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Original Article

An anti-coagulation agent Futhan preferentially targets $GABA_A$ receptors in lung epithelia: implication in treating asthma

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Abstract: Futhan is a serine protease inhibitor and medicine in the treatment of disseminated intravascular coagulation (DIC) and acute pancreatitis. It is metabolized quickly in vivo. Here we show that Futhan reversibly inhibits NMDA receptors in hippocampal neurons and $GABA_A$ receptors both in hippocampal neurons and in A549 cells, a human alveolar epithelial cell line. The effect of Futhan on $GABA_A$ receptors in A549 cells is much more potent than its effect on $GABA_A$ receptors in hippocampal neurons (IC_{50}: 0.9 μM v.s. 7.3 μM). Since $GABA_A$ receptors are also expressed in various non-neuronal tissues, particularly in airway epithelia and GABA promotes mucus production during asthma, our findings indicate that Futhan may be developed as a novel aerosolized therapeutic to treat asthma through blocking $GABA_A$ receptors in the lung.

Keywords: Asthma, $GABA_A$ receptors, NMDA receptors, Futhan, ion channels

Introduction

A-type gamma-aminobutyric acid receptors (GABAA receptors or GABAARs) are hetero-oligomeric complexes which form chloride-permeable ion channels. $GABA_AR$s represent major fast inhibitory receptors in the central nervous system (CNS). Distinct isoforms of $GABA_AR$s have different pharmacological, developmental, and physiological feature and are localized to specific cellular compartments [1, 2]. The binding of GABA to its ionic receptors causes a change of conformation that permits the influx/efflux of chloride ions which, in adult neurons, shunt excitatory input and render neurons less excitable [1]. Generally, enhancing $GABA_AR$ activities by agents such as sedatives, or general anaesthetics 'soothes' the brain [3], while blocking $GABA_AR$s with GABA antagonists disinhibits and excites the nervous system. However, $GABA_A$ receptors also distribute to non-neuronal tissues such as liver, lung and pancreas [4-7], participating in a variety of functions such as secretion and fluid balance. For example, GABAergic system has been recently characterized in airway epithelia of the lung [4, 6, 8]. Activation of $GABA_A$ receptors in lung, in contrast to the brain, leads to depolarization of lung airway cells facilitating epithelial secretion [6]. In addition, $GABA_AR$s are responsible for mucus overproduction and the activation of $GABA_AR$ promotes asthma [6]. Conversely, blockade of $GABA_AR$s by GABA antagonists prevents mucus secretion. These findings implicate that airway $GABA_AR$s may serve as novel targets for treating asthma [9]. However, due to their CNS effect, most antagonists of $GABA_AR$ are proconvulsive, which limits their therapeutic potential in combating $GABA_AR$-associated disorders in non-neuronal tissues [10-13].

Futhan (FUT-175, or Nafamostat Mesylate, chemical name: 6-Amidino-2-naphthyl-4-guanidinobenzobate dimethanesulfonate) is a synthetic, competitive, reversible serine protease inhibitor and has potent inhibition on a wide

spectrum of proteases, including trypsin, thrombin, plasmin, kallikreins, factors B and D, factor Xa, and tryptase [14-18]. This inhibitory spectrum confers Futhan capacity to intervene the coagulation-fibrinolysis system, the kallikrein-kinin system and the complement system. Therefore Futhan functions as an anti-inflammation and anti-coagulation agent [14, 19, 20]. Futhan is clinically used for the treatment of DIC and acute pancreatitis and also serves as an anticoagulant agent in extracorporeal circulation (ECC) [19-21].

As for its chemical structure, Futhan is a highly polarized, di-cationic agent governed by an amidine group at one end and a guanidine group at the other [15]. It is highly hydrophilic and cannot readily penetrate the BBB to enter the brain [14, 15]. Moreover, Futhan is an unstable drug as an ester conjugate of 6-amidino-2-naphthol (AN) and p-guanidinobenzoic acid (p-GBA). It can be rapidly hydrolyzed into AN and p-GBA in vivo by esterase in the liver and blood [14].

Accumulating evidence suggests that besides conventional clinical usage, Futhan also demonstrates a myriad of other beneficial pharmacological activities, such as anti-tumor [22, 23], pain relief [24], organ protection [21, 25]. Some effect such as anti-cancer application is being tested under human clinical trials [23]. Moreover, Futhan has been shown to have anti-asthma effect in mice model [26, 27]. The pharmacological profiles of Futhan, however, need to be clearly elucidated in order to avoid its adverse effects and fully explore its clinical potential.

Here we show that Futhan reversibly and completely inhibits both NMDA receptors and $GABA_A$ receptors in primarily cultured hippocampal neurons. In addition, Futhan more potently blocks $GABA_A$ receptors endogenously expressed in A549, a human alveolar epithelial cell line. As Futhan does not readily cross the blood-brain-barrier (BBB) and is metabolized rapidly in vivo, it may be developed into a novel medicine for treating asthma.

Materials and methods

Calcium imaging

Fura-2 fluorescent Ca^{2+} imaging was performed as described previously [35]. Cortical or hippocampal neurons grown on 25 mm round glass coverslips were washed three times with ECF and incubated with 5 μM Fura-2-AM for ~ 40 min at room temperature. Neurons were then washed three times and incubated in normal ECF for 30 min. Coverslips with Fura-2-loaded neurons were transferred to a perfusion chamber on the stage of an inverted microscope (Nikon TE300). Cells were illuminated using a xenon lamp (75W) and observed with a 40 x UV fluor oil-immersion objective lens. Video images were obtained using a cooled CCD camera (Sensys KAF 1401, Photometrics, Tucson, AZ). Digitized images were acquired, stored, and analyzed in a PC controlled by Axon Imaging Workbench software (AIW2.1, Axon Instruments, Sunnyvale, CA). The shutter and filter wheel (Lambda 10-2, Sutter Instrument, Novato, CA) were also controlled by AIW to allow timed illumination of cells at 340 and 380 nm excitation wavelengths. Fura-2 fluorescence was detected at an emission wavelength of 510 nm. Ratio images of 340/380 nm were analyzed by averaging pixel ratio values in circumscribed regions of cells in the field of view. The values were exported from AIW to SigmaPlot for further analysis and plotting.

Electrophysiology

All animal experiments were carried out according to guidelines approved by the University of Toronto Animal Care Committee. Primary cultures of mouse hippocampal and cortical neurons were prepared as previously described[28]. Electrophysiological recordings were made from cultured A549 cells or cultured mouse hippocampal neurons, 14–21 days after plating. The extracellular solution (ECS) was composed of (in mM): 140 NaCl, 2 $CaCl_2$, 1 $MgCl_2$ 25 N-2-Hydroxyethylpiperazine-N'-thanesulfonic acid (HEPES), 33 glucose, 5.4 KCl and 0.0002 tetrodotoxin with pH of 7.3-7.4 and osmolarity ranging from 320-330 mOsm. To record NMDA-receptor-mediated current, magnesium was not added in ECS. The intracellular solution for voltage clamp recording consisted of (in mM):140 CsF (or 140 CsCl where indicated), 11 ethyleneglycol-bis-(α-amino-ethyl ether) N,N'-tetra-acetic acid (EGTA) as intracellular calcium chelating buffer, 10 HEPES, 2 $MgCl_2$, 2 tetraethyl ammonium chloride (TEA-Cl), 1 $CaCl_2$, and 4 K_2ATP. Pipette resistance ranges were 2-4 MW when filled with this intracellular solution. All recordings were performed at room temperature. Membrane potential was held at -60 mV

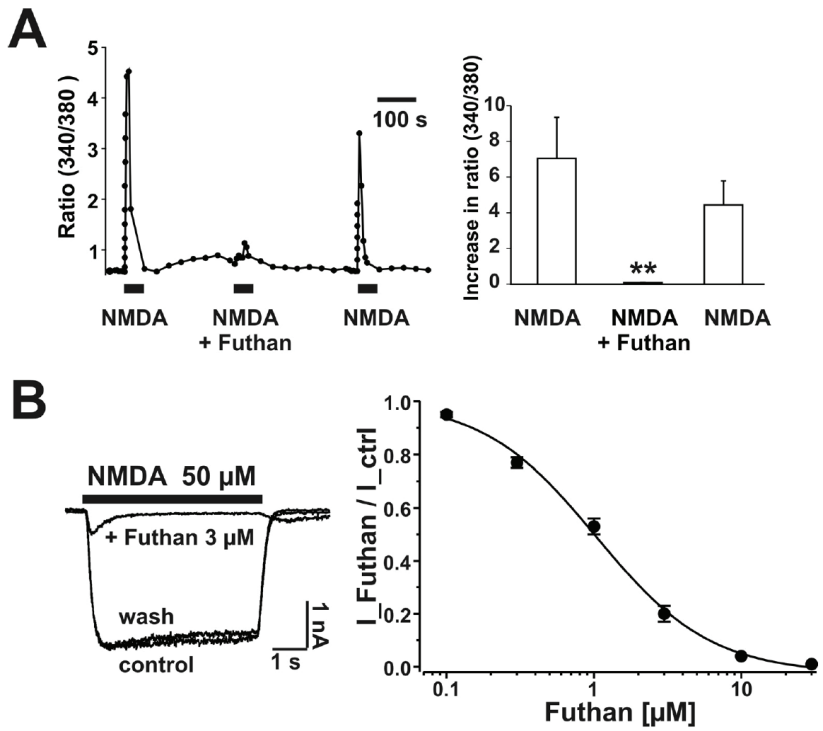

Figure 1. Futhan abolishes NMDA-induced calcium influx and blocks NMDA receptors in hippocampal neurons. A, calcium imaging. Effect of Futhan on NMDA-induced increase of intracellular Ca^{2+} in cultured mouse hippocampal neurons. Left, representative changes in 340/380 nm ratio by perfusion of 100 μM NMDA in the absence and presence of 50 μM Futhan. Right, summary data showing the reduction of increase of 340/380 nm ratio by Futhan, n = 9, ** $p<0.01$. B, Futhan directly blocks NMDA receptor in cultured hippocampal neurons. Left, examplar traces of NMDA evoked-current blocked by Futhan. Bath and testing solution contained no added magnesium. Right, concentration response of Futhan blockade on NMDA receptors; IC_{50}: 1.0 ± 0.1 μM, n = 6.

throughout the recording if not otherwise indicated. Access resistance was monitored by applying a voltage step of -5 mV. $GABA_A$ (or NMDA) receptor - mediated current was elicited by rapid application of GABA (or NMDA) delivered from a multi-barrelled fast perfusion system for 5 seconds and repeated every minute. AMPA receptors-mediated current in hippocampal neurons is elicited by application of 50 μM glutamate at the presence of 50 μM D-APV so as to block NMDA receptors. The perfusion rate of the solution was approximately 1 ml per minute. Whole-cell currents were recorded using an Axopatch-1D amplifier (Molecular Devices, Sunnyvale, CA). Electrophysiological recordings were filtered at 2 kHz and digitized at 5-10 kHz using a Digidata 1332A or/and simultaneously through MiniDigi 1A, and acquired online with pClamp8.2 (Axon Instruments) or/and Axoscope 9.2 (Axon instruments). GABA-induced currents in cultured A549 cells were recorded similarly as in cultured hippocampal neurons.

Data analysis

Data were analyzed with Clampfit 9.2 (Axon Instruments, Sunnyvale, CA), Excel 2002 (Microsoft Corporation, Seattle, WA, USA), Origin 5.0 (Microcal Software, Northampton, MA) and final illustrated using CorelDraw13 (Corel Corporation, Mountain View, CA). Currents were normalized to the amplitude of control responses. NM inhibitory concentration-response plots was fitted to the logistic equation: $I = (A_{max}-A_0)/[1+(X/IC_{50})^n]+A_0$, where I is the normalized current amplitude, X is the antagonist concentration; n is Hill coefficient; IC_{50} is the concentration of antagonist that generate 50% of maximal inhibition. Data were presented as mean ± SEM. Futhan was purchased from BioMol (Plymouth meeting, PA, USA) and its metabolites p-GBA and AN were from TCI (Portland, Oregon, USA).

Results

Futhan strongly blocks NMDA receptors in hippocampal neurons

To test if Futhan blocks some key neuronal receptors in the brain, we first used calcium-imaging technique to monitor calcium entry upon NMDA challenge in primarily cultured hippocampal neurons. As shown in **Figure 1A**, NMDA-induced increase of $[Ca^{2+}]_i$ was inhibited by Futhan in cultured neurons. The 340/380 ratio was 7.04 ± 2.31 in the absence of Futhan,

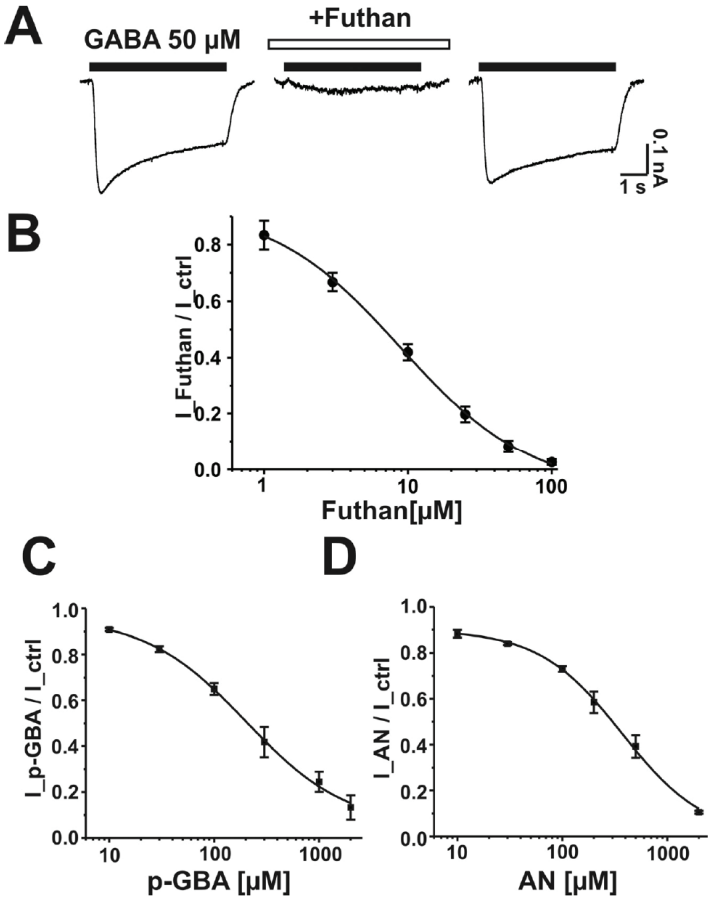

Figure 2. Futhan reversibly blocks $GABA_A$ receptors in cultured hippocampal neurons. A, Futhan reversibly and completely blocks $GABA_A$ receptors. Representative traces of GABA-activated current blocked by Futhan (100 μM), n = 6. B, concentration response of Futhan blockade on $GABA_A$ receptors, GABA-currents at the presence of Futhan were normalized to control GABA-response before Futhan application. C and D, AN and p-GBA weakly blocks $GABA_A$ receptors in hippocampal neurons. C, concentration response of p-GBA blockade on $GABA_A$ receptors; IC_{50}: 335 ± 40 μM, n = 5. D, concentration response of AN blockade on $GABA_A$ receptors; IC_{50}:236 ± 47 μM, n = 4.

0.08 ± 0.02 in the presence of 50 μM Futhan (n = 9, $p < 0.01$), 5.20 ± 1.44 after washout of Futhan, respectively, suggesting that Futhan inhibits NMDA receptors. Next we used whole-cell voltage clamp to verify if Futhan affects NDMA receptor-mediated currents. Indeed Futhan inhibits NMDA currents in cultured hippocampal neurons (**Figure 1B**). It reversibly blocks NMDA-current in a concentration-dependent manner and the IC_{50} is 1.0 ± 0.1 μM (n = 6).

Futhan blocks $GABA_A$Rs in cultured hippocampal neurons

Next we examined whether Futhan inhibits $GABA_A$ receptors in cultured hippocampal neurons. **Figure 2A** shows that Futhan (100 μM) reversibly and completely blocks $GABA_A$ receptors. The inhibition was concentration-dependent and the IC_{50} was 7.3 ± 0.6 μM (n = 7, **Figure 2B**), indicating that $GABA_A$ receptors are also targets of Futhan. Futhan is a linear dicationic ester conjugate represented by AN and p-GBA [29]. AN and p-GBA are two metabolites of Futhan *in vivo* [30]. Next we tested whether these metabolites retain Futhan's effects in blocking $GABA_A$ receptors in the hippocampal neurons. **Figure 2C** and **2D** show that AN and p-GBA only weakly and reversibly blocks $GABA_A$ receptors in hippocampal neurons. AN blocks $GABA_A$Rs with an IC_{50} of 335 μM and p-GBA blocks it with an IC_{50} of 236 μM. The weaker effects of AN and p-GBA on $GABA_A$ receptors suggest that the whole structure of Futhan is essential for retaining the potent inhibition on $GABA_A$ receptors.

Futhan potently inhibits $GABA_A$ receptors expressed in lung A549 cells

$GABA_A$ receptors are also expressed in peripheral tissues such as lung [4, 6], pancreas [7, 31], and liver [5]. For example, GABAergic system was recently characterized in lung airway

and alveolar epithelial cells [4, 8]. It has been shown that GABA promotes mucus production [6]. $GABA_A$ receptors expressed in peripheral tissues differ from neuronal $GABA_A$ receptors in many aspects including expression level, current kinetics, agonist affinity and pharmacology [6, 9]. Therefore, we compared GABA-evoked current in cultured hippocampal neurons with that in A549, a cell line derived from lung type II alveolar cell. **Figure 3A** shows typical GABA-currents recorded from hippocampal neurons and A549 cells. Under the same recording conditions, the peak current amplitude of GABA in neurons is 18-folds higher than that in A549 cells. Moreover, GABA-current in A549 cells demonstrates only very weak desensitization while GABA-evoked current in neurons desensitizes much faster. The ratio of steady-state current amplitude to peak current amplitude is 0.66 ± 0.02 (n = 23) in A549 cells, significantly higher than that recorded in hippocampal neurons (0.27 ± 0.02, n = 23). These data confirm that $GABA_A$ receptor subunit composition in lung cells is distinct from that in hippocampal neurons, suggesting that their pharmacology may differ too.

It has been shown that Futhan has anti-asthma effect [26] [27]. To test if anti-asthma effect of Futhan is probably mediated through its anti-GABAergic effect in the lung, we examined if Futhan inhibits $GABA_A$ receptors endogenously expressed in A549 cells. **Figure 3B** shows that Futhan blocks $GABA_A$ receptors expressed in A549. Futhan (10 μM) completely abolished the GABA-current that fully recovered after washout. At this concentration, Futhan only blocks ~ 40% GABA-current in hippocampal neurons (**Figure 2B**). The IC_{50} of blockade on $GABA_A$ receptors of A549 was 0.9 μM (**Figure 3C**), indicating that the inhibition of Futhan on non-neuronal $GABA_ARs$ is much more potent than that of neuronal $GABA_ARs$. These data suggest that the anti-asthma effect of Futhan could be partially explained by the blockade of $GABA_A$ receptors in the lung.

Discussion

Futhan inhibits a number of serine proteases [15, 16, 18]. It is clinically used to treat DIC, ECC and acute pancreatitis [14]. In this study, we report novel off-target effects of this medicinal compound: Futhan strongly blocks both $GABA_A$ receptors and NMDA receptors in cul-

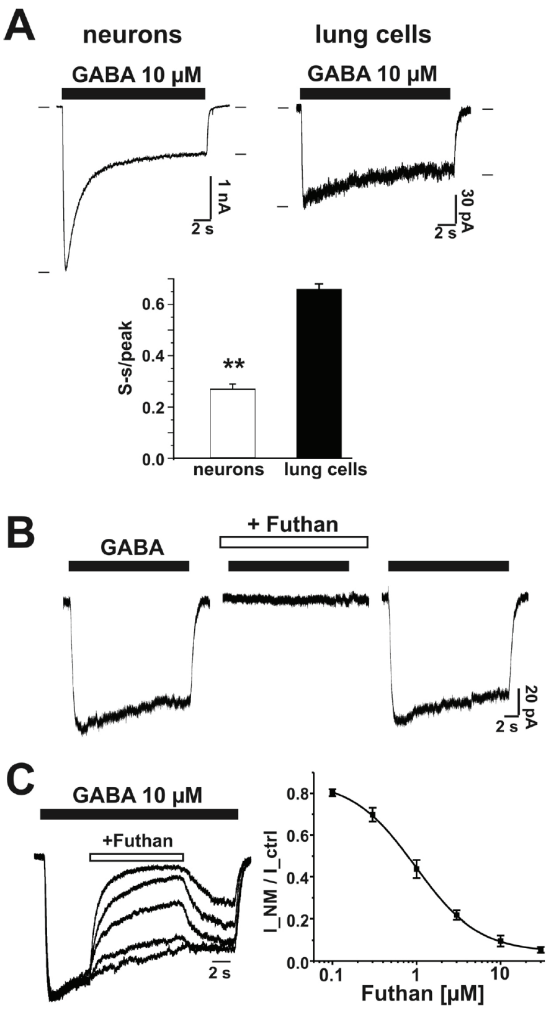

Figure 3. Futhan potently blocks $GABA_A$ receptors endogenously expressed in A549 cells. A. Top, representative GABA response in cultured hippocampal neurons (left) and in lung A549 cells (right). Intracellular pipette solution was filled with 140 mM CsCl and other components (see methods). The peak amplitude of GABA-currents were 4.0 ± 0.7 nA in hippocampal neurons (n = 9) and 0.22 ± 0.04 nA in A549 cells (n = 23). Bottom, bar graph shows ratio of steady-state current to peak current amplitude (S-s/peak) in neurons and in A549 cells, respectively. GABA-current in A549 desensitized less strongly than in neurons. ** p<0.01. B, representative GABA-current trace recorded from A549 cells; Futhan (10 μM) reversibly and completely abolished the response evoked by GABA, n = 6. C, concentration response of Futhan blockade on $GABA_A$ receptors in A549 cells. Left, current trace representative (scaled and superimposed), various concentrations of Futhan were mid-applied, indicated by empty bar. Right, concentration response of Futhan inhibition, IC_{50}:0.9 ± 0.1 μM, n = 7.

tured hippocampal neurons. Interestingly, Futhan preferentially blocks $GABA_A$ receptors expressed in lung epithelial cells.

Although Futhan blocks acid-sensing ion channels [32-34], TRPM7 [35], NMDA receptors, AMPA receptors (with an IC_{50} of 321 ± 49 μM, unpublished) and $GABA_A$ receptors that are critical for neuronal functions, so far there are no reports to demonstrate obvious pro-convulsive effects or other neurological complications of this drug [14]. This is probably due to several facts. First, Futhan is a double-charged chemical, making it very difficult to enter the brain to encounter these neuronal receptors. Secondly, Futhan is metabolized rapidly in the body [36, 37]. The system retention time of Futhan is very short (5~8 min after hemodialysis) [14], which decrease its effective concentration *in vivo*. Thirdly, Futhan is the only active form and its molecular integrity appears to be essential for retaining its anti-protease activities [14]. Similarly, our data demonstrates that Futhan's metabolites p-GBA and AN only affect $GABA_A$ receptors weakly. Fourthly, the potency of Futhan on lung $GABA_A$ receptors is ~ 8-fold higher than on neuronal receptors. These features largely explain why Futhan remains as a relatively safe medicine even if Futhan strongly blocks NMDA receptors and $GABA_A$ receptors in neurons. On the other hand, as Futhan can effectively target $GABA_A$ receptors that are distributed to peripheral non-neuronal tissues, this may partly account for its beneficial effects. Indeed $GABA_A$ receptors are widely expressed in non-neuronal tissues such as lung [4, 6], pancreas [7, 31], and liver [5], participating in a plethora of (patho-)physiological functions like asthma [6], diabetes [12, 13], hepatic encephalopathy and systemic hypotension [5]. In this regard, Futhan could exert its beneficiary impacts in vivo through targeting $GABA_A$ receptors.

Our study indeed suggests that Futhan may be a good drug candidate to intervene diseases involving non-neuronal GABAergic system such as asthma [6] through a combination of its anti-GABAergic and anti-protease properties. Futhan protects lung from various insults [38, 39]. Moreover, Futhan inhibits airway eosinophilic inflammation and airway epithelial remodeling in a murine model of allergic asthma [26]. In line with this finding, Futhan attenuates allergen -induced airway inflammation and eosinophilia [27]. But these studies have not clearly defined which mechanism contributes to the anti-asthma effect of Futhan [40]. We found that Futhan potently blocks $GABA_A$ receptors expressed in lung cells. As activation of $GABA_A$Rs promotes mucus secretion during asthma [6], Futhan (aerosolized or intravenously administered) can be developed to combat asthma through blocking GABAergic system in the airway.

Cells in different tissues have different chloride reversal potentials, controlled by distinct chloride transporters [41, 42], which accounts for diverse and even opposite functions of $GABA_A$Rs at different locations. Generally in the nervous system, GABA dampens neuronal excitation in adults and GABA-potentiating agents such as sedatives, narcotics, and general anaesthetics are widely used for therapeutic purposes [3, 43-45]. Conversely $GABA_A$ receptor-blocking agents often have harmful or pro-convulsive effects. However, in non-neuronal tissues, activation of $GABA_A$ receptors tends to increase cellular activities [6, 8]. This study indicates that Futhan can be developed as a novel medicine for non-neuronal GABA-associated diseases, like asthma, because it can preferentially target non -neuronal $GABA_A$ receptors and largely restrict its activity in the periphery.

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