contains a benzothiazole moiety [N-methyl-1,3-benzothiazole-2-amine (R-like)] related toriluzole and a phenoxy-propranol-amine moiety [(RS)-1-(3,4-difluorophenoxy)-3-(piperidin-1-yl)propan-2-ol (A-core)] recalling propranolol. Both riluzole and propranolol are efficient sodium channel blockers. We studied in detail the effects of lubeluzole (racemic mixture and single isomers), the aforementioned lubeluzole moieties, and riluzole on sodium channels to increase our knowledge of drug-channel molecular interactions. Compounds were tested on hNav1.4 sodium channels, and on F1586C or Y1593C mutants functionally expressed in human embryonic kidney 293 cells, using the patch-clamp technique. Lubeluzole blocked sodium channels with a remarkable effectiveness. No stereoselectivity was found. Compared with mexiteline, the dissociation constant for inactivated channels was ~600 times lower (~11 nM), conferring to lubeluzole a huge use-dependence of great therapeutic value. The F1586C mutation only partially impaired the use-dependent block, suggesting that additional amino acids are critically involved in high-affinity binding. Lubeluzole moieties were modest sodium channel blockers. Riluzole blocked sodium channels efficiently but lacked use dependence, similar to R-like. F1586C fully abolished A-core use dependence, suggesting that A-core binds to the local anesthetic receptor. Thus, lubeluzole likely binds to the local anesthetic receptor through its phenoxy-propranol-amine moiety, with consequent use-dependent behavior. Nevertheless, compared with other known sodium channel blockers, lubeluzole adds a third pharmacophoric point through its benzothiazole moiety, which greatly enhances high-affinity binding and use-dependent block. If sufficient isofrom specificity can be attained, the huge use-dependent block may help in the development of new sodium channel inhibitors to provide pharmacotherapy for membrane excitability disorders, such as myotonia, epilepsy, or chronic pain.

Introduction

Lubeluzole is the S-enantiomer of a benzothiazole derivative initially proposed as a neuroprotector in stroke. After encouraging initial results in experimental models, the drug was retired because clinical trials failed to find sufficient evidence of benefits in terms of survival after ischemic stroke injury (Gandolfo et al., 2002). The exact mechanism exerted by lubeluzole in vivo to produce neuroprotective effects is not clearly defined. The drug may act on multiple molecular targets, at least in vitro. It inhibits glutamate release, nitric oxide synthesis, and blocks voltage-gated calcium channels (Lesage et al., 1996; Scheller et al., 1997; Marrannes et al., 1998). In addition, the drug blocks voltage-gated sodium channels in brain neurons and cardiomyocytes (Osikowska-Evers et al., 1995; Le Grand et al., 2003).

Structurally, lubeluzole contains two distinct portions: one benzothiazolic moiety related to riluzole, and one phenoxy-propranol-amine moiety, recalling β-adrenergic drugs like priporanol (Fig. 1). Both riluzole and propranolol are potent sodium channel blockers. Blocking of sodium channels by riluzole is likely involved in the clinical efficacy of the drug in amyotrophic lateral sclerosis (Benoit and Escande, 1991; Hebert et al., 1994; Song et al., 1997; Wang et al., 2008). Propranolol induces a use-dependent block of various sodium channel subtypes in a manner reminiscent of local anesthetics (Fischer, 2002; Desaphy et al., 2003; Wang et al., 2010). We thus decided to analyze in detail the effects of lubeluzole, its R isomer, a racemic mixture, the aforementioned isolated

ABBREVIATIONS: A-core, (RS)-1-(3,4-difluorophenoxy)-3-(piperidin-1-yl)propan-2-ol; FC, F1586C mutation; HEK293, human embryonic kidney 293; hp, holding potential; h value, proportion of channels in the resting state; INa, whole-cell sodium currents; KAPP, apparent dissociation constants; Kd, dissociation constant of lubeluzole for block of inactivated channels; KR, dissociation constant for lubeluzole block of resting channels; LA, local anesthetic; nH, slope factor; R-like, N-methyl-1,3-benzothiazol-2-amine; TB, tonic block; V, conditioning pulse potential; WT, wild type.
lubeluzole moieties, and riluzole on human voltage-gated sodium channels expressed in a mammalian cell line. The drugs were also tested on sodium channels mutated at Phe1586 [F1586C mutant (FC)] and Tyr1593 (Y1593C), which are thought to be part of the local anesthetic receptor within the channel ion pore (Ragsdale et al., 1994). These mutations usually impair the high-affinity, use-dependent binding of local anesthetics (Ragsdale et al., 1994; Desaphy et al., 2009, 2010). The results define the molecular interaction of lubeluzole with sodium channels, and underscore its potential in the treatment of membrane excitability disorders.

Materials and Methods

**Sodium Current Recordings.** The generation of permanent HEK293 (human embryonic kidney 293) cell lines expressing the human skeletal muscle subtype of voltage-gated sodium channels (hNav1.4), and its F1586C or Y1593C mutants, has been previously reported (Desaphy et al., 2009, 2010).

Whole-cell sodium currents (INa) were recorded at room temperature (20–22°C) using an Axopatch 1D amplifier (Axon Instruments, Union City, CA). Voltage clamp protocols and data acquisition were performed with pCLAMP 6.0 software (Axon Instruments) through a 12-bit Analogic-Digital/Digital-Analogic interface (Digidata 1200; Axon Instruments). Pipettes made with Corning 7052 glass (Garner Glass, Claremont, CA) had resistance ranging from 1 to 3 MΩ. Pipette currents were low-pass filtered at 2 kHz (−3 dB) using the four-pole Bessel filter of the amplifier, and digitized at 10–20 kHz. After rupturing the patch membrane, a 25-ms-long test pulse to −20 mV from a holding potential (hp) of −120 mV was applied to the cell at a low frequency until stabilization of INa amplitude and kinetics was achieved (typically 5 minutes). Only data from cells exhibiting peak current amplitudes of 0.6 to 6 nA and series resistance errors <5 mΩ were considered for analysis. Little (<5%) or no rundown was observed within the experiments. Voltage clamp protocols and analysis details are reported in the Results section.

**Drugs and Solutions.** Patch-clamp pipette solution contained 120 mM CsF, 10 mM CsCl, 10 mM sodium chloride, 5 mM EGTA, and 5 mM Heps, and the pH was set to 7.2 with CsOH. Bath solution for patch-clamp recordings contained 150 mM sodium chloride, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM Heps, and 5 mM glucose. The pH was set to 7.4 with NaOH.

Riluzole (salt-free) was purchased from Sigma-Aldrich (Milan, Italy). All other exploratory compounds were synthesized in our laboratories either as hydrochloride salts or salt-free (Bruno et al., 2006; see also supplemental material for a detailed description of medicinal chemistry). The drugs were dissolved in 0.2% dimethylsulfoxide [riluzole, N-methyl-1,3-benzothiazole-2-amine (R-like), and (RS)-1-(3,4-difluorophenoxy)-3-(piperidin-1-yl)propan-2-ol (A-core)] or up to 3% cyclodextrin (lubeluzole), then diluted in bath solution at the desired final concentration. No effect of 0.2% dimethylsulfoxide or 3% cyclodextrin was found on sodium currents. The patched cell was continuously exposed to a stream of control or drug-supplemented bath solution flowing out from a plastic capillary.

**Results**

**State-Dependent Block of Sodium Channels by Lubeluzole.** To permit direct comparison with sodium channel blockers tested in previous studies, block of hNav1.4 channels by lubeluzole, its R isomer, and the racemic mixture was evaluated by measuring the reduction of INa elicited from a holding potential of −120 to −30 mV at frequency stimulations of 0.1 and 10 Hz. Sodium current traces recorded in a representative HEK293 cell before (control) and during application of 10 μM R,S-lubeluzole are illustrated in Fig. 2A. With 10 μM R,S-lubeluzole, the amplitude of peak INa was reduced by 28% ± 6% at 0.1 Hz and 94% ± 2% at 10 Hz (n = 5), indicating a huge use dependence. The time course of the use-dependent block at 10 Hz by R,S-lubeluzole is displayed in Supplemental Fig. 1, showing exponential decay time constants ranging from 0.28 to 1.6 seconds as a function of drug concentration. Lubeluzole effects were fully reversed upon drug removal. The concentration-response curves were constructed for R,S-lubeluzole and its single enantiomers and fitted with a first-order binding function:

$$I_{DRUG}/I_{CTRL} = 1/\left(1 + ([DRUG]/IC_{50})^{nH}\right)$$  \hspace{1cm} (1)

where IC₅₀ is the half-maximum inhibitory concentration and nH is the slope factor. The fit parameters are given in Table 1. No significant difference was found between
To estimate the apparent dissociation constant for lubeluzole resting, closed channels (Hille, 1977a; Desaphy et al., 2010), for open and/or inactivated channels with respect to the racemic R,S-lubeluzole only. Discerned, the next experiments were performed using the 2003, 2004, 2009, 2010). Since no stereoselective activity was tested so far in these experimental conditions (Desaphy et al., 2003, 2010). Since no stereoselective activity was tested so far in these experimental conditions (Desaphy et al., 2003, 2010). Since no stereoselective activity was tested so far in these experimental conditions (Desaphy et al., 2003, 2010). Since no stereoselective activity was tested so far in these experimental conditions (Desaphy et al., 2003, 2010). Since no stereoselective activity was tested so far in these experimental conditions (Desaphy et al., 2003, 2010). Since no stereoselective activity was tested so far in these experimental conditions (Desaphy et al., 1994; Pless et al., 2011; Desaphy et al., 2012).

lubeluzole enantiomers and the racemic mixture. The half-maximum inhibitory concentrations (IC_{50}) were about 35 μM at 0.1 Hz and 1.8 μM at 10 Hz. Lubeluzole appears among the more efficient and use-dependent sodium channel blockers tested so far in these experimental conditions (Desaphy et al., 2003, 2004, 2009, 2010). Since no stereoselective activity was discerned, the next experiments were performed using the racemic R,S-lubeluzole only.

According to the modulated receptor hypothesis, the use-dependent block may result from a greater affinity of the drug for open and/or inactivated channels with respect to the resting, closed channels (Hille, 1977a; Desaphy et al., 2010). To estimate the apparent dissociation constant for lubeluzole block of resting channels (K_R), we measured tonic block (TB) of hNav1.4 on the first test pulse applied at ~30 mV after the cells were maintained at a holding potential of ~180 mV for 120 seconds (Fig. 3, A and B). Then, use-dependent block occurred during subsequent test pulses, especially at the 10-Hz frequency. All effects were fully reversed upon drug washout. Representative current traces shown in Fig. 3C illustrate TB and use-dependent block of hNav1.4 channels at 10 Hz by 1 mM lubeluzole. On average, 1 mM lubeluzole induced 49% ± 3% of TB, 60% ± 9% of block at 0.1 Hz, and 98% ± 1% of block at 10 Hz (n = 3). The concentration-TB relationship determined with hp = −180 mV is shown in Fig. 3D. Because the entire population of sodium channels are in the resting state at a holding potential of ~180 mV, the IC_{50} value calculated with eq. 1 provides a good estimate of K_R, which was 843 ± 242 μM. This value is very similar to the K_R value of mexiletine (~800 μM; Desaphy et al., 2004).

To calculate the apparent dissociation constant of lubeluzole for block of inactivated channels (K_I), we measured the TB at an hp of ~90 or ~60 mV using the protocol shown in Fig. 3A. A 100-ms-long prepulse at ~180 mV was required to allow channels to recover from voltage-dependent inactivation developed at ~90 or ~60 mV, before assessing drug-induced channel block at ~30 mV. The apparent dissociation constants (K_{APP}), calculated as the IC_{50} for TB at a holding potential of ~90 or ~60 mV, result from the block of a mixed population of resting and inactivated channels with different affinities (Fig. 3D). The proportion of channels in the resting state at hps of ~90 and ~60 mV (h value) was determined from the voltage-dependent availability relationship constructed as described in Fig. 5C. Then, the value for K_I was estimated from the equation developed by Bean et al. (1983):

\[ \frac{I}{K_{APP}} = (1 - h)/K_I \cdot h/K_R. \]

Using an hp of ~90 mV, the K_{APP} value was 0.45 ± 0.04 μM and the h value was 0.933, thus the calculated K_I of lubeluzole was 0.030 ± 0.002 μM and the h value was 0.120, thus the estimated K_I of lubeluzole was 0.011 μM.

It should be noted that the 100-ms-long prepulse at ~180 mV (Fig. 3A) was needed to allow sodium channels to fully recover from voltage-dependent inactivation, especially at an hp of ~60 mV, but it also allowed a proportion of drug-bound channels to unbind lubeluzole. Indeed, from the measure of the time course of recovery from inactivation in the absence and presence of 30 μM lubeluzole (Fig. 4), it appears that about 17% of sodium channel block is removed after 100 ms at ~180 mV. Thus, the value of TB measured as in Fig. 3 may be underestimated, and consequently, the calculated K_I value may be slightly overestimated.

Overall, these results indicate that lubeluzole is a very potent blocker of inactivated sodium channels, with a K_I close to 10 nM. For comparison, the estimated K_I for mexiletine was 6 μM (Desaphy et al., 2004).

**Lubeluzole Binding Site May Overlap That of Local Anesthetics.** Most of the use-dependent blockers of sodium channels bind to the local anesthetic (LA) receptor located within the ion-conducting pore. In particular, a phenylalanine residue located in domain IV segment 6 (Phe1586 in hNav1.4) is critical for high-affinity binding by interacting with the protonated amine group of drugs through the π-cation link (Ragsdale et al., 1994; Pless et al., 2011; Desaphy et al., 2012).
To verify whether lubeluzole binds to the LA receptor, we tested the drug on sodium currents in HEK293 cells permanently transfected with the F1586C mutant. We previously showed that the F1586C mutation completely prevented block of inactivated channels by mexiletine and other use-dependent sodium channel blockers (Desaphy et al., 2009, 2010). Figure 5A illustrates F1586C sodium current traces in the presence of 10 μM lubeluzole to be compared with the wild-type (WT) current traces shown in Fig. 2A. No significant drug effect was observed at 0.1 Hz, whereas a reduction of 48% ± 6% (n = 7) was measured at a stimulation frequency of 10 Hz. These effects were reversed upon drug washout. The dose-response curves on the F1586C mutant indicate IC50 values of 107 ± 11 μM at 0.1 Hz and 10.4 ± 1.3 μM at 10 Hz (Fig. 5B). Thus, although reduced, the use-dependent block of sodium channels persisted despite the F1586C mutation. The limited channel block may arise from either direct alteration of drug binding or altered sodium channel gating by the F1586C mutation. Indeed, mutation of this residue has been shown to shift the channel availability voltage-dependence toward less negative voltage, which might reduce apparent drug affinity (Ragsdale et al., 1994; Desaphy et al., 2009). We thus measured the voltage dependence of channel inactivation using a standard two-pulse protocol (Fig. 5E). Sodium currents were elicited by a 20-ms test pulse at −30 mV after 50-ms conditioning pulses to potentials ranging from −150 to −30 mV in 10-mV increments. The holding potential was −180 mV, and the interval duration between two pulses was 15 seconds. The normalized peak sodium current recorded during the test pulse (INa/INa,max) was plotted against the conditioning pulse potential (V), and the relationships were fitted using the Boltzmann equation:

\[
\frac{I_{Na}}{I_{Na, max}} = \frac{1}{1 + \exp\left[\frac{V-V_{1/2}}{S}\right]}.
\]  

(3)

where V1/2 is the half-maximum inactivation potential and S is the slope factor. In this study, the half-maximum inactivation voltage was indeed shifted from −72.6 ± 0.2 mV (n = 34) for WT channels to −59.4 ± 0.4 mV (n = 30) for F1586C. Note that little difference in V1/2 was found using 50-, 250-, or 500-ms-long conditioning pulses (see Supplemental Fig. 2). We thus calculated the KR and KI values for lubeluzole on F1586C (Fig. 5F). The K_R value determined at an hp of −180 mV was 703 ± 179 μM. The K_I calculated using an hp of −60 mV was 1.4 ± 0.2 μM. Thus, considering a h value of 0.52 at an hp of −60 mV, the K_I value calculated from eq. 2 was 0.64 μM, which is ~60-fold higher than the K_I of lubeluzole for WT channels. Altogether, these results suggest that Phe1586 may be involved in lubeluzole high-affinity binding, but is less critical for use-dependent block with respect to other sodium channel blockers.

Another amino acid potentially involved in the high-affinity receptor for LA-like drugs is the tyrosine in position 1593 of the hNav1.4 channel (Desaphy et al., 2010). This residue was originally proposed to interact with the aromatic tail of sodium channel blockers (Ragsdale et al., 1994), but this hypothesis was recently challenged (Ahern et al., 2008). Indeed, mutations of Y1593 can modify sodium channel gating, which complicates the interpretation of pharmacological data. For instance, we observed a significant ~20% reduction of Y1593 sodium currents at 10 Hz in the absence of drug (dashed line in Fig. 5B), which suggests the slowing of recovery from inactivation (O’Reilly et al., 2000; Xiao et al., 2001; Desaphy et al., 2012). Note, however, that the corresponding Y1767C in the cardiac hNav1.5 isoform accelerates the recovery from inactivation, which potentiates mutant channel inhibition by the open-channel blocker ranolazine (Huang et al., 2011). We tested R,S-lubeluzole on the Y1593C mutant (Fig. 5, C and D). To take into account use-dependent inhibition occurring at 10 Hz in the absence of drug, an additional variable, namely, Imax, was introduced in eq. 1 to fit the concentration-response relationship (Desaphy et al., 2012). The calculated Imax value ± the S.E. of the fit was 0.82 ± 0.04. The calculated values for IC50 and nH parameters are reported in Table 1. Compared with WT, the Y1593C mutation had little effect on lubeluzole block at 0.1 Hz, but increased the IC50 at 10 Hz by about 2.5-fold, suggesting that Y1593C may contribute to the high-affinity receptor for lubeluzole.

Table 1 presents IC50 values and slope factors (nH) for lubeluzole and its moieties, namely, R-like and A-core, to verify whether they are efficient blockers of sodium channels.

### Table 1: Half-maximum inhibitory concentrations (IC50) for the exploratory compound on wild-type hNav1.4 channels, F1586C, and Y1593C hNav1.4 mutants

<table>
<thead>
<tr>
<th>Channel type</th>
<th>Drug</th>
<th>IC50 (μM)</th>
<th>nH</th>
<th>IC50 (μM)</th>
<th>nH</th>
<th>IC50 (μM)</th>
<th>nH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.1 Hz</td>
<td></td>
<td>10 Hz</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>Lubeluzole</td>
<td>39.7 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>26.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R isomer</td>
<td>35.7 ± 3.7</td>
<td>1.2 ± 0.1</td>
<td>2.8 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R,S-lubeluzole</td>
<td>30.6 ± 5.1</td>
<td>0.9 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>18.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A-core</td>
<td>457 ± 121</td>
<td>0.9 ± 0.2</td>
<td>221 ± 53</td>
<td>1.1 ± 0.3</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A-like</td>
<td>712 ± 51</td>
<td>2.0 ± 0.3</td>
<td>615 ± 86</td>
<td>2.0 ± 0.4</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Riluzole</td>
<td>50.0 ± 7.8</td>
<td>1.7 ± 0.4</td>
<td>42.8 ± 3.2</td>
<td>1.7 ± 0.2</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>F1586C</td>
<td>R,S-lubeluzole</td>
<td>107 ± 11</td>
<td>1.3 ± 0.1</td>
<td>10.4 ± 1.3</td>
<td>0.8 ± 0.1</td>
<td>10.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A-core</td>
<td>523 ± 45</td>
<td>0.9 ± 0.1</td>
<td>472 ± 41</td>
<td>0.9 ± 0.1</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Riluzole</td>
<td>127 ± 10</td>
<td>2.5 ± 0.5</td>
<td>111 ± 7</td>
<td>3.1 ± 1.0</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Y1593C</td>
<td>R,S-lubeluzole</td>
<td>36.1 ± 1.0</td>
<td>1.7 ± 0.1</td>
<td>4.6 ± 0.8</td>
<td>1.1 ± 0.1</td>
<td>7.8</td>
<td></td>
</tr>
</tbody>
</table>

We next synthesized the single moieties of lubeluzole, namely, R-like and A-core, to verify whether they are efficient blockers of sodium channels. The R-like compound was compared with riluzole. Representative effects of 300 μM A-core on the wild-type hNav1.4 sodium channels.
channel and F1586C mutant are shown in Fig. 6, and the concentration-effect relationships were determined. Compared with lubeluzole, A-core was a modest sodium channel blocker, showing a 12-fold increased IC\textsubscript{50} at 0.1 Hz (Table 1). Also, use-dependent block was greatly reduced, since the 0.1:10-Hz IC\textsubscript{50} ratio was reduced from ∼20 for lubeluzole to ∼2 for A-core. The F1586C mutation did not affect block at 0.1 Hz, but completely abolished use-dependent block.

The R-like compound was ∼20 times less efficient than lubeluzole in blocking sodium channels at 0.1 Hz, and completely missed use dependency with a 0.1:10-Hz IC\textsubscript{50} ratio close to unity (Fig. 7; Table 1). Also, riluzole fully lacks use dependence, but affinity at 0.1 Hz was comparable to that of lubeluzole, with an IC\textsubscript{50} of 50 \(\mu\)M. Of note are the slope factors of concentration-response curves for R-like and riluzole, which were close to 2, suggesting either a 2:1 drug:channel stoichiometry or a nonlinear relationship between the drug concentration in solution and that in proximity to the receptor. Compared with wild-type channels, riluzole was 2-fold less efficient in blocking F1586C channels, with an IC\textsubscript{50} of 127 \(\mu\)M at 0.1 Hz (Fig. 7). Again, in the cells patched for riluzole assay, the FC channels inactivated at potentials about 10 mV less negative than WT channels (not shown), suggesting that differences in voltage dependence of inactivation between the two channels may contribute substantially to variation in IC\textsubscript{50} (Song et al., 1997). We cannot, however, definitely exclude that Phe1586 may be involved directly or indirectly in riluzole binding.

The two moieties, R-like and A-core, were tested individually or together on hNav1.4 sodium currents (Fig. 8). As expected, inhibition by 300 \(\mu\)M A-core was use-dependent, whereas inhibition by 300 \(\mu\)M R-like was not. The simultaneous application of 300 \(\mu\)M A-core and 300 \(\mu\)M R-like produced sodium channel inhibition very similar to that exerted by 300 \(\mu\)M A-core alone and remained, by far, inferior to that exerted by 300 \(\mu\)M lubeluzole, suggesting that the integrity of the lubeluzole molecule is critical to obtain clinically relevant block.

**Fig. 3.** Determination of the apparent dissociation constants of R,S-lubeluzole for resting, closed (K\textsubscript{R}), and inactivated (K\textsubscript{I}) hNav1.4 sodium channels. (A) Description of the voltage-clamp protocol. (B) Time course of hNav1.4 current amplitude in a representative cell exposed to 1 mM lubeluzole at a holding potential of -180 mV. For TB, the drug was applied while the cells were maintained at the holding potential for 120 seconds without depolarization (0 Hz). Tonic block was measured on the first test pulse at -30 mV, while use-dependent block developed at frequency stimulations of 0.1 and 10 Hz. No current variation was observed with the same protocol in the absence of drug [control (CTRL)]. Drug effects were fully reversed upon washout. (C) Current traces recorded in the same cell as in (B). (D) Concentration-TB relationships were determined using hp values of -180, -90, or -60 mV. Each data point is the mean ± S.E.M. of at least three cells. The relationships were fitted with eq. 1, in which IC\textsubscript{50} corresponds to K\textsubscript{R} (hp = -180 mV) or K\textsubscript{APP} (hp = -90 and hp = -60 mV). The calculated K\textsubscript{R} value ± S.E. of the fit was 843 ± 242 \(\mu\)M (nH = 0.63 ± 0.12). The calculated K\textsubscript{APP} value ± S.E. of the fit was 0.45 ± 0.04 \(\mu\)M (nH = 0.51 ± 0.02) at an hp of -90 mV and 0.013 ± 0.002 \(\mu\)M (nH = 0.76 ± 0.08) at an hp of -60 mV.
The IC₅₀ values calculated for skeletal muscle channels using an hp of -120 mV (35 µM at 0.1 Hz and 2 µM at 10 Hz) are quite comparable to those measured for cardiac (9.5 µM) and neuronal (3.1 µM) channels using a less negative hp, suggesting little selectivity among sodium channel subtypes. Most probably, the relative safety of lubeluzole in vivo would be related to voltage- and use-dependent mechanisms, allowing a selective action of the drug on sodium channels in pathologically depolarized or overexcited cells. Although the affinity of lubeluzole for resting channels was similar to that of the class Ib antiarrhythmic mexiletine, its affinity for inactivated channels was 200 times greater, allowing a formidably use-dependent block of potentially great therapeutic value. Indeed, although some compounds have been shown to display relative selectivity among sodium channel subtypes (Jarvis et al., 2007; Williams et al., 2007), use-dependent block remains today the main quality allowing a selective action of sodium channel blockers on pathologic overactive tissues in vivo.

Importantly, we observed little difference in sodium channel inhibition between lubeluzole, its R isomer, or the racemic mixture. Since neuroprotective effects of lubeluzole, both in vitro and in vivo, are stereospecific with the R isomer being inefficient (De Ryck et al., 1996; Scheller et al., 1997; Culmsee et al., 1998), our results indicate that sodium channel blockade is not a major contributor to the lubeluzole mechanism of action in neuroprotection.

The integrity of the lubeluzole molecule is needed to obtain sodium channel blockade at clinically relevant concentrations, since both moieties, A-core and R-like, exerted only modest sodium channel blockade. Experiments with moiety mixture suggest that the two moieties may bind to independent but neighboring receptor sites, and that linking A-core and R-like together in the single molecule of lubeluzole results in a powerful synergism.

It is noteworthy that riluzole is a sodium channel blocker, but differences have been reported regarding its selectivity among sodium channel subtypes or in-between fast and persistent sodium currents (Song et al., 1997; Urbani and Belluzzi, 2000). In our hands, riluzole appeared to be a very efficient blocker of functionally expressed human skeletal muscle sodium channels, confirming the results obtained by others in differentiated human skeletal muscle cells (Wang et al., 2008). Although lubeluzole has been reported as a preferential blocker of inactivated channels (Song et al., 1997), we did not observe any use-dependent block with a stimulation frequency up to 10 Hz, suggesting that the drug may unbind the channel very quickly at negative potential with a dissociation time constant inferior to 100 ms at a holding potential of -120 mV. The trifluoromethoxy group in riluzole appeared of critical importance, since its removal, as in R-like, greatly impaired sodium channel blocking activity.

Lubeluzole shares many chemical properties and sodium channel blocking mechanisms with local anesthetics. Voltage and use-dependent block are the hallmarks of sodium channel inhibition by LAs. Structurally, lubeluzole contains an aryloxy moiety and a protonable amine linked by a short carbon chain, conferring hydrophobicity (log P = 4.08 ± 0.01) and basicity (pKa = 7.14 ± 0.02), respectively. The pKa of lubeluzole is close to that of lidocaine (pKa = 7.9; Hille, 1977b), but is less basic than that of mexiletine (pKa = 9.3; Desaphy et al., 2012). Thus, the partition of lubeluzole molecules between neutral and charged forms at pH 7.4 is quite similar to lidocaine, close to a 1-to-1 ratio. The log P of lubeluzole is 2-fold that of mexiletine (log P = 2.2; Desaphy...
The ability of lubeluzole to access its receptor within the pore lumen would be easy, either through the closed channel (owing to the elevated hydrophobicity and the abundance of neutral form at pH 7.4) or through the open channel. This is quite in accord with our results. Another consideration is that the low affinity of lubeluzole for the resting channel (high $K_R$) may be due to a limited interaction of the drug with the channel in the resting state, rather than to a reduced ability of the drug to access its receptor, as previously observed with pilsciazone (Desaphy et al., 2010). Altogether, these observations suggest that lubeluzole may bind to the local anesthetic receptor within the sodium channel ion-conducting pore. The key component of high-affinity

---

**Fig. 5.** Effect of R,S-lubeluzole on F1586C (FC) and Y1593C hNav1.4 sodium channel mutants. (A and B) Representative F1586C and Y1593C sodium current traces are shown. Currents were elicited from a holding potential of −120 mV to a test potential of −30 mV in the absence of drug (control (CTRL)), in the presence of 10 μM R,S-lubeluzole at a frequency stimulation of 0.1 or 10 Hz, and after drug washout. Note that, in contrast to F1586C, the Y1593C mutant displays significant use-dependent block at 10 Hz in the absence of drug (dashed line in (B)). (C and D) Concentration-response relationships were constructed at frequency stimulations of 0.1 and 10 Hz for F1586C and Y1593C. Each point is the mean ± S.E.M. from at least three cells. The dashed line in (D) shows the concentration-response relationship at 10 Hz normalized with respect to use-dependent current inhibition in the absence of drug. The relationships were fitted with eq. 1, and the calculated IC$_{50}$ and $nH$ values ± S.E. of the fit are reported in Table 1. (E) The voltage-dependence of the steady-state availability of the wild-type hNav1.4 sodium channel and F1586C mutant was determined by measuring sodium currents elicited by a 20-ms-long test pulse to −30 mV after 50-ms-long conditioning pulses to potentials ranging from −150 to −30 mV in 10-mV increments. The holding potential was −180 mV, and the interval duration between two pulses was 15 seconds. The normalized peak sodium current recorded during the test pulse was plotted against the conditioning pulse potential. The relationships were fitted with eq. 3, giving a half-maximum inactivation potential ($V_{1/2} ± S.E.$ of the fit) of −72.6 ± 0.2 mV for the wild-type hNav1.4 and −59.4 ± 0.4 mV for the F1586C mutant (n = 30). The slope factors ($S ± S.E.$ of the fit) were 6.4 ± 0.2 mV for the wild-type hNav1.4 and 6.7 ± 0.4 mV for the F1586C mutant. (F) The tonic block of the F1586C mutant by lubeluzole was determined at an hp of −180 mV to calculate $K_R$ or −60 mV to calculate $K_{APP}$, as in Fig. 3. Each data point is the mean ± S.E.M. of at least three cells. The concentration-TB relationships were fitted with eq. 1. The calculated IC$_{50}$ values ± S.E. of the fit were 703 ± 179 μM (hp = −180 mV) and 1.35 ± 0.22 μM (hp = −60 mV). The slope factors ($nH ± S.E.$ of the fit) were 0.8 ± 0.1 μM (hp = −180 mV) and 0.7 ± 0.1 μM (hp = −60 mV).
binding to the inactivated channel is a phenylalanine side chain that may interact electrostatically with the charged amine of LA drugs (π-cation interaction) (Ragsdale et al., 1994; Pless et al., 2011; Desaphy et al., 2012). Nonconservative mutations of this residue disrupt binding of LAs to inactivated sodium channels, resulting in a complete abolition of use-dependent block. The situation was different with lubeluzole, since voltage and use-dependent block of F1586C channels

Fig. 6. Effects of A-core on the wild-type hNav1.4 channel and F1586C (FC) sodium channel mutant. (A and B) Representative sodium current traces are shown. Currents were elicited from a holding potential of −120 mV to a test potential of −30 mV in the absence of drug [control (CTRL)] and in the presence of 300 μM A-core at a frequency stimulation of 0.1 or 10 Hz. All effects were fully reversible (not shown). (C) Concentration-response relationships were constructed at frequency stimulations of 0.1 and 10 Hz. Each point is the mean ± S.E.M. from at least three cells. The relationships were fitted with eq. 1, and the calculated IC50 and nH values ± S.E. of the fit are reported in Table 1. For comparison, violet lines report fits of concentration-response curves for R,S-lubeluzole at 0.1 Hz (plain line) and 10 Hz (dashed line), as detailed in Fig. 1.

Fig. 7. Effect of R-like and riluzole on the wild-type hNav1.4 channel and F1586C (FC) sodium channel mutant. (A–C) Representative sodium current traces are shown. Currents were elicited from a holding potential of −120 mV to a test potential of −30 mV in the absence of drug [control (CTRL)] and in the presence of 1 mM R-like or 100 μM riluzole at a frequency stimulation of 0.1 or 10 Hz. (D) Concentration-response relationships were constructed at frequency stimulations of 0.1 and 10 Hz. Each point is the mean ± S.E.M. from at least three cells. The relationships were fitted with eq. 1, and the calculated IC50 and nH values ± S.E. of the fit are reported in Table 1. For comparison, violet lines report fits of concentration-response curves for R,S-lubeluzole at 0.1 Hz (plain line) and 10 Hz (dashed line), as detailed in Fig. 1.
We observed that the Y1593C mutation reduced use-dependent block by lubeluzole, suggesting that Y1593 may also contribute to the lubeluzole high-affinity receptor. Nevertheless, since the Phe1586 mutation reduced high-affinity binding only partially, we hypothesize that lubeluzole may present an additional pharmacophoric element, the benzothiazole moiety, that interacts with amino acids located somewhere within the open pore lumen (Fig. 9). This third pharmacophoric point alone may not be sufficient to induce use dependence, since inhibition by R-like or riluzole was not use-dependent, but may greatly potentiate use dependence induced by the $\pi$-cation interaction at Phe1586. If such a hypothesis were confirmed, this specific trait may open the way to the design of new, interesting drugs in view of improving pharmacotherapy of sodium channel–related pathologies. For instance, because pathogenic sodium channel mutations may impair the effects of specific LAs, drugs alternative to traditional LAs may prove useful in sodium channelopathies (Desaphy et al., 2001, 2004).

Lubeluzole was developed for the prevention of damage from acute stroke, but a number of clinical trials failed to demonstrate significant benefit in humans (Gandolfo et al., 2002). Moreover, higher doses were shown to lengthen the QT interval on electrocardiograms, raising concerns about cardiac safety. As said earlier, our results suggest that neuroprotection by lubeluzole is likely not triggered by sodium channel inhibition. Because the drug exerts use-dependent sodium channel block at very low concentrations, it remains a possibility that such low doses may be able to produce sufficient inhibition of overexcited cells in pathologic conditions, such as myotonic syndromes, epilepsies, and chronic pain, without interfering with cardiac function. This hypothesis may merit further investigation in animal models before returning to human studies.
Mechanism of Na Channel Block by Lubeluzole


Address correspondence to: Jean-François Desaphy, Section of Pharmacology, Department of Pharmacy, University of Bari-Aldo Moro, via Orabona 4 – campus, I-70125 Bari, Italy. E-mail: jfdesaphy@farmbiol.uniba.it

References


